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STRUCTURE OF THE CAPSULAR POLYSACCHARIDE OF *Klebsiella* SEROTYPE K53*

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ABSTRACT

Klebsiella serotype K53 is one of three strains belonging to the chemotype whose capsular polysaccharides consist of D-glucuronic acid, D-galactose, D-mannose, and L-rhamnose residues, and the structure of its polysaccharide was found to be of the "5 + 1 type" shown, of the same structural pattern as the capsule from *Klebsiella* K52.

$$\rightarrow 4)\beta\text{DGlc}p\text{A}(1\rightarrow 2)\alpha\text{DMan}p(1\rightarrow 2)\alpha\text{DMan}p(1\rightarrow 3)\beta\text{DGal}p(1\rightarrow 2)\alpha\text{LRha}p(1\rightarrow$$
$$\begin{array}{c} 3 \\ \uparrow \\ 1 \\ \alpha\text{LRha}p \end{array}$$

INTRODUCTION

Nimmich has analyzed[1] the capsular polysaccharide of *Klebsiella* K53 and has shown[2] that it belongs to the chemotype that also comprises K40 and K80. As part of our continuing investigation of these polysaccharides, we now report the structure of the capsular antigen of *Klebsiella* K53; the structures of those from K40 and K80 are, at present, unknown.

RESULTS AND DISCUSSION

Composition and n.m.r. spectra. — *Klebsiella* K53 bacteria were grown on an agar medium, and the capsular polysaccharide was purified by one precipitation with Cetavlon. The product moved as one band during electrophoresis, and had $[\alpha]_D$ $-12°$, which compares well to the value of $[\alpha]_D$ $+2.3°$ calculated by using Hudson's rules of isorotation[3]. The molecular weight of the polysaccharide was determined by gel chromatography to be 1.2×10^6.

Paper chromatography of an acid hydrolyzate of the polysaccharide showed the presence of rhamnose, galactose, mannose, and glucuronic acid. Methanolysis of

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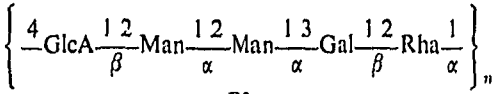
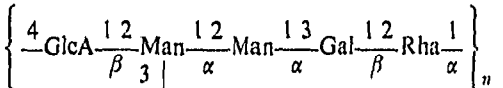
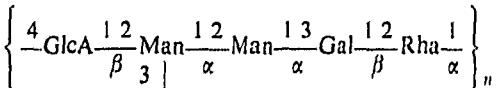
TABLE I

N.M.R. DATA FOR *Klebsiella* K53 CAPSULAR POLYSACCHARIDE, AND DERIVED POLY- AND OLIGO-SACCHARIDES

Compound[a]	*A*[b]	$J_{1,2}$[c] *(Hz)*	^{1}*H-N.m.r. data*		^{13}*C-N.m.r. data*	
			Integral, proton	*Assignment*[d]	*p.p.m.*[b]	*Assignment*[e]
GlcA$\frac{1\,2}{\beta}$Man-OH	5.30	2	0.7	α-Man-OH	102.49	β-GlcA
	4.99	s	0.3	β-Man-OH	92.91	α,β-Man-OH
A1	4.57	7	1	β-GlcA	61.40	C-6 of Man
GlcA$\frac{1\,2}{\beta}$Man$\frac{1\,2}{\alpha}$Man-OH	5.37	s	0.7	α-Man-OH	102.36	β-GlcA
	5.25	s	0.3	unknown origin	100.84	α-Man-Man
	5.16	1	1	α-Man-Man	93.40	α,β-Man-OH
A2	4.93	s	0.3	β-Man-OH	61.84 }	C-6 of Man
	4.58	6	1	β-GlcA	61.38 }	
GlcA$\frac{1\,2}{\beta}$Man$\frac{1\,2}{\alpha}$Man$\frac{1\,3}{\alpha}$Gal-OH	5.29	s	1.2	α-Man-Gal	102.32	β-GlcA
	5.18	s	1	α-Man-Man	101.02	α-Man-Man
	4.65 }	7 }	1.8	{ β-Gal-OH	97.21	β-Gal-OH
	4.61 }	7 }		{ β-GlcA	95.41 }	α-Man-Gal
A3	1.29	s	0.7	unknown origin	95.12 }	
					93.12	α-Gal-OH
					61.71	C-6 of Man,Gal
GlcA$\frac{1\,2}{\beta}$Man$\frac{1\,2}{\alpha}$Man$\frac{1\,3}{\alpha}$Gal$\frac{1\,2}{\beta}$Rha-OH	5.35	s	0.5	α-Rha-OH	105.44	β-Gal-Rha
	5.26	s	1	α-Man-Gal	102.31	β-GlcA
	5.19	s	1	α-Man-Man	101.11	α-Man-Man
	4.84	s	0.5	β-Rha-OH	95.53	α-Man-Gal
	4.63 }	7 }	2	{ β-Gal	93.91	α-Rha-OH
	4.59 }	7 }		{ β-GlcA	93.68	β-Rha-OH
A4	signal for CH_3 of Rha (δ 1.28 was not recorded)				61.79	C-6 of Man,Gal
					17.63	C-6 of Rha

 **P2**	[1]H-n.m.r. spectrum was not recorded				105.27 102.20 101.12 100.43 95.50 61.72 17.28	β-Gal-Rha β-GlcA α-Man-Man α-Rha-GlcA α-Man-Gal C-6 of Man,Gal C-6 of Rha
 **P1a**	5.27 5.20 5.03 4.55 1.28	s s s b 6 ($J_{5,6}$)	3.2 2 4	α-unassigned β-unassigned CH_3 of Rha	105.27 103.44 102.23 101.16 100.37 96.77 95.51 61.73 17.42	β-Gal-Rha β-GlcA α-Man-Man α-Rha-GlcA α-Rha terminal α-Man-Gal C-6 of Man,Gal C-6 of Rha
 **P1**	5.29[f] 5.23 5.05 4.55 1.28	b b b b 6 ($J_{5,6}$)	4 2 6	α-unassigned β-unassigned CH_3 of Rha	105.32[f] 103.35 101.28 100.44 97.31 96.14 61.91 17.42	β-Gal-Rha β-GlcA α-Man-Man α-Rha-GlcA α-Rha terminal α-Man-Gal C-6 of Man,Gal C-6 of Rha

[a]For origin of compounds **A1–A4**, **P1**, **P1a**, and **P2**, see text. [b]Chemical shift relative to internal acetone; δ 2.23 for ^{1}H-n.m.r., and 31.07 p.p.m. for ^{13}C-n.m.r. downfield from sodium 4,4-dimethyl-4-silapentane-1-sulfonate. [c]Key: b = broad, s = singlet. [d]For example, α-Man-OH = proton on C-1 of α-linked D-Man residue; for disaccharides, the data refer to the glycosidic proton [e]As for [d], but for anomeric, ^{13}C nuclei [f]Spectra recorded without internal standard; chemical shift assigned relative to those of CH_3 of rhamnose; δ 1.28 for ^{1}H-n.m.r., and 17.42 p.p.m. for ^{13}C-n.m.r.

K53 (refs. 4 and 5), reduction of the hydrolyzate, and analysis of the derived alditol acetates by gas–liquid chromatography confirmed the presence of L-rhamnose, D-mannose, D-galactose, and D-glucose in the approximate molar ratios of 2.3 : 1.9 : 1 : 1. Configurations were determined according to the sign[6] of the c.d. curves of the alditol acetates; a partially methylated derivative was used for galactitol.

The ^{1}H-n.m.r. spectrum of the native polysaccharide (**P1**) exhibited a strong doublet at δ 1.28, $J_{5,6}$ 6 Hz, and four broad signals in the anomeric region, at δ 4.55, 5.05, 5.23, and 5.29 (see Table I). The signal at δ 1.28 was assigned to methyl protons of the rhamnosyl units, that upfield of δ 5.0 to β-linkages, and those downfield to α-linkages. The integral ratio indicated that the repeating unit contains six monosaccharide constituents, two of which are rhamnose, and that two are β-linked and four α-linked. The absence of signals at δ 1.5 and 2.2 showed that the polysaccharide does not contain any pyruvic acetal or *O*-acetyl group[7].

The ^{13}C-n.m.r. spectrum of K53 corroborated the results obtained in the ^{1}H-n.m.r. experiment. Six signals appeared in the anomeric region, at 96.14, 97.31, 100.44, 101.28, 103.35, and 105.32 p.p.m. The signal at 61.91 p.p.m., attributable to C-6 of mannose and galactose, indicated that these positions are unsubstituted[8]. The signal at 17.42 p.p.m. was assigned to the deoxy group of rhamnose.

N.m.r. spectra (^{1}H and ^{13}C) were also recorded for a sample (**P1a**) of K53 that had undergone mild hydrolysis, but the treatment did not greatly improve the ^{1}H-n.m.r. spectrum. The ^{13}C spectrum was similar to that of the native polysaccharide,

TABLE II

METHYLATION ANALYSES OF K53 CAPSULAR POLYSACCHARIDE, AND DERIVED POLY- AND OLIGO-SACCHARIDES

Methylated sugars[a] *(as alditol acetates)*	T[b] *Column (ECNSS-M)*	*Mole %*[c]							
		I[d]	*II*	*III*	*IV*	*V*	*VI*	*VII*	*VIII*
2,3,4-Rha	0.44	18 6	19.5						30.1
3,4-Rha	0.89	19.0	18.4	18 8				16.2	
2-Et-4,6-Man	1.82								20 5
3,4,6-Man	1.94	17 6	18.9	45.1	50.8	68 3	54 0	45.3	26.1
2,4,6-Gal	2.27	17.2	15.4	30 5			21.4	20.5	23.3
2,3,4-Glc	2.48				49.2	31.7	24.6	18.0	
2,3,6-Glc	2.50		11.6						
4,6-Man	3.31	16 0	14 1						
2,3-Glc	5.32	11.6	2.0	5.6					

[a]2,3,4-Rha = 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl-L-rhamnitol, etc. [b]Retention time relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol, on an ECNSS-M column, isothermal at 170°. [c]Values are corrected by use of the effective, carbon-response factors given by Albersheim *et al.*[25]. [d]I, methylated, original polysaccharide **P1**; II, methylated polysaccharide, reduced, and remethylated; III, methylated polysaccharide **P2**; IV, aldobiouronic acid **A1**; V, aldotriouronic acid **A2**; VI, aldotetraouronic acid **A3**; VII, aldopentaouronic acid **A4**; VIII, ethylated product from uronic acid degradation.

except that seven signals appeared in the anomeric region, instead of six. However, two signals, at 102.23 and 103.44 p.p.m., were assigned to the glucuronic acid residue.

Precise assignment of the signals was achieved after study of the ^{1}H- and ^{13}C-n.m.r. spectra of oligosaccharides obtained by partial hydrolysis; see Table I.

Methylation analysis. — Methylation[9,10] of K53 polysaccharide, reduction, hydrolysis, conversion into alditol acetates, and analysis by g.l.c.–m.s.[11,12] indicated that K53 is composed of a hexasaccharide repeating-unit (see Table II, column I). The presence of a di-*O*-methyl-D-mannosyl residue is attributable to a branch point, and that of a tri-*O*-methyl-L-rhamnosyl unit to the terminal sugar of a side chain. The 2,3-di-*O*-methyl-D-glucose arose from the D-glucuronic acid; this was shown by the appearance, in the g.l.c. tracing, of a peak corresponding to the acetate of 2,3,6-tri-*O*-methylglucitol, and the almost complete disappearance of the peak for 2,3-di-*O*-methylglucitol acetate after the permethylated polysaccharide was reduced and remethylated (see Table II, column II). A small proportion of the di-*O*-methyl-D-glucose found in this second analysis was attributable to incomplete methylation.

Partial hydrolysis. — The oligosaccharides obtained by partial hydrolysis of the K53 polysaccharide were separated by means of an ion-exchange resin into acidic and neutral components, and the former were partially resolved by gel-filtration chromatography. Pure fractions were isolated by descending paper-chromatography; four oligosaccharides were thus collected, namely, compounds **A1**–**A4**. The structures of these compounds were established by n.m.r. spectroscopy (see Table I) and by methylation (see Table II). Compound **A4** was thus shown to be βGlc*p*A(1→2)-αMan*p*(1→2)αMan*p*(1→3)βGal*p*(1→2)Rha (**A4**), **A3**, **A2**, and **A1** being the related aldotetrao-, aldotrio-, and aldobio-uronic acids (see Table I).

The ^{13}C-n.m.r. spectrum of **A3** merits brief comment. For this compound, five signals were to be expected in the anomeric region of the spectrum, but six were observed. The possibility that **A3** was a mixture of oligosaccharides was precluded by checking the purity of the fraction by paper chromatography, and by determining the end group(s) by the aldononitrile method of Morrison[13]. However, for some β-D-glucopyranosyl-L-rhamnose disaccharides, Colson and King reported[14] that, when linked to O-2 of rhamnose, the anomeric signal of the glycopyranosyl residue, is split by the mutarotational equilibrium of the reducing group. A similar result has been observed[15] for an oligosaccharide, isolated from *Klebsiella* K74, having the same two terminal sugars as **A4**. On the other hand, a hexasaccharide isolated by depolymerization of *Klebsiella* K18 by use of a viral enzyme showed such signal splitting in the ^{1}H-, but not in the ^{13}C-, spectrum[16].

Uronic acid degradation. — The position of the mannose residue carrying the side chain was established by subjecting the permethylated K53 polysaccharide to base-catalyzed degradation[17,18]. The product was ethylated, and subsequent analysis demonstrated the presence of 2-*O*-ethyl-4,6-di-*O*-methylmannose, not 2-*O*-ethyl-3,4,6-tri-*O*-methylmannose. Degradation of the glucuronic acid was accompanied by loss of the 3,4-di-*O*-methylrhamnose residue (see Table II, column VIII) These results, taken in conjunction with the structure of **A4**, indicated that the side chain

consists of a single sugar unit (rhamnose), and this was confirmed by partial depolymerization. described next.

Location of the side chain. — Hydrolysis of the native, K53 polysaccharide with acid for a short time gave a nondialyzable product (**P2**) which, on methylation analysis, showed the complete loss of 2,3,4-tri-*O*-methylrhamnose, and replacement of 4,6-di-*O*-methylmannose by a second molecule of 3,4,6-tri-*O*-methylmannose (see Table II, column III).

The linear. degraded product **P2** had $[\alpha]_D$ +14.2°, in good agreement with the value of $[\alpha]_D$ +15.2° calculated by using Hudson's rules of isorotation[3]. Comparison of the ^{13}C-n.m.r. spectra of **P1** and **P2** indicated the disappearance of a signal at 97.31 p p.m., which was thus assigned to the α-L-rhamnopyranosyl group in the side chain; hence, the signal at 100.44 p.p.m. is attributable to the in-chain rhamnosyl residue. Removal of the side chain caused the chemical shift of the D-glucuronic acid to move upfield, from 103.35 to 102.20 p.p.m.

CONCLUSION

Data obtained in the present structural investigation demonstrate that the capsular polysaccharide from *Klebsiella* serotype K53 is composed of hexasaccharide repeating-units having the following structure.

$$\rightarrow 4)\beta\text{D-Glc}p\text{A}(1\rightarrow 2)\alpha\text{D-Man}p(1\rightarrow 2)\alpha\text{D-Man}p(1\rightarrow 3)\beta\text{D-Gal}p(1\rightarrow 2)\alpha\text{L-Rha}p(1\rightarrow$$

$$\begin{array}{c} 3 \\ \uparrow \\ 1 \\ \alpha\text{L-Rha}p \end{array}$$

Of the *Klebsiella* capsular polysaccharides reported to date, only K52 (ref. 19) has the same structural pattern, but it is not of the same chemotype[2].

Capsular polysaccharides K53 and K52 cross-react heavily in anti-K47 serum[20]. Both antigens, as well as the capsular polysaccharide K47 (ref. 21), contain a common disaccharide, α-L-Rha*p*-(1→4)-β-D-Glc*p*A. In K47, this disaccharide constitutes the lateral side-chain, whereas, in K52 and K53, it is part of the backbone. The serological behavior suggests that this dimer is an antigenic determinant. Cross-reactivity of K53 with anti-PnXXIII also suggests that the (nonreducing) α-L-rhamnosyl lateral end-group could be an immunodominant sugar, as this structural feature is common to the K53 and PnXXIII polysaccharides[20].

EXPERIMENTAL

General methods. — Solutions were evaporated under diminished pressure at temperatures not exceeding 40°. Optical rotations were measured at room temperature in a Perkin–Elmer model 141 polarimeter, using a 10-cm cell. Circular dichroism measurements were made with a Jasco J-20 automatic recording spectropolarimeter,

with a quartz cell of path length 0.01 cm. Infrared spectra were recorded with a Perkin–Elmer 457 spectrophotometer.

Descending paper-chromatography was conducted by using Whatman No. 1 paper for analytical purposes, and Whatman 3MM for preparative paper-chromatography. The following solvent systems (v/v) were used: (*A*) 18:3:1:4 ethyl acetate–acetic acid–formic acid–water, (*B*) 8:2:1 ethyl acetate–pyridine–water, and (*C*) 2:1:1 1-butanol–acetic acid–water. Chromatograms were developed by using an alkaline silver nitrate reagent[22].

Gel-filtration chromatography was conducted on a column (100 × 3 cm) of Bio-Gel P-2 (100–200 mesh). The column was irrigated with 500:5.2 water–pyridine–acetic acid at a flow rate of ~10 mL/h. Fractions (2.0–2.5 mL) were collected, freeze-dried, weighed in tared tubes, and the results plotted on graph paper to produce a chromatogram.

Analytical, g.l.c. separations were performed by using a Hewlett–Packard model 5710A gas chromatograph fitted with dual, flame-ionization detectors. A stainless-steel column (1.8 m × 3 mm) of 5% of ECNSS-M on Gas Chrom Q (100–120 mesh), used exclusively, was operated at 170° (isothermal), except as otherwise stated. Preparative g.l.c. was performed on an F & M model 720 gas chromatograph equipped with dual, thermal-conductivity detectors. A column (1.8 m × 6.3 mm) of 5% of Silar 10C on Gas Chrom Q (100–120 mesh) was used for preparative separations An Infotronics CRS-100 electronic integrator was used to measure peak areas.

G.l.c.–m.s. was performed with a 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°.

^{1}H-N m.r. spectra were recorded with a Varian XL-100 spectrometer at ~90° and, in some cases, at ambient temperature (for suppression of the water peak). Samples in D_2O were hydrogen-exchanged and freeze-dried three or four times in 99.7% D_2O. ^{13}C-N.m.r. spectra were recorded with a Varian CFT-20 instrument at ambient temperature for solutions in 1:1 H_2O–D_2O. In all instances, acetone (δ 2.23 for ^{1}H-n.m.r., and 31.07 p.p.m. for ^{13}C-n.m.r. spectra, measured against aqueous sodium 4,4-dimethyl-4-silapentane-1-sulfonate) was used as the internal standard. In addition, a ^{13}C-n.m.r. spectrum of the native polysaccharide (**P1**), recorded at 95°, was obtained by courtesy of Dr. M. Vignon, CERMAV/CNRS, Grenoble, France, with a Cameca 250-MHz instrument. Photocopies of the n.m r. spectra recorded in Table I are available[23].

Isolation and purification of Klebsiella *K53 polysaccharide.* — A culture of *Klebsiella* K53 (1756/51) was obtained from Dr. I. Ørskov, Copenhagen, and grown on a 3% sucrose–yeast extract–agar medium composed of NaCl (5 g), K_2HPO_4 (2.5 g), $MgSO_4 \cdot 7\ H_2O$ (0.62 g), $CaCO_3$ (0.5 g), sucrose (75 g), Bacto yeast extract (5 g), and agar (37.5 g) in water (2.5 L). The cells were harvested after 3 d, and 1 L of a very viscous slime was collected. The slime was diluted to 3 L with 1% aqueous phenol, and centrifuged, in batches, for 8–12 h at 30,000 r.p.m. in a

Beckman model L3-50 ultracentrifuge fitted with rotor type 35. The clear, supernatant liquids were decanted, combined (~2 L), and the polysaccharide precipitated by pouring into ethanol (10 L). The crude polysaccharide was dissolved in water (1 L), and precipitated with 10% Cetavlon solution. The precipitate was isolated by centrifugation, dissolved in 4M NaCl (1 L), and reprecipitated by pouring into ethanol. The purified polysaccharide was collected, dissolved in water, and dialyzed against running tap-water for 3 days. Freeze-drying of this solution yielded 13 g of the sodium salt of the capsular polysaccharide, $[\alpha]_D$ −12° (*c* 0.24, water). The purity of the polysaccharide was checked by electrophoresis, using a 1% solution on a cellulose acetate strip (Sepraphore III: 15 × 2.5 cm) in Veronal buffer, pH 8.6 (LKB-Produkter AB, Stockholm 12, Sweden) at 300 V for 60 min, and then development in Alcian Blue. Homogeneity was also confirmed by gel chromatography by courtesy of Dr. S. C. Churms, University of Cape Town, South Africa, and the molecular weight of the K53 polysaccharide was determined to be 1.2×10^6.

Some spectroscopic analyses were performed on K53 polysaccharide that had been partially depolymerized to lower the viscosity: this was achieved by mild hydrolysis in 0.5M trifluoroacetic acid for 30 min at 95°, and then, under diminished pressure, several additions and evaporations of water to eliminate the excess of acid. The hydrolyzate was dissolved in water, dialyzed overnight against running tap-water, and freeze-dried to give **P1a**.

Analysis of component sugars. — The polysaccharide was hydrolyzed overnight with 2M trifluoroacetic acid (TFA) at 95°. After evaporation, the hydrolyzate was found, by paper chromatography in solvents *A* and *B*, to contain rhamnose, galactose, mannose, and glucuronic acid.

Methanolysis of *Klebsiella* K53 capsular polysaccharide (30 mg) with 3% methanolic hydrogen chloride, and subsequent treatment with sodium borohydride in anhydrous methanol, depolymerized the substrate and reduced the uronic acid residues[4,5]. Total hydrolysis, reduction of the free sugars to alditols with sodium borohydride, and acetylation with 1:1 acetic anhydride–pyridine overnight at room temperature yielded rhamnitol pentaacetate and the hexaacetates of mannitol, galactitol, and glucitol, which were separated by using a column of 5% of ECNSS-M, programmed at 170° for 8 min, and then at 4°/min to 190°.

Methylation analysis — (*a*) *Native polysaccharide* **P1**. Dried K53 capsular polysaccharide (~500 mg) was dissolved in anhydrous dimethyl sulfoxide (50 mL) with ultrasonic agitation, and methylated by treatment with dimethylsulfinyl anion (10 mL) for 6 h, and then with methyl iodide (8 mL) for 1 h (refs. 9 and 10). After removal of the excess of the reagents by dialysis against running tap-water for three days, the methylated polysaccharide was obtained by freeze-drying. Subsequent Purdie[24] treatment with silver oxide and methyl iodide gave a permethylated product that showed no hydroxyl-group absorption at 3600 cm^{-1} in the infrared spectrum; yield 373 mg.

A sample (55 mg) of this dried material was reduced with lithium aluminum hydride in refluxing oxolane (15 mL) for 3 h and then overnight at room temperature.

The white precipitate of aluminum hydroxide was dissolved with 4% hydrochloric acid, and the reduction product was extracted with chloroform (5 × 10 mL). The extracts were combined, washed with water (3 × 10 mL), and evaporated to dryness, and the reduced polysaccharide (45 mg) was divided into halves. One half was hydrolyzed with 2M trifluoroacetic acid overnight at 95°, the hydrolyzate reduced with sodium borohydride, and the alditols acetylated with 1:1 acetic anhydride–pyridine overnight at room temperature. The other half was remethylated by one Purdie treatment[24], and converted into the corresponding alditol acetates as for fraction one. Analysis of both mixtures of partially methylated alditol acetates by g.l.c. and g.l.c.–m.s allowed the assignments given in Table II, columns I and II.

(*b*) *Partially degraded, K53 polysaccharide* (**P2**). The side chain was selectively removed from a sample (215 mg) of K53 polysaccharide by hydrolysis with 2M trifluoroacetic acid for 30 min at 95°. The hydrolyzate was concentrated under diminished pressure, evaporated several times with water to eliminate the excess of acid, and the residue dissolved in water, and the solution dialyzed overnight against running tap-water. Freeze-drying of the solution yielded 165 mg of polymeric material (**P2**), $[\alpha]_D$ +14.2° (*c* 0.82, water). Methylation of **P2** (16 mg) by the Hakomori procedure[9,10], derivatization to the corresponding alditol acetates as for the original polysaccharide, and g.l.c. analysis gave the results listed in Table II, column III.

Partial hydrolysis. — A batch hydrolysis was performed on K53 polysaccharide (640 mg) with 0.5M trifluoroacetic acid for 5 h at 95°. After removal of the acid by addition and evaporation of several portions of water, paper chromatography (solvent *A*) of the hydrolyzate showed the presence of several oligosaccharides. The total material was applied to the top of a column (20 × 2 cm) of Bio-Rad AG1-X2 (formate) resin, and the column was eluted with water (700 mL), and then with 10% formic acid (500 mL) Paper chromatography (solvent *B*) of the neutral fraction (288 mg) showed that it contained mainly monosaccharides, and very little oligosaccharide

The acidic fraction (322 mg) was separated by gel-filtration chromatography on a column (100 × 3 cm) of Bio-Gel P-2. The column was irrigated with a buffer, 500:5:2 water–pyridine–acetic acid, and fractions (2.0–2.5 mL) were collected, freeze-dried, and weighed in tared tubes. Fractions 20–26 were pooled, as well as fractions 29–34, 35–40, and 43–46, and then purified by descending paper-chromatography on Whatman 3 MM paper, using solvent *C* for 3–6 days; four pure oligosaccharides were thus collected, namely, **A1**, **A2**, **A3**, and **A4**.

Methylation analysis of these oligosaccharides was performed as follows. Dried samples (7–10 mg each) were each dissolved in anhydrous dimethyl sulfoxide (5 mL), treated with dimethylsulfinyl anion (3 mL) for 6 h, and then with methyl iodide (4 mL) for 1 h (refs. 9 and 10). The mixtures were diluted with water, made neutral with 10% acetic acid, transferred to separatory funnels, and extracted with chloroform (5 × 10 mL). The extracts were combined, back-extracted with water (3 × 10 mL), and evaporated to dryness under diminished pressure. Permethylated oligosaccharides were reduced with lithium aluminum hydride, and the alditols

converted into the corresponding acetates, as previously described for the methylation analysis of the original polysaccharide. G.l.c. analysis of the mixture of partially methylated, alditol acetates allowed the assignments given in Table II (columns IV–VII), and the results were confirmed by analysis by g.l.c.–m.s.

Uronic acid degradation. — A sample (100 mg) of dried, methylated polysaccharide and *p*-toluenesulfonic acid (1 mg) were dissolved in 19:1 (v/v) dimethyl sulfoxide–2,2-dimethoxypropane (20 mL) in a flask sealed with a rubber cap. The flask was flushed with nitrogen, dimethylsulfinyl anion (10 mL) was added with a syringe, and the mixture was stirred overnight at room temperature. The substrate was then directly re-alkylated by adding ethyl iodide (7 mL) with external cooling, and the mixture was stirred for a further 1 h. The mixture was made neutral with 10% acetic acid, and extracted with chloroform (4 × 10 mL). The extracts were combined, washed with water (3 × 10 mL), and evaporated to dryness. Hydrolysis of the degraded product with 2M trifluoroacetic acid, reduction with sodium borohydride, acetylation with 1:1 (v/v) acetic anhydride–pyridine overnight at room temperature, and g.l.c. analysis of the corresponding alditol acetates, gave the results listed in Table II, column VIII; the results were confirmed by g.l.c.–m.s. analysis[11,12].

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