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STRUCTURAL INVESTIGATION OF *Klebsiella* SEROTYPE K62 POLYSACCHARIDE

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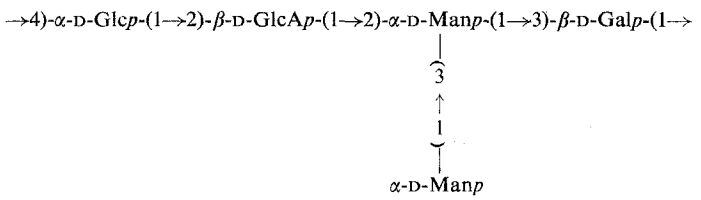
ABSTRACT

By methylation analysis and, in particular, by characterization of the aldobio- to aldotetrao-uronic acids obtained by partial hydrolysis, the polysaccharide has been shown to contain the pentasaccharide repeating unit given. The configuration of the anomeric linkages follows from the ^{1}H-n.m.r. spectroscopy of the intact polysaccharide and of the isolated oligomers. Serotype K62 is one of ~20 strains of the same chemotype, which includes K7 and K28 (whose structures are already known).



INTRODUCTION

In the genus *Klebsiella*, 80 strains are recognized serologically on the basis of their antigenic, capsular polysaccharides. Nimmich[1,2] has qualitatively analyzed each of the strains, and his results may be grouped according to the monosaccharides present, *i.e.*, by chemotype[3]. When this is done, it is found that certain combinations are unique (*e.g.*, for[4] K32 or[5] K63), whereas, in other instances, one chemotype may embrace more than 15 strains. In the latter case, of which K62 is an example, it is clearly of particular immunochemical interest to determine the primary structure of the different serotypes within the same chemotype. With this in mind, the structure of the capsular polysaccharide of serotype K62 has been determined, and compared with those of[6] K7 and[7] K28.

Because of the diversity of structural patterns encountered in these *Klebsiella* polysaccharides, determination of the exact monosaccharide sequence in the repeating unit has sometimes presented certain problems. In the present study on K62, the proposed structure was readily elucidated by methylation and, in particular, by thorough characterization of the acidic oligosaccharides obtained by partial hydrolysis.

RESULTS AND DISCUSSION

Isolation, composition, and n.m.r. spectrum of K62 polysaccharide

The capsular polysaccharide, purified by one precipitation with Cetavlon, had a neutralization equivalent of 806, whereas one uronic acid residue to four hexose residues requires 826. The ^{1}H-n.m.r. (p.m.r.) spectrum of a 2% solution showed relatively poor definition, because of the viscosity of the solution (see Fig. 1A). When a sample of the polysaccharide was mildly depolymerized by a short treatment with acid, and the product examined by p.m.r. spectroscopy, the resolution was greatly improved (see Fig. 1B), and the spectrum showed signals for five anomeric protons[8], corresponding to three α-linkages (τ 4.58, 4.81, and 4.85) and two β-linkages (τ 5.35 and 5.49).

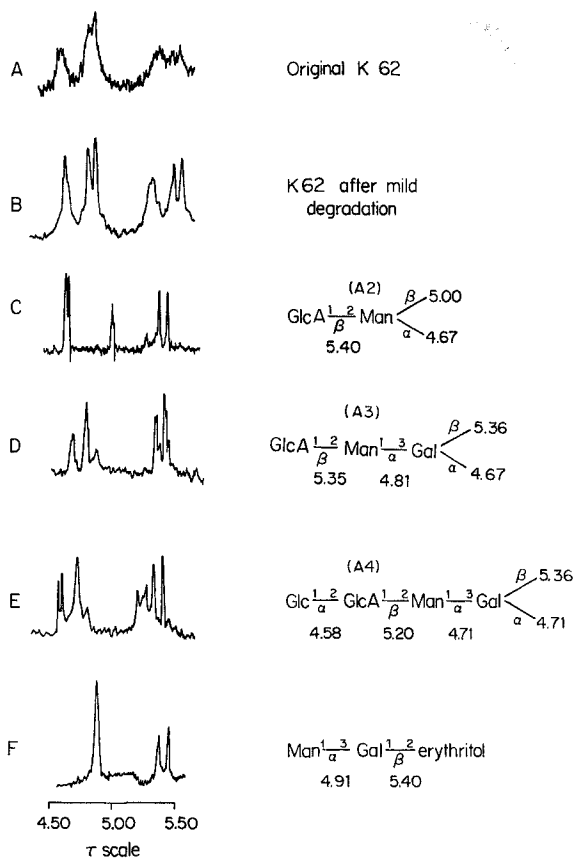


Fig. 1. P.m.r. spectra at 100 MHz of solutions in D_2O at ~90°; only the region of the anomeric protons is shown. [A and B, K62 polysaccharide; C, D, and E, acidic oligomers (A_2, A_3, and A_4) isolated by partial hydrolysis; F, disaccharide glycoside obtained by Smith degradation. For further details, see text.]

After hydrolysis of the polysaccharide with either hydrochloric or trifluoroacetic[9] acid, the hydrolyzate contained D-mannose, D-glucose, D-galactose, a substantial proportion of free D-glucuronic acid, and lesser proportions of acidic oligomers. This result confirmed those of Nimmich[1,2], and also indicated that the aldobiouronic acid was relatively easily cleaved. Following reduction of the uronic acid, quantitative analysis for the constituents showed that the ratios of D-mannose: D-glucose: D-galactose were 2:2:1, consistent with a pentasaccharide repeating unit, as suggested by the neutralization equivalent and, particularly, by the p.m.r. spectrum. The configuration of each sugar was proved by the sign of the circular dichroism spectrum[10] of the alditol acetates in the case of the first two named; of the lactone, for D-glucuronic acid; and of a methylated alditol acetate subsequently isolated, for D-galactose.

Methylation analysis

Fully methylated polysaccharide was readily obtained by one treatment according to the method of Hakomori[11], followed by reaction under the respective conditions of Kuhn and Purdie. Hydrolysis of the resulting material gave a mixture of neutral and acidic compounds which was separated by use of ion-exchange resins. The neutral sugars were tentatively identified by qualitative, paper chromatography and, more completely, by g.l.c.–m.s. of their alditol acetates[14,15], with the results shown in Table IA. It should be noted that, by starting with a chromatographic tank unsaturated with solvent, it is possible to distinguish between 2,3,4,6-tetra-*O*-methylmannose and the glucose isomer (see Experimental).

TABLE I

METHYLATION ANALYSIS OF *Klebsiella* TYPE K62 CAPSULAR POLYSACCHARIDE

Methylated sugar[a]	*Retention times*[b] (*min*)		*Sample*[c]		
	a	b	*A*	*B*	*C*
2,3,4,6-Me_4-Man	8.6	25	++	tr	+
2,3,6-Me_3-Glc	21	33	++	+	+
2,4,6-Me_3-Gal	18	37	++	+	+
4,6-Me_2-Man	26	40	+	++	+
3,4-Me_2-Glc	42	49		++	+

[a]2,3,4,6-Me_4-Man = 2,3,4,6-tetra-*O*-methylmannose, etc. [b]Column *a*, 3% of ECNSS-M, at 195°, He at 71 ml/min; column *b*, 20% of Apiezon L, at 212°, He at 97 ml/min. [c]Methylated polysaccharide, neutral fraction (A); acidic fraction (B); after reduction of uronic ester (C). Approximate, relative molar proportions indicated; tr = trace.

Preparative, paper chromatography permitted the isolation of individual components, of which 2,3,4,6-tetra-*O*-methylmannose was identified by the retention times in g.l.c. analysis of the derived methyl mannosides, but, more specifically, by demethylation [17] and conversion of the product into crystalline[18] mannitol hexa-

acetate, m.p 120–122°. The 2,3,6-tri-*O*-methyl-D-glucose was obtained cystalline[19], m.p. 115–117°, as was 2,4,6-tri-*O*-methyl-D-galactose[20], m.p. 103–105°. The 4,6-di-*O*-methylmannose was characterized by its R_F value on paper, and by g.l.c.–m.s. of the derived alditol acetate.

The acidic fraction was best examined after conversion into the ester glycosides, reduction ($LiAlH_4$), and hydrolysis. Paper-chromatographic examination was of little use, as the principal component overlapped with 4,6-di-*O*-methylmannose. In g.l.c., however, these two compounds were widely separated, and the unknown compound was identified as 3,4-di-*O*-methylglucose by m.s. of the alditol acetate (see Table IB). With the characterization of the sugar derived from the uronic acid, each of the five units anticipated in the repeating structure was accounted for. In order to determine their relative proportions, a sample of the fully methylated polysaccharide was carboxyl-reduced[21], and analyzed; each component was found in equimolar proportion.

The isolation of one mole of tetra-*O*-methylmannose and an equivalent quantity of di-*O*-methylmannose shows that the two mannose residues correspond to a terminal group and a branch-point residue, respectively, whereas the other three sugars found represent in-chain residues. The complete sequence of the repeating pentasaccharide was established by examination of the oligosaccharides obtained by partial hydrolysis of the original polysaccharide.

Partial hydrolysis, and characterization of oligosaccharides

Preliminary experiments on autohydrolysis showed that, at pH 3.5, for periods of up to 37 h at 100°, only D-mannose was released, with the formation of insignificant amounts of oligomeric material; this result is consistent with the methylation results, namely, that one unit of D-mannose is a nonreducing end-group.

Hydrolysis with 1M trifluoroacetic acid (TFA) for 5 h gave mainly aldobiouronic acid as the only oligosaccharide, but, when the time of hydrolysis was lessened to 1 h, oligosaccharides ranging from aldobio- to aldopentao-uronic acids were obtained. These oligomers were freed of monosaccharides by passage through a short column of charcoal–Celite[22], and then separated, by gel filtration[23] on Sephadex G15, into four fractions: A_2, A_3, (A_4+A_5), and A_6. The two components present in the third fraction were separated by paper chromatography in solvent C for seven days.

Each of the oligosaccharides was examined by p.m.r. spectroscopy[8], and, by comparing the spectra (see Fig. 1C,D,E) of the successive compounds A_2 to A_4, it was possible to assign the anomeric configuration of each glycosidic linkage in the oligomers and, thus, in the original polysaccharide. The specific optical rotation of each oligomer was consistent with these assignments. The structure of each oligomer A_2 to A_4 is given in Fig. 1C,D,E, and is based on (*a*) total and partial hydrolysis, and (*b*) methylation analysis of the acidic and/or reduced oligosaccharide. These procedures are described in the Experimental section, and the results are given in Table II. In addition, the aldobiouronic acid (A_2) was found to undergo enzymic hydrolysis by β-D-glucosiduronase, confirming the deductions from optical rotation[24]

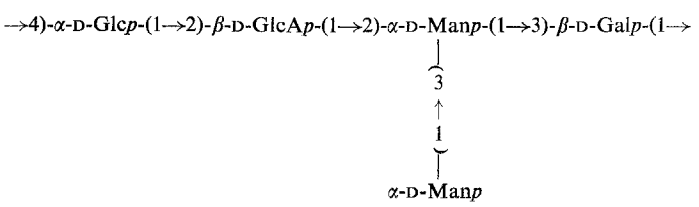
TABLE II

ANALYSIS OF ACIDIC OLIGOSACCHARIDES FROM *Klebsiella* K62 CAPSULAR POLYSACCHARIDE

Oligomer	A		B	C	D	E	
	$[\alpha]_D$ (*in* H_2O)		*Hydrolysis*	*Reduction and hydrolysis*	*Methylated sugars* (R_F, *solvent D*)	*Methylated alditol acetates* (*retention time*[a], *min*)	
	(*degrees*)	*concn.*					
Aldobiouronic acid (A_2)	−32.0	6.37	Man[b]	Man	3,4,6-Man[c] (0.56)	3,4,6-Man	(15)
			GlcA	Glc	[2,3,4-Glc (0.58)][d]	2,3,4-Glc	(18)
Aldotriouronic acid (A_3)	+32.1	10.9	Gal	Gal	3,4,6-Man (0.58)	3,4,6-Man	(12)
			Man	Man	2,4,6-Gal (0.41)	2,4,6-Gal	(14)
			GlcA	Glc	[2,3,4-Glc (0.60)][d]	2,3,4-Glc	(16)
Aldotetraouronic acid (A_4)	+56.2	4.45	Gal	Gal	2,3,4,6-Glc (0.84)	2,3,4,6-Glc	(10)
			Man	Man	3,4,6-Man (0.60)	3,4,6-Man	(12)
			Glc	Glc	2,4,6-Gal (0.41)	2,4,6-Gal	(14)
			GlcA		2,5,6-Gal (0.68)		
Fraction A_5	+19.4	1.08	Gal		2,3,4,6-Glc (0.84)		
			Man		2,3,4,6-Man (0.82)		
					3,4,6-Man (0.56)		
			Glc		2,3,6-Glc (0.55)		
					2,4,6-Gal (0.40)		
			GlcA		4,6-Man (0.30)		

[a]Column *a*; for details, see text. [b]Man = mannose, etc. [c]3,4,6-Man = 3,4,6-tri-*O*-methylmannose, etc. [d]After reduction of uronic ester.

and p.m.r. spectroscopy concerning the anomeric configuration of the glycosidic linkage. Only very small quantities of A_5 and A_6 were obtained, and the methylation analysis suggested that each was a mixture. As the nature of A_4 and the lower oligomers is sufficient to establish the sequence in the repeating unit, A_5 and A_6 were not examined further. The methylation analysis of the original polysaccharide, in conjunction with the formula of A_4, permits the structure of the pentasaccharide repeating unit of K62 capsular polysaccharide to be formulated as follows.



Periodate oxidation

Confirmation of this structure was sought from the results of periodate oxidation. The intact polysaccharide consumed 0.82 mole of periodate per molar proportion of hexose residue (theoretical, 0.80). Total hydrolysis of the derived polyalcohol gave mannose, galactose, erythritol, and glycerol in equimolar proportions (paper chromatography, and g.l.c. of the alditol acetates).

Partial hydrolysis (Smith degradation[25]) of the polyalcohol with Amberlite IR-120 (H^+) resin, followed by gel filtration, gave a disaccharide glycoside whose p.m.r. spectrum is shown in Fig. 1F. Analysis by g.l.c. of a hydrolyzate thereof gave the crystalline alditol acetates of D-mannose, D-galactose, and erythritol, and showed the three components to be present in equimolecular proportions.

CONCLUSIONS

Comparison of the structures of the capsular polysaccharides of K-types 7 (ref. 6), 28 (ref. 7), and 62 confirms the great variety of structural patterns that may be found in the antigens of this genus of bacterium. Type K7 is distinguished in this trio by having pyruvic acid bound as an acetal (*i.e.*, as a 1-carboxyethylidene group) with a single, side-chain unit of D-galactose. In K28, the side chain consists of a pseudoaldobiouronic acid.

Heidelberger and Nimmich[26] studied the immunochemical relationships between Pneumococci and *Klebsiella*, but in the case of K62, found only a weak cross-reaction with anti-Pn V. They pointed out that D-mannose has not been found in any pneumococcal, type-specific substance and, thus, with K62, strong cross-reaction would not be anticipated. It was proposed[26] that the weak cross-reaction observed was probably due to a 2-*O*-substituted D-glucosyluronic acid residue, a structural feature confirmed by the present study.

EXPERIMENTAL

General methods. — All evaporations were conducted under diminished pressure at a bath temperature not exceeding 40° (except as noted). Optical rotations were measured at 23 ±1° with a Perkin–Elmer model 141 polarimeter. Melting points were determined with a Fisher–Johns apparatus and are uncorrected. Analytical, paper chromatography was performed on Whatman No. 1 filter paper. Zones on chromatograms were detected with silver nitrate–sodium hydroxide[27] for reducing and non-reducing sugars, and *p*-anisidine in 1-butanol[28] for reducing and methylated sugars; the developers used were solvent A, 18:3:1:4 ethyl acetate–acetic acid–formic acid–water; B, 4:1:1 ethyl acetate–pyridine–water; C, 2:1:1 1-butanol–acetic acid–water; D, 2-butanone–water azeotrope; and E, 4:1:5 1-butanol–ethanol–water. For preparative, paper chromatography, Whatman No. 3MM filter paper was used. Thin-layer chromatography was performed with silica gel as the adsorbent. Gas–liquid chromatography was conducted on an F and M 720 instrument with the following columns: *a*, 3% of ECNSS-M on 100–120 mesh Gas Chrom Q, (1.25 m × 6.25 mm o.d.), and *b*, 20% of Apiezon L on 60–80 mesh Diatoport S (2.2 m × 6.25 mm o.d.); peak areas were determined with an Infotronics CRS-100 electronic integrator. Infrared spectra were recorded with a Perkin–Elmer model 457 infrared spectrophotometer. P.m.r. spectra were recorded with Varian T-60 and HA-100 instruments, with tetramethylsilane as the external standard, except as noted.

Isolation and properties of K62 capsular polysaccharide. — *Klebsiella* K62 (5711-52) was grown in the medium[29] as for K5, and harvested to give 400 ml of slime and cells from 4 liters of agar medium. Phenol (5 g) in water (100 ml) was added to the slime, and the mixture was diluted to 1.5 liters with water. Centrifugation of the diluted slime at 27,000 r.p.m. (68,000 *g*) for 1 h yielded a clear, light-yellow, supernatant liquor, which was treated with Cetavlon (10%, 25 ml) to precipitate the acidic polysaccharide. The neutral polysaccharide remaining in the supernatant liquor was dialyzed and freeze-dried, yielding 0.7 g; this material was not examined further. The precipitated acidic polysaccharide was dissolved in sodium chloride (2M), and re-precipitated by pouring the solution into five volumes of ethanol. The precipitate, collected by centrifugation, was dissolved in water, decationized, dialyzed against distilled water, and freeze-dried, to give 3.7 g of pure, acidic polysaccharide; ash content nil, nitrogen nil. The electrophoretic mobility on cellulose acetate was 2.4 cm in 30 min at pH 8.8 and 300 V. The equivalent weight of this polysaccharide was 806 by titration with 0.01M sodium hydroxide (phenolphthalein).

The p.m.r. spectrum of a 2% solution of the original polysaccharide in D_2O at 95° showed signals of anomeric protons at τ 5.49 (1 H), 5.35 (1 H), 4.85 (1 H), 4.81 (1 H), and 4.58 (1 H). A mildly degraded, K62 polysaccharide was obtained by heating the original polysaccharide with trifluoroacetic acid (0.2M) for 30 min at 97°, followed by dialyzing against tap water for 24 h. The material in the dialysis tubing was freeze-dried, and its p.m.r. spectrum in D_2O showed signals for the anomeric

protons similar to those of the original polysaccharide, but with improved resolution: τ 5.49 ($J_{1,2}$ 7 Hz, 1 H), 5.35 ($J_{1,2}$ 5 Hz, 1 H), 4.85 (1 H), 4.81 (1 H), and 4.58 (1 H) (see Fig. 1B).

Analysis of constituent sugars. — The capsular polysaccharide (4 mg) was hydrolyzed with 2M trifluoroacetic acid for 9 h at 100°. After evaporation, the hydrolyzate was examined by paper chromatography in solvent B, revealing three major spots for mannose, glucose, and galactose, and an acidic component at the origin. Paper chromatography in solvent A showed the presence of glucuronic acid, an aldobiouronic acid (R_{Glc} 0.45), and a trace of an aldotriouronic acid (R_{Glc} 0.16), in addition to the neutral hexoses.

The polysaccharide (10 mg) was heated in hydrochloric acid (2M) on a steam bath for 8 h. The solution was passed through a column of Duolite A-4 (OH^-) resin, to neutralize the hydrochloric acid and to remove the acidic sugars. After evaporation, the neutral fraction was analyzed, and found to contain mannose, galactose, and glucose in the ratios of ~2:1:1, as determined by g.l.c. of the alditol acetates on column *a*. The individual components were collected, and identified as mannitol hexaacetate. m.p. 119–121°; galactitol hexaacetate, m.p. 161–163°; and glucitol hexaacetate, m.p. 93–95°. The D configuration of mannose and glucose was found by dissolving, in acetonitrile, the mannitol hexaacetate and the glucitol hexaacetate collected from g.l.c. The positive, circular dichroism curves at 213 nm were identical to those given by authentic samples[10].

A sample of polysaccharide (10 mg) was refluxed in methanolic hydrogen chloride (3%) for 10 h on a steam bath. After neutralization (silver carbonate), and reduction (sodium borohydride in methanol), the mixture was made neutral with Amberlite IR-120 (H^+) cation-exchange resin, and distilled three times with methanol. The residue was further hydrolyzed (2M trifluoroacetic acid), reduced and, acetylated. The acetylated mixture was found by g.l.c. analysis on column *a* to contain mannitol hexaacetate (m.p., and, mixed with D-mannitol hexaacetate, 121–123°), galactitol hexaacetate (m.p. and mixed m.p. 162–164°), and glucitol hexaacetate (m.p., and, mixed with D-glucitol hexaacetate, 95–97°) in the ratios of ~2:1:2.

Methylation of the capsular polysaccharide. — To a solution of dry polysaccharide (1 g) in anhydrous dimethyl sulfoxide (40 ml) was added methylsulfinyl anion (25 ml, 3M), and the mixture was stirred for 12 h. Methyl iodide (8 ml) was slowly added while stirring and keeping the temperature below 20°. After 0.5 h, the solution was dialyzed against running water for 24 h, and then extracted with chloroform (3 × 100 ml), to give 0.9 g of methylated polysaccharide that showed hydroxyl-group absorption at 3600 cm^{-1}. The incompletely methylated polysaccharide was dissolved in *N*,*N*-dimethylformamide (8 ml) and methyl iodide (20 ml). While stirring, silver oxide (5 g) was added. Another portion (5 g) of silver oxide was added 10 h later, and stirring was continued for 12 h more. The recovered material was methylated with Purdie's reagents, to give a product showing no absorption at 3600 cm^{-1}, $[\alpha]_D$ +59.7° (*c* 2.3, carbon tetrachloride).

Methylated polysaccharide (0.1 g) was heated in formic acid (90%) for 1 h at 100°, and the solution was evaporated to dryness. The residue was dissolved in 2M hydrochloric acid, and the solution was heated for 6 h at 100°, and evaporated to dryness; analysis of the hydrolyzate by paper chromatography (solvents D and E), showed the presence of 2,3,4,6-tetra-*O*-methylmannose (R_F 0.80, solvent D; 0.82, solvent E), 2,3,6-tri-*O*-methylglucose (R_F 0.55, D; 0.72, E), 2,4,6-tri-*O*-methylgalactose (R_F 0.40, D; 0.63, E), 4,6-di-*O*-methylmannose (R_F 0.28, D; 0.56, E), and an acidic component (origin, D: R_F 0.29, E). Separation on Duolite A-4 anion-exchange resin gave a neutral fraction which was reduced with sodium borohydride, and the product acetylated. G.l.c. analysis on column *a* (195°, helium flow-rate, 71 ml/min) and column *b* (212°, helium flow-rate, 97 ml/min) gave the results shown in Table IA. The components were identified by their retention times and mass spectra.

A separate portion of the methylated polysaccharide (0.7 g) was heated in formic acid (90%) on a steam bath for 40 min. After evaporation to dryness, the mixture was further hydrolyzed in 2M trifluoroacetic acid on a steam bath for 3 h. After removal of trifluoroacetic acid by evaporation, the hydrolyzate was separated into a neutral fraction (0.42 g) and an acidic fraction (0.21 g) by using Duolite A-4 (OH^-) ion-exchange resin.

The neutral fraction was separated into four components by preparative, paper chromatography using solvent D.

Fraction I (51 mg) had chromatographic mobility in solvent D identical to that of 2,3,4,6-tetra-*O*-methyl-D-mannose, and could be differentiated from 2,3,4,6-tetra-*O*-methylglucose in solvent D if the chromatographic tank was initially not saturated with respect to the solvent system and had several drops of ammonium hydroxide on the bottom of the tank. Under these special conditions, a longer time (~10 h) was needed to develop the chromatogram, but ready separation of 2,3,4,6-tetra-*O*-methylmannose and 2,3,4,6-tetra-*O*-methylglucose into two distinct spots (with a ratio of mobility Me_4-Man/Me_4-Glc = ~0.94) was achieved.

A portion of this fraction (5 mg) was methanolyzed with 3% methanolic hydrogen chloride; after neutralization (silver carbonate) and concentration, the product was examined on column *a*, at 150° and a helium flow-rate of 60 ml/min. Two peaks were obtained in the ratio of ~10:1, identical in ratio and retention times to methyl 2,3,4,6-tetra-*O*-methyl-α-D-mannoside (20.9 min) and methyl 2,3,4,6-tetra-*O*-methyl-β-D-mannoside (27.5 min) obtained by methyl glycosidation of an authentic sample of 2,3,4,6-tetra-*O*-methyl-D-mannose.

A separate portion of fraction I (5 mg) in dichloromethane (1 ml) was treated with boron trichloride (2 ml) for 30 min at −75° and then allowed to stand for 16 h at room temperature. After evaporation, and distillations with methanol, the de-*O*-methylated product was reduced, the alditol acetylated, and the product separated on column *a* programmed from 185–220° at 1°/min, with a helium flow rate of 100 ml/min, to give a peak identical to that of authentic D-mannitol hexaacetate (18 min). A sample

was collected, and the m.p., and mixed m.p. with D-mannitol hexaacetate, was 120–122°.

Fraction II (30 mg) had the same chromatographic mobility as 2,3,6-tri-*O*-methyl-D-glucose (R_F 0.55, solvent D). The m.p. was 115–117° (from ethyl ether), and this was undepressed on admixture with an authentic sample of 2,3,6-tri-*O*-methyl-D-glucose[19].

Fraction III (32 mg) had the same chromatographic mobility as 2,4,6-tri-*O*-methyl-D-galactose (R_F 0.40, solvent D); it was crystallized from chloroform, and recrystallized from ethyl ether, to give crystals of m.p. 103–105°, undepressed on admixture with an authentic sample of 2,4,6-tri-*O*-methyl-D-galactose[20]. The c.d. spectrum of 2,4,6-tri-*O*-methylgalactitol triacetate was identical to that of a standard sample of 2,4,6-tri-*O*-methyl-D-galactitol triacetate.

Fraction IV had the same chromatographic mobility as 4,6-di-*O*-methyl-D-mannose (R_F 0.28, solvent *D*). Attempted crystallization of fraction IV was not successful and it was subsequently reduced, the alditol acetylated, and the acetate examined on column *a* (195°, helium flow-rate 71 ml/min), to give a component identical in retention time (26.1 min) and mass spectrum to that of the alditol acetate of authentic 4,6-di-*O*-methyl-D-mannose.

The acidic fraction (20 mg) was hydrolyzed in 2M hydrochloric acid on a steam bath for 8 h. After evaporation, the hydrolyzate was shown by paper chromatography to contain 4,6-di-*O*-methylmannose (R_F 0.28, solvent D) as the major, neutral component, together with small amounts of 2,3,6-tri-*O*-methylglucose (R_F 0.55, solvent D) and 2,4,6-tri-*O*-methylgalactose (R_F 0.40, solvent D), in addition to acidic components at the origin.

A separate portion of the acidic fraction (100 mg) was heated under reflux overnight with 3% methanolic hydrogen chloride (25 ml), to give the ester glycosides, which were reduced by refluxing with lithium aluminum hydride (0.5 g) in tetrahydrofuran (15 ml) for 4 h. The excess of lithium aluminum hydride was decomposed by adding ethyl acetate, the solution was evaporated to dryness, the residue was extracted with chloroform, the extract was evaporated, and the residue was hydrolyzed with 2M trifluoracetic acid. The hydrolyzate was examined by paper chromatography; this showed 4,6-di-*O*-methylmannose overlapped with another component (R_F 0.28, solvent D), 2,3,6-tri-*O*-methylglucose (R_F 0.55, solvent D), and 2,4,6-tri-*O*-methylgalactose (R_F 0.40, solvent D). No acidic components were observed at the origin. The hydrolyzate was subsequently reduced, the alditols acetylated, and the acetates examined by g.l.c. on column *a*, with the results shown in Table IB. The slowest component was characterized by the fragmentation pattern of its mass spectrum as 3,4-di-*O*-methylglucitol tetraacetate.

Reduction of the methylated polysaccharide. — To a solution of the methylated K62 (0.1 g) in tetrahydrofuran (30 ml) was added lithium aluminum hydride (0.16 g) in tetrahydrofuran (8 ml), and the mixture was boiled under reflux overnight. The reduction product (0.09 g) was heated with formic acid (90%) on a steam bath for 1 h, and the solution evaporated; the residue was further hydrolyzed with 2M trifluoroace-

tic acid for 4 h. Quantitative analysis of the derived alditol acetates by g.l.c. on columns *a* and *b* showed the components (listed in Table IC) to be present in equimolecular amounts. The identity of the methylated sugars was confirmed by mass spectrometry.

Partial hydrolysis of the capsular polysaccharide. — Autohydrolysis of the capsular polysaccharide was conducted at pH 3.5 and 100° for up to 37 h. D-Mannose (as the only neutral monosaccharide) and traces of oligosaccharides were found by paper chromatography in solvents A and C.

Polysaccharide (0.4 g) was hydrolyzed with M trifluoroacetic acid on a steam bath for 5 h. After evaporation, the hydrolyzate was found, by paper chromatography in solvent A, to contain mannose, glucose, galactose, glucuronic acid, and a component having the mobility of an aldobiouronic acid (R_{Man} 0.37). The hydrolyzate was separated into neutral and acidic fractions by using ion-exchange resins [Amberlite IR-120 (H^+) and Duolite A-4 (OH^-)]. The acidic fraction (100 mg) was applied to a column (110 × 2 cm) of Sephadex G 15 which was irrigated with water at a flow rate of ~5 ml/h. The eluate was collected in fractions (~2 ml), and the content of each tube was examined on paper in solvent C. The component corresponding to an aldobiouronic acid, A_2, (60 mg) was eluted at ~140 ml, followed by fractions containing a mixture of an aldobiouronic acid and glucuronic acid, and, finally, fractions containing only glucuronic acid (10 mg). The glucuronic acid was evaporated, with the addition of a few drops of concentrated hydrochloric acid. The c.d. curves of the isolated glucuronic acid and that of a standard of D-glucuronic acid were both positive ($\Delta\varepsilon_{219}^{MeCN}$ +2.82 and +3.32°, respectively).

In order to obtain oligosaccharides higher than the aldobiouronic acid (A_2), milder conditions of hydrolysis were used by heating the polysaccharide (0.8 g) in M trifluoroacetic acid for 1 h. The solution was concentrated, and evaporated three times with water, and the residue was fractionated on a charcoal–Celite column prepared by pouring a mixture of active carbon (30 g) and Celite (30 g) into a Büchner funnel (10 cm i.d.) to make a column 1.4 cm thick[22]. Monosaccharides were eluted by distilled water (2.8 liters), and a series of oligosaccharides was eluted with 20% aqueous ethanol (2 liters) and 10% aqueous isopropyl alcohol (500 ml); yield of oligosaccharides, ~0.34 g. The mixture of oligosaccharides was separated (100 mg per run) on a column (110 × 2 cm) of Sephadex G 15 into four fractions (R_{Man} 0.46, 0.19, 0.12, and 0.06, solvent C), namely di- (A_2, 4 mg), tri- (A_3, 52 mg), tetra-and penta-saccharides (50 mg), and A_6 (31 mg). A successful separation of the third fraction into tetrasaccharide (A_4 28 mg) and A_5 (13 mg) was achieved by prolonged paper-chromatography (7 days) in solvent C.

Examination of acidic oligosaccharides. — The data on the oligomers A_2 to A_4 and the mixture A_5 are given in Table II. These results were obtained as follows.

(*i*) *Hydrolysis.* Partial hydrolysis of A_3 with 0.2M trifluoroacetic acid for 4 h at 97° showed galactose and A_2; similarly A_4 gave glucose, galactose, A_3, and A_2. More-complete hydrolysis was achieved by using 2M HCl for 8 h at 97°. Chromatograms were obtained in solvents A and B, most conveniently by developing for several hours in B, drying, and redeveloping in A (see Table IIB). The hydrolyzates were

converted into methyl ester methyl glycosides, these were reduced with sodium borohydride in methanol, the products were hydrolyzed (M trifluoroacetic acid for 4 h at 97°), and the products analyzed by g.l.c. as their alditol acetates (see Table IIC). In the case of A_2 only, the acetates were collected, and characterized as mannitol hexaacetate (m.p., and mixed with D-mannitol hexaacetate, 120–122°) and glucitol hexaacetate (m.p., and mixed with D-glucitol hexaacetate, 95–97°).

(*ii*) *Methylation.* The oligomers were methylated by the method of Hakomori, and about one-third was hydrolyzed (2M HCl) and the hydrolyzate examined on paper (solvent D). The neutral sugar(s) are shown in Table IID and, in all cases, there was an acidic component having $R_F \sim 0.2$. For A_4, the whole sample was hydrolyzed, and only the neutral sugars were analyzed by g.l.c. of the alditol acetates. Otherwise, the remaining two-thirds of A_2 and A_3 were methylated with Purdie's reagents, the products reduced with sodium borohydride in methanol, and the products hydrolyzed. Paper chromatography (solvent D) was of little use, as 2,3,4-tri-*O*-methylglucose and 3,4,6-tri-*O*-methylmannose have similar mobilities, but it did permit the identification of the third sugar in A_3. Analysis of the reduced hydrolyzate as alditol acetates on column *a* (180°, He at 43 ml/min for A_2; 185°, He at 60 ml/min for A_3 and A_4) gave good separations, and the identity of each component was confirmed by mass spectrometry (see Table IIE). Satisfactory, quantitative relationships were obtained for A_2 to A_4, but A_5 was clearly a mixture.

(*iii*) *P.m.r. spectroscopy.* Each oligomer was exchanged twice with D_2O, and the spectrum was recorded for a solution in D_2O at 90°; relevant chemical shifts are shown in Fig. 1C,D,E.

Periodate oxidation. — Capsular polysaccharide (0.86 g) was dissolved in 0.05M sodium periodate (200 ml) in the dark at 1°. The uptake of periodate per sugar residue leveled off (at 0.82 mole) in 360 h. The excess of periodate and iodate ions was precipitated with barium hydroxide, and the mixture was centrifuged. The supernatant liquor was reduced (sodium borohydride), and decationized (Amberlite IR-120), evaporated to dryness, and the residue treated by three additions and evaporations of methanol. The yield of polyalcohol was 0.442 g.

The polyalcohol (15 mg) was hydrolyzed with 2M hydrochloric acid for 6 h at 97°. Paper chromatography in solvent A showed galactose, mannose (R_{Gal} 1.24), erythritol (R_{Gal} 1.97), and glycerol (R_{Gal} 2.34). Analysis of the alditol acetates by g.l.c. on column *a* programmed from 150° at 3°/min, with a helium flow-rate of 50 ml/min, gave four peaks identical in retention times to the acetates of authentic glycerol (8.8 min) and erythritol (19.0 min, m.p. and mixed m.p. 80–82°), and the alditol acetates from D-mannose (41.0 min, m.p. and mixed m.p. 120–122°), and D-galactose (43.9 min, m.p. and mixed m.p. 160–162°) in the ratios of 1:1:1:1.

A solution of the polyalcohol was acidified with Amberlite IR-120 (H^+), and evaporated at 30°; the product showed, by paper chromatography in solvent A, a component having R_{Gal} 0.53. A solution of the polyalcohol in 2M trifluoroacetic acid at room temperature showed an increased release of glycerol and erythritol, together with an oligosaccharide having R_{Gal} 0.53 (solvent A). A solution of the polyalcohol

(0.11 g) was acidified with Amberlite IR-120 (H^+), and concentrated; the concentrate was passed through a column (110 × 2.0 cm i.d.) of Sephadex G 15 at a water flow-rate of ~4 ml/h. Besides higher oligomers, a disaccharide glycoside [24 mg, $[\alpha]_D$ +52.2° (*c* 4.48, water)] and glycerol (14.3 mg) were collected. The identity of glycerol was confirmed by the retention time of the acetate in g.l.c. (column *a*).

Paper chromatography of the hydrolyzate of the disaccharide glycoside gave galactose, mannose, and erythritol (solvent A). G.l.c. analysis of the alditol acetates on column *a* (programmed from 200° at 1°/min, with a helium flow-rate of 50 ml/min) gave peaks identical to those for erythritol tetraacetate (8.4 min, m.p. and mixed m.p. 80–82°), D-mannitol hexaacetate (39.9 min, m.p. and mixed m.p. 121–123°), and galactitol hexaacetate (43.5 min, m.p. and mixed m.p. 160–162°).

The p.m.r. spectrum of the disaccharide glycoside (D_2O, 95°) showed signals of anomeric protons at τ 5.40 ($J_{1,2}$ 7 Hz, 1 H) and 4.91 (1 H); see Fig. 1F.

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