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THE MOLECULAR STRUCTURE OF THE CAPSULAR POLYSACCHARIDE FROM *Klebsiella* TYPE 27

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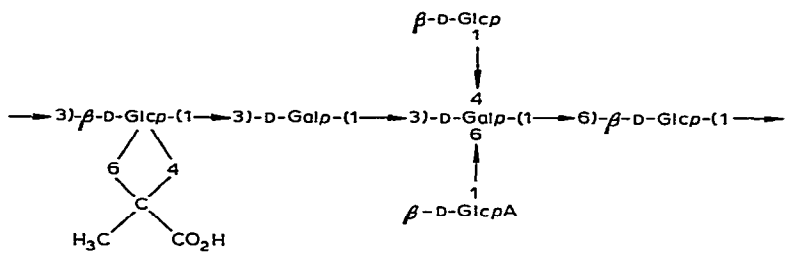
ABSTRACT

The capsular polysaccharide of *Klebsiella* serotype K27 has been investigated by techniques involving methylation analysis, autohydrolysis, and graded hydrolysis with acid. The anomeric configurations of the sugar constituents were determined, where possible, on the basis of p.m.r. spectroscopy and optical rotation data. The results of these studies are consistent with a primary structure in which the repeating-unit is the doubly branched hexasaccharide:



INTRODUCTION

The group of *Klebsiella* K-serotypes that produce capsular polysaccharides consisting of residues of D-glucuronic acid, D-galactose, and D-glucose includes *Klebsiella* type 27 and six other strains. Branched-tetrasaccharide repeating-units have been found in the polysaccharides from K8[1], K11[2], K25[3], and K51[4]; the primary structures of the remaining polysaccharides of this chemotype are under investigation[5]. We now report studies of the polysaccharide from *Klebsiella* K27, which contains a 1-carboxyethylidene group (pyruvic acid acetal) in addition to the three types of sugar.

RESULTS AND DISCUSSION

The acidic polysaccharide (*A*), obtained from a culture (N.C.T.C. 9147), had $[\alpha]_D +5°$, $\overline{M}_w$ 940,000, and was monodisperse according to gel-permeation chromatog-

TABLE I

PARTIAL HYDROLYSIS OF *Klebsiella* K27 CAPSULAR POLYSACCHARIDE IN 0.01M TRIFLUOROACETIC ACID

Time (h)	*Molecular-weight[a] distribution*	$\bar{M}_w$[c]	$[\alpha]_D$[d] *(degrees)*
0	940,000[b]	940,000	+5
1	720,000 (92); $\leqslant 10^4$ (8)	720,000	+8
4	450,000 (84); $\leqslant 10^4$ (16)	450,000	+10
7	n.d.	n.d.	+12
24	15,000 (28); 10,000 (8); 6,000 (20); 4,000 (21); 2,000 (11); 1,000 (3); $\leqslant$ 300 (9)	4,900	+17
48	6,000 (9); 5,000 (6), 4,000 (8); 3,000 (16); 2,000 (15); 1,000 (30); $\leqslant$ 300 (16)	2,700	+22

[a]By gel-permeation chromatography on 6% agarose (0–7 h) or Bio-Gel P-10 (24 h, 48 h); proportions by weight given in parentheses after molecular weights corresponding to peaks in chromatogram. [b]Single peak. [c]Of portion within fractionation range of gel only. [d]Concentration, 0.48%.

raphy and ultracentrifugation. The p.m.r. spectrum of the polysaccharide showed the presence of one pyruvic acid acetal group and six anomeric protons; no acetyl groups were detected. The neutral sugar components were found by g.l.c. analysis of an hydrolysate to be galactose and glucose in the molar ratio 2:3. D-Configurations were assigned to these sugars by the procedures outlined later.

Graded hydrolysis with acid; molecular weight distribution. — On graded hydrolysis with acid, monitored by paper chromatography, glucose and pyruvic acid were the first components to be released, followed more slowly by galactose and two neutral disaccharides. Glucuronic acid and several other acidic products of higher molecular weight were then detected, including an aldobiouronic acid that was chromatographically indistinguishable from 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose.

Gel-permeation chromatography of samples removed at intervals during mild hydrolysis with acid showed that the breakdown of the polysaccharide was slow in comparison with that of most of the other *Klebsiella* polysaccharides that have been examined by this method, the rate being comparable with that for the K64 polysaccharide[6]. Gel-permeation chromatography on agarose revealed a slow decrease in $\bar{M}_w$ to 450,000 during the first 4 h of hydrolysis in 0.01M acid at 100°; after hydrolysis for 24 h and more, Bio-Gel P-10 chromatograms of the products (see Table I) showed multiple peaks, at elution volumes corresponding to a molecular weight of 1000 and integral multiples thereof (up to 10,000). This result indicates the presence of a repeating unit having a molecular weight of ~1000 (*i.e.*, a hexasaccharide).

TABLE II

FURTHER HYDROLYSIS OF DEGRADED *Klebsiella* K27 POLYSACCHARIDE IN 0.1M TRIFLUOROACETIC ACID

Time (h)	*Molecular-weight[a] distribution (%)*	$\bar{M}_w$[c]	$[\alpha]_D$[d] *(degrees)*
1	5,000 (2); 4,000 (3); 3,000 (6); 2,000 (7); 1,000 (35); 660 (11); 520 (10); ⩽ 300 (26)	1,400	+25
3	2,000 (9); 1,000 (36); 660 (21); 520 (8); ⩽ 300 (26)	980	+28
5	1,000 (31); 660 (23); 520 (12); ⩽ 300 (34)	690	+29
24	[b]	[b]	+40

[a]By chromatography on Bio-Gel P-10. [b]Di- and mono-saccharides only. [c]Of portion within fractionation range of gel. [d]Concentration, 0.46%.

TABLE III

METHYL ETHERS FROM THE HYDROLYSATES OF THE ORIGINAL (*A*) AND DEGRADED (*B*) POLYSACCHARIDES FROM *Klebsiella* K27

Sugar[a]	T[b]	*Molar proportion (%)*	
		A[c]	B[c]
2,3,4,6-Glc	1.01	20	19
2,4,6-Gal	1.19	22	19
2,4,6-Glc	1.34	—	18
2,3,4-Glc	1.62	19	19
2,4-Gal	1.80	—	7
2-Gal	2.48	19	18
2-Glc	2.76	20	—

[a]2,3,4,6-Glc = 2,3,4,6-tetra-*O*-methyl-D-glucose, *etc.* [b]Retention time of the corresponding (Me_3Si) alditol relative to that of 2,3,4,6-tetra-*O*-methyl-D-galactose on an SE-52 column at 140°. [c]Hydrolysates examined after hydrolysis for 18 h.

After hydrolysis of the polysaccharide for 48 h in 0.01M acid, the product having molecular weight 1000 was present in the hydrolysate to the extent of 30% by weight, but 54% of the mixture consisted of material of molecular weight 2000–6000 ($\bar{M}_w$ 2700). The products having molecular weights >1000 disappeared only after further hydrolysis in 0.1M acid for 5 h, at which stage much of the hexasaccharide had broken down to lower oligosaccharides (Table II), so that its proportion remained at only ~30% by weight under these conditions.

Autohydrolysis of the capsular polysaccharide. — Autohydrolysis of polysaccharide *A* yielded pyruvic acid and traces of glucose, which were separated by dialysis from the polysaccharide product *B*, $[\alpha]_D$ +2°, $\overline{M}_w$ 840,000 (by gel-permeation chromatography), the p.m.r. spectrum of which showed the absence of pyruvic acid acetal.

Methylation analysis of polysaccharides A *and* B. — Samples of polysaccharides *A* and *B* were methylated and hydrolysed, and the sugars present in the hydrolysates were analysed by paper chromatography and by g.l.c.[7]. The results are given in Table III.

It is evident that the number of branch-points in the structure cannot be accounted for solely by the D-glucosyl groups as indicated by these analyses. The identification of 2,4,6-tri-*O*-methyl-D-galactose and 2,3,4-tri-*O*-methyl-D-glucose shows that these residues represent 3- and 6-linked sugars in the chain, while the small proportion of 2,4-di-*O*-methyl-D-galactose formed from the degraded polysaccharide *B* is commensurate with loss of some D-glucosyl end-groups during the autohydrolysis, and is evidence that these are linked to O-4 of D-galactose. This same D-galactosyl residue must be linked to glucuronic acid through O-6, and through O-3 in the polysaccharide chain, in order to account for the 2-*O*-methyl-D-galactose found in both hydrolysates. The formation of 2,4,6-tri-*O*-methyl-D-glucose from polysaccharide *B* indicates the removal, during autohydrolysis, of pyruvic acid from O-4 and O-6 of a D-glucosyl residue, which is also linked through O-3 in the polysaccharide chain.

Confirmation that the glucuronic acid residues are in a terminal position was obtained by methanolysis of fully methylated K27 polysaccharide followed by g.l.c. of the resulting methyl glycosides[8]. In addition to the neutral sugars given in Table III, there were two peaks corresponding to methyl 2,3,4-tri-*O*-methylglucuronate. The glucuronic acid groups were shown to have the D configuration by the procedure outlined later.

Partial hydrolysis of polysaccharide B *with acid; isolation and characterisation of oligosaccharides.* — Hydrolysis of a sample of polysaccharide *B* yielded a number of oligosaccharides (**1**–**8**), which were isolated by preparative paper chromatography and examined by gel-permeation chromatography on Bio-Gel P-10. Of the acidic oligosaccharides isolated, one fraction (**8**) contained two components, having molecular weights 3000 (30% by weight) and 2000, respectively; the other fractions each gave a single peak, at molecular weights 1000 (*i.e.*, the structurally significant hexasaccharide), 850, 660, 520, and 360. These fractions resemble the components referred to earlier (Table II), with the addition of the one of molecular weight 850, which was not observed for polysaccharide *A*. The two neutral components were disaccharides (molecular weight, 340), as indicated also by their mobilities in paper chromatography. Where possible, the structures of oligosaccharides **1**–**8** were determined by standard techniques, as described later (Table IV).

Neutral disaccharide **1** was indistinguishable in paper chromatography from 3-*O*-β-D-glucopyranosyl-D-glucose, in three different solvent systems (*A*, *B*, and *C*),

TABLE IV

ANALYSIS OF THE OLIGOSACCHARIDES FROM AUTOHYDROLYSED POLYSACCHARIDE *B* FROM *Klebsiella* K27

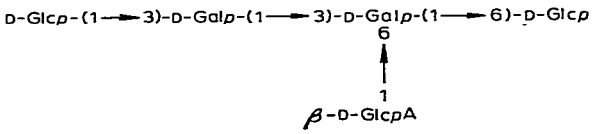
Fraction	*Oligomer*	$[\alpha]_D$ *(degrees)*	R_{GLC} *(Solvent A)*	*Mol. wt.*	*Neutral sugars (molar proportions)*	*Methylated (Me_3Si) alditols (molar proportions)*
1	Neutral disaccharide	+16	0.73	340	Glc	2,3,4,6-Glc (1.0) 2,4,6-Glc (1.0)
2	Neutral disaccharide	+22	0.59	340	Glc (1.0) Gal (1.0)	2,3,4,6-Glc (1.0) 2,4,6-Gal (1.0)
3	Aldobiouronic acid	−7	0.40	360	Gal	2,3,4-Gal
4	Aldotriouronic acids	+5	0.23	520	Glc (1.0) Gal (0.8)	Indicated that fraction was a mixture
5	Aldotetraouronic acids	+4	0.13	660	Glc (2.0) Gal (1.8)	Indicated that fraction was a mixture
6	Acidic pentasaccharide	+16	0.07	850	Glc (2.0) Gal (1.8)	2,3,4,6-Glc (1.0) 2,4,6-Gal (0.8) 2,3,4-Glc (0.7) 2,4-Gal (0.7)
7	Acidic hexasaccharides	+6	0.03	1000	Glc (3.0) Gal (2.5)	2,3,4,6-Glc (1.0) 2,4,6-Gal (1.1) 2,4,6-Glc (0.5) 2,3,4-Glc (0.7) 2,4-Gal (0.4)
8	Acidic oligosaccharides	+18	0.00	2000 (70%) + 3000 (30%)	Glc (3.0) Gal (2.6)	2,3,4,6-Glc (1.0) 2,4,6-Gal (1.1) 2,4,6-Glc (0.6) 2,3,4-Glc (0.8) 2,4-Gal (0.4) 2-Gal (0.6)

and the optical rotation and paper-chromatographic mobility of neutral disaccharide **2** were in good agreement with those reported[9] for 3-*O*-β-D-glucopyranosyl-D-galactose. The optical rotations of the aldobiouronic acid **3** and of its barium salt ($[\alpha]_D$ −3°) accorded with those reported for 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose[10]; this structural unit is common in plant gums, but has not been reported previously in work on *Klebsiella* polysaccharides.

The non-integral value obtained for the molar proportion of D-galactose in the hydrolysates of the acidic oligosaccharides after treatment for 8 h with 2M trifluoroacetic acid at 100° is consistent with this hexose's being part of the aldobiouronic acid. The more vigorous conditions (2M trifluoroacetic acid, 100°, 18 h) used in acid hydrolysis of polysaccharide *A* gave the proportions of neutral sugars reported earlier.

The methylation analyses of the fractions **4** and **5** (molecular weights 520 and 660, respectively) indicated that they were mixtures of linear and branched oligosaccharides, which were not fully characterised.

The methylation analysis of fraction **6** is consistent with the acidic pentasaccharide structure shown, and the methylated sugars obtained on hydrolysis of the fully methylated fraction **7** indicate that both structures **7a** and **7b** could be present.



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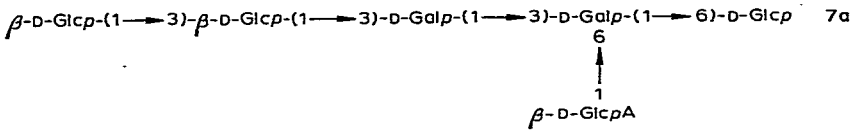
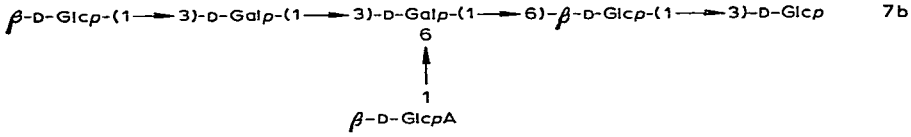


7a



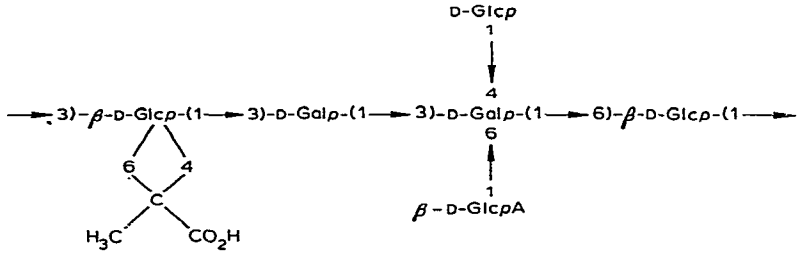
7b

Methylation analysis of oligosaccharides **8** shows that a large number of the D-glucosyl groups linked to O-4 of D-galactosyl residues in the polysaccharide chain are not removed under the conditions of acid hydrolysis used to generate the above oligosaccharides. The low proportion of 2,4-di-*O*-methyl-D-galactose formed from the higher oligosaccharides further confirms that this D-galactosyl residue is linked to D-glucuronic acid, through O-6, to form the aldobiouronic acid **3**.

Postulated structure of repeating unit in Klebsiella *K27 capsular polysaccharide.*— The methylation analyses show that the repeating unit in the K27 capsular polysaccharide consists of chain residues of 3- and 6-linked D-glucose and of 3-linked D-galactose, to which are attached terminal units of D-glucuronic acid, pyruvic acid, and D-glucose. Consideration of these results, together with the structures of the oligosaccharides mentioned above, particularly 3-*O*-β-D-glucopyranosyl-D-glucose, shows that, in order to obtain this neutral disaccharide and 3-*O*-β-D-glucopyranosyl-D-galactose, the polysaccharide chain must contain the sequence of sugar residues →3)-β-D-Glc*p*-(1→3)-D-Gal*p*-(1→3)-D-Gal*p*-(1→6)-β-D-Glc*p*-(1→.

The pyruvic acid acetal has been shown to be attached to the only 3-linked D-glucosyl residue, but the terminal units of D-glucuronic acid and D-glucose could be linked to either one of the D-galactosyl residues in the chain. However, since neutral disaccharides were obtained having (1→3) linkages between glucose and glucose and between glucose and galactose, but not between galactose and galactose, it appears that the D-galactosyl residue carrying the terminal groups occurs directly before the 6-linked glucosyl residue in the polysaccharide chain. On the basis of the

foregoing argument, **9** represents the sugar sequence present in the structure of the *Klebsiella* K27 polysaccharide.



9

The nature of certain anomeric linkages in **9** could not be determined by consideration of the oligosaccharides **1–8**. P.m.r. spectroscopy of the original (*A*) and degraded (*B*) polysaccharides revealed only one anomeric signal corresponding to an α-D linkage, the remainder being indicative of β-D linkages. This α-D linkage may be assigned to one of the D-galactosyl chain-residues or to the D-glucosyl end-group in the repeating sequence. In order to assign these linkages unambiguously, it is necessary to isolate the pure acidic tri- and tetra-saccharide components liberated by partial hydrolysis of polysaccharide *B* with acid; however, attempts to fractionate the mixtures **4** and **5** were not successful.

Comparison with other Klebsiella *capsular polysaccharides.* — Of the structures known for *Klebsiella* polysaccharides, that of K64[11], which has a hexasaccharide repeating-unit with a double branch on a D-mannosyl residue, is the closest analogue to that of K27, although recent studies have shown[12] that the pentasaccharide repeating-unit of the polysaccharide from K33 contains a D-mannosyl residue that is substituted at O-3, O-4, and O-6. The rates of degradation of both the K27 polysaccharide and that from K64 under conditions of mild, acid hydrolysis are comparatively low; acidic oligosaccharides of fairly high molecular weight (from K27, 1000 and 850; from K64, 1100 and 930) are thereby released, together with two neutral disaccharides which have proved crucial in formulating the repeating units. Both polysaccharides contain the structural unit 4,6-*O*-(1-carboxyethylidene)-D-glucose, which forms a side chain in the case of the K64 polysaccharide. Autohydrolysis of both polysaccharides yields pyruvate-free products having high d.p. The only major differences in the general structural patterns of the K27 and K64 polysaccharides are the positions of the pyruvic acid and D-glucuronic acid groups.

EXPERIMENTAL

General methods. — Paper chromatography (p.c.) was performed with Whatman No. 1 paper and the following solvent systems (all v/v): *A*, 1-butanol–acetic acid–water (2:1:1); *B*, 1-butanol–ethanol–water (4:1:5, upper phase); *C*, ethyl

acetate–pyridine–water (8:2:1); *D*, ethyl acetate–acetic acid–formic acid–water (18:3:1:4); and *E*, butanone–water azeotrope. Detection was effected by heating the papers at 110° for 5–10 min after spraying with *p*-anisidine hydrochloride in aqueous 1-butanol or *o*-phenylenediamine in aqueous trichloroacetic acid (for pyruvic acid)[13]. Other general methods and methylation analysis were as previously described[11].

Properties of Klebsiella *K27 capsular polysaccharide.* — The capsular polysaccharide (*A*), isolated and purified as described elsewhere[6], had $[\alpha]_D^{20}$ +5° (*c* 0.7) (Found: N, 0.4%) and moved as a single component on ultracentrifugation and gel-permeation chromatography on agarose. The p.m.r. spectrum of a 2% solution of the polysaccharide in D_2O showed a sharp singlet at δ 1.52 (Me of the pyruvic acid acetal group), and signals for 6 anomeric protons[14], corresponding to one α linkage at δ 5.0 (*J* 3 Hz) and five β linkages at δ 4.38–4.86. The chemical shifts and coupling constants of the β linkages could not be assigned to specific residues, because of the close proximity of the signals.

Hydrolysis of polysaccharide A. — (*a*) After hydrolysis (2M trifluoroacetic acid, 100°, 18 h) of polysaccharide *A*, p.c. (solvents *A* and *C*) showed the presence of D-galactose, D-glucose, D-glucuronic acid, and an aldobiouronic acid. Analysis of the neutral sugars present was carried out by g.l.c. of the derived alditol acetates[15]. Preparative g.l.c. of the acetates allowed recovery of the individual compounds and identification of D-glucitol hexa-acetate by comparison of its circular dichroism spectrum with that of a standard sample. The D-galactose derivative was shown to be achiral by this method. After isolation of D-galactose from the hydrolysate by preparative p.c. (solvent *C*), methylation, hydrolysis, and reduction with sodium borohydride gave 2,3,4,6-tetra-*O*-methyl-D-galactitol (identified by the g.l.c. retention time of the trimethylsilyl derivative). Acetylation yielded 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-galactitol, which was purified by preparative g.l.c. In acetonitrile solution, this derivative gave a positive c.d. curve, identical to that of the authentic compound, thus confirming the D configuration[16] of the parent sugar.

(*b*) Graded acid hydrolysis of polysaccharide *A* was performed as previously described[6].

(*c*) *Autohydrolysis.* A solution of polysaccharide *A* (180 mg) in distilled water (3 ml) was heated in a sealed tube under nitrogen for 3 h at 100°. P.c. (solvent *D*) of the concentrated solution showed a fast-moving spot, chromatographically identical with pyruvic acid and having the same characteristic fluorescence as the authentic material when sprayed with *o*-phenylenediamine and examined under ultraviolet light. Detection with *p*-anisidine showed the presence of D-glucose and a large amount of material at the origin. The autohydrolysate was diluted with water and dialysed against distilled water (3 litres) for 24 h. The non-dialysable material (132 mg) was recovered by freeze-drying of the aqueous solution. A sample of the polysaccharide *B* obtained by this process was methylated (Hakomori method)[17].

Methylation analyses. — Samples of the fully methylated polysaccharides *A* and *B* were hydrolysed with 2M trifluoroacetic acid at 100° for 18 h. The hydrolysates

were chromatographed on paper (solvents *B* and *E*), and analysed by g.l.c. of the trimethylsilylated, derived alditols. The sugars in the hydrolysates were converted into their methylated alditol acetates and recovered by preparative g.l.c. The identity of each acetate was confirmed by mass spectrometry.

Partial hydrolysis of polysaccharide B. — Polysaccharide *B* (100 mg) was treated with 0.1M trifluoroacetic acid (5 ml) for 3 h at 100°. The structures of the oligosaccharides obtained were investigated by partial hydrolysis with acid and methylation analysis, optical rotatory power being used to assign the configurations of anomeric linkages. The results obtained are shown in Table IV.

After hydrolysis (2M trifluoroacetic acid, 100°, 8 h) of the acidic, higher oligosaccharides **4**–**8**, p.c. of each hydrolysate (solvents *A* and *C*) revealed D-galactose, D-glucose, D-glucuronic acid, and a component having the same mobility as 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose. The proportions of the neutral sugars present in the hydrolysates were determined by g.l.c. (alditol acetate method). The lower oligosaccharides **1**–**3** were characterised as follows:

Methanolysis of the fully methylated disaccharide **1** gave (g.l.c. analysis) the methyl glycosides of 2,3,4,6-tetra-*O*-methyl-D-glucose and 2,4,6-tri-*O*-methyl-D-glucose. The identities of these sugars were confirmed by hydrolysis of the methylated disaccharide, followed by g.l.c. of the trimethylsilyl derivatives of the anomers of the tetra- and tri-*O*-methyl sugars.

After reduction of disaccharide **2** with sodium borohydride, hydrolysis (p.c., solvent *C*) gave D-glucose only. Methanolysis of the fully methylated disaccharide gave (g.l.c.) the methyl glycosides of 2,3,4,6-tetra-*O*-methyl-D-glucose and 2,4,6-tri-*O*-methyl-D-galactose.

Methanolysis of the fully methylated aldobiouronic acid **3** gave (g.l.c.) the methyl glycosides of methyl 2,3,4-tri-*O*-methyl-D-glucuronate (removed by saponification) and 2,3,4-tri-*O*-methyl-D-galactose.

Methylated **3** (6 mg) was reduced[18] with lithium aluminium hydride (20 mg) in tetrahydrofuran (5 ml) for 18 h. After addition of aqueous ethanol and evaporation to dryness, the residue was extracted with chloroform (3 × 5 ml); the extracts were combined, the chloroform was evaporated, the residue was hydrolysed, and the resulting sugars were converted into their derived alditol acetates. Analysis by g.l.c. then revealed products derived from 2,3,4-tri-*O*-methyl-D-glucose and 2,3,4-tri-*O*-methyl-D-galactose in approximately equal proportions. Recovery of the glucose derivative by preparative g.l.c., followed by demethylation[19], gave glucitol, which was shown to have the D configuration by the c.d. spectrum of its hexa-acetate. Since the 2,3,4-tri-*O*-methyl-D-glucose was derived from 2,3,4-tri-*O*-methylglucuronic acid, the acid residues in the methylated aldobiouronic acid must have the D configuration.

The p.m.r. spectrum of the barium salt of **3** showed anomeric signals at δ 5.22 (*J* 3 Hz), 4.64 (*J* 8 Hz), and 4.45 (*J* 8 Hz), consistent with those expected from α- and β-D-galactose and β-D-glucuronic acid moieties.

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