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AN ARABINOGLUCURONOMANNAN FROM SUSPENSION-CULTURED CELLS OF *Nicotiana tabacum*

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ABSTRACT

From the soluble polysaccharides of suspension-cultured, tobacco cells (*Nicotiana tabacum*), an arabinoglucuronomannan (AGM) was isolated from the extract with phosphate buffer. Application of partial hydrolysis with acid, carboxyl reduction, methylation analysis, and ^{1}H- and ^{13}C-n.m.r. spectroscopy indicated AGM to consist of the tetrasaccharide repeating-unit

$$\begin{array}{ccc} \rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{A-}(1\rightarrow 2)\text{-}\alpha\text{-D-Man}p\text{-}(1\rightarrow . \\ \quad 3 \qquad\qquad\quad 3 \\ \quad\uparrow \qquad\qquad\quad \uparrow \\ \quad 1 \qquad\qquad\quad 1 \\ \alpha\text{-L-Ara}f \qquad\quad \alpha\text{-L-Ara}f \end{array}$$

INTRODUCTION

Polysaccharides that are easily extractable with water or buffer are present in many higher plants: arabinans[1,2], arabinogalactans, and arabinogalactan–proteins[3] have been isolated and characterised. We have reported[4,5] on some of the extracellular polysaccharides (ECP) secreted into the medium of suspension-cultured cells of tobacco (*Nicotiana tabacum*). We now report on the polysaccharides which can be solubilised from the cells with neutral buffer of high ionic strength, and on a structural investigation of one of these polysaccharides, namely, an arabinoglucuronomannan.

RESULTS AND DISCUSSION

Extraction of the suspension-cultured cells. — Fresh cells were extracted with 0.1M phosphate buffer containing 0.4M potassium chloride, and the extract was eluted from DEAE-cellulose (AcO^- form), stepwise with water, 0.1, 0.25, 0.5, and M sodium acetate, and 0.1M sodium hydroxide. The yields and analyses of some of the fractions are listed in Table I; the fractions eluted with M sodium acetate and 0.1M sodium hydroxide had low contents of carbohydrate.

TABLE I

DATA ON DEAE-CELLULOSE FRACTIONS OF THE BUFFER EXTRACT[a,b]

Fractions	*Wt.*[a] *(mg)*	*Protein (%)*	*Uronic acid (%)*	*Carbo-hydrate (%)*	*Neutral-sugar composition (mole %)*					
					Rha	*Ara*	*Xyl*	*Man*	*Gal*	*Glc*
Extract	2820.0	26.0	7.9	38.5	3.4	50.4	7.8	9.6	23.6	5.3
Water eluate	74.0	5.2	2.1	49.1	—	50.9	—	2.2	42.1	4.8
0.1M buffer eluate	790.3	23.5	4.8	51.9	5.4	45.6	2.7	—	46.2	—
0.25M	639.2	5.5	9.4	34.5	7.3	48.5	5.7	26.9	8.7	2.8
0.5M	324.5	6.8	7.1	34.3	—	55.0	2.4	34.2	4.5	3.9

[a]From 3 kg of fresh, wet cells. [b]See Experimental for details of analytical procedures.

The buffer extracted 2.6% of the weight of the cells, and the material contained protein[6] (26%) which could have arisen from cytoplasmic materials. The extracted material was rich in arabinose (50.4%), and the molar ratio of arabinose and galactose was 2.1 : 1. Hori *et al.*[7] extracted an arabinogalactan (–protein) with buffer from suspension-cultured, tobacco cells that contained arabinose and galactose in the ratio 0.8 : 1. By comparing these data, the presence of an arabinose-rich polysaccharide, which differed from arabinogalactan, in the buffer extract was suggested. Mannose (9.6%), which rarely appears in suspension-cultured cells, was also present.

The neutral fraction contained no xylose and only a small proportion of glucose, indicating the absence of starch and xyloglucan, in contrast to the extracellular polysaccharides which contain considerable proportions of xyloglucan[8].

The fraction eluted with 0.1M sodium acetate was mainly composed of the typical acidic arabinogalactan. The fractions eluted with 0.25 and 0.5M sodium acetate contained ~50% of arabinose, relatively little galactose, and considerable proportions of mannose.

Purification of the fractions eluted with 0.25 and 0.5M sodium acetate. — The 0.25M-fraction (639.2 mg) was deproteinised by partition[9] between phenol and 0.1M borate buffer (pH 9.8), and then eluted from DEAE-Sephadex A-50 (Cl^- form) by using a linear gradient of sodium chloride. A single, major peak was eluted at 0.33M salt and there were small peaks due to the contaminants.

Because the 0.5M-fraction (324.5 mg) showed the same behaviour as the 0.25M-fraction in ion-exchange chromatography and electrophoresis, the two fractions were combined.

The purified, acidic polysaccharide (AGM, 300 mg) had $[\alpha]_D^{23}$ +16° (*c* 0.55); it was homogeneous by electrophoresis, and gel-permeation chromatography revealed a single peak corresponding to a molecular weight of 5×10^5. AGM contained 33% of uronic acid (3-hydroxybiphenyl method[10]) and, on complete hydrolysis with acid, yielded arabinose, mannose, glucuronolactone, and galactose in the molar ratios 59.5 : 15.5 : 22.3 : 2.7 (dithioacetal method[11], Table II).

TABLE II

COMPONENT-SUGAR ANALYSIS[a] OF ORIGINAL AND MODIFIED ARABINOGLUCURONOMANNAN

	Mole %			
	AGM[b]	*AGM-R*[c]	*GM*[d]	*GM-R*[e]
Arabinose	59.5	49.6	—	—
Glucuronic acid[f]	22.3	14.4	51.8	22.2
Mannose	15.5	23.4	48.2	51.0
Galactose	2.7	2.3	—	—
Glucose	—	10.3	—	26.8

[a]Determined by the diethyl dithioacetal method (see Experimental). [b]Arabinoglucuronomannan [c]Carboxyl-reduced arabinoglucuronomannan. [d]Glucuronomannan main-chain. [e]Reduced glucuronomannan. [f]Corrected values (see Experimental).

TABLE III

METHYLATION ANALYSIS OF ORIGINAL AND MODIFIED ARABINOGLUCURONOMANNAN

Methylated sugars[a]	T[b]		*Mole %*			
	Column A (OV-101)	*Column B (SP-1000)*	*AGM*	*AGM-R*	*GM*	*GM-R*
2,3,5-Ara	0.71	0.60	53.4	51.4	—	—
2,3,4,6-Gal	1.00	1.00	2.4	2.3	—	—
3,4,6-Man	1.11	1.24	8.9	7.7	100.0	74.0
4,6-Man	1.21	1.54	21.9	22.1	—	—
2,3,6-Glc	1.13	1.34	—	3.4	—	26.0
2,6-Glc	1.20	1.57	—	6.0	—	—
2-Glc	1.29	1.95	13.4	7.1	—	—

[a]2,3,5-Ara = 1,4-Di-*O*-acetyl-2,3,5-tri-*O*-methylarabinose, *etc.* [b]Retention times relative to that of 2,3,4,6-tetra-*O*-methyl-D-galactitol diacetate.

Methylation analysis of AGM (Table III) revealed terminal arabinofuranosyl (2,3,5-Me_3-Ara), 2- and 2,3-substituted mannopyranosyl (3,4,6-Me_3- and 4,6-Me_2-Man), and terminal galactopyranosyl (2,3,4,6-Me_4-Gal) residues in the molar ratios 53.4:8.9:21.9:2.4. Methylation analysis, after carboxyl-reduction[12], revealed 4- and 3,4-substituted glucopyranosyl (2,3,6-Me_3- and 2,6-Me_2-Glc) residues, which were derived from the glucosyluronic acid residues of AGM.

Since all of the arabinosyl residues were terminal and furanosidic, these units could be removed preferentially[13] with 0.05M trifluoroacetic acid (TFA) at 100° for 90 min. Elution of the hydrolysate from Bio-Gel P-2 gave a monosaccharide fraction containing mainly arabinose and a trace of galactose. The optical rotation established that the arabinose was L.

The material in the void volume contained glucuronic acid and mannose in the molar ratio 1 : 1, suggesting that the main chain of AGM was a glucuronomannan (GM). Methylation analysis, before and after carboxyl-reduction, showed that GM contained 2-substituted mannosyl and 4-substituted glucosyluronic acid residues.

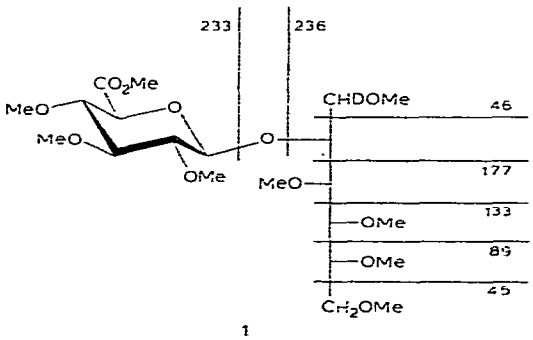
Hydrolysis of GM, followed by ion-exchange and cellulose-column chromatography, gave mannose and glucuronic acid, the optical rotations of which indicated each to be D.

Thus, AGM possesses a main chain consisting of (1→4)-linked glucosyluronic acid and (1→2)-linked mannosyl residues, most of which are substituted at O-3 with arabinofuranosyl residues, but some with galactopyranosyl residues.

In the methylation analyses (Table III) of the AGM and the carboxyl-reduced (AGM-R), a 2-*O*-methylhexose derivative was detected in considerable proportions, the alditol acetate of which could be distinguished from 2-*O*-methylmannitol pentaacetate[14] by g.l.c. Thus, it may have the *gluco* configuration and have been formed from a 3,4-disubstituted glucopyranosyluronic acid residue.

Isolation and characterisation of aldobiouronic acid. — AGM was partially hydrolysed with M TFA at 120° for 1 h. The acidic products contained (t.l.c.) mainly one saccharide, which was purified by chromatography on cellulose. Acid hydrolysis of the saccharide gave equimolar amounts of glucuronic acid and mannose, and borohydride reduction followed by hydrolysis converted all of the mannose into mannitol. Thus, the saccharide was an aldobiouronic acid (glucosyluronic acid–mannose).

The aldobiouronic acid was reduced with sodium borodeuteride, and then methylated and subjected to g.l.c.–m.s. The mass spectrum contained peaks for the glucopyranosyluronic acid (*m/z* 233) and mono-substituted mannitol (*m/z* 236) residues. Consideration of the deuterium-labelled (*m/z* 46) and other fragments (*m/z* 45, 89, 133, and 177) indicated[15,16] that the mannitol residue was linked through O-2 (see **1**).



1

The ^{1}H-n.m.r. spectrum of the reduced aldobiouronic acid showed a single anomeric-proton signal at δ 4.65, the $J_{1,2}$ value (7.3 Hz) of which demonstrated that the D-glucosyluronic acid residue had the β configuration. Thus, the aldobiouronic

TABLE IV

^{13}C-N.M.R. DATA[a] OF ARABINOGLUCURONOMANNAN, GLUCURONOMANNAN, AND THE ALDOBIOURONIC ACID

	Chemical shifts (p.p.m.)		$[^1J_{C-1,H-1}$ *(Hz)*$]$	
	C-6	*C-1*		
	GlcpA	*Araf*	*GlcpA*	*Manp*
Arabinoglucuronomannan	176.0	110	103.5	100.3
Glucuronomannan[b]	173.5	—	103.3 [160]	100.4 [170]
Aldobiouronic acid	174.9	—	102.7	93.0

[a]See Experimental. [b]Esterified by diazomethane, to increase the solubility.

acid was 2-*O*-(β-D-glucopyranosyluronic acid)-D-mannose. This aldobiouronic acid has been isolated from various sources[17-19] and its ^{1}H-n.m.r. spectrum was consistent with the literature data[18,19]. The isolation of a single aldobiouronic acid in high yield suggests that the dimer may be the repeating unit of the main chain.

^{13}C-N.m.r. spectroscopy of AGM. — The ^{13}C-chemical shifts of the signals for the anomeric and carboxyl carbon atoms for AGM, GM, and the aldobiouronic acid are listed in Table IV. The spectrum of AGM (Fig. 1) indicated that the carboxyl groups (176.0 p.p.m.) were unsubstituted.

AGM showed three signals for anomeric carbon atoms, together with signals for

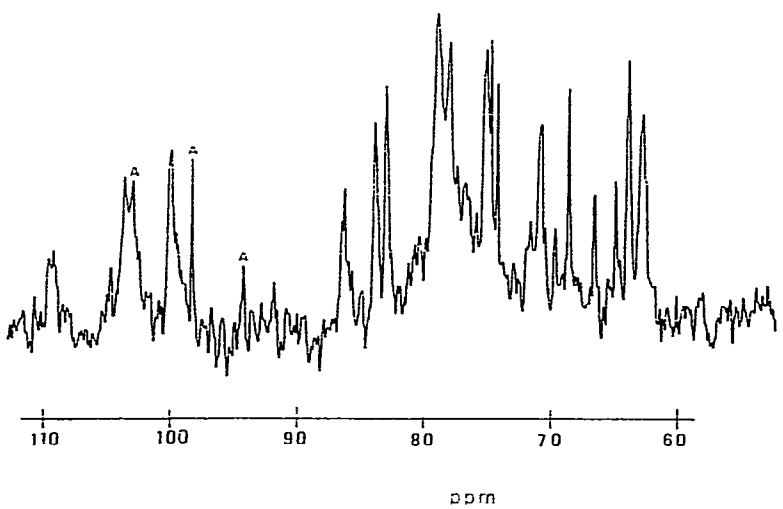


Fig. 1. ^{13}C-N.m.r. spectrum of the arabinoglucuronomannan in 0.9% NaCl in D_2O at 60°; 45° pulse; repetition time, 1 s; 100,000 accumulations. The carboxyl signal was observed at 176.0 p.p.m. The anomeric signals due to the free arabinose cleaved from the polymer during the n.m.r. measurement are indicated by A.

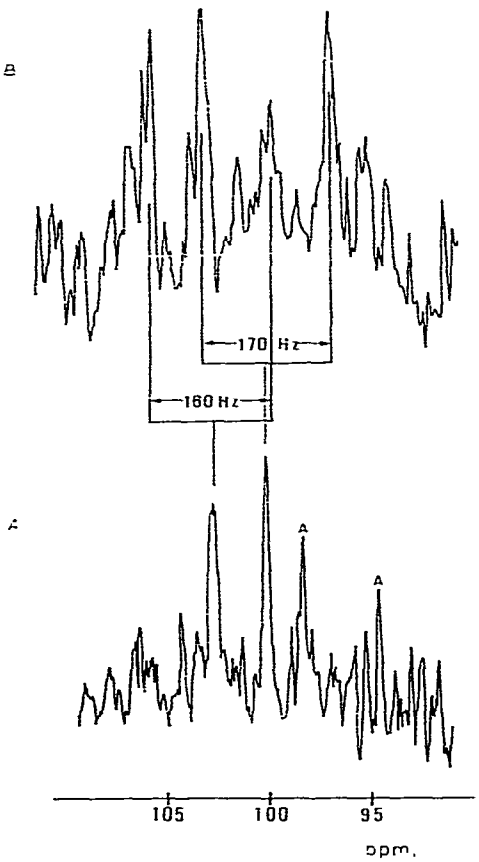
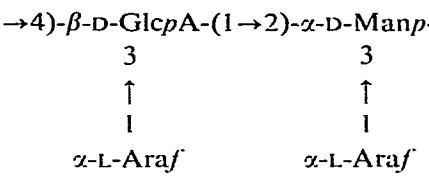


Fig. 2. The ^{13}C-n.m.r. spectral region of anomeric carbon atoms of the glucuronomannan: *A*, ^{1}H-decoupled; *B*, ^{1}H-coupled (n.O.e. mode). The signals from contaminating, free arabinose are marked A.

free arabinose, presumably liberated from the polymer by autohydrolysis at the elevated temperature of the measurement. The signal at 110 p.p.m., which was absent in the spectrum of GM (Fig. 2), was readily assigned[13] to the α-L-arabinofuranosyl residues. The aldobiouronic acid gave two signals for anomeric carbons. Having regard to the ^{1}H-n.m.r. data, the signal at 102.7 p.p.m. was assigned to the β-D-glucopyranosyluronic acid residue[20], and that at 93.0 p.p.m. to C-1 of the mannosyl residue. The signals of α and β anomers probably overlap. By comparison with these data, the signal at 103.5 p.p.m. given by AGM was assigned to the β-D-glucopyranosyluronic acid residue, and that at 100.3 p.p.m. to the D-mannopyranosyl residue. The $J_{C\text{-}1,H\text{-}1}$ values of 170 and 160 Hz, respectively, for the anomeric carbon atoms of the D-mannosyl and D-glucosyluronic acid residues of GM suggested[21] α and β configurations.

Thus, the structure of AGM is tentatively assigned as follows:



Aspinall *et al.*[22–24] have reported the presence of glucuronomannans in various plant gums. However, the glucuronomannan moieties were minor components of the polymers, and arabinogalactans were attached as side chains.

A polysaccharide having a glucuronomannan interior-chain has been obtained from the extracellular polysaccharides of suspension-cultured, tobacco cells[5], but the precise structure of the side chain is not yet known.

EXPERIMENTAL

General methods. — Concentrations were performed under diminished pressure at <40°. Optical rotations were measured at 23° with a Jasco DIP-181 polarimeter. T.l.c. was conducted on cellulose (Merck) with *A*, 1-butanol–pyridine–water (6:4:3); *B*, ethyl acetate–acetic acid–formic acid–water (18:8:3:9); *C*, ethyl acetate–acetic acid–water[25] (10:5:6); and detection with aniline hydrogen phthalate. Cellulose chromatography was performed on a column (20 × 2 cm) of Whatman Cellulose powder CF 11, and the fractions were monitored by t.l.c. Zone electrophoresis was performed on Whatman GF/A, glass-microfibre paper (57 × 5 cm) with *A*, 0.1M sodium tetraborate buffer[26] (pH 9.3) at 1500 V for 60 min; *B*, 10% pyridine–0.3% acetic acid–0.01M disodium ethylenediaminetetra-acetate buffer[27] (pH 6.5) at 2000 V for 60 min; and detection with 50% sulphuric acid. High-performance, gel-permeation chromatography was conducted with a Waters Solvent Delivery System 6000 constant-flow pump and a Waters R-401 differential refractometer for monitoring the column effluent. Separations were performed on a column of TOYO SODA TSK-Gel G4000SW, which was calibrated with Dextran T-series (Pharmacia). G.l.c. was performed with a Shimadzu GC-7-A or GC-4CM instrument fitted with a flame-ionisation detector and *A*, a glass column (200 × 0.3 cm) containing Gas Chrom P (100/200 mesh) coated with a mixture of 0.2% of poly(ethylene glycol adipate), 0.2% of poly(ethylene glycol succinate), and 0.4% of silicone XF-1150; *B*, a glass-capillary column (40 m × 0.28 mm) coated with 3% of OV-101; and *C*, a glass-capillary column (40 m × 0.28 mm) coated with 3% of SP-1000. Carbohydrate was determined by the phenol–sulphuric acid method[28], uronic acid by the modified[10] 3-hydroxybiphenyl method, and protein by the Lowry method[6] with bovine serum alubumin as the standard.

Neutral-sugar analysis was performed by g.l.c. (column *A*) of the alditol acetates[29,30].

Acid hydrolysis of AGM and the derivatives was performed in 2M trifluoro-

acetic acid (TFA) at 120° for 4 h. Neutral sugars and uronic acids were analysed simultaneously by g.l.c. (column *B*) of their diethyl dithioacetal trimethylsilyl derivatives[11]. Since uronic acids are degraded during acid hydrolysis, appropriate calibration curves were used.

Methylation and g.l.c.–m.s. (columns *B* and *C*) were performed essentially as described previously[29,30]. Methylation of acidic carbohydrates was completed by the Hakomori method. 1,3,4,5,6-Penta-*O*-acetyl-2-*O*-methyl-D-mannitol was a gift from Dr. K. Kanamaru[14].

The g.l.c.–m.s. of the methylated oligosaccharide was conducted with a Hitachi RM-50GC spectrometer, using a glass column (1 m × 3 mm) packed with Chromosorb W(AW-DMCS) coated with 5% of OV-101.

N.m.r. spectra (^{13}C, 25.1 MHz; ^{1}H, 99.6 MHz) were obtained in the Fourier-transform mode, using a Jeol FX-100 n.m.r. spectrometer. For ^{1}H-n.m.r. spectra, the sample (~5 mg) was deuterium-exchanged (thrice) and dissolved in 0.03 ml of deuterium oxide containing 0.9% of sodium chloride. The measurements were performed in 1-mm tubes at 60°. Chemical shifts (δ values) were measured downfield from that of internal sodium 4,4-dimethyl-4-silapentane-1-sulphonate. Natural-abundance, ^{13}C-n.m.r. spectra were obtained with complete proton-decoupling at 60° or 80°. The spectral width was 6 kHz; acquisition time, 1 s, data points, 8 k; and pulse width, 7 μs (45°). The samples were dissolved in 0.9% sodium chloride in deuterium oxide. The spectrum of the polymeric sample (~50 mg) was obtained with a 10-mm tube and external Me_4Si, and the oligomeric sample with a 1-mm tube and internal MeOH (49.8 p.p.m. from Me_4Si) as the standard. Chemical shifts were expressed in p.p.m. downfield from the standards, and $^{1}J_{C,H}$ values were determined by the gated, ^{1}H-decoupler sequence to retain the n.O.e.

Suspension-cultured cells. — Suspension culture of tobacco cells (*Nicotiana tabacum*, cv. BY-2) was carried out as described previously[31]. The extracellular polysaccharides (ECP) were removed by repeated washing (thrice) with 0.1M potassium phosphate buffer (pH 7.0).

The ECP-free cells (3 kg, fresh wt.; 110 g, dry wt.) were frozen and thawed, dissolved in 4 vol. of 0.1M phosphate buffer (pH 7.0) containing 0.4M potassium chloride and 0.01M 2-mercaptoethanol, and homogenised in a Waring Blendor for 10 min. The homogenate was centrifuged for 10 min at 2000 *g*. The extraction was repeated thrice. All of the above procedures were carried out at 5°. The supernatant solution was boiled under reflux for 30 min to denature the hydrolytic enzymes (almost all of the arabinofuranosyl linkages were not cleaved under these conditions[13]), and then concentrated, filtered, dialysed, and freeze-dried.

A solution of the extract (2.82 g) in water was applied to a column (50 × 7 cm) of DEAE-Cellulose (Brown, OAc^- form), and eluted stepwise with water, 0.1, 0.25, 0.5, and M potassium acetate (pH 6.0), and 0.1M sodium hydroxide. Each fraction was dialysed and freeze-dried.

The combined fractions eluted with 0.25 (639.2 mg) and 0.5M (324.5 mg) potassium acetate were dissolved in 40 vol. of 0.05M sodium tetraborate buffer

(pH 9.8), and 20 vol. of phenol were added. The mixtures were stirred vigorously and then centrifuged, and the buffer layers were dialysed and freeze-dried. The deproteinised fractions were redissolved into 0.01M Tris–HCl buffer (pH 8.5), applied to a column (10 × 2 cm) of DEAE-Sephadex A-50 (Cl^- form) conditioned with the same buffer, and eluted with a linear gradient of NaCl. Each of the fractions showed a single, major peak at 0.33M NaCl, with small peaks due to contamination. The main fractions were collected, combined, dialysed, and freeze-dried, to give the arabinoglucuronomannan (AGM, 300 mg).

Investigation of AGM. — (*a*) *Carboxyl-reduction.* AGM was reduced[12] with sodium borohydride. Three treatments achieved ~50% reduction of the uronic acid, as checked by the dithioacetal method.

(*b*) *Partial hydrolysis with acid.* (*i*) A solution of AGM (50 mg) in 0.05M TFA (10 ml) was kept at 100° for 90 min under nitrogen, and then eluted from a column (150 × 1.5 cm) of Bio-Gel P-2 with water. The glucuronomannan (GM) (27 mg) eluted in the void volume was collected and freeze-dried, and shown to contain glucuronic acid and mannose in the molar ratio of 1 : 1.

The GM was reduced by the same procedure as used for AGM (50% conversion in three treatments).

Prior to ^{13}C-n.m.r. spectroscopy, the GM was esterified[27] with diazomethane, to increase the solubility.

(*ii*) A sample (12 mg) of AGM was hydrolysed with M TFA (2 ml) at 120° for 60 min. The hydrolysate was applied to a column (5 × 1 cm) of Dowex 1 (OAc^-) resin and eluted with water and 4M acetic acid. T.l.c. (solvents *B* and *C*) of the latter eluate revealed a single spot with traces of glucuronolactone and acidic, higher oligosaccharides. The main product was purified on a cellulose column (solvent *C*), to yield pure disaccharide (5 mg) that was homogeneous in t.l.c. (R_{GlcA} 0.73 and 0.83 in solvents *B* and *C*, respectively). The disaccharide contained glucuronic acid and mannose in the molar ratio 49.4 : 50.6. Borohydride reduction followed by acid hydrolysis gave glucuronic acid and mannitol in the ratio 56.5 : 43.5. Reduction with sodium borodeuteride and then methylation gave a single product (g.l.c.), the mass spectrum of which contained, *inter alia*, the following fragments: *m/z* 45 (100%), 46 (44), 89 (51), 133 (8), 169 (9), 177 (3), 201 (50), 233 (4), 236 (12), and 319 (3).

The ^1H-n.m.r. spectrum of the aldobiouronic acid contains four signals for anomeric protons at δ 5.28 (0.8 H, *J* 1.5 Hz), 4.95 (0.2 H, *J* 1 Hz), 4.64 (0.2 H, *J* 6.8 Hz), and 4.55 (0.8 H, *J* 7.1 Hz). In contrast, the reduced aldobiouronic acid showed a single signal for anomeric protons at δ 4.65 (*J* 7.3 Hz), indicating that the non-reducing glucosyluronic acid residue was β. From ^1H-n.m.r. data for D-mannose, the signals at δ 5.28 and 4.95 were assigned to α- and β-D-mannose moieties. The peak areas indicated the $\alpha\beta$-ratio to be 4 : 1. The splitting of the signal for H-1 of the β-D-glucosyluronic acid residue was probably due to the neighbouring, reducing residue[32, 33]. The ^1H-n.m.r. data for the aldobiouronic acid accord with those reported by Dutton and Yang[18].

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