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Klebsiella K82: CHEMICAL PROOF OF A NEW STRAIN*

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ABSTRACT

A strain of *Klebsiella* isolated during an epidemiological survey in the U.S.A. was classified as a new strain of *Klebsiella pneumoniae* on the grounds that it did not react with any antiserum against the K antigens already known. The new strain did, however, exhibit capsular quellung with *Klebsiella* K8 and K20.

The structure of the capsular polysaccharide (K antigen) of *Klebsiella* K82 has been established as having the tetrasaccharide repeating-unit shown ("three plus one" type). This is a novel structure among the *Klebsiella* K antigens, thus providing chemical confirmation that K82 is indeed a new strain. The presence of a lateral (D-glucosyluronic acid)-D-galactosyl unit common to K8, K20, and K82 explains the capsular quellung reaction observed.

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

INTRODUCTION

Qualitative analyses of the capsular antigens of *Klebsiella* have been reported for strains K1-72 (ref. 1), K73-80 (ref. 2), and K81 (ref. 3). In 1977, Ørskov and Fife-Asbury proposed[4] the deletion of certain K numbers on the grounds that either (*a*) the strains were identical, or closely related, to already known strains, or (*b*) the bacteria had been incorrectly identified as *Klebsiella*. In the same paper, they reported the isolation of a new *Klebsiella pneumoniae*, to which they assigned the number K82, and the biochemical reactions of which were described[4]. The designation of K82 as a new strain was based on the observation that it does not react with any antiserum against previously known K antigens. A K82 antiserum, however, does give capsular quellung with K8 and K20, but at very low titer levels[4].

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In continuation of our chemical examination of this genus, we now report the primary structure of the capsular polysaccharide of *Klebsiella* K82.

RESULTS AND DISCUSSION

Composition and n.m.r. spectra. — *Klebsiella* K82 bacteria were grown on an agar medium, and the acidic polysaccharide was purified by one precipitation with cetyltrimethylammonium bromide[5,6]. The product was monodisperse by gel-permeation chromatography ($\bar{M}_w = 2 \times 10^6$) and had $[\alpha]_D$ +31°, which compares well with the value of +32° calculated by using Hudson's rules of isorotation[7]. Analysis of the acidic polysaccharide gave galactose and glucose in the ratio of 1.5:1 (see Table I, column I). When the uronic acid was reduced (following methanolysis[8], or in the intact polysaccharide[9]), galactose and glucose were obtained in equimolar proportions (see Table I, columns II and III). The composition of the K82 polysaccharide is thus galactose:glucose:glucuronic acid in the ratios of 2:1:1. The three sugars were shown to be of the D configuration by the circular dichroism curves of the derived alditol acetates and of a methylated derivative[10].

The ^{1}H-n.m.r. spectrum confirmed the presence of a tetrasaccharide repeating-unit and showed that three units are β- and one is α-linked. These results were confirmed by the ^{13}C-n.m.r. spectrum, which also indicated that the O-6 atoms of the hexoses are unsubstituted (see Table II).

Methylation analyses[11,12]. — Analyses were conducted (*a*) on the original polysaccharide, (*b*) with reduction of the uronic ester following methylation, (*c*) with remethylation of product *b*, and (*d*) on the neutral polysaccharide, obtained by carbodiimide[9] reduction. The results, presented in Table III, columns I to V, confirm the concept of a tetrasaccharide repeating-unit, and show that the branch point is a galactosyl residue linked at O-3 and O-4, and that the glucuronic acid is a terminal, nonreducing group. A β-elimination experiment[13] demonstrated that the uronic acid is directly linked to the galactose at O-4 (see Table III, column V).

TABLE I

SUGAR ANALYSIS OF *Klebsiella* K82 POLYSACCHARIDE AND DERIVED PRODUCTS

Sugars[a] *(as alditol acetates)*	*Mol ratio*						
	I[b]	*II*	*III*	*IV*	**A2**	**A3**	**A4**
Galactose	1.5	1.0	1.09	2.2	1.0	1.0	1.08
Glucose	1.0	0.83	1.0	1.0	0.87	1.94	1.0
Glycerol	—	—	—	0.15[c]	—	—	—

[a]Using an SP-2340 column programmed from 195° for 4 min, and then at 2°/min to 260°. [b]I, original acidic polysaccharide; II, carboxyl-reduced polysaccharide; III, carbodiimide-reduced polysaccharide; IV, polyol from periodate oxidation; **A2**, **A3**, and **A4**, acidic oligosaccharides obtained from partial hydrolysis (after carboxyl reduction). [c]Quantitation of glycerol triacetate is inaccurate, due to its high volatility.

TABLE II

N.M.R. DATA FOR *Klebsiella* K82 CAPSULAR POLYSACCHARIDE AND THE DERIVED OLIGOSACCHARIDES

Compound[a]	*^{1}H-N.m.r. data*				*^{13}C-N.m.r. data*	
	Δ[b]	*J_{12}*[c] *(Hz)*	*Integral proton*	*Assignment*[d]	*p.p.m.*[e]	*Assignment*[f]
GlcAβ(1→4)Gal-OH	5.29	3	0.3	4-Gal-α-OH	103.54	GlcA-β
A2	4.75	8	1.0	GlcA-β	93.40	4-Gal-α-OH
	4.61	8	0.7	4-Gal-β-OH		
	4.50	b	1.0	H-5 (GlcA)		
Glcβ(1→3)Gal~OH 4↓β 1 GlcA					172.29	C=O (GlcA)
	5.30	3	0.6	3,4-Gal-α-OH	105.46	Glc-β
	4.97	8	1.0	GlcA-β	103.32	GlcA-β
	4.69	8	0.4	3,4-Gal-β-OH	97.47	3-Gal-β-OH
A3	4.65	8	1.0	Glc-β	93.44	3-Gal-α-OH
	4.45	b	1.0	H-5 (GlcA)		
Glcβ(1→3)Galα(1→3)Gal~OH 4↓β 1 GlcA	5.31	3	1.0	3,4-Gal-α		
	4.95	8	1.0	GlcA-β		
	4.77	8	1.0	3-Gal-β-OH		
A4	4.66	8	1.0	Glc-β		
	4.55	b	1.0	H-5 (GlcA)		
Glcβ(1→3)Galα(1→3)Galactitol 4↓β 1 GlcA	5.50	s	1.0	3,4-Gal-α		
	4.73	8	1.0	GlcA-β		
	4.54	8	1.0	Glc-β		
A4 (reduced)	4.50	b	1.0	H-5 (GlcA)		
3←Glcβ(1→3)Galα(1→3)Galβ(1→	5.40	s	1.0	3-Gal-α	105.29	3-Glc-β
SD	4.75	8	1.0	3-Gal-β	105.15	3-Gal-β
	4.71	8	1.0	3-Glc-β	100.35	3-Gal-α
					62.00	C-6 of hexoses

TABLE II *(continued)*

N.M.R. DATA FOR *Klebsiella* K82 CAPSULAR POLYSACCHARIDE AND THE DERIVED OLIGOSACCHARIDES

Compound[a]	*^{1}H-N.m.r. data*				*^{13}C-N.m.r. data*	
	Δ[b]	J_{12}[c] *(Hz)*	*Integral proton*	*Assignment*[d]	*p.p.m.*[e]	*Assignment*[f]
K82 capsular polysaccharide (mildly hydrolyzed)	5.40	s	0.3	3,4-Gal—$\underset{\alpha}{}$	171.30	C=O (GlcA)
	5.30	s	0.3	Gal—$\underset{\alpha}{}$OH	105.31	β-Glc
	4.96	s	2.0	GlcA—$\underset{\beta}{}$	103.33	β-GlcA
	4.71	s		3-Gal—$\underset{\beta}{}$	105.02	β-Gal
			2.4	Gal—$\underset{\beta}{}$OH	99.83	α-Gal
	4.64	s		3-Glc—$\underset{\beta}{}$	61.52	C-6 of hexoses
	4.50	b	1.0	H-5 (GlcA)		

[a]For the origin of compounds **A2**, **A3**, **A4**, and **SD**, 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]Key: b = broad, unable to assign accurate coupling constant; s = singlet. [d]For example, 3-Gal—$\underset{\alpha}{}$ 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. [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.

TABLE III

METHYLATION ANALYSES OF *Klebsiella* K82 POLYSACCHARIDE AND DERIVED PRODUCTS

Methylated sugar[a] *(as alditol acetate)*	*Mol %*[b]							
	I[c]	*II*[c]	*III*[c]	*IV*[d]	*V*[e]	*VI*[e]	*VII*[d]	*VIII*[e]
2,3,4,6-Glc			19	23			23	36
2,3,4-Glc		22					28	30
2,4,6-Glc	32	24	28	25	34	34		
2,4,6-Gal	37	28	30	25	66	66	10	
2,5,6-Gal							15	
2,6-Gal	31	26	23	27			27	34

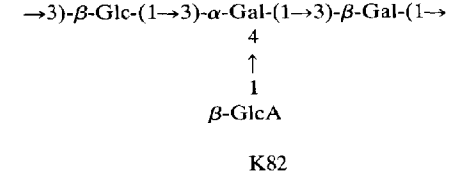
[a]2,3,4,6-Glc = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol, *etc.* [b]Values are corrected by use of the effective, carbon-response factors given by Albersheim *et al.*[21]. [c]Using an ECNSS-M column programmed from 180° for 4 min, and then at 2°/min to 200°. [d]Using an SP-1000 column at 200° isothermal. [e]Using an ECNSS-M column at 170° isothermal. I, original acidic polysaccharide; II, reduction of uronic ester; III, remethylation after reduction of uronic ester; IV, carbodiimide-reduced capsular polysaccharide; V, product from β-elimination and remethylation; VI, product from Smith degradation; VII, methylated and reduced tetrasaccharide; and VIII, methylated and reduced trisaccharide.

Smith degradation[14,15]. — Carboxyl-reduced K82 polysaccharide[9] consumed two mol of periodate per repeating unit, in agreement with theory, and analysis[16] showed that only the glucuronic acid was oxidized. The recovered material (**SD**) was polymeric, confirming that the uronic acid is laterally attached, and the p.m.r. spectrum showed the disappearance of a β-signal at δ 4.96 ($J_{1,2}$ 8 Hz), in addition to a peak, at δ 4.50, attributed to H-5 of the acid. Other examples are known where H-5 of glucuronic acid may give a signal in the region normally associated with anomeric protons[17]. Methylation of the Smith-degraded product gave results identical to those obtained by β-elimination (see Table III, column VI). Two possible structures were consistent with the results obtained so far, but a choice between them could be made on the basis of the fragments obtained by partial hydrolysis.

Partial hydrolysis. — Preliminary experiments revealed that optimal production of oligosaccharides occurred on treatment of K82 polysaccharide with M trifluoroacetic acid for 1.5 h on a steam bath. Separation on an ion-exchange column of the products in the hydrolyzate gave a neutral and an acidic fraction. The former contained glucose, galactose, and a trace of an oligosaccharide. The latter fraction was separated by gel-permeation chromatography into two portions. One was shown to be polymeric, and was not further examined. The other was separated by paper chromatography, and gave three pure oligosaccharides, designated **A2**, **A3**, and **A4**. The structures of these compounds, presented in Table II, were established by analysis (see Table I), n.m.r. spectroscopy (see Table II), and methylation (see Table III, columns VII and VIII). In addition, the aldobiouronic acid **A2** was found to be hydrolyzed by β-D-glucosiduronase, and the p.m.r. spectrum of the alditol derived from **A4** was also recorded, in order to determine the anomeric configuration of the internal galactosyl unit. It appeared from the p.m.r. spectrum of **A4** that the terminal (reducing) galactose residue existed essentially in the β configuration.

CONCLUSION

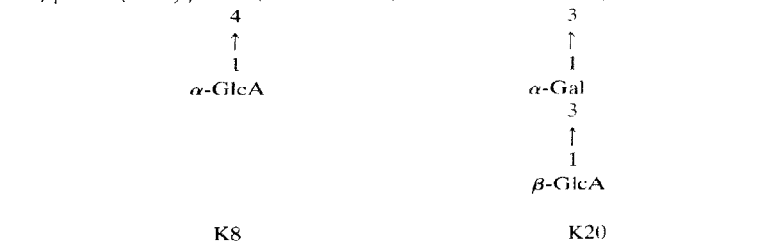
The sum of these experiments demonstrates that the structure of the capsular polysaccharide from *Klebsiella* K82 is based on the tetrasaccharide repeating unit shown.



K82

This structure resembles that of K8 (ref. 18) and, in common with K20 (ref.

19), it has a lateral, β-D-glucosyluronic acid group as a terminal unit. These similarities in chemical structure thus explain the capsular quellung reaction observed between these three strains. Despite the similarity between the K8 and K82 antigens, the structure of the K82 polysaccharide is novel in this series, thus corroborating the observation by Ørskov and Fife-Asbury[4] that K82 does not react with any of the antisera from the other K-types. Furthermore, the p.m.r. spectrum of a sample of K8 polysaccharide was shown to differ from that of K82.



EXPERIMENTAL

General methods. — Solutions were evaporated at bath temperatures not exceeding 40°. Frozen solutions were obtained by using a Dry Ice–acetone mixture, and lyophilized in a Unitrap II freeze-dryer. De-ionizations were performed on a column of Amberlite IR-120 (H^+) resin. Optical rotations were measured on aqueous solutions at 23–25° in a 10-cm cell with a Perkin–Elmer model 141 polarimeter. Circular dichroism (c.d.) spectra were recorded with a Jasco J20 automatic recording spectropolarimeter for solutions in a quartz cell of 0.3 mL capacity and a path length of 0.1 cm. Compounds were dissolved in spectroscopic-grade acetonitrile, and the spectra were recorded in the range of 210–240 nm. The infrared (i.r.) spectra of methylated derivatives were recorded with a Perkin–Elmer model 457 spectrophotometer for solutions in spectroscopic-grade carbon tetrachloride.

Analytical paper-chromatography was performed by the descending method, using Whatman No. 1 paper and the following solvent systems: (*1*) 18:3:1:4 ethyl acetate–acetic acid–formic acid–water, (*2*) freshly prepared 2:1:1 1-butanol–acetic acid–water, (*3*) 8:2:1 ethyl acetate–pyridine–water, and (*4*) the upper phase of 4:1:5 1-butanol–ethanol–water. Preparative paper-chromatography was conducted by using Whatman No. 3 paper and either solvent system *1* or *2*. Analytical paper-electrophoresis was conducted on a Savant high-voltage (5 kV) system (model LT-48A). Kerosene was the coolant, and the buffer system used was 5:2:743 pyridine–acetic acid–water, pH 5.3. Whatman No. 1 paper (77 cm × 20 cm) was used for all such experiments, with an applied current of 25–50 mA. Chromatograms were de-

veloped either with alkaline silver nitrate or by heating at 110° for 10 min after being sprayed with a solution of *p*-anisidine hydrochloride in aqueous 1-butanol.

Preparative gel-permeation chromatography was performed in columns (2.5 × 100 cm) of Bio-Gel P-2 (400 mesh). The void volume of the column, and the efficiency of packing, were determined by using Blue Dextran (0.2%).

The concentration of the samples applied to the column ranged from 40–100 mg/mL. The eluant used was 500:5:2 water–pyridine–acetic acid at a flow-rate of 10 mL/h. Fractions (2.5 mL) were collected, freeze-dried, and weighed, and the elution profile was obtained. Sephadex LH-20 was used to purify carbohydrate material soluble in organic solvents, *e.g.*, permethylated oligo- and poly-saccharides.

Analytical, g.l.c. separations were performed with a Hewlett–Packard 5700 instrument, fitted with dual flame-ionization detectors, on alditol acetate derivatives. An Infotronics CRS-100 electronic integrator was used to quantify the peak areas. Stainless-steel columns (1.8 m × 3 mm) were used with a nitrogen carrier-gas flow-rate of 20 mL/min. The columns used were: (*A*) 3% of SP-2340 on Supelcoport (100–120 mesh), programmed from 195° for 4 min, and then at 2°/min to 260°, (*B*) 5% of ECNSS-M on Gas Chrom Q (100–120 mesh), isothermal at 170°, or programmed from 180° for 4 min, and then at 2°/min to 190°, (*C*) 3% of OV-225 on Gas Chrom Q (100–120 mesh), isothermal at 170°, or programmed from 180° for 4 min, and then at 2°/min to 190°, and (*D*) 5% of SP-1000 on Gas Chrom Q (100–120 mesh), isothermal at 220°. Preparative g.l.c. was conducted with an F and M model 720 dual-column instrument fitted with thermal conductivity detectors, and a helium carrier-gas flow-rate of 60 mL/min. Stainless-steel columns (1.8 m × 6.3 mm) used were: (*E*) 3% of SP-2340 on Supelcoport (100–120 mesh), programmed from 200–260° at 4°/min, and (*F*) 5% of OV-225 on Supelcoport (100–120 mesh), programmed from 190–260° at 1°/min. G.l.c.–m.s. was performed with a Micromass 12 instrument fitted with a Watson–Biemann separator, and a helium carrier-gas flow-rate of 25 mL/min. Spectra were recorded at 70 eV with an ionization current of 100 μA and an ion-source temperature of 200°. The columns used for separations were *B*, *C*, and *D*.

^{1}H-N.m.r. spectra were recorded with a Bruker WH-400 instrument. Spectra were recorded either at ambient or elevated temperatures (95 ±5°) and acetone (2.23 p.p.m.) was used as an internal standard. All values are given relative to that of external sodium 4,4-dimethyl-4-silapentane-1-sulfonate. Samples were prepared by dissolving them in D_2O and freeze-drying (three times). Carbohydrate samples (10–20 mg) dissolved in D_2O were examined in 5-mm diameter, n.m.r. tubes. ^{13}C-N.m.r. experiments were conducted with a Bruker WH-400, a Bruker WP-80, or a Varian CFT-20 instrument. All spectra were recorded at ambient temperature, and acetone was used as the internal standard (at 31.07 p.p.m.). Samples were dissolved in the minimum volume of D_2O, and examined in n.m.r. tubes of 5 or 10 mm diameter.

Preparation and properties of K82 capsular polysaccharide. — A culture of *Klebsiella* K82, obtained from Dr. Ida Ørskov, Copenhagen, was grown as previ-

ously described[5,9], and the polysaccharide was purified by one precipitation with cetyltrimethylammonium bromide. The purified polysaccharide (6 g from 10 L of medium) had $[\alpha]_D$ +31° (*c* 0.29, water), and analysis by gel chromatography showed it to be homogeneous, with an average molecular weight of 2×10^6. N.m.r. spectroscopy was performed on the original material, but better spectra (^1H and ^{13}C) were obtained after treatment of the polysaccharide with 0.5M trifluoroacetic acid (TFA) for 30 min on a steam bath. The principal signals and their assignments are recorded in Table II. The spectrum of K8 polysaccharide, recorded under identical conditions, showed anomeric protons at δ 5.58, 5.24, 4.8e, and 4.76.

Analysis of K82 polysaccharide. — A sample (25 mg) of K82 polysaccharide was hydrolyzed with 2M TFA overnight on a steam bath, and the hydrolyzate was examined by paper chromatography and by g.l.c. The former technique showed the presence of glucose, galactose, and an aldobiouronic acid (R_{Glc} 0.41, solvent *1*); the g.l.c. analysis is shown in Table I, column I. A portion of K82 polysaccharide (20 mg) was methanolyzed[8] overnight in 3% HCl–MeOH, the products reduced with sodium borohydride, and hydrolyzed, and the products analyzed, with the results shown in Table I, column II. Alditol acetates for c.d. measurements[10] were obtained by g.l.c., using column *F*.

Reduction of K82 polysaccharide. — Following the published procedure[9], K82 polysaccharide (1.05 g) was reduced by using 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (8 g) and aqueous 2M sodium borohydride (300 mL). Neutral polysaccharide (1.03 g) was obtained after two treatments, and had the composition shown in Table I, column III.

Methylation analysis. — A sample of K82 polysaccharide in the free acid form (300 mg) was methylated by the Hakomori procedure[11,12], and the product, isolated by dialysis and lyophilization, was treated with the Purdie reagents[20] for 5 d, to give the fully methylated (t.r.) polysaccharide. One part (60 mg) was hydrolyzed with 2M TFA, and paper chromatography in solvent *4* then revealed four components, namely 2,4,6-tri-*O*-methylglucose (R_{TMG}, 0.77), 2,4,6-tri-*O*-methylgalactose (0.57), 2,6-di-*O*-methylgalactose (0.47), and an acid compound (0.13). Analytical figures for the neutral sugars are presented in Table III, column I. A second portion (120 mg) of the methylated polysaccharide was reduced with lithium aluminum hydride in refluxing oxolane. The product (110 mg) was divided into halves, one of which was hydrolyzed and analyzed, with the results shown in Table III, column II. The other half was remethylated[11,12], and hydrolyzed, paper chromatography then showed the presence of an additional compound, namely 2,3,4,6-tetra-*O*-methylglucose (R_{TMG}, 1.00). The g.l.c. results are given in Table III, column III. The neutral polysaccharide (30 mg) obtained by carbodiimide reduction[9] was also subjected to methylation analysis, with the results shown in Table III, column IV.

Uronic acid degradation[13]. — Methylated K82 polysaccharide (80 mg), dried *in vacuo*, was dissolved in 19:1 Me_2SO–2,2-dimethoxypropane (20 mL) containing a trace of *p*-toluenesulfonic acid, and stirred under nitrogen. Sodium methylsul-

finylmethanide (2M, 15 mL) was added, and the mixture was stirred for 18 h at room temperature. The mixture was frozen, methyl iodide was added, the mixture melted, and stirred for 1.5 h at room temperature, and the methyl iodide evaporated. The methylated, degraded product was isolated by partition between chloroform and water (3 × 15 mL), the product dried, and hydrolyzed with 2M TFA, and the sugars released were analyzed as described previously for the methylation analysis (see Table III, column V).

Periodate oxidation[16] *and Smith degradation*[14,15] *of neutral K82 polysaccharide.* — To a solution of the carbodiimide-reduced K82 polysaccharide (26 mg) in water (5 mL) was added 0.03M sodium metaperiodate solution (5 mL). The reaction was conducted at room temperature and in the dark. Aliquots (0.1 mL) were withdrawn periodically, and diluted 250 times with water. The absorbances[16] at 223 nm of the resulting solutions were measured in a Gilford spectrophotometer, model 240. The periodate consumption reached a plateau after ~100 h, when ~0.08 mmol of IO_4^- had been consumed, equivalent to 2 mol of IO_4^- per mol of repeating unit. Ethylene glycol (0.1 mL) was added, to decompose the excess of periodate, the polyaldehyde was reduced with sodium borohydride, the base was neutralized with 50% acetic acid, and the solution dialyzed, and lyophilized, to yield the polyalcohol (13.5 mg). Analysis by g.l.c. gave the results shown in Table I, column IV.

A solution of carbodiimide-reduced K82 polysaccharide (270 mg) in water (150 mL) was mixed with 0.2M sodium metaperiodate solution (150 mL), and kept in the dark at room temperature. After 125 h, the excess of periodate was decomposed by the addition of ethylene glycol (5 mL). After dialysis for 2 d, the lyophilized polyaldehyde in water (50 mL) was reduced to the polyol with sodium borohydride, the base was neutralized with 50% acetic acid, and the solution dialyzed, and freeze-died. A second treatment was conducted, and, after similar processing, the polyalcohol was recovered (yield 205 mg). A sample of the polyalcohol (150 mg) was subjected to Smith hydrolysis with 0.5M TFA for 28 h at room temperature. The freeze-dried product (105 mg), recovered after dialysis, was a polysaccharide whose n.m.r. spectrum contained three anomeric signals (see Table II). A sample of the Smith-hydrolyzed polysaccharide (30 mg) was subjected to methylation analysis, and it gave 2,4,6-tri-*O*-methylgalactose and 2,4,6-tri-*O*-methylglucose in the ratio of 2:1 (see Table III, column VI).

Partial hydrolysis. — The K82 polysaccharide (530 mg) was hydrolyzed with M TFA on a steam-bath for 1.5 h. After removal of the TFA by successive evaporations with water, the mixture was separated on a column of Bio-Rad AG1-X2 (formate) ion-exchange resin, to give a neutral (170 mg) and an acidic fraction (330 mg). The neutral fraction was eluted with distilled water, and the acidic fraction with formic acid (10%). The acidic fraction was applied to a column of Bio-Gel P-2 and the effluent was collected in 2.5-mL fractions. These were freeze-dried and weighed; the elution profile obtained showed two main fractions, 1 and 2. Fraction 1, which was eluted immediately after the void volume (320 mL, Blue Dextran)

was shown by paper chromatography to be polymeric. Further separation of fraction 2 by paper chromatography (solvent *1*), gave an aldobiouronic acid **A2** (15 mg, R_{Glc} 0.44), an aldotriouronic acid **A3** (32 mg, R_{Glc} 0.33), and an aldotetraouronic acid **A4** (24 mg, R_{Glc} 0.2). Paper chromatography of the neutral fraction showed mainly galactose and glucose, and an insignificant amount of a neutral disaccharide (R_{Glc} 0.43).

The analyses performed on each oligosaccharide were as follows. (*a*) *Sugar analysis.* Acidic oligosaccharides were treated with 3% methanolic hydrogen chloride for 8 h on a steam bath. The methyl ester obtained was reduced with sodium borohydride in anhydrous methanol[8], followed by hydrolysis with 2M TFA, sodium borohydride reduction to the alditols, and acetylation with 1:1 acetic anhydride–pyridine. Data for the g.l.c. analyses (column *A*) are given in Table III. (*b*) *Methylation analysis.* All methylations were conducted by the method of Hakomori[11,12] (the acidic oligosaccharides being reduced with lithium aluminum hydride in anhydrous oxolane after methylation); hydrolysis with 2M TFA, sodium borohydride reduction, and acetylation gave the partially methylated alditol acetates, which were analyzed by g.l.c. and g.l.c.–m.s. (see Table III). The n.m.r. data for each oligosaccharide are given in Table II.

Enzymic hydrolysis. — (*a*) *β*-D-*Glucosidase.* Compound **A4** (3 mg) was dissolved in acetate buffer (2 mL, pH 7.0), *β*-D-glucosidase (Sigma, 1 mg) was added, and the solution was incubated for 2 d at 37°. No glucose was detected by paper chromatography (solvent *1*); however, the enzyme was active on cellobiose. (*b*) *β*-D-*Galactosidase.* Compound **A4** (3 mg) was used, and the procedure followed was as for (a). No galactose was detected by paper chromatography; the enzyme was active on lactose. (*c*) *β*-D-*Glucosiduronase.* Compound **A2** (2 mg) was dissolved in acetate buffer (4 mL, pH 7.0), *β*-D-glucosiduronase (Sigma, 1 mg) was added, and the solution was incubated for 1 d at 37°. Glucuronic acid and galactose were detected by paper chromatography (solvent *1*); the enzyme was active on phenylphthalein mono-*β*-D-glucosiduronic acid.

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