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THE STRUCTURAL ELUCIDATION OF THE CAPSULAR POLYSACCHARIDE OF *Klebsiella* K68*

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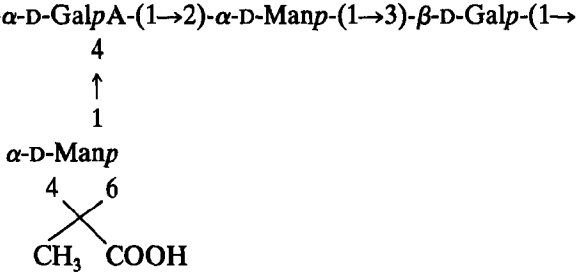
ABSTRACT

The structure of the capsular polysaccharide isolated from *Klebsiella* K68 bacteria has been elucidated by both chemical and spectroscopic methods. The structure is of the "3 + 1" type, similar in pattern to the structures of the polysaccharides of *Klebsiella* K11 and K57, having a single branch point on the uronic acid. The polysaccharide is shown to consist of the following repeating unit:



INTRODUCTION

In an earlier study[1] it was indicated that K68 polysaccharide belonged to a unique chemotype in the *Klebsiella* series of capsular polysaccharides, being composed of glucuronic acid, glucose, galactose, mannose and fucose. Our work shows this to be incorrect, and in fact reveals the K68 polysaccharide as structurally very similar to that of *Klebsiella* K57 (ref. 2), containing galacturonic acid, galactose and mannose. The *Klebsiella* K11 (ref. 3), K57 (ref. 2), and now K68 polysaccharides exhibit the "3 + 1" pattern of structure, but K68 is the first polysaccharide in the *Klebsiella* series to have a pyruvic-acetal group attached to a side-chain mannose unit. The present report describes the elucidation of the structure of the *Klebsiella* K68 polysaccharide.

*Dedicated to Professor Luis F. Leloir on the occasion of his 80th birthday.

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RESULTS AND DISCUSSION

Composition and n.m.r. spectra. — The capsular polysaccharide from *Klebsiella* K68 was isolated from the bacteria by methods described previously[4]. The polysaccharide was purified via its cetyltrimethylammonium salt and shown to be homogeneous by gel-permeation chromatography. ^{1}H-N.m.r. spectroscopy of the partially autohydrolyzed polysaccharide (K68 P.A.) showed six signals in the anomeric region (δ 4.5–5.5) as well as a signal at δ 1.68 indicating the presence of pyruvate (~10%) (see Table I). The spectrum was well resolved, and demonstrated that the polysaccharide is composed of a distinct repeating unit. The presence of one pyruvic acetal per repeating unit was established from the ^{1}H-n.m.r. spectrum of the native polysaccharide, despite the spectrum being poorly resolved because of the high viscosity of the solution. ^{13}C-N.m.r. spectroscopy of the partially autohydrolyzed polysaccharide showed four anomeric signals (95–105 p.p.m.) as well as a signal in the carboxyl region (see Table I, K68 P.A.).

Acid hydrolysis of the polysaccharide and g.l.c. analysis of the derived peracetylated aldononitriles showed the presence of mannose and galactose in the molar ratio 2.0:1.0. Methanolysis followed by reduction of the uronic acid, then hydrolysis and derivatization of the sugars as peracetylated aldononitriles, showed mannose and galactose in the molar ratio 2.0:1.9, indicating the presence of one mole of galacturonic acid in the tetrasaccharide repeating unit. Thus, of the six signals in the δ 4.5–5.5 region of the ^{1}H-n.m.r. spectrum of K68 P.A. (Fig. 1), only four are due to anomeric protons. The other two signals are due to H-5 of α-galacturonic acid and H-2 of one of the mannose units (see comments on partial hydrolysis products **A1** and **A2**). All sugar components of the polysaccharide were found to be of the D-configuration by measurement of their optical rotations after isolation by paper chromatography.

Methylation analysis. — A portion of the original polysaccharide was methylated by the Hakomori method[5] and, on hydrolysis and g.l.c.–m.s. of the derived alditol acetates, gave the results shown in Table II, column I. The methylated polysaccharide was reduced with lithium aluminum hydride (LAH), remethylated by the Kuhn method[6], and, on analysis of the derived alditol acetates by g.l.c.–m.s., gave the results shown in Table II, column II. In order to confirm the position of the pyruvic acetal in the polymer, the polysaccharide was autohydrolyzed for a short period and then methylated. On hydrolysis and g.l.c. analysis (as alditol acetates) the appearance of 2,3,4,6-tetra-*O*-methylmannose (0.71 mole) and a decrease in the proportion of 2,3-di-*O*-methylmannose (0.23 mole) were observed (Table II, column III). These results show that the K68 polysaccharide consists of a tetrasaccharide repeat unit with galacturonic acid as the branch point and pyruvate carried on position 4 and 6 of a terminal mannose unit.

Periodate oxidation and Smith degradation[7]. — When a portion of polysaccharide was oxidized with sodium metaperiodate, 2.06 moles were consumed per mole of repeating unit, confirming the methylation analysis. Smith degradation of

a larger sample of polysaccharide yielded an acidic product (**SD**, $[\alpha]_D^{20}$ +52.2°), which was isolated by paper chromatography. Fraction **SD** was methylated using the Hakomori method, methanolyzed, carboxyl-reduced and hydrolyzed. G.l.c. analysis of the derived alditol acetates indicated, however, a complex mixture. The ^{1}H-n.m.r. spectrum of **SD** showed signals for α-galacturonic acid (δ 5.43), β-galactose (δ 4.81) and a new signal at δ 5.09 (1 H)*. It was thought that this signal could have arisen from the proton of an acetal or hemiacetal which had formed between the glyceraldehyde resulting from Smith degradation of a 2-linked mannose, and other free hydroxyl groups present. This phenomenon has been noted before[8,9], and it is known that glyceraldehyde rarely exists in monomeric form but in fact dimerizes and hydrates readily[10]. No aldehyde proton was observed. This would explain the difficulty in interpreting the methylation analysis of **SD**. Reduction of **SD** with sodium borohydride (16 h) gave a product (**SD**-reduced) having its ^{1}H-n.m.r. signal at δ 5.09 reduced in intensity to 0.5 H. As a precautionary measure the product was again reduced (6 h) and, without further n.m.r.-spectral analysis, was methylated. After carboxyl reduction and hydrolysis, g.l.c. analysis of the derived alditol acetates showed 2,3,4,6-tetra-*O*-methylgalactose and 3,4-di-*O*-methylgalactose in the molar ratio 1.00:0.62 (Table II, column IV). Thus it can be concluded that **SD**-reduced is a disaccharide having the following structure:

β-Gal*p*-(1→2)-α-Gal*p*A-(1→2)-Glycerol

Partial hydrolysis. — The polysaccharide was partially hydrolyzed with acid, and two acidic oligosaccharides (**A1** and **A2**) were separated from the hydrolyzate by paper chromatography. Both **A1** and **A2** were found to have galactose at the reducing end by the method of Morrison[11] as modified by McGinnis[12]. Product **A1** had a d.p. of four, and **A2** a d.p. of three. Methylation, reduction, and hydrolysis of **A1** and **A2** and g.l.c.–m.s. analysis of the derived alditol acetates gave the results shown in Table II, columns V and VI, thus confirming that **A1** is a tetrasaccharide and **A2** is a trisaccharide. Products **A1**, $[\alpha]_D^{20}$ +92° (*c* 0.37, water), and **A2**, $[\alpha]_D^{20}$ +109° (*c* 0.48, water), have the following partial structures:

A1 Gal*p*A-(1→2)-Man*p*-(1→3)-Gal
4
↑
1
Man*p*

A2 Gal*p*A-(1→2)-Man*p*-(1→3)-Gal

^{1}H-N.m.r. spectroscopy of **A1** showed anomeric signals corresponding to α-

*For the assignment of the anomeric signals, refer to the section on partial hydrolysis.

TABLE I

N.M.R. DATA FOR *Klebsiella* K68 POLYSACCHARIDE AND DERIVED OLIGOSACCHARIDES

Compound[a]	*^1H data*				*^{13}C data*	
	δ[b] *(p.p.m.)*	$J_{1,2}$ *(Hz)*	*Integral (no. of H)*	*Assignment*[c]	*δ*[d] *(p.p.m.)*	*Assignment*[e]
$[\xrightarrow{2}\text{GalA}\xrightarrow[\alpha]{1\,2}\text{Man}\xrightarrow[\alpha]{1\,3}\text{Gal}\xrightarrow[\beta]{1}]_n$	1.68		0.3	CH_3 of pyruvate	173.12	COOH of GalA
4 | α	4.52		1	H-2 of $\xrightarrow{2}$Man-α-		
1 |	4.65	8	1	$\xrightarrow{3}$Gal-β-	105.08	$\xrightarrow{3}$Gal-β-
Man	4.72		1	H-5 of GalA		
\ /	4.95		1	Man-α-	100.98	Man-α-
Pyr (10%)	5.21		1	$\xrightarrow{2}$Man-α-	95.52	$\xrightarrow{2}$Man-α-
(K68 P.A.)	5.46		1	$\xrightarrow{2}$GalA-α- 4|	102.47	$\xrightarrow{2}$GalA-α- 4|
$\text{GalA}\xrightarrow[\alpha]{1\,2}\text{Man}\xrightarrow[\alpha]{1\,3}\text{Gal}$	4.45		1	H-2 of $\xrightarrow{2}$Man-α-		
4 | α	4.56		1	H-5 of GalA		
1 |	4.62	8	0.6	$\xrightarrow{3}$Gal*p*-β-	97.20	$\xrightarrow{3}$Gal-β-
Man	4.93		1	Man-α-	101.77	Man-α-
(A1)	5.21		1	$\xrightarrow{2}$Man-α-,	95.55	$\xrightarrow{2}$Man-α-*f*
	5.23		1	$\xrightarrow{4}$GalA-α-	102.26	$\xrightarrow{4}$GalA-α-
	5.26	2	0.1	$\xrightarrow{3}$Gal*f*-α-	93.09	$\xrightarrow{3}$Gal-α-
	5.29	3	0.3	$\xrightarrow{3}$Gal*p*-α-	95.24	$\xrightarrow{2}$Man-α-*f*

GalA$\frac{1\,2}{\alpha}$Man$\frac{1\,3}{\alpha}$Galactitol 4 | α 1 | Man	4.47		1	H-2 of —Man-α-		
	4.70		1	H-5 of GalA		
	4.93		1	Man-α		
	5.21		1	$\frac{2}{}$Man-α-		
(**A1**-reduced)	5.28		1	$\frac{4}{}$GalA-α-		
GalA$\frac{1\,2}{\alpha}$Man$\frac{1\,3}{\alpha}$Gal	4.37		1	H-2 of $\frac{2}{}$Man-α-	101.86	GalA-α-
	4.60		1	H-5 of GalA	95.17	$\frac{2}{}$Man-α-[f]
(**A2**)	4.61	8	0.6	$\frac{3}{}$Gal*p*-β-	97.16	$\frac{3}{}$Gal-β-
	5.22		2	GalA-α- $\frac{2}{}$Man-α-	95.47	$\frac{2}{}$Man-α-[f]
	5.27	2	0.1	$\frac{3}{}$Gal*f*-α-	93.06	$\frac{3}{}$Gal-α-
	5.29	3	0.3	$\frac{3}{}$Gal*p*-α-		
GalA$\frac{1\,2}{\alpha}$Man$\frac{1\,3}{\alpha}$Galactitol	4.36		1	H-2 of $\frac{2}{}$Man-α-		
	4.68		1	H-5 of GalA		
(**A2**-reduced)	5.21		1	$\frac{2}{}$Man-α-		
	5.26		1	GalA-α-		
Gal$\frac{1\,2}{\beta}$GalA$\frac{1\,2}{\alpha}$Glycerol	4.58	8	1	Gal-β-	105.68	Gal-β-
	4.81		1	H-5 of $\frac{2}{}$GalA-α-		
(**SD**-reduced)	5.08		0.5			
	5.43		1	$\frac{2}{}$GalA-α-	103.28	$\frac{2}{}$GalA-α-

[a]For the sources of K68 P.A., **A1, A2,** and **SD**-reduced, see text. [b]Chemical shift downfield from sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS). [c]$\frac{2}{}$Man-α- refers to the anomeric proton of a 2-linked mannosyl residue in the α-anomeric configuration. [d]Chemical shift in p.p.m. downfield from DSS, measured from internal acetone at 31.07 p.p.m. [e]As for *c*, but for ^{13}C nuclei. [f]Twinned signals due to mannosyl residue interior to reducing end galactose.

TABLE II

METHYLATION ANALYSES OF K68 POLYSACCHARIDE AND DERIVED POLY- AND OLIGO- SACCHARIDES

Methylated sugars[a] *(as alditol acetates)*	*T*[b] *on DB-225*	*Molar ratios*[c]					
		I	*II*	*III*	*IV*	*V*	*VI*
2,3,4,6-Man	0.98			0.71		0.76	
2,3,4,6-Gal	1.10				1.00		
3,4,6-Man	1.55	0.97	0.90	0.83		1.00	1.00
2,5,6-Gal	1.62					0.46	0.50
2,4,6-Gal	1.70	1.00	1.00	1.00		0.27	0.24
2,3,4-Gal	2.21						0.93
2,3-Gal	3.30					0.63	
2,3-Man	2.86	0.94	0.96	0.23			
3,4-Gal	3.75				0.62		
3,6-Gal	2.73		0.60				

[a]2,3,4,6-Man = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylmannitol, etc. [b]Retention times relative to 2,3,4,6-Glc on column DB-225 (J & W fused-silica capillary column, 0.25 μm film thickness, 30 m × 0.25 mm) at 200°. [c]I, methylated native polysaccharide; II, methylated, reduced, remethylated polysaccharide; III, methylated, partially autohydrolyzed polysaccharide; IV, methylated **SD**-reduced; V, methylated, reduced **A1**; VI, methylated, reduced **A2**.

galacturonic acid, α-mannose, and reducing-end galactose, as well as the signals of H-5 of galacturonic acid and H-2 of mannose (Table I). In the spectrum of **A2**, two one-proton anomeric signals were observed, corresponding to α-galacturonic acid and α-mannose, and also signals for reducing-end galactose, H-5 of galacturonic acid, and H-2 of mannose. The comparison of these two spectra allows the assignment of the signal at δ 4.95 in the spectrum of the partially autohydrolyzed polysaccharide (K68 P.A.) to the terminal mannose unit. The resonance arising from H-2 of a mannose unit is present in the proton spectra of both **A1** and **A2**, thus one can conclude that this resonance is due to H-2 of the 2-linked mannose unit and not the terminal one. Carver and Grey[13] have shown that glycosylation at position 2 of an α-mannose unit causes a dramatic deshielding of the C-2 proton of this residue. Interestingly, the signal at δ 5.46 corresponding to H-1 of α-galacturonic acid in the spectrum of K68 P.A. is shifted 0.25 p.p.m. to higher field in the spectra of the oligomers **A1** and **A2**, where this sugar is no longer a branch point. A similar but smaller shift is also observed for the H-5 signal.

Comparison of the spectra of **A1, A2**, and **SD**-reduced allows all the signals in the anomeric area of the spectrum of K68 P.A. to be assigned (Fig. 1). The signal at δ 4.72 is thus due to H-5 of galacturonic acid. This is in keeping with the results of a previous study in which the H-5 signal of α-galacturonic acid is shown to occur to lower field than that of the β anomer[14].

The signals in the ^{13}C-n.m.r. spectrum of K68 P.A. were assigned by comparison with the spectra of **A1, A2**, and **SD**.

The anomeric configuration of the terminal mannose residue cannot be

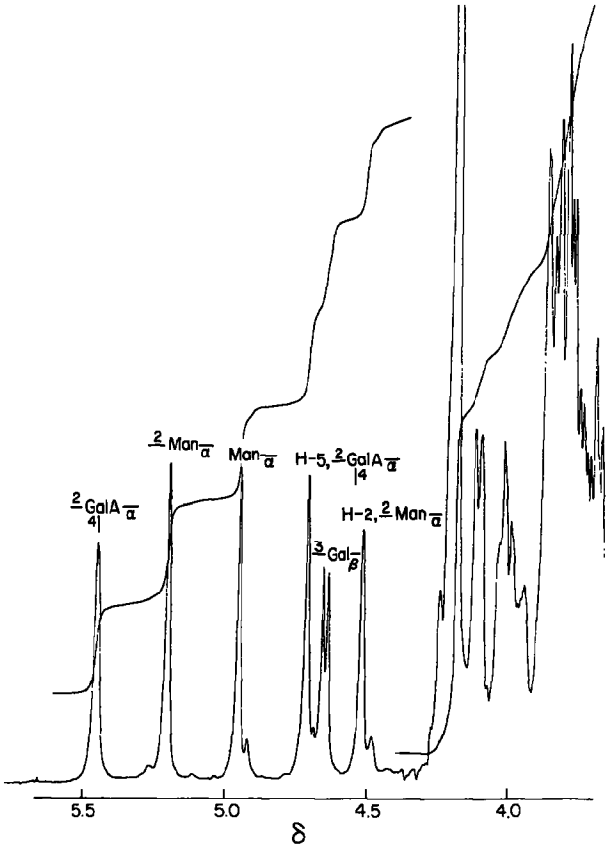

Fig. 1. Anomeric region (δ 4.0–5.5) of the ^{1}H-n.m.r. spectrum (400 MHz) of partially autohydrolyzed *Klebsiella* K68 polysaccharide.

deduced from the positions of the signals for H-1 and C-1 in the n.m.r. spectra, because these signals occur in between the distinct areas of resonance of α- and β-anomeric protons and carbons. To ascertain the nature of the linkage, the difference in the molar rotation of **A1** and **A2** was calculated. The molar rotation of **A1** is +626° and that of **A2** +565°, the difference being +61°. Since the only difference between the two oligomers is the terminal mannose units (mass 162), the contribution by the mannose to the rotation of **A1** is $+61 \div 1.62 = +37.7°$. Methyl α-D-mannopyranoside has $[\alpha]_D$ +79.2°, and the β anomer −49°. Thus it is clear that the terminal mannose branch in the K68 polysaccharide is α-linked to the galacturonic acid unit. This is confirmed[15] by the value of 171.9 Hz for the ^1J, C-1–H-1 coupling of the mannose signal at 100.98 p.p.m., obtained from the proton-coupled ^{13}C-n.m.r. spectrum.

CONCLUSION

The polysaccharide of *Klebsiella* K68 thus has the structure shown in the abstract. The only differences between it and the polysaccharide of *Klebsiella* K57 are that K68 contains pyruvate, and that in K57 the linkage of the β-galactose unit to the galacturonic acid is 1→3 and not 1→2. The pattern exhibited by the repeating structure is of the "3 + 1" type, of which *Klebsiella* K11 (ref. 3) is an example, and the polysaccharide falls into the same chemotype as those of *Klebsiella* K3 (ref. 16), K49 (ref. 17) and K57 (ref. 2).

EXPERIMENTAL

General methods. — Optical rotations were measured in a Perkin–Elmer model 141 polarimeter employing a 1 cm cell. Solutions were evaporated under diminished pressure at temperatures not exceeding 40°. Paper chromatography was conducted by the descending method using Whatman No. 1 paper and the following solvent systems: I, 8:2:1 v/v ethyl acetate–pyridine–water; and II, 2:1:1 v/v 1-butanol–acetic acid–water.

Chromatograms were developed with silver nitrate[18] (A) and periodate-benzidine[19] (B) reagents. Analytical g.l.c. separations were performed using a Hewlett–Packard model 5710 A gas chromatograph fitted with dual flame ionization detectors. A stainless steel column (1.8 m × 3 mm) packed with 3% OV-225 on Gas Chrom Q (100–120 mesh) was used, and operated at a temperature of 190° (isothermal) except as otherwise stated. A Hewlett–Packard model 5890 A gas chromatograph fitted with a flame ionization detector was also used, with a J & W Scientific fused-silica, bonded-phase (0.25 μm film thickness) DB 225 capillary column (0.25 mm × 30 m). G.l.c.–m.s. was performed with a Micromass-12 instrument equipped 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- And ^{13}C-n.m.r. spectra were recorded on a Bruker WH-400 FT spectrometer at ambient temperature, or in some cases at 95° for ^{1}H-spectra. Samples in D_2O were hydrogen exchanged and freeze-dried 3 or 4 times in 99.7% D_2O. In all cases, acetone (δ 2.23 for ^{1}H-n.m.r. and 31.07 p.p.m. for ^{13}C-n.m.r., with reference to aqueous sodium 4,4-dimethyl-4-silapentane-1-sulfonate) was used as the internal standard.

Isolation and purification of the polysaccharide. — A culture of *Klebsiella* K68 was obtained from Drs. F. and I. Ørskov, Copenhagen, and propagated on a sucrose-rich nutrient agar. The capsular polysaccharide was isolated by Cetavlon precipitation and purified as described previously[4], to yield 5.6 g, shown to be monodisperse by gel-permeation chromatography. It had $M_R = 9 \times 10^6$ (calibration with dextrans) and $[\alpha]_D$ +114.9° (*c* 1.4, water).

Analysis of component sugars. — Polysaccharide (9.4 mg) was hydrolyzed overnight with 2M trifluoroacetic acid (TFA) at 100°. After evaporation of the acid,

the liberated sugars were transformed into peracetylated aldononitriles according to the method of McGinnis[12], and analyzed by g.l.c. on a column of OV-225 at 230°. To ascertain the identity of the uronic acid, the polysaccharide (15 mg) was methanolyzed with 3% methanolic hydrogen chloride, reduced overnight with sodium borohydride (40 mg) in anhydrous methanol, and hydrolyzed with 2M TFA. G.l.c. of the derived, peracetylated aldononitriles as before gave the identity of the uronic acid.

Methylation analysis. — The polysaccharide (206 mg) was methylated[5] and a portion (9.5 mg) was hydrolyzed with 2M TFA (16 h, 100°). The hydrolyzate was reduced with sodium borohydride to alditols and acetylated with 1:1 v/v acetic anhydride–pyridine (1 h, 100°). Another portion of methylated polymer (50 mg) was carboxyl-reduced with lithium aluminum hydride in anhydrous tetrahydrofuran. The mixture was acidified with aqueous tartaric acid (500 mg), stirred, and processed in the usual way. The methylated reduced polysaccharide was then remethylated[6], and the product was hydrolyzed (2M TFA, 100°, 16 h) and converted into alditol acetates as just described.

Periodate oxidation and Smith degradation[7]. — A solution of K68 polysaccharide (60.3 mg) in water (10 mL) was mixed with a 0.03M solution of $NaIO_4$ (10 mL), and the reaction was allowed to proceed at room temperature in the dark. The consumption of periodate, followed spectrophotometrically[7], reached a plateau after 24 h. For the Smith degradation, a solution of polymer (416 mg) in water (50 mL) was mixed with reagent (0.4M $NaClO_4$ and 0.1M $NaIO_4$, 50 mL) and allowed to stand in the dark at room temperature. After 96 h the reaction was terminated with ethylene glycol (2 mL) and the oxidized polysaccharide was reduced with sodium borohydride. After dialysis and freeze drying the product was again exposed to the periodate–perchlorate solution, reduced, dialyzed, and freeze-dried. The resulting polyalcohol was hydrolyzed with 0.5M TFA (50 mL) at room temperature for 48 h, and the TFA was removed by evaporation.

Paper chromatography of the Smith-degraded product (**SD**) using solvent I (developing agent B) showed only traces of mannose and galactose, whereas solvent II showed a compound having $R_{cellobiose}$ (48 h) of 1.13. Product **SD** was then applied to large papers (46 × 57 cm), which were developed in solvent I for 48 h. The position of **SD** was located with reagent B, and it was extracted into water (3×) and freeze-dried to yield 73.7 mg (17.7%) of material having $[\alpha]_D$ +52.2° (*c* 0.96, water). A portion (10 mg) was methylated by the Hakomori method, and the product was purified on a Sephadex LH-20 column (5 × 0.5 cm) then reduced ($NaBH_4$ in dry methanol, 16 h). After carboxyl-reduction, hydrolysis, and derivatization, g.l.c. of the alditol acetates showed a complex mixture. A portion of **SD** was therefore reduced with $NaBH_4$ (16 h) and a ^1H-n.m.r. spectrum acquired. It was further reduced (6 h) and the steps of methylation (Hakomori), reduction ($NaBH_4$), and acetylation (1:1 v/v acetic anhydride–pyridine) were repeated.

Partial hydrolysis. — Polysaccharide (35 mg) in 0.5M TFA was heated at 100°, and aliquots were removed every 0.5 h. Paper chromatography of the hydrolyzate

after 2.5 h, using solvent II and reagent A, revealed two oligosaccharides as well as galactose and mannose. The process was repeated using 500 mg of polysaccharide (0.5M TFA, 3 h, 100°), and after removal of the acid by evaporation the hydrolyzate was applied to large papers (46 × 57 cm), which were developed as before. The oligosaccharides were extracted into water (3×), filtered, and freeze-dried to give **A1** (53 mg) and **A2** (45 mg). Fragment **A1** had $R_{cellobiose}$ 0.34 and **A2** had $R_{cellobiose}$ 0.53 (48 h). Portions of **A1** (1 mg) and **A2** (1.3 mg) were reduced ($NaBH_4$, 1 h), hydrolyzed (2M TFA, 16 h, 100°), and converted to peracetylated aldononitriles according to McGinnis[12]. G.l.c. analysis showed galactitol hexaacetate and mannononitrile pentaacetate in both cases.

Oligosaccharides **A1** (7 mg) and **A2** (8 mg) were methylated (Hakomori), the products were purified on a Sephadex LH-20 column (5 × 0.5 cm), reduced with $NaBH_4$ in dry methanol (16 h), hydrolyzed, and converted into alditol acetates as before. For direct reduction to alditols, **A1** and **A2** were treated with $NaBH_4$ for 1 h.

Identification of the absolute configurations of the sugar components. — A portion of polysaccharide (72 mg) was methanolyzed (3% methanolic HCl, 16 h, 80°), reduced ($NaBH_4$, 16 h), and hydrolyzed (2M TFA, 16 h, 100°). Paper chromatography using solvent I and reagent A gave good separation of the components. The hydrolyzate was applied to a large paper (46 × 57 cm), which was developed for 36 h. After extraction into water (3×), filtration, and lyophilization the optical rotations of the sugars were measured and found to correspond well with values for authentic D-galactose and D-mannose.

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