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STRUCTURAL INVESTIGATION OF *Klebsiella* SEROTYPE K46 POLYSACCHARIDE*

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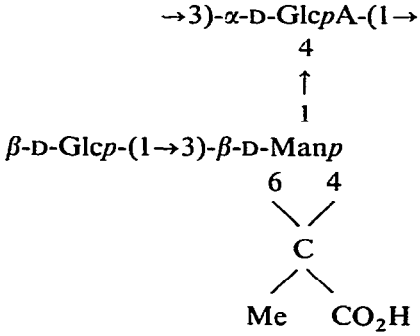
ABSTRACT

The structure of the capsular polysaccharide from *Klebsiella* type K46 has been investigated by using the techniques of methylation analysis, periodate oxidation, and partial hydrolysis. The anomeric linkages were determined by ^{1}H- and ^{13}C-n.m.r. spectroscopy of the polysaccharide and of derived poly- and oligo-saccharides obtained through degradative procedures. ^{1}H-N.m.r. spectroscopy of the polysaccharide in D_2O showed clearly a ratio of one (1-carboxyethylidene) group (CH_3, δ 1.47) to six anomeric protons (δ 4.62–5.29). The polysaccharide was shown to consist of the following hexasaccharide repeating unit, which is unique in this series in having a (1-carboxyethylidene) acetal group on a lateral, but nonterminal, sugar residue.



INTRODUCTION

Of the approximately eighty serologically different strains of *Klebsiella* bacteria, the structural analyses of ~45 of the capsular polysaccharides produced by these bacteria have thus far been reported. *Klebsiella* K46 polysaccharide has been shown to contain residues of mannose, galactose, glucose, glucuronic acid, and pyruvic acid[1,2]. It is one of 11 serologically different K-types having this same qualitative composition.

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TABLE I

N.M.R. DATA FOR *Klebsiella* K46 CAPSULAR POLYSACCHARIDE, AND COMPOUNDS ISOLATED THEREFROM

Compound[a]	Δ[b]	$J_{1,2}$[c] *(Hz)*	^{1}*H-N.m.r. data* *Integral proton*	*Assignment*[d]	^{13}*C-N.m.r. data* *p.p.m.*[e]	*Assignment*[f]
GlcA$\underset{\alpha}{\overset{1\,3}{---}}$Man-OH	5.32	4	1	α-GlcA	101.44	α-GlcA
	5.18	s	0.6	α-Man-OH	94.85	α-Man-OH
A-1	4.92	s	0.4	β-Man-OH	94.73	β-Man-OH
GlcA$\underset{\alpha}{\overset{1\,3}{---}}Man\underset{\alpha}{\overset{1\,3}{---}}$Gal-OH	5.34[g]	4	1.5	α-GlcA	101.50	α-GlcA
	5.33[g]	—		α-Gal-OH	97.23	α-Gal-OH
A-2	5.05	4	1	α-Man		β-Gal-OH
	4.66	7	0.5	β-Gal-OH	97.07	α-Man
Glc$\underset{\beta}{\overset{1\,3}{---}}$Man-OH	5.23	s	0.6	α-Man-OH		
	4.90	s	0.4	β-Man-OH		
N-1	4.60	8	1	β-Glc		
Gal$\underset{\alpha}{\overset{1\,3}{---}}$Gal-OH	5.32	2	0.4	α-Gal-OH		
	5.14	3	1	α-Gal		
N-2	4.66	6	0.6	β-Gal-OH		

$\frac{3}{}$GlcA$\frac{1\ 3}{\alpha}$Man$\frac{1\ 3}{\alpha}$Gal$\frac{3}{\alpha}$Gal$\frac{1}{\beta}$ 4| β| 1| 4 Me Man > C < 6 CO_2H	5.33 5.20 5.09 4.89 1.53	b b s b s	1 1 1 2 3	α-GlcA α-Gal α-Man {β-Gal {β-Man CH_3 of pyruvate	103.16 101.17 100.16 97.05 95.99 25.52	β-Gal α-GlcA β-Man α-Man α-Gal CH_3 of pyruvate
P-1						
$\frac{3}{}$GlcA$\frac{1\ 3}{\alpha}$Man$\frac{1\ 3}{\alpha}$Gal$\frac{1\ 3}{\alpha}$Gal$\frac{1}{\beta}$	5.31 5.19	b s	1 1	α-GlcA α-Gal	103.82 101.53	β-Gal α-GlcA
P-2	5.07 4.77	s b	1 1	α-Man β-Gal	97.12 96.05	α-Man α-Gal
$\frac{3}{}$GlcA$\frac{1\ 3}{\alpha}$Man$\frac{1\ 3}{\alpha}$Gal$\frac{1\ 3}{\alpha}$Gal$\frac{1}{\beta}$ 4| β| 1| 4 Me Man > C < 3| 6 CO_2Na β| 1| Glc	5.29 5.20 5.05 4.88 4.62	b b b b 8	1 1 1 2 1	α-GlcA α-Gal α-Man {β-Gal {β-Man β-Glc	103.16 100.77 100.16 97.16 96.16	β-Gal α-GlcA {β-Glc {β-Man α-Man α-Gal
K46 Native polysaccharide	1.47	s	3	CH_3 of pyruvate	25.47	CH_3 of pyruvate

[a]For the origin of compounds **A-1**, **A-2**; **N-1**, **N-2**; and **P-1**, **P-2**, see text. [b]Chemical shift relative to internal acetone: δ 2.23, downfield from sodium 4,4-dimethyl-4-silapentane-1-sulfonate (D.S.S.). [c]s = singlet. [d]For example, α-GlcA = proton on C-1 of α-linked D-GlcA residue. [e]Chemical shift, in p.p.m., downfield from Me_4Si, relative to internal acetone; 31.07 p.p.m. downfield from D.S.S. [f]As for [d], but for anomeric, ^{13}C nuclei. [g]These signals consisted of a clear doublet centered on δ 5.34; $J_{1,2}$ 4 Hz, with a distinct shoulder at δ 5.31.

Another nine strains have capsular polysaccharides of the same qualitative sugar composition, but lacking pyruvic acid[3]. We now report the results of our structural investigation of the polysaccharide from *Klebsiella* K46.

RESULTS AND DISCUSSION

Composition and n.m.r. spectra. — After purification by precipitation with Cetavlon, the capsular polysaccharide from *Klebsiella* K46 had $[\alpha]_D$ +116°. ^{1}H-N.m.r. spectroscopy of the polysaccharide showed the presence of six anomeric protons, and also indicated one (1-carboxyethylidene) group per six sugar residues. Of the six anomeric signals, the ^{1}H-n.m.r. spectrum revealed that three arise from α-linked, and three from β-linked sugars[4]. The ^{13}C-n.m.r. spectrum clearly indicated the presence of a (1-carboxyethylidene) group, as well as signals from six anomeric carbon atoms. Assignment of the anomeric signals was achieved by examination of the ^{1}H- and ^{13}C-n.m.r. spectra of poly- and oligo-saccharides derived from the K46 polysaccharide during the structural investigation (see Table I). Hydrolysis of the polysaccharide with acid, and paper chromatography of the hydrolyzate, indicated the presence of mannose, galactose, glucose, glucuronic acid, and acidic oligomer **A-1** (see later). Methanolysis of the polysaccharide, reduction with sodium borohydride, and hydrolysis, yielded a mixture of sugars. Analysis by g.l.c. confirmed the presence of mannose, galactose, and glucose (glucuronic acid) in the ratios of 1 : 1 : 1. These results indicate that the K46 polysaccharide contains mannose, galactose, glucose, and glucuronic acid residues in the ratios of 2 : 2 : 1 : 1, and that the polysaccharide consists of a hexasaccharide repeating-unit (see Table II, column 1). The mannose, glucose, and glucuronic acid were shown to be of the D configuration by the circular dichroism curves of the corresponding alditol acetates[5]. The configuration of the galactose was shown to be D by the circular dichroism curve of the 2,4,6-tri-*O*-methyl derivative obtained from the methylation analysis.

TABLE II

SUGAR ANALYSIS OF *Klebsiella* K46 CAPSULAR POLYSACCHARIDE, AND COMPOUNDS DERIVED THEREFROM

Sugars (as alditol acetates on column 1)	*Mole %*					
	I[a]	*II*[b]	*III*[b]	*IV*	*V*	*VI*
Mannose	34	1.9	1.0	38	25	49
Galactose	34	2.3	2.1	39	49	—
Glucose (glucuronic acid)	32	1.0	1.1	23	25	50
Erythritol[c]	—	—	+[d]	—	—	—
Glycerol[c]	—	+[d]	—	—	—	—

[a]I, original, capsular polysaccharide; II, polyol from first periodate oxidation; III, polyol from second periodate oxidation; IV, **P-1**; V, **P-2**; VI, **N-1**. [b]Relative proportions. [c]Column *2*. [d]The symbol + = present, but not quantitated.

TABLE III

METHYLATION ANALYSIS OF NATIVE, AND DEGRADED, K46 CAPSULAR POLYSACCHARIDE

Methylated sugars[a] *(as alditol acetates)*	*Mole %*[b]					
	I[c]	*II*	*III*	*IV*	*V*	*VI*
2,4,6-Man	14.9	8.5	39	53	41	—
2,4,6-Gal	38.3	50.3	6	—	—	41
2,3,4-Glc	—	—	—	47	—	—
2,3,4,6-Glc	11.1	15.2	—	—	59	—
2,3,4,6-Gal	—	—	—	—	—	59
2-Man	17.4	26.0	—	—	—	—
2-Glc	18.4	—	59	—	—	—

[a]2,4,6-Man = 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylmannitol, etc. [b]Column 2; values were corrected by use of the effective, carbon response-factors given by Albersheim *et al.*[17]. [c]I, original polysaccharide, methylated and reduced; II, methylated, neutral fraction; III, methylated, acidic fraction; IV, compound A-1; V, compound N-1; VI, compound N-2.

Methylation analysis. — Methylation[6–10] of K46 polysaccharide was hindered by the low solubility of the polysaccharide in dimethyl sulfoxide. Hakomori treatment and a subsequent Purdie methylation were needed in order to achieve a product that showed no hydroxyl absorption in the infrared spectrum. The fully methylated polysaccharide was carboxyl-reduced with lithium aluminum hydride, and the product hydrolyzed. Methylation analysis supported the concept of a six-sugar repeating-unit (see Table III, column 1). The appearance of 2,6-di-*O*-methylglucose and the loss of 2-*O*-methylglucose, after the methylated, carboxyl-reduced polysaccharide had been remethylated, confirmed that the 2-*O*-methylglucose was derived from glucuronic acid, and the presence of 2,3,4,6-tetra-*O*-methylglucose indicated that the glucose unit is the terminal glycosyl group of the side chain.

When a sample of the fully methylated polysaccharide was hydrolyzed, a neutral and an acidic fraction were obtained by separation on an ion-exchange resin. No 2-*O*-methylglucose (which would have arisen from glucuronic acid in the original carbohydrate) was found in the neutral fraction. The ratio of 2,4,6-tri-*O*-methylmannose to 2-*O*-methylmannose was 0.3:1, consistent with the concept that the mannosyl residue substituted at O-3 remained bound to the uronic acid. The small proportion of 2,4,6-tri-*O*-methylmannose undoubtedly arose by partial cleavage of the aldobiouronic acid linkage. The formation of 2-*O*-methylmannose shows that the (1-carboxyethylidene) group must be linked to the mannose as an acetal, and the methylation analysis also shows that glucuronic acid is a branch point in the chain (see Table III, column II).

The acidic fraction of the hydrolyzate was methanolyzed, the products reduced, and the materials hydrolyzed, to give 2,4,6-tri-*O*-methylmannose and 2-*O*-methylglucose (from glucuronic acid in the original substance) together with a small pro-

portion of 2,4,6-tri-*O*-methylgalactose. The absence of 2,4,6-tri-*O*-methylmannose from the neutral fraction, and its presence in the acidic fraction, show that the aldobiouronic acid is 3-*O*-(D-glucopyranosyluronic acid)-D-mannose, and that the aldotriouronic acid may be 3-*O*-(D-glucopyranosyluronic acid)-3-*O*-(D-mannopyranosyl)-D-galactose (see Table III, column III).

Isolation of oligosaccharides. — The initial hydrolysis of K46 polysaccharide gave an acidic oligosaccharide (**A-1**), together with a mixture of monosaccharides. Compound **A-1** was separated, purified, and then shown, by n.m.r. and methylation, to be 3-*O*-(α-D-glucopyranosyluronic acid)-D-mannose (see Table I, and Table III, column IV). Partial hydrolysis of K46 polysaccharide gave acidic and neutral oligosaccharides which were separated by use of an ion-exchange resin. The acidic compound **A-2** was similarly shown (see Table I) to be 3-*O*-(α-D-glucopyranosyluronic acid)-3-*O*-α-D-mannopyranosyl-D-galactose.

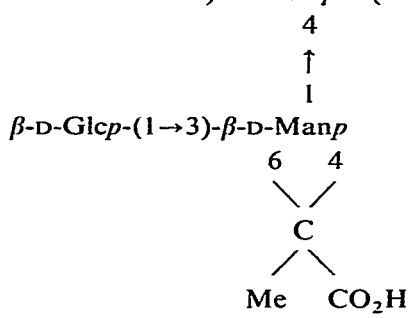
The neutral fraction was separated by paper chromatography into two components, **N-1** and **N-2**. Compound **N-1** was found to be 3-*O*-β-D-glucopyranosyl-D-mannose, and **N-2** to be 3-*O*-D-galactopyranosyl-D-galactose (see Table I, and Table III, columns V and VI).

Periodate oxidation. — Periodate oxidation of the polysaccharide[11,12], and subsequent reduction, yielded a polyalcohol. Total sugar analysis of the derived polyol showed the presence of mannose, galactose, glucuronic acid, and glycerol, *i.e.*, loss of glucose (see Table II, column II). As only the terminal sugar unit of the polysaccharide was susceptible to periodate oxidation, it was selectively removed by Smith degradation, to give a polysaccharide (**P-1**) having a five-sugar repeating unit (see Table II, column IV). The ^{1}H- and ^{13}C-n.m.r. spectra of **P-1** revealed the presence of a (1-carboxyethylidene) group and, when compared with those spectra of the original polysaccharide, showed the absence of a signal corresponding to one β-linked sugar. Methylation analysis of compound **P-1** yielded 2,3-di-*O*-methylmannose, 2,4,6-tri-*O*-methylmannose, 2,4,6-tri-*O*-methylgalactose, and 2-*O*-methylglucose (from glucuronic acid in the original polysaccharide), confirming that the (terminal) glucosyl group in the original polysaccharide had been attached to O-3 of mannose, and that the (1-carboxyethylidene) group is present as an acetal spanning O-4 and O-6 of the mannosyl residue.

Periodate degradation of **P-1**, and subsequent reduction, yielded a second polyalcohol, analysis of which gave mannose, galactose, glucuronic acid, and erythritol; the first two sugars were present in the ratio of 1 : 2, *i.e.*, there had been a loss of mannose (see Table II, column III). It was, again, only the terminal unit that was oxidized, and, hence, the terminal mannose was selectively removed by Smith degradation, to give polysaccharide **P-2** (see Table II, column V). The identification of 2,4,6-tri-*O*-methylmannose, 2,4,6-tri-*O*-methylgalactose, and 2,4-di-*O*-methylglucose (glucuronic acid in the original) resulting from methylation analysis of **P-2** established that the terminal mannosyl group of **P-1** had been attached to O-4 of glucuronic acid. The ^{1}H- and ^{13}C-n.m.r. spectra of **P-2** indicated the presence of one β-linked sugar less in **P-2** than in **P-1**, demonstrating that the mannosyl residue in

P-2 is also β-linked. No further oxidation occurred when compound **P-2** was treated with periodate.

From the results reported here, it is deduced that the structure of the repeating unit of *Klebsiella* K46 capsular polysaccharide is as shown. The isolation of six different oligo- and poly-saccharides from K46 polysaccharide is in agreement with this structure.



EXPERIMENTAL

General methods. — Optical rotations were measured at 23–25° with a Perkin–Elmer model 141 polarimeter for solutions in a 10-cm cell. Circular dichroism (c.d.) spectra were recorded with a Jasco J-20 automatic, recording spectropolarimeter. Infrared (i.r.) spectra were recorded with a Perkin–Elmer 457 spectrophotometer.

Paper chromatography was conducted by the descending method, using Whatman No. 1 paper and the following solvent systems (v/v): (*A*) 18:3:1:4 ethyl acetate–acetic acid–formic acid–water, and (*B*) 2:1:1 1-butanol–acetic acid–water. Chromatograms were developed with *p*-anisidine trichloroacetate[13] or with silver nitrate[14].

^{1}H-N.m.r. spectra were recorded with a Varian XL-100 instrument at 95°. The hydrogen in samples dissolved in D_2O was exchanged with deuterium and the product freeze-dried three or four times in D_2O, and then dissolved in D_2O. Acetone was used as the internal standard (δ 2.23). ^{13}C-N.m.r. spectra were recorded with a Varian CFT-20 instrument. Samples for ^{13}C-n.m.r. spectroscopy were dissolved in 1:1 H_2O–D_2O. Acetone was used as the internal standard (31.07 p.p.m.).

Gel-filtration chromatography was performed on a column (2.5 × 93 cm) of Bio-Gel P-2 which was eluted with 500:5:2 water–pyridine–acetic acid at a flow rate of 5.2 mL.h^{-1}. Fractions (2 mL) were collected and lyophilized, and analyzed by paper chromatography.

Analytical g.l.c. separations were performed with a Hewlett–Packard 5700 instrument fitted with dual flame-ionization detectors. An Infotronics CRS-100 electric integrator was used to measure peak areas. Stainless-steel columns (1.8 m × 3 mm) were used, with nitrogen as the carrier gas at a flow rate of 15 mL.min^{-1}. The

columns used were (*1*) 3% of SP-2340 on Gas Chrom Q (100–120 mesh), programmed from 195° for 4 min and then at 2°/min to 260°; (*2*) 5% of ECNSS-M on Gas Chrom Q (100–200 mesh), programmed from 160° for 4 min and then at 2°/min to 200°; (*3*) 3% of OV-225 on Gas Chrom Q (100–200 mesh), programmed from 160° for 4 min and then at 2°/min to 200°. Preparative g.l.c. was conducted in an F and M model 720 dual-column instrument equipped with thermal-conductivity detectors. Columns (1.8 m × 6.3 mm) of 3% of SP-2340 (*4*), and 5% of ECNSS-M (*5*) on Gas Chrom Q (100–200 mesh) were used. G.l.c.–mass spectrometry was conducted with a Micromass 12 instrument fitted with a Watson–Biemann separator. The mass spectra were recorded at 70 eV, with an ionization current of 100 μA and ion-source temperature of 200°.

Preparation and properties of Klebsiella *K46 capsular polysaccharide.* — A culture of *Klebsiella* K46 (5281) was obtained from Dr. Ida Ørskov (WHO International *Escherichia* Center, Copenhagen, Denmark), and was grown on a sucrose–yeast extract–agar medium for 3 d at room temperature. The medium had the following composition: sucrose (75 g), Bacto yeast–extract (5 g), NaCl (5 g), K_2HPO_4 (2.5 g), $MgSO_4 \cdot 7\ H_2O$ (0.62 g), $CaCO_3$ (0.5 g), Bacto agar (37.5 g), and water (2.5 L). The cells were harvested, and diluted to 2.7 L with water containing 1% of phenol. This suspension was then centrifuged for 5 h at 30,000 r.p.m. in a Beckman L3-50 centrifuge fitted with rotor type 35. The clear, supernatant liquor (2 L) was separated, and the polysaccharide was precipitated by pouring into 19:1 ethanol–methanol (6 L). The crude polysaccharide, isolated by decantation, was dissolved overnight in water (2 L), and then precipitated with 10% Cetavlon solution. The precipitate was isolated by centrifugation, washed with a small volume of cold water, and dissolved in 4M NaCl (1 L). The polysaccharide was precipitated by pouring the solution into 19:1 ethanol–methanol (3 L), with stirring. The Cetavlon-purified polysaccharide was isolated by centrifugation, dissolved in water (2.5 L), and then dialyzed for 2–3 days against running tap-water. Freeze-drying of this solution yielded 8 g of the polysaccharide (~1 g/L of medium). The polysaccharide had $[\alpha]_D$ +116° (*c* 1.3, water), and was examined by ^1H- and ^{13}C-n.m.r. spectroscopy (see Table I). The ^1H-n.m.r. spectrum of K46 polysaccharide was recorded for a solution of 3 mg per mL of D_2O at 95°, and the ^{13}C-n.m.r. spectrum for a solution of 10 mg per mL of 1:1 H_2O–D_2O at room temperature.

Sugar analysis. — The polysaccharide (50 mg) was hydrolyzed with 2M trifluoroacetic acid (TFA) overnight on a steam bath. The acid was removed by several evaporations under diminished pressure with water, and the mixture was passed through a column of Amberlite AG 1-X2 (formate) resin to separate the neutral from the acidic sugars. The acidic components were eluted from the resin with 10% formic acid, and the formic acid was removed by evaporation with water. Paper chromatography of the neutral and acidic sugars (solvents *A* and *B*) revealed three major spots, of mannose, galactose, and glucose, for the neutral fraction, and two spots, of glucuronic acid and an aldobiouronic acid, for the acidic fraction.

A sample of polysaccharide (20 mg) in methanolic hydrogen chloride (3%)

was refluxed overnight on a steam bath. After neutralization (lead carbonate), and reduction (sodium borohydride in anhydrous methanol), the mixture was made neutral with Amberlite IR-120 (H^+) cation-exchange resin, and filtered, and the filtrate evaporated three times with methanol in order to remove borate ion. The residue was further hydrolyzed with 2M TFA, and the product reduced, and the alditols were acetylated. The alditol acetates of mannose, galactose, and glucose (glucuronic acid) were separated by g.l.c. (column *1*), and found to be present in the ratios of 1 : 1 : 1 (Table II, column I).

Samples for c.d. analysis were obtained by preparative g.l.c. (column *4*). By comparison with authentic standards[5], the c.d. curves of the alditol acetates corresponding to mannose and glucose (glucuronic acid) showed these sugars to have the D configuration. The configuration of the galactosyl residue was determined by examining the c.d. curve of the 2,4,6-tri-*O*-methylgalactose as the alditol acetate obtained during methylation analysis, and prepared by preparative g.l.c. (column *5*). By comparison with an authentic standard, the galactose was shown to be of the D configuration.

Methylation analysis. — Methylation of K46 capsular polysaccharide in the sodium salt form (600 mg) was performed by the Hakomori procedure[6]. Difficulty was encountered in dissolving the polysaccharide in dimethyl sulfoxide (50 mL), and agitation in a sonicator for two days at room temperature was needed in order to achieve complete dissolution. Methylation was found to be incomplete, and a Purdie[15] treatment with silver oxide and methyl iodide was needed. The final product (320 mg) showed no i.r. absorption at 3600 cm^{-1} (OH), and strong absorption at 1745 cm^{-1} (methyl ester CO). The ^{1}H-n.m.r. spectrum of this material showed a singlet at δ 1.5 (CH_3 of acetal).

Methylated K46 polysaccharide (184 mg) was reduced with lithium aluminum hydride in refluxing oxolane overnight, and the product (42 mg) showed no carbonyl absorption (1745 cm^{-1}) in its i.r. spectrum. Hydrolysis of this material (28 mg) with 2M TFA overnight on a steam bath, and subsequent reduction with sodium borohydride, followed by acetylation with acetic anhydride–pyridine, yielded a mixture of partially methylated, alditol acetates which was analyzed by g.l.c. (columns *2* and *3*), and g.l.c.–m.s. (ref. 16). The alditol acetates of 2,3,4,6-tetra-*O*-methylglucose, 2,4,6-tri-*O*-methylmannose, 2,4,6-tri-*O*-methylgalactose, 2-*O*-methylmannose, and 2-*O*-methylglucose were identified, and found to be present in the ratios of 0.6 : 0.8 : 2 : 1 : 0.9 (see Table III, column I). Methylated, carboxyl-reduced polysaccharide was remethylated, and the product hydrolyzed, and the derived sugars were converted into alditol acetates. G.l.c. analysis gave peaks corresponding to 2,3,4,6-tetra-*O*-methylglucose, 2,4,6-tri-*O*-methylmannose, 2,4,6-tri-*O*-methylgalactose, 2,6-di-*O*-methylglucose, and 2-*O*-methylmannose.

A separate portion of the methylated polysaccharide (86 mg) was heated in 2M TFA on a steam bath overnight. The acid was removed by evaporation with several portions of water, and the hydrolyzate was separated into a neutral fraction (46 mg) and an acidic fraction (37 mg) by using Amberlite AG 1-X2 (formate)

ion-exchange resin. The alditol acetates of the neutral fraction were examined by g.l.c. (columns *2* and *3*), and g.l.c.–m.s. The alditol acetates of 2,3,4,6-tetra-*O*-methylglucose, 2,4,6-tri-*O*-methylmannose, 2,4,6-tri-*O*-methylgalactose, and 2-*O*-methylmannose were identified, and found present in the ratios of 0.6:0.3:1.9:1.0 (see Table II, column II).

The acidic fraction in methanolic hydrogen chloride (3%) was refluxed overnight on a steam bath, the acid neutralized, the product reduced with sodium borohydride in dry methanol, the base neutralized with Amberlite IR-120 (H^+) resin, the solution evaporated, and the residue evaporated three times with methanol. The residue was further hydrolyzed with 2M TFA, the product reduced, and the alditols acetylated. The alditol acetates were examined by g.l.c. (column *2*) and g.l.c.–m.s. The alditol acetates from 2,4,6-tri-*O*-methylmannose, 2,4,6-tri-*O*-methylgalactose, and 2-*O*-methylglucose were identified, and found present in the ratios of 0.7:0.1:1.0 (see Table III, column III).

Isolation and characterization of oligosaccharides. — *Klebsiella* K46 polysaccharide (500 mg) was hydrolyzed in 2M TFA (100 mL) overnight on a steam bath. The hydrolyzate was dialyzed against water (1 L), the TFA removed by evaporation with several portions of water, and the mixture separated into neutral and acidic fractions by using Amberlite AG 1-X2 (formate) resin. An acidic fraction (50 mg) was eluted with 10% formic acid (100 mL), and the eluate applied to a column of Bio-Gel P-2. The effluent was collected in fractions (2 mL), and the contents of each tube were examined by paper chromatography (solvent *A*); gel chromatography did not completely separate the components. Pure oligosaccharide **A-1** (27 mg) was obtained by paper chromatography (R_{Glc} 0.49, solvent *A*); $[\alpha]_D$ +85° (*c* 1.35, water). The ^{1}H-n.m.r. spectrum showed anomeric signals at δ 5.34 (1 H, 4 Hz), 5.18 (0.6 H, singlet), and 4.92 (0.4 H, singlet). The ^{13}C-n.m.r. spectrum showed a signal at 61.75 p.p.m. resulting from C-6 of mannose, and two anomeric signals, at 101.4 p.p.m. attributable to C-1 of α-glucuronic acid and at 94.8 and 94.3 p.p.m. corresponding to C-1 of the (reducing) mannose residue (see Table I).

Compound **A-1** (10 mg) was methylated by the Hakomori method, and the product reduced with lithium aluminum hydride in refluxing oxolane. Hydrolysis (2M TFA, for 5 h on a steam bath), reduction with sodium borohydride, and acetylation yielded alditol acetates identified by g.l.c. (columns *2* and *3*) and g.l.c.–m.s. as 2,4,6-tri-*O*-methylmannose and 2,3,4-tri-*O*-methylglucose, present in the ratio of ~1:1 (see Table III, column IV).

In order to obtain oligosaccharides other than compound **A-1**, milder conditions of hydrolysis were used. The polysaccharide (1 g) in 0.5M TFA was heated for 45 min on a steam bath, and the solution was dialyzed against water (1 L) overnight; the acid was removed by several successive evaporations with water, and the dialyzable material was separated into neutral (373 mg) and acidic (568 mg) components by using Amberlite AG 1-X2 (formate) ion-exchange resin. The acidic fraction was eluted with 10% formic acid, and applied to a column of Bio-Gel P-2. The eluate was collected in fractions (2 mL), and the contents of each tube were examined by

paper chromatography (solvent *A*), but good separation was not achieved. Paper chromatography yielded pure oligosaccharide **A-2** (10 mg; R_{Glc} 0.37, solvent *A*) whose ^{1}H-n.m.r. spectrum had signals for anomeric protons at δ 5.34 (4 Hz), 5.33 (singlet), 5.05 (1 H, 4 Hz), and 4.66 (0.5 H, 7 Hz). The ^{13}C-n.m.r. spectrum showed two signals, at 61.7 and 61.8 p.p.m., due to C-6 of hexoses, and three signals in the anomeric region; at 101.5 p.p.m., attributable to C-1 of α-glucuronic acid, 97.2 p.p.m. corresponding to C-1 of the (reducing) galactose residue, and 97.07 p.p.m., corresponding to C-1 of the α-mannosyl residue (see Table I).

Paper chromatography (solvents *A* and *B*) of the neutral fraction gave two pure, oligosaccharide components **N-1** (20 mg) and **N-2** (20 mg).

Compound **N-1** (R_{Glc} 0.38, solvent *A*) gave a ^{1}H-n.m.r. spectrum having anomeric protons at δ 5.23 (0.6 H, singlet), 4.90 (0.4 H, singlet), and 4.60 (1 H, 8 Hz). Paper chromatography (solvent *A*) of the hydrolyzate of **N-1** (5 mg) (2M TFA for 5 h on a steam bath) revealed the presence of mannose and glucose, and g.l.c. analysis of the alditol acetates (column *I*) showed them to be present in the ratio of 1 : 1 (see Table II, column VI). Hakomori methylation of **N-1** (10 mg), and subsequent hydrolysis (2M TFA for 5 h on a steam bath), reduction, and acetylation yielded the alditol acetates corresponding to 2,3,4,6-tetra-*O*-methylglucose and 2,4,6-tri-*O*-methylmannose, in the ratio of 1.0:0.7 (see Table III, column V).

Compound **N-2** (R_{Glc} 0.26, solvent *A*) gave a ^{1}H-n.m.r. spectrum having anomeric protons at δ 5.32 (0.4 H, 2 Hz), 5.14 (1 H, 3 Hz), and 4.66 (0.6 H, 6 Hz). Paper chromatography (solvents *A* and *B*) of the hydrolyzate of **N-2** (5 mg) (2M TFA for 5 h on a steam bath) revealed the presence of galactose only, and this was confirmed by g.l.c. of the alditol acetate. Hakomori methylation of **N-2** (10 mg), and subsequent hydrolysis (2M TFA for 5 h on a steam bath), reduction, and acetylation yielded the alditol acetates corresponding to 2,3,4,6-tetra-*O*-methylgalactose and 2,4,6-tri-*O*-methylgalactose in the ratio of 1.0:0.7 (see Table III, column VI).

Sequential, Smith periodate degradations. — A sample of polysaccharide (1 g) was subjected to periodate oxidation with 0.05M sodium periodate (100 mL) for 4 days at 5° in the dark. The excess of periodate was decomposed with ethylene glycol (10 mL), the mixture was dialyzed overnight against running tap-water, and the material remaining was reduced overnight with sodium borohydride (1 g). Following decomposition of the hydride with acetic acid, the solution was dialyzed and freeze-dried, to yield 950 mg of the derived polyol. Methanolysis of a sample (20 mg) of this polyol with methanol–HCl (3%) overnight on a steam bath, and subsequent reduction with sodium borohydride in anhydrous methanol, hydrolysis, and derivatization of the liberated monosaccharides as their alditol acetates gave, by g.l.c. (column *I*), peaks corresponding to mannose, galactose, and glucose (glucuronic acid) in the ratios of 2:2:1 (see Table II, column II). The acetate of glycerol was also observed.

The rest of the polyol was then subjected to Smith hydrolysis with 0.5M TFA overnight at room temperature, and the product dialyzed and freeze-dried (527 mg; compound **P-1**). Compound **P-1** had $[\alpha]_D$ +109° (*c* 3.0, water), and its ^{1}H-n.m.r.

spectrum showed a sharp singlet at δ 1.53, characteristic of the CH_3 of pyruvate, and anomeric signals at δ 4.89–5.33 (see Table I for details). The ^{13}C-n.m.r. spectrum also showed the presence of pyruvate (CH_3 at 31.7 p.p.m.); for details, see Table I.

Sugar analysis of **P-1** was performed by methanolysis, reduction with sodium borohydride in anhydrous methanol, hydrolysis, reduction, and acetylation. The alditol acetates derived from mannose, galactose, and glucose (glucuronic acid) were separated by g.l.c. (columns *1* and *2*), and found to be present in the ratios of 2:2:1. No glycerol was observed (see Table II, column IV). Methylation of **P-1** was performed by the Hakomori procedure, followed by treatment with the Purdie reagents. Methylated **P-1** was reduced overnight with lithium aluminum hydride in refluxing oxolane and, following conversion into the alditol acetates, was analyzed by g.l.c.–m.s. The alditol acetates of 2,4,6-tri-*O*-methylmannose, 2,4,6-tri-*O*-methylgalactose, 2,3-di-*O*-methylmannose, and 2-*O*-methylglucose were identified (columns *2* and *3*).

Compound **P-1** (400 mg) was subjected to a second periodate oxidation, with 0.05M sodium periodate (50 mL) for 4 days at 5° in the dark. Sugar analysis of this second polyol (20 mg) was performed by successive methanolysis, reduction, hydrolysis, reduction, and acetylation. Comparative g.l.c. (column *1*) gave alditol acetates of mannose, galactose, and glucose (glucuronic acid) in the ratios of 1:2:1 (see Table II, column III); the alditol acetate of erythritol was also observed. An attempted, Smith degradation using 0.5M TFA overnight at room temperature was unsuccessful. Smith hydrolysis of the polyol with 0.1M TFA for 1 h on a steam bath, followed by dialysis and then freeze-drying, gave polysaccharide **P-2**. Compound **P-2** showed $[\alpha]_D$ +184° (*c* 2.0, water). The ^1H- and ^{13}C-n.m.r. data for **P-2**, recorded in Table I, showed that the (1-carboxyethylidene) acetal group had been eliminated. Methanolysis of **P-2**, and subsequent reduction, hydrolysis, reduction, and acetylation, yielded a mixture of alditol acetates of mannose, galactose, and glucose (glucuronic acid) in the ratios of 1:2:1 (see Table II, column V) by g.l.c. analysis (column I). Hakomori methylation of **P-2**, and subsequent reduction with lithium aluminum hydride in refluxing oxolane, hydrolysis, reduction, and acetylation, yielded the alditol acetates of 2,4,6-tri-*O*-methylmannose, 2,4,6-tri-*O*-methylgalactose, and 2,4-di-*O*-methylglucose, identified by comparative g.l.c. (columns *2* and *3*) and g.l.c.–m.s.

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