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STRUCTURAL STUDIES OF THE CAPSULAR POLYSACCHARIDE OF *Klebsiella* TYPE 81

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ABSTRACT

The structure of the capsular polysaccharide from *Klebsiella* Type 81 has been investigated. Methylation analysis, uronic acid degradation, Smith degradation, and graded acid hydrolysis were the principle methods used. The anomeric nature of the glycosidic linkages was determined by characterization of fragments obtained from the various methods of degradation used. One of the L-rhamnosidic linkages was not present in any of these fragments, but is assumed to be an α-linkage from considerations of optical-rotation data. These studies show that the polysaccharide consists of the following hexasaccharide repeating-unit: →2)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→4)-β-D-GlcA*p*-(1→2)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→3)-β-D-Gal*p*-(1→.

INTRODUCTION

The structures of the capsular polysaccharides from *Klebsiella* Types 9[1], 47[2], and 52[3], all consisting of D-glucuronic acid, D-galactose, and L-rhamnose, are different, thus reflecting their different serological specificity. The same sugars were identified in the capsular polysaccharide of a *Klebsiella* strain isolated from a patient having a urinary-tract infection. The strain proved to be serologically different from the hitherto known 80 serotypes and has been designated[4] K81. We now report structural studies of this polysaccharide.

RESULTS AND DISCUSSION

The polysaccharide, $[\alpha]_{578}$ −52°, was isolated as previously reported for other *Klebsiella* K-antigens[5]. It contained only traces of nitrogen and phosphorus, and, from its i.r. and n.m.r. spectra, no *O*-acetyl or other *O*-acyl groups. An acid hydrolysate of the carboxyl-reduced[6] polysaccharide contained L-rhamnose, D-galactose, and D-glucose in the relative proportions 4.1:1.1:1.0. These sugars accounted

for 94% of the polysaccharide material. Galactose and rhamnose were isolated and proved to have the D and L configuration, respectively. The glucuronic acid has the D configuration, as the derived glucose was oxidized by D-glucose oxidase. The proportions of the three sugars, indicating a hexasaccharide repeating-unit, were confirmed by the n.m.r. spectrum of the original polysaccharide, which showed signals for methyl protons (δ 1.2–1.4 p.p.m.), from the L-rhamnose residues, and anomeric protons (δ 4.5–5.4 p.p.m.) in the ratio 12:6.

Methylation analysis[7,8] of the original and carboxyl-reduced polysaccharide (Table I, columns A and B) shows that the polysaccharide is linear, and further strongly supports the assumption of a hexasaccharide repeating-unit. It also demonstrates that, except for the D-glucuronic acid residues, the sugar residues are pyranosidic. That the D-glucuronic acid residues are pyranosidic is, however, evident from the results of the uronic-acid degradation, as discussed below.

TABLE I

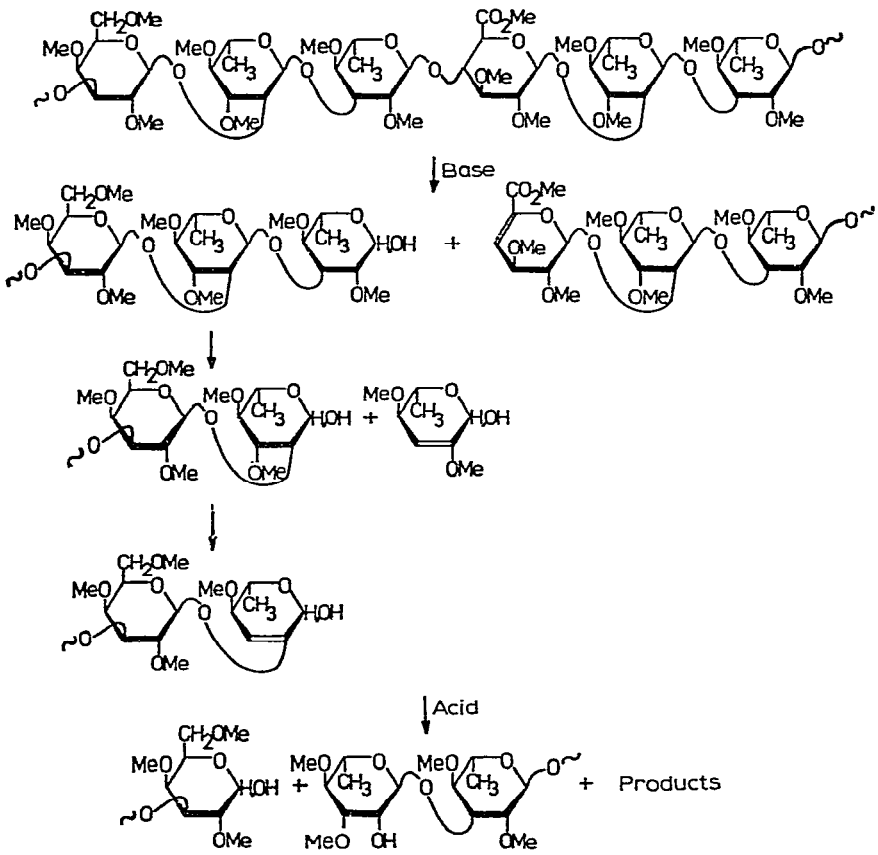
METHYLATION ANALYSES OF ORIGINAL AND MODIFIED TYPE 81 CAPSULAR POLYSACCHARIDES OF *Klebsiella*

Methylated sugar[a]	T[b]	*Mole %*[c]					
		A	*B*	*C*	*D*	*E*	*F*
1,2,4,5,6-Gal[d]	0.42	—	—	—	7[f]	—	—
2,3,4-Rha[d]	0.45	—	—	—	31[g]	—	56[h]
3,4-Rha	0.92	42	35	34	10	—	—
2,4-Rha	0.98	34	33	37	36	67	—
2,4,6-Gal	2.28	24	16	29[e]	17	33	44
2,3,6-Glc	2.49	—	16	—	—	—	—

[a]1,2,4,5,6-Gal = 1,2,4,5,6-penta-*O*-methyl-D-galactitol; 2,3,4-Rha = 2,3,4-tri-*O*-methyl-L-rhamnose, etc. [b]Retention time of the corresponding alditol acetate relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol on an ECNSS-M column at 170°. [c]Polysaccharide: A, original; B, carboxyl-reduced before methylation; C, degraded polysaccharide (uronic acid degradation, see Text); D, degraded, trideuteriomethylated polysaccharide (uronic acid degradation, see Text); E, carboxyl-reduced, periodate-oxidized, reduced, and methylated polysaccharide (see Text); F, degraded, trideuteriomethylated polysaccharide (Smith degradation, see Text). [d]Part of this volatile ether and derivatives was probably lost during work-up. [e]≃50% Monodeuterated at C-1. [f]Monodeuterated at C-1 and trideuteriomethylated at O-1 and O-5. [g]Trideuteriomethylated at O-2. [h]Trideuteriomethylated at O-3.

The fully methylated and esterified polysaccharide, obtained by methylation according to Hakomori[7,8], was subjected to a degradation[9] of uronic acid, that is, treatment with strong base followed by acid hydrolysis under mild conditions. The product was reduced with sodium borodeuteride, then a portion was hydrolysed, and the resulting methylated sugars were analysed by g.l.c.–m.s. as the alditol acetates[8,10] (Table I, column C). Part of the reduced product was remethylated using trideuteriomethyl iodide, then hydrolysed, and analysed in the same way (Table I, column D). Even if the result of the degradation shows that some of the consecutive reactions

have not gone to completion, it is evident that three of the six sugar residues have not been degraded. One of the L-rhamnose residues becomes terminal and appears as tri-*O*-methyl-L-rhamnose (Table I, column D). This sugar has a trideuteriomethyl group at O-2, demonstrating that the uronic acid residue was linked to this position and was released during the mild hydrolysis with acid. The D-galactose residue becomes a reducing terminus after the mild hydrolysis with acid, as indicated both by the monodeuteration at C-1 in the derived alditol and by the formation of a D-galactitol pentamethyl ether after remethylation of the reduced product. It is further evident from the analyses that one of the 3-linked L-rhamnose residues is unaffected. The D-glucuronic acid residue and the three undegraded sugar residues are consequently linked as indicated in the partial structure **1**. Both the L-rhamnose residue linked to O-4 of the D-glucuronic acid residue and also the adjacent L-rhamnose residue are degraded, thereby demonstrating that the former is linked to O-3 and the latter to O-2, and not *vice versa*. Thus, the complete sequence **2** is established from this degradation, the course of which is summarized in Scheme 1.



Scheme 1

→4)-D-GlcA*p*-(1→2)-L-Rha*p*-(1→3)-L-Rha*p*-(1→3)-D-Gal*p*-(1→

1

→2)-L-Rha*p*-(1→3)-L-Rha*p*-(1→4)-D-GlcA*p*-(1→2)-L-Rha*p*-(1→3)-L-Rha*p*-(1→3)-D-Gal*p*-(1→

2

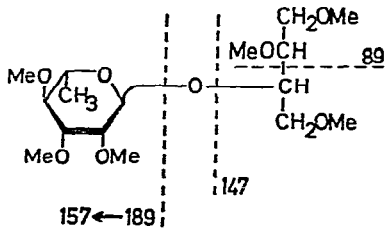
The main part of the material that had been subjected to degradation of uronic acid and reduction was remethylated, using non-deuterated reagent. The resulting, fully methylated oligosaccharide alditol was isolated by high-speed liquid chromatography. Acid hydrolysis of the product yielded comparable amounts of 1,2,4,5,6-penta-*O*-methyl-D-galactitol (deuterium labelled at C-1), 2,3,4-tri-*O*-methyl-L-rhamnose, and 2,4-di-*O*-methyl-L-rhamnose, in agreement with results discussed above. The structure of the methylated trisaccharide alditol **3** was also confirmed by g.l.c.–m.s.[10]. From the optical rotation of the fully methylated trisaccharide alditol, $[\alpha]_{578}$ −40°, it is inferred that both L-rhamnose residues are α-linked. This was confirmed by the n.m.r. spectrum, which showed signals for the anomeric protons at δ 5.17 and 5.05 p.p.m., with $J_{1,2}$ 1.5 Hz for both. Structure **3**, fully methylated, is therefore proved for the degradation product, thus establishing the anomeric natures of two L-rhamnose residues in the hexasaccharide repeating-unit.

α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→3)-D-Galactitol

3

The carboxyl-reduced polysaccharide was subjected to a modified Smith-degradation, involving periodate oxidation, borohydride reduction, methylation, hydrolysis under mild conditions, and borohydride reduction[11,12]. A hydrolysate of the polyalcohol contained L-rhamnose and D-galactose in the ratio 1.8:1, the recovery being 63% of the theoretical value. On complete hydrolysis, the methylated polyalcohol yielded 2,4-di-*O*-methyl-L-rhamnose and 2,4,6-tri-*O*-methyl-D-galactose as expected. The ratio between these sugars, determined by g.l.c.–m.s.[8,10], was 2:1 (Table I, column E). A sample of methylated polyalcohol that had been subjected to mild hydrolysis with acid followed by reduction and remethylation with trideuteriomethyl iodide yielded, on complete hydrolysis, 2,3,4-tri-*O*-methyl-L-rhamnose, trideuteriomethylated at O-3, and 2,4,6-tri-*O*-methyl-D-galactose, in the ratio ~5:4 (Table I, column F). The low percentage of the tri-*O*-methyl-L-rhamnose is not unexpected, as this substance and its derivatives are volatile and may be lost during concentrations. The main part of the material subjected to the modified Smith-degradation described above was remethylated with methyl iodide. The major components were separated by high-speed liquid chromatography. The first yielded 2,3,4-tri-*O*-methyl-L-rhamnose on hydrolysis and, from its m.s.[10] and other evidence presented above, was a fully methylated 2-*O*-L-rhamnopyranosyl-D-erythritol. The origins of some pertinent fragments are indicated in formula **4**. The product showed $[\alpha]_{578}$ −52°, demonstrating that the L-rhamnose residue is α-linked. In agreement with

this, the signal for the anomeric proton in the n.m.r. spectrum appeared at δ 4.98 p.p.m. ($J_{1,2}$ 1.5 Hz). The structural element **5** in the repeating unit is thereby established.

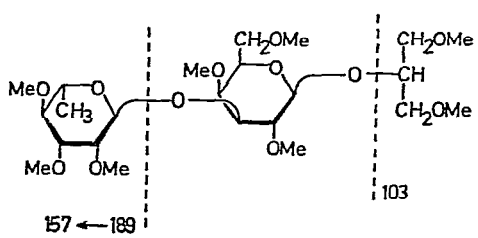


4

→3)-α-L-Rha*p*-(1→4)-D-GlcA*p*-(1→

5

The second component yielded 2,3,4-tri-*O*-methyl-L-rhamnose and 2,4,6-tri-*O*-methyl-D-galactose on hydrolysis. The mass spectrum[10] was that expected for fully methylated L-rhamnopyranosyl-(1→3)-D-galactopyranosyl-(1→2)-glycerol (**6**). It showed $[\alpha]_{578}$ −42°, demonstrating that the L-rhamnose residue is α-linked and the D-galactose residue β-linked. In agreement with this conclusion, two signals for anomeric protons were observed in the n.m.r. spectrum. One at δ 5.22 p.p.m. ($J_{1,2}$ 1.5 Hz) derives from the α-L-rhamnopyranose residue, the other, at δ 4.40 p.p.m. ($J_{1,2}$ 7.2 Hz), comes from the β-D-galactopyranose residue. The glycoside **6** thus derives from the structural element **7** of the hexasaccharide repeating-unit. The 3-substituted L-rhamnose residue is the same as in partial structure **3**, thus confirming the α-configuration of this residue.



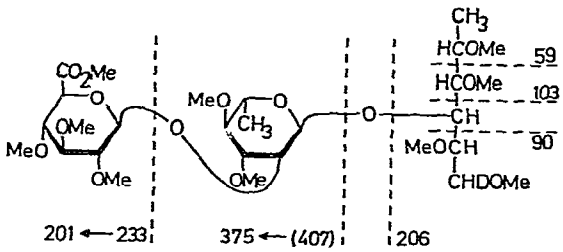
6

→3)-α-L-Rha*p*-(1→3)-β-D-Gal*p*-(1→2)-L-Rha*p*-(1→

7

Part of the polysaccharide was subjected to partial hydrolysis, and an acidic component was isolated by ion-exchange chromatography followed by gel filtration.

Methylation analysis of the derived, carboxyl-reduced alditol yielded comparable amounts of 1,2,4,5-tetra-*O*-methyl-L-rhamnitol, 3,4-di-*O*-methyl-L-rhamnose, and 2,3,4-tri-*O*-methyl-D-glucose, thus establishing the structure of the aldotriouronic acid. G.l.c.–m.s.[10] of the fully methylated alditol methyl ester (**8**) confirmed this result. The origins of some pertinent fragments are indicated in the formulae.



8

The original aldotriouronic acid showed $[\alpha]_{578}$ −38°, demonstrating that the L-rhamnosidic residue is α-linked and the D-glucuronic acid residue is β-linked. In agreement with this conclusion, the signals for the anomeric protons in the n.m.r. spectrum of the derived alditol appeared at δ 5.21 ($J_{1,2}$ 1.5 Hz) and 4.61 p.p.m. ($J_{1,2}$ 7.0 Hz). These results demonstrate the partial structure **9** in the hexasaccharide repeating-unit. The central L-rhamnose residue in **9** is the same as the terminal L-rhamnose residue in **3**, thus confirming the α-configuration of this residue.

→4)-β-D-GlcA*p*-(1→2)-α-L-Rha*p*-(1→3)-L-Rha*p*-(1→

9

The isolation of the fragments **3**, **4**, and **6** and the aldotriouronic acid by different types of degradation furnishes strong evidence of a regular structure with a repeating unit in the polysaccharide. It should be noted that the partial structures **5**, **7**, and **9**, obtained by Smith degradation and partial hydrolysis studies, overlap. The sequence of the six sugar residues in the repeating unit **2** is therefore independently defined by these studies.

From the studies reported above, the anomeric natures of five of the sugars in the hexasaccharide repeating-unit are determined. The remaining residue, the L-rhamnose residue with D-galactose linked to its 2-position, is not present in any of the fragments studied, but only as the glycerol moiety of **6**. From the low optical rotation of the polysaccharide, $[\alpha]_{578}$ −52°, it seems most probable, although not actually proved, that this L-rhamnose residue is α-linked as well as the three others. With this reservation, we propose the structure **10** for the hexasaccharide repeating-unit of the *Klebsiella* Type 81 capsular polysaccharide.

→2)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→4)-β-D-GlcA*p*-(1→2)-α-L-Rha*p*-(1→3)-
α-L-Rha*p*-(1→3)-β-D-Gal*p*-(1→

10

Extracellular, bacterial polysaccharides are often composed of oligosaccharide repeating-units, containing two or more sugar residues. The same is true for the O-specific side-chains of bacterial lipopolysaccharides. Hexasaccharide units seem to be the largest for which unambiguous structures have been published. The present structure is, to the best of our knowledge, the first example of a linear hexasaccharide repeating-unit.

EXPERIMENTAL

General methods. — Concentrations were carried out under reduced pressure, at bath temperatures which did not exceed 40°. For g.l.c., a Perkin–Elmer 900 instrument fitted with flame-ionisation detector was used. Separations were performed on glass columns (180 × 0.15 cm) containing (*a*) 3% of ECNSS-M on Gas Chrom Q (100/120 mesh) at 170° (for partially methylated alditol acetates) or 190° (for alditol acetates); (*b*) 3% of OV-1 on the same support (for permethylated oligosaccharide derivatives). For quantitative evaluation of the g.l.c., a Hewlett–Packard 3370B integrator was used. G.l.c.–m.s. was performed with a Perkin–Elmer 270 instrument (for alditol acetates and derivatives) or a Varian MAT 311–SS 111 MS m.s.–computer system (for oligosaccharide derivatives). Spectra were recorded at 70 eV, with an ionisation current of 80 μA (Perkin–Elmer) and 1000 μA (Varian MAT), and ion-source temperatures of 80° and 180°, respectively. For analytical and preparative paper chromatography, FILTRAK FN-4 paper and the solvent systems (*a*) ethyl acetate–acetic acid–water (3:1:1) and (*b*) 1-butanol–pyridine–water (6:4:3) were used. The compounds were detected with aniline hydrogen phthalate. High-speed liquid chromatography was performed with a Waters Solvent Delivery System 6000 constant-flow pump, and a Waters R 401 differential refractometer was used for monitoring the column effluents. Separations were performed on two Waters Microporasil columns (30 × 0.5 cm) connected in series, with ethyl acetate as eluant. N.m.r. were recorded on a Varian HA-100 instrument. The spectra were recorded for solutions in $CDCl_3$ or D_2O, using Me_4Si and sodium 2,2,3,3-tetradeuterio-4,4-dimethyl-4-silapentane-1-sulphonate as internal standard, respectively. I.r. spectra were recorded on a Perkin–Elmer 257 instrument, and optical rotations on a Perkin–Elmer 141 instrument using 100-mm semi-micro cells.

Isolation of the polysaccharide from Klebsiella *K-type 81 (strain 370).* — This was performed as described[5] earlier. The polysaccharide showed $[\alpha]_{578}^{22}$ −52° (*c* 0.1, water). In the i.r. spectrum (KBr), no significant absorptions at ~1735 cm^{-1} (*O*-acyl region) were observed. The percentages of nitrogen (0.33%) and phosphorus (0.08%) were insignificant. The n.m.r. spectrum of the polysaccharide was recorded on a solution of 80 mg/ml of D_2O at 80°.

Sugar and methylation analyses. — These were performed essentially as described

before[1,7–10]. In sugar analyses, D-arabinose (0.5 mg) was used as internal standard. The partially methylated and trideuteriomethylated alditol acetates gave mass spectra in which fragments containing a trideuteriomethoxy group were recognized by the shift of three mass-units. Rhamnose, galactose, and glucuronic acid were isolated from a hydrolysate of the polysaccharide (see below). The rhamnose showed $[\alpha]_{589}^{20}$ +9° (*c* 2, water), and the galactose $[\alpha]_{589}^{20}$ +77° (*c* 1, water). Glucose obtained by reduction of the glucuronic acid was oxidized by D-glucose oxidase.

Carboxyl-reduction of the native polysaccharide. — This was performed by the procedure of Taylor and Conrad[6]. Two consecutive treatments were needed to obtain complete reduction.

Uronic acid degradation of methylated polysaccharide. — A solution of carefully dried, methylated polysaccharide (180 mg) and toluene-*p*-sulphonic acid (5 mg) in a mixture (32 ml) of methyl sulphoxide and 2,2-dimethoxypropane (19:1) was prepared in a serum vial sealed with a rubber cap. The vial was flushed with nitrogen and kept in an ultrasonic bath for 30 min. 2M Methylsulphinyl anion in methyl sulphoxide (15 ml) was added and the solution sonificated for another 30 min. The vial was kept at room temperature overnight. 50% Aqueous acetic acid (30 ml) was added, and the product was recovered by partition between chloroform and water, and treated with 10% aqueous acetic acid for 1 h at 100°. The material was recovered by freeze-drying and dissolved in a mixture (50 ml) of *p*-dioxane and ethanol (8:3). Sodium borodeuteride (200 mg) was added and the mixture was stirred overnight. The mixture was then treated with Dowex-50 (H^+, prewashed with *p*-dioxane–ethanol) resin and concentrated, and boric acid was removed from the residue by repeated distillations with methanol (4 × 10 ml). Part (5%) of the recovered material was hydrolysed, and the resulting sugars were analysed, as their alditol acetates, by g.l.c.–m.s.[8,10] (Table I, column C). Another part (5%) was remethylated using trideuteriomethyl iodide, and the material was recovered by partition between chloroform and water. The product was then hydrolysed and the sugars were analysed, as their alditol acetates, by g.l.c.–m.s.[8,10] (Table I, column D). The main portion (90%) of the degraded and reduced material was remethylated using methyl iodide, and the product was recovered by partition between chloroform and water, and purified by liquid chromatography, yielding permethylated α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→3)-D-galactitol (**3**) (4.5 mg). On t.l.c. (ethyl acetate), the compound showed R_F 0.35, and on g.l.c. (OV-1 column at 200°), T_{MEL} 4.7 (retention time relative to permethylated melibiitol). The mass spectrum showed, *inter alia*, the following peaks (relative intensities in brackets): *m/e* 71(30), 75(88), 88(100), 89(32), 90(17), 101(100), 125(20), 129(30), 133(8), 145(20), 157(47), 172(15), 189(86), 205(13), 236(12), 331(0.3), and 363(0.5). In the n.m.r. spectrum ($CDCl_3$), the signals from the anomeric protons occurred at δ 5.17 and 5.05 p.p.m. (both $J_{1,2}$ 1.5 Hz). The compound showed $[\alpha]_{589}^{22}$ −40° (*c* 0.5, chloroform). Part of the material was hydrolysed, and the sugars were analysed, as their alditol acetates, by g.l.c.–m.s.[8,10]. 1,2,4,5,6-Penta-*O*-methyl-D-galactitol (deuterium label at C-1), 2,3,4-tri-*O*-methyl-L-rhamnose, and 2,4-di-*O*-methyl-L-rhamnose were obtained in comparable amounts in this analysis.

Smith degradation of the polysaccharide. — Carboxyl-reduced polysaccharide (210 mg) was dissolved in 0.1M sodium acetate buffer of pH 3.9 (300 ml), and 0.3M sodium metaperiodate (75 ml) and 1-propanol (4 ml) were added. The reaction mixture was stirred in the dark at 4° for 120 h. Excess periodate was destroyed by adding ethylene glycol (10 ml), and the mixture was dialysed overnight. The solution was concentrated to ~100 ml and sodium borohydride (2 g) was added. After stirring for 9 h at room temperature, the excess of borohydride was destroyed by addition of 90% aqueous acetic acid. The solution was then dialysed. Part (1%) of the recovered material was subjected to sugar analysis, using D-arabinose (0.5 mg) as internal standard. The main portion (99%) was freeze-dried and then subjected to two consecutive methylations using methyl iodide. Part (5%) of the methylated material was hydrolysed, and the sugars were analysed, as their alditol acetates, by g.l.c.–m.s.[8,10] (Table I, column E). The main portion (95%) was treated with 90% formic acid (30 ml) for 1 h at 40°. The solution was evaporated to dryness, suspended in water, and freeze-dried. The recovered product was dissolved in a mixture (50 ml) of *p*-dioxane–ethanol (8:3). Sodium borohydride (250 mg) was added and the reaction mixture was stirred overnight. After conventional work-up, a portion (10%) was remethylated using trideuteriomethyl iodide. The recovered material was hydrolysed, and the sugars were analysed, as their alditol acetates, by g.l.c.–m.s.[8,10] (Table I, column F). The main portion (90%) was remethylated using methyl iodide, and the product fractionated by liquid chromatography, yielding permethylated α-L-Rha*p*-(1→2)-D-erythritol (**4**, 9.2 mg) and permethylated α-L-Rha*p*-(1→3)-β-D-Gal*p*-(1→2)-glycerol (**6**, 15.1 mg). Compound **4** showed R_F 0.50 on t.l.c. (ethyl acetate), and T_{GG} 1.02 (retention time relative to permethylated α-D-Gal*p*-(1→2)-glycerol) on g.l.c. (OV-1 column at 225°). The mass spectrum showed, *inter alia*, the following peaks: *m/e* 88(100), 89(25), 101(28), 125(4), 147(9), 157(4), 189(8), and 207(20). In the n.m.r. spectrum ($CDCl_3$), the signal from the anomeric proton occurred at δ 4.98 p.p.m. ($J_{1,2}$ 1.5 Hz). The compound showed $[\alpha]_{589}^{22}$ −52° (*c* 0.3, chloroform). Part of the material was hydrolysed, and the sugars were analysed, as their alditol acetates, by g.l.c.–m.s.[8,10]. Only 2,3,4-tri-*O*-methyl-L-rhamnose was obtained in this analysis (the volatile D-erythritol derivative was lost during concentrations). Compound **6** showed R_F 0.28 on t.l.c. (ethyl acetate), and T_{MEL} 1.07 on g.l.c. (OV-1 column at 225°). The mass spectrum showed, *inter alia*, the following peaks: *m/e* 88(100), 101(75), 103(17), 125(9), 157(13), 163(9), 189(47), and 367(2). In the n.m.r. spectrum ($CDCl_3$), the signals from the anomeric protons occurred at δ 5.22 ($J_{1,2}$ 1.5 Hz) and 4.40 p.p.m. ($J_{1,2}$ 7.2 Hz). The compound showed $[\alpha]_{589}^{22}$ −48° (*c* 0.5, chloroform). Part of the material was hydrolysed, and the sugars were analysed, as their alditol acetates, by g.l.c.–m.s.[8,10]. 2,3,4-Tri-*O*-methyl-L-rhamnose and 2,4,6-tri-*O*-methyl-D-galactose were obtained in equimolecular proportions in this analysis.

Partial acid hydrolysis of the polysaccharide, and isolation and characterization of oligosaccharide. — Native polysaccharide (1.0 g) was hydrolysed with 0.13M sulphuric acid for 40 min at 100°. After neutralisation with barium carbonate, the hydrolysate was fractionated on a column of Dowex-1 x8 (acetate form) resin. The column was

first eluted with water, yielding neutral sugars (see above), and then with M acetic acid, yielding acidic oligosaccharides. One of the fractions was further purified on a column of Sephadex G-15, yielding the pure aldotriouronic acid (50 mg). On paper chromatography in systems (*a*) and (*b*), the compound showed R_{RHA} 0.22 and 0.20, respectively. The compound showed $[\alpha]_{578}^{22}$ −38° (*c* 0.3, water). The aldotriouronic acid was transformed into its alditol by reduction with sodium borodeuteride. In the n.m.r. spectrum (D_2O at 95°), the signals from the anomeric protons were obtained at δ 5.21 ($J_{1,2}$ 1.5 Hz) and 4.61 p.p.m. ($J_{1,2}$ 7.0 Hz). The alditol was methylated and recovered by partition between chloroform and water. On g.l.c.–m.s., the permethylated aldotriouronic acid alditol showed (OV-1 column at 210°) T_{MEL} 4.2, and the mass spectrum contained, *inter alia*, the following fragments: *m/e* 46(19), 59(97), 88(62), 90(23), 101(96), 103(30), 169(70), 201(100), 206(6), 233(9), 266(6), and 375(12). Part of the permethylated sample was reduced with sodium borodeuteride in *p*-dioxane–ethanol (8:3), hydrolysed, transformed into alditol acetates, and analysed by g.l.c.–m.s.[8,10]. 2,3,4-Tri-*O*-methyl-D-glucose (dideuterated at C-6), 3,4-di-*O*-methyl-L-rhamnose, and 1,2,4,5-tetra-*O*-methyl-L-rhamnitol (monodeuterated at C-1) were obtained in comparable amounts in this analysis.

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