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STRUCTURAL STUDIES OF THE EXTRACELLULAR POLYSACCHARIDES OF *Rhizobium japonicum* STRAINS 71A, CC708, AND CB1795*

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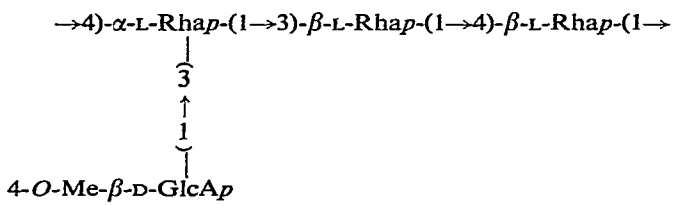
ABSTRACT

Each of the serologically related, title strains produced extracellular polysaccharides that were mixtures of an acidic polysaccharide and smaller proportions of a glucan. These were separated by column chromatography and the structures of the acidic polysaccharides investigated by alkylation, specific sequential degradation, and periodate oxidation in conjunction with ^{1}H-n.m.r. spectroscopy. The polysaccharides from the three strains appear to be identical rhamno-4-*O*-methylglucuronans, having the following tetrasaccharide repeating-unit.



The molecular weight of the polysaccharides of strains CC708 and CB1795 was of the order of 65,000–70,000.

INTRODUCTION

The extracellular polysaccharides of strains of *Rhizobium japonicum* and the other "slow-growing" rhizobia have been found to be diverse in their composition, often varying from strain to strain[1–3], unlike the "fast-growing" group of rhizobial organisms (*R. trifolii*, *R. leguminosarum*, *R. phaseoli*, and *R. meliloti*) in which all strains of a species appear to produce similar polysaccharides[1]. Preliminary studies in this laboratory of the qualitative composition of the total, crude, extracellular polysaccharides of *R. japonicum* strains have shown that they may be arranged in groups according to the nature and number of their constituent sugars. This paper describes the structural analysis of the polysaccharides of three strains that contain

*Dedicated to Dr. Allene Jeanes on the occasion of her retirement.

the same three sugars, namely glucose, rhamnose, and an unknown uronic acid subsequently identified as 4-*O*-methyl-D-glucuronic acid.

RESULTS AND DISCUSSION

The polysaccharides studied here, from *R. japonicum* strains 71a, CC708, and CB1795, were found by paper chromatography (p.c.) to contain similar sugars: glucose, a component that migrated faster than glucose, and another component having the behaviour of a uronic acid but which did not co-migrate with glucuronic or galacturonic acid standards. Because the slow-growing group of rhizobia, to which *R. japonicum* belongs, produce polysaccharides that characteristically contain methylated sugars[1–3], which can be difficult to identify, care was given to the identification of the unknown constituents in the present polysaccharides. The constituents were confirmed to be L-rhamnose and 4-*O*-methyl-D-glucuronic acid by analysis of the sugars isolated from the 71a polysaccharide; their identity in the other two polysaccharides was established by the identical behaviour of the three polysaccharides and their constituents in the subsequent analyses. L-Rhamnose and 4-*O*-methyl-D-glucuronic acid have been reported previously[4,5] in *Rhizobium* polysaccharides, but this is the first time that their identity has been confirmed.

The quantitative composition of the polysaccharides is shown in Table I. The constancy of the relative amounts of L-rhamnose and 4-*O*-methyl-D-glucuronic acid and the variability of the glucose content in the crude polysaccharide suggested the presence of two different polysaccharides—a glucan and a rhamno-4-*O*-methyl-glucuronan—in each crude preparation. Unsuccessful attempts were made to separate the neutral from the acidic polysaccharides by such methods as fractionation on an ion-exchange (Deacidite FF) column, precipitation by cetylpyridinium chloride on a cellulose column[6], and hydrophobic affinity-chromatography[7] on columns of Sephadex LH-20 and octyl-Sepharose 4B. It was found possible, however, to remove the glucan from the acidic polysaccharide of strain 71a by affinity chromatography on concanavalin A–Sepharose[8]. Subsequently, it was found that the glucan could be removed more simply by gel-filtration chromatography[9] on Sephadex G-200, and this method was used with the CC708 and CB1795 polysaccharides. The acidic polysaccharides of the three strains appeared to have identical compositions after removal of the glucan (Table I) and to contain three L-rhamnose residues for each 4-*O*-methyl-D-glucuronic acid group. The small proportions of glucose in the purified, acidic polysaccharides are regarded as arising from contamination by small amounts of residual glucans and are not considered further here.

Purified glucans were recovered from each polysaccharide in small amounts, insufficient for further analysis. They did not appear to exist as fibrils when examined by electron microscopy[10]. Glucans have been reported to be present in the extracellular polysaccharides of *R. lupini*[11] and in the cell-wall polysaccharides of *R. leguminosarum*[12], and it is possible that they may be found to be of more common occurrence among rhizobia than has been recognised.

TABLE I

COMPOSITION[a] OF POLYSACCHARIDES BEFORE AND AFTER REMOVAL OF GLUCAN, AND AFTER CARBOXYL-REDUCTION

Strain	*Before*			*After*				*Molar ratio*			$[\alpha]_D$[b]
	Rha %	*4MeGlcA* %	*Glc* %	*Rha* %	*4MeGlcA* %	*Glc* %	*4MeGlc* %	*Rha*	*4MeGlcA*	*4MeGlc*	
71a	59	24	17	71	29	1		3.1	1		−46°
CC708	67	27	6	70	30	<1		3.0	1		−37°
CB1795	64	26	10	70	30	<1		3.0	1		−41°
71a reduced[c]				75		2	23	4.25		1	

[a]Determined by methanolysis[1]. [b]Optical rotation of purified polysaccharides, in a 1-dm tube (*c* 0.3–0.7, water). [c]Significant proportions of 3-acetyl-2,6-dimethylpyran-4-one were present in the reduced polysaccharide.

The acidic, major polysaccharides of the three strains were examined by ^{1}H-n.m.r. spectroscopy[13] and identical spectra were obtained from each. Four anomeric protons were detected; those having signals at δ 4.70 ($J_{1,2}$ low), 4.67 ($J_{1,2}$ low), and 4.53 ($J_{1,2}$ 7.5 Hz) were assigned to two β-linked L-rhamnose and a β-linked 4-*O*-methyl-D-glucuronic acid residues, respectively. The anomeric nature of the remaining proton of rhamnose [giving rise to the most downfield signal at δ 4.99 ($J_{1,2}$ low)] was more ambiguous, but α-linked L-rhamnose residues have been reported[14] to have this chemical shift; the present signal was confirmed by sequential, specific-degradation experiments to arise from α-linked L-rhamnose residues. The n.m.r. spectra confirmed the absence of pyruvyl and acetyl substituents.

G.l.c. analysis of the alditol acetates[15] of the methylated polysaccharides[16,17] on a wide range of columns showed the presence of only two peaks. M.s. analysis[18] showed that the slower-moving peak arose from the alditol acetate of 2-*O*-methyl-L-rhamnose, whereas the faster peak was not a single component but a mixture of the alditol acetates of 2,3- and 2,4-di-*O*-methyl-L-rhamnose. Because it was not possible to separate these isomers as the alditol acetates, other derivatives capable of allowing their separation, and thereby separate estimation, were sought. These di-*O*-methyl-L-rhamnose isomers were readily separated as the methyl glycosides, obtained by methanolysis of the polysaccharides; their m.s. fragmentation-patterns[19] identified methyl 2,4-di-*O*-methyl-L-rhamnoside as having a shorter retention time than the 2,3 isomer. Equal amounts of the 2,3- and 2,4-di-*O*-methyl-L-rhamnose derivatives were found in the methylated polysaccharides (Table II). Two other peaks were obtained by g.l.c. of the methanolysates of the methylated polysaccharides, but as the 4-*O*-methyl-D-glucuronic acid and 2-*O*-methyl-L-rhamnose derivatives were not separated from each other, these latter peaks could not be used quantitatively; however, the identification by m.s.[20] of the uronic acid derivative as methyl (methyl 2,3,4-tri-*O*-methyl-D-glucosid)uronate indicated that the uronic acid residues were in non-reducing, terminal positions in the polysaccharides.

TABLE II

METHYLATION ANALYSIS OF *Rhizobium japonicum* 71A, CC708, AND CB1795 NATIVE POLYSACCHARIDES

Strain	*Alditol acetates*		*Methyl glycosides*	
	2,3-Rha[a] + *2,4-Rha* (T *0.90*[b]) (*mol %*)	*2-Rha* (T *1.21*) (*mol %*)	*2,4-Rha* (T *0.78*[c]) (*mol %*)	*2,3-Rha* (T *1.00*) (*mol %*)
71a	67	33	33	34
CC708	66	34	33	33
CB1795	67	33	33	34

[a]2,3-Rha = 2,3-di-*O*-methyl-L-rhamnose, and so on. [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 OV-225 column at 185° (*T* 1.0 = 3.3 min). [c]Retention time of the corresponding methyl glycoside relative to methyl 2,3,4,6-tetra-*O*-methyl-α-D-glucoside on an XF1150/EGS column at 125° (*T* 1.0 = 2.95 min).

To avoid the need for two separate g.l.c. analyses (of the alditol acetates and methyl glycosides, respectively), made necessary because the methylated sugars could not be completely separated as one type of derivative, the possibility of achieving better separations of the alditol acetates of the ethylated derivatives[21] was investigated. Partial separation of the 2,3- and 2,4-di-*O*-ethyl-L-rhamnose peaks was obtained, together with that of 2-*O*-ethyl-L-rhamnose, allowing the three constituents to be determined in one experiment (Table III). Butylation was also investigated, but substitution of the hydroxyl groups in the polysaccharide was incomplete and the peaks that were obtained were not identified because of the greater complexity of their mass spectra.

TABLE III

ETHYLATION ANALYSIS OF *Rhizobium japonicum* 71A NATIVE AND CARBOXYL-REDUCED POLYSACCHARIDES

Sugars and location of ethoxy groups[a]	*Native*[c]		*Reduced*[d]	
	T[b]	*Mol %*	T[b]	*Mol %*
2,3-Rha	0.71	32	0.84	28
2,4-Rha	0.77	36	0.90	28
2-Rha	1.23	32	1.32	25
2,3,4*,6-Glc			1.05	19

[a]2,3-Rha=2,3-di-*O*-ethyl-L-rhamnose, and so on. The asterisk indicates the location of the naturally occurring methyl group derived from 4-*O*-methyl-D-glucuronic acid. [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 the respective columns. [c]Analysed on an ECNSS-M column at 185° (*T* 1.0=6.6 min). [d]Analysed on an OV-225 column at 165° (*T* 1.0=24.2 min). Significant amounts (~8%) of 3-acetyl-2,6-dimethylpyran-4-one (*T* 0.34) were present in the reduced polysaccharide but have been omitted from these results.

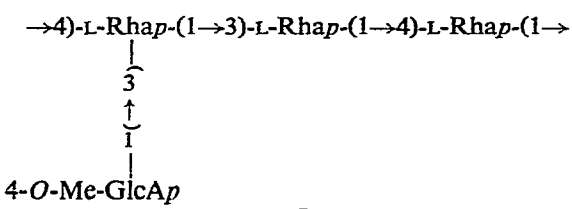
In order to confirm the location at O-4 of the naturally occurring methyl group of the uronic acid, and to confirm the position of these uronic acids as terminal non-reducing residues, the 71a polysaccharide was reduced in aqueous solution by the carbodiimide method[22]. To establish that the reduction was complete, samples of the reduced polysaccharide were methanolysed and hydrolysed, respectively. G.l.c. analysis of the former showed that reduction was complete because no 4-*O*-methyl-D-glucuronic acid was detectable; 4-*O*-methyl-D-glucose was present in the hydrolysate but its amount was insufficient to account for all of the uronic acid (Table I). However, an additional peak, having a very short retention-time (*T* 0.05, relative to the alditol acetate of glucose) was found in the alditol acetate preparation from the hydrolysate; its size was approximately equal to the difference between the expected and recovered values for 4-*O*-methyl-D-glucose. This component was identified as 3-acetyl-2,6-dimethylpyran-4-one by its mass[23] and u.v. spectra, and m.p. The origin of this compound is unknown and remains to be examined.

The reduced 71a polysaccharide was ethylated and analysed as the alditol acetate derivatives by g.l.c. (Table III). The recovery of the ethylated derivatives of

L-rhamnose was close to the molar ratios expected, but the recovery of the glucose derivative was again low; 3-acetyl-2,6-dimethyl-pyran-4-one was present in this material also. The identification of the glucose derivative as 1,5-di-*O*-acetyl-2,3,6-tri-*O*-ethyl-4-*O*-methylglucitol confirmed that the naturally occurring methyl group is at O-4 and that the uronic acid is present in non-reducing, terminal positions in the native polysaccharide.

The presence of four types of structural constituents (**1**–**4**) was thus established by these experiments. The order in which they are linked in the polysaccharides was determined by specific, sequential degradation of the 71a polysaccharide, beginning with the identification of the point of attachment of the terminal uronic acid residues (**4**).

Elimination of the 4-*O*-methyl-D-glucuronic acid residues from the methylated polysaccharide by treatment with sodium methylsulphinylmethylide[24], followed by remethylation with trideuteriomethyl iodide, allowed identification of the hydroxyl group liberated by the uronic acid residues eliminated. The detection of 2-*O*-methyl-3-*O*-deuteriomethyl-L-rhamnose as the only deuteriomethylated residue in the remethylated, degraded polysaccharide indicated that **4** is linked to O-3 of **3**. The rhamnose residue (**1** or **2**) to which the glycosidic group of **3** is attached was identified by submitting a sample of the methylated polysaccharide, from which the 4-*O*-methyl-D-glucuronic acid had been eliminated, to specific oxidation[25] of the liberated hydroxyl group at C-3 of **3** followed by treatment with base to eliminate the oxidized rhamnose residue. Ethylation and analysis of the polysaccharide residue after this treatment showed the presence of 2,4-di-*O*-methyl-3-*O*-ethyl-L-rhamnose and established that **3** is linked glycosidically to **2**. The following linkage sequence of the structural elements in the repeating unit (**5**) was thus established.



5

The nature of the anomeric linkages was determined by periodate oxidation and by ^{1}H-n.m.r. spectroscopy. The spectra of the native polysaccharides (see foregoing)

had indicated the presence of one α- and three β-linked residues. Periodate oxidation of the 71a polysaccharide followed by mild acid hydrolysis yielded a product which, from inspection of **5** and n.m.r. spectroscopy, was identified as **6**.

L-Rha*p*-(1→3)-L-Rha*p*-(1→3)-1-deoxyerythritol.

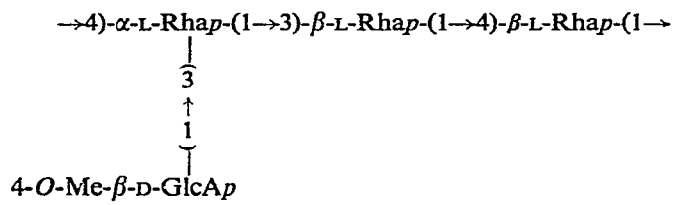
6

The n.m.r. spectrum of **6** showed the presence of two anomeric protons giving signals at δ 4.99 ($J_{1,2}$ 1.5 Hz) and 4.70 ($J_{1,2}$ 1 Hz), interpreted as indicating an α- and a β-linked rhamnose, respectively. The two residues (**1** and **4**) lost by periodate oxidation were thus both β-linked. Periodate oxidation of **6**, followed by mild acid hydrolysis, eliminated the terminal rhamnose residue to leave a product (**7**) which, from its n.m.r. spectrum and optical rotation, was β-linked. The rhamnose moiety **3**, from which the terminal residue in **6** was derived, is therefore concluded to be α-linked; this assignment is supported by the difference in optical rotation of **6** ($[\alpha]_D$ +13.6°) and **7** ($[\alpha]_D$ +18°).

β-L-Rha*p*-(1→3)-1-deoxyerythritol

7

The repeating unit of the 71a polysaccharide is therefore concluded to be the structure **8**.



8

The polysaccharide of strain 71a was the only one to be subjected to complete structural analysis here, but the evidence suggests that the polysaccharides of strains CC708 and CB1795 have the same structure. The results of methylation analysis and ^{1}H-n.m.r. spectra of the three polysaccharides were identical, and the close relationship of the three *R. japonicum* strains was demonstrated by serological analysis[26]. The extracellular polysaccharides of this organism do not react with antisera, but immunodiffusion analysis does allow the relationship of the strains to be compared (on the basis of their somatic or *O*-antigens, which are the dominant antigens in these bacteria). In reactions with anti-CB1795 antiserum, 71a was antigenically identical with CB1795, whereas CC708 was found to be a related, cross-reacting strain. These strains were initially isolated from soybeans grown at widely separated locations in the United States and there is little reason to believe them to have been connected with each other (D. F. Weber, personal communication). Whether they can be regarded as identical or not will require comparisons of many biological and

symbiotic characters, and it is wiser to regard strains that are of separate origin as being distinct until they are exhaustively proven otherwise.

EXPERIMENTAL

Microorganisms and polysaccharide production. — The strains of *Rhizobium japonicum* used (and their original designations) were: 71a (USDA, Beltsville, Maryland; strain 3I1b71a), CB1795 (USDA, Beltsville, Maryland; strain 3I1b46), and CC708 (Nitragin Company, Milwaukee, Wisconsin; strain 61A76). Cultures were maintained on yeast extract–mannitol agar slopes and grown in liquid defined medium for the production of extracellular polysaccharide as described previously[1].

General methods. — Standard analytical methods were used as described previously[1]. Hydrolyses were performed in 0.5M sulphuric acid for 16 h at 100° and methanolyses were conducted in M dry methanolic hydrogen chloride for 16 h at 90°. Paper chromatography (p.c.) was performed by descending elution in the following solvents (*A*) 10:3:3 1-butanol–pyridine–water and (*B*) 18:3:1:4 ethyl acetate–acetic acid–formic acid–water, and sugars were detected by the silver nitrate–sodium hydroxide, *p*-anisidine, and aniline phthalate sprays. Gas–liquid chromatographic (g.l.c.) separations were performed on a Pye series 104, model 64, dual-column instrument equipped with flame-ionization detector and stainless-steel analytical (3 m × 2 mm i.d.) and preparative (2 m × 4 mm i.d.) columns. The column packings used were (*A*) 3% of ECNSS-M on Gas-Chrom Q (80–100 mesh); (*B*) 1.5% of XF-1150 and 1.5% of ethylene glycol succinate on Chromosorb W(AW) (80–100 mesh); (*C*) 3% of OV-101 on the Chromosorb support; (*D*) 1.5% of OV-17 on the Gas–Chrom Q support; (*E*) 3% of OV-225 on the Gas-Chrom Q support; (*F*) 3% of OV-275 on the Gas-Chrom Q support. For mass spectrometry (m.s.), samples from g.l.c. were separated on a stream splitter, collected in capillaries, and analysed by electron impact in an AEI MS-902 instrument equipped with a cooled insertion probe. ^{1}H-N.m.r. spectra were recorded on Varian A-60 (60-MHz) and Brucker HX-270 (270-MHz) instruments, with D_2O as solvent and 1,4-dioxane as the internal standard (δ 3.70).

Identification of 4-O-methyl-D-glucuronic acid. — A sample (1 g) of the 71a polysaccharide was methanolysed (100 ml of reagent), neutralised with silver carbonate and hydrolysed (100 ml of reagent). The hydrolysate was brought to pH 3.5 with barium hydroxide at 55°, centrifuged, and the supernatant titrated to pH 9 with M sodium hydroxide[27]. The hydrolysate was passed through columns (60 × 1 cm) of Zeokarb 225(H^+) and Deacidite FF (acetate) resins (Permutit Co., London; these resins are equivalent to Dowex 50 and Dowex 1, respectively) mounted in tandem[27], and the effluent was saved for analysis of the neutral component (see later). The columns were washed with water (400 ml) and the Deacidite column was eluted with 0.05M sodium acetate–acetic acid buffer (pH 5.8), collecting 10-ml fractions, samples of which were tested with the carbazole reagent[28]. A single, symmetrical uronic acid peak was detected; the fractions giving positive results were combined, treated with

Zeokarb 225(H^+) resin, and evaporated to low volume; yield (carbazole reaction) 46 mg. The n.m.r. spectrum at 60 MHz included a strong signal at δ 3.45, indicating the presence of an O-CH_3 group.

The isolated uronic acid (2 mg) was converted into the methyl glycoside methyl ester by treatment with methanolic hydrogen chloride, derivatised (Me_3Si), and compared with authentic 4-*O*-methyl-D-glucuronic acid similarly treated. Single peaks having identical retention times were obtained with the derivatives of the unknown uronic acid and 4-*O*-methyl-D-glucuronic acid on ECNSS-M (column *A*, 150°)[29], OV-17 (column *D*, 150°)[29], and OV-225 (column *E*, programmed from 100° at 3°/min)[1]; their retention times, relative to the main peak of the methyl glycoside methyl ester of D-glucuronic acid[1] on these columns, were 0.91, 0.54, and 0.96, respectively.

Attempts to demethylate the uronic acid directly were not successful and it was necessary to reduce it first. A sample of the methyl glycoside methyl ester (20 mg) was dried over phosphorus pentaoxide, dissolved in tetrahydrofuran (10 ml), and added dropwise to lithium aluminium hydride (180 mg) suspended in stirred tetrahydrofuran (10 ml). The mixture was boiled for 2 h under reflux, cooled, the excess of hydride decomposed with ethyl acetate, water added, the mixture centrifuged, and the turbid supernatant filtered. The filtrate was treated with Zeokarb 225(H^+) and Deacidite FF (acetate) resins and hydrolysed. P.c. (solvent *A*) showed the presence of a single component (R_{Glc} 1.85), different from 4-*O*-methyl-D-galactose (R_{Glc} 1.54), but no sample of 4-*O*-methyl-D-glucose was available for comparison. A sample of the reduced product was acetylated[15] and analysed by g.l.c. on ECNSS-M (column *A*, 190°); it gave a single peak having a retention time identical to that of the alditol acetate of 4-*O*-methyl-D-galactose[1] (*T* 0.83, relative to the alditol acetate of glucose). M.s. analysis gave peaks diagnostic for a monomethyl hexitol pentaacetate substituted at O-4 (or O-3) (*m/e* 189, 201). The remainder of the reduced uronic acid material was demethylated[30] without solvent, at −80°, with boron trichloride (2 ml). Glucose was detected by p.c. in solvent *A* as the product of demethylation. The uronic acid component isolated from the polysaccharide had $[\alpha]_D$ +35.3° (*c* 3.06, water) and was therefore identified as 4-*O*-methyl-D-glucuronic acid (lit.[31] $[\alpha]_D$ +36°).

Identification of L-*rhamnose.* — The effluent from the foregoing ion-exchange columns was analysed by p.c. and found to contain glucose and larger proportions of a faster-moving component having the mobility of L-rhamnose (R_{Glc} 2.3, solvent *A*); the latter component also gave the same colours as rhamnose with aniline phthalate and *p*-anisidine sprays. The fast-migrating component was isolated by preparative p.c. and its n.m.r. spectrum examined at 60 MHz; its spectrum included signals at δ 5.14 (0.65 H, $J_{1,2}$ low), 4.84 (0.35 H, $J_{1,2}$ low), and 1.29 (3 H, $J_{5,6}$ 6.4), identical with those of L-rhamnose. The isolated material also behaved identically with rhamnose standards when compared as different derivatives (Me_3Si, Me_3Si of the methyl glycosides, and alditol acetates) by g.l.c. on different columns (OV-101, OV-225 and ECNSS-M), and the optical rotation $[\alpha]_D$ +10.3° (*c* 2.4, water) confirmed that it was the L enantiomer.

Purification of polysaccharides. — The crude polysaccharide of strain 71a was purified by affinity chromatography. The polysaccharide (450 mg) was dissolved in 60 ml of an acetate buffer (0.1M pH 6) containing sodium chloride (0.1M) and the chlorides of calcium, magnesium, and manganese (each mM). The centrifuged solution was applied to a column (10 × 2.5 cm) of concanavalin A–Sepharose (Pharmacia) at room temperature, and this was eluted with the same buffer at 12 ml/h, collecting 10-ml fractions. The anthrone reaction was used to detect the polysaccharide in the fractions. The acidic polysaccharide emerged at 70 ml and was completely eluted after 200 ml; the appropriate tubes were combined and dialysed against repeated changes of distilled water. After 340 ml had passed through the column, methyl α-D-mannoside (0.1M) was added to the buffer and it was detected in the effluent after a further 60 ml had emerged; elution was continued for a further 300 ml. This latter volume was combined and dialysed to remove the mannoside. The dialysed polysaccharide solutions were deionised by treatment with Zeokarb 225(H^+) and lyophilised. Analysis of the two polysaccharides by methanolysis, derivatisation (Me_3Si), and g.l.c. on OV-225 (column *E*, programmed from 100° at 3°/min) showed that the first-eluted fraction, consisting of L-rhamnose and 4-*O*-methyl-D-glucuronic acid, now contained about 1% of glucose (Table I), whereas the polysaccharide retained by the column until eluted by methyl α-D-mannoside contained only glucose; yields: rhamno-4-*O*-methylglucuronan, 330 mg; glucan, 3 mg.

The crude polysaccharides of strains CC708 and CB1795 were purified by gel-filtration chromatography. These polysaccharides (300 mg) were dissolved in 30 ml of phosphate-buffered saline (sodium chloride, 0.1M; sodium phosphate buffer, 0.01M, pH 7, and sodium azide 0.03M), and applied to a column (86 × 5 cm) of Sephadex G-200 superfine (Pharmacia) by upward flow. Elution was continued with the same solution at 13.8 ml/h, collecting 10-ml fractions and using the anthrone reaction to detect the polysaccharides eluted. Comparison of intensities of colour in the reaction after 4 and 10 min of heating, respectively, distinguished between the rhamno-4-*O*-methylglucuronan and glucan peaks because the former polysaccharide gave a stronger reaction in 4 min, whereas the latter was more intense after 10 min. The acidic polysaccharides of both strains emerged (peak at 620 ml) close to the void volume (510 ml), in contrast to the glucans, which were eluted close to the total bed-volume (1740 ml). Calibration of the column with a range of dextrans of known molecular weights (Pharmacia dextran T fractions) indicated that the acidic polysaccharides were in the molecular weight range 65,000–70,000. These polysaccharides were dialysed, deionized and lyophilised; yields of rhamno-4-*O*-methylglucuronan: 241 mg from strain CC708 and 160 mg from strain CB1795. The yield of glucan was less than 10 mg in each case. These low recoveries of glucan are believed to be due to their adherence to the column gels.

For n.m.r. spectroscopy[13], the polysaccharides were converted into the salt form by titration to pH 7 with 0.01M potassium hydroxide, lyophilized, exchanged twice with, and finally dissolved in, deuterium oxide to give gels of 5–8% polysaccharide, and their spectra were recorded at 90° at 270 MHz. The spectra of the

three polysaccharides were identical and included four anomeric signals at δ 4.99 (1 H, $J_{1,2}$ low), 4.70 (1 H, $J_{1,2}$ low), 4.67 (1 H, $J_{1,2}$ low), and 4.53 (1 H, $J_{1,2}$ 7.5 Hz). A signal arising from O-CH_3 was present at δ 3.45, and a group of signals from C–CH_3 groups of rhamnose at δ 1.31, 1.29, and 1.26 (each 3 H, $J_{5,6}$ 6 Hz). No signals indicative of pyruvyl or acetyl substituents were detected, and the absence of these groups was confirmed by negative results from the appropriate chemical reactions[1].

Methylation analysis of native polysaccharides. — The polysaccharides (20–80 mg), in the free-acid form, were methylated in dimethyl sulphoxide with sodium methylsulphinylmethylide as described by Sandford and Conrad[16], and were subsequently treated with the Purdie reagents[17]. The solutions of methylated polysaccharide were dialysed, evaporated to dryness, washed with hexane to remove impurities, and the hexane-insoluble residues were used in the subsequent analyses. The completeness of methylation was confirmed by the absence of hydroxyl absorption in the i.r. spectra of solutions of the methylated polysaccharides in chloroform.

The methylated polysaccharides (1–5 mg) were hydrolysed in 90% formic acid for 2 h at 100°. The solutions were evaporated to dryness and the residues hydrolysed in 0.25M sulphuric acid for 12 h at 100°. The hydrolysates were neutralised with washed barium carbonate and converted into the corresponding alditol acetates[15]. The products were analysed by g.l.c. on a range of columns: ECNSS-M (column *A*, 165°), XF-1150+EGS (column *B*, 185°), OV-101 (column *C*, 180°), OV-225 (column *E*, 165° and 185°), and OV-275 (column *F*, 185°). Two peaks were observed in all cases; the results from analysis on OV-225 (185°) were used for quantitation (Table II). M.s. analysis[18] showed that the first peak contained the alditol acetates of 2,3-di-*O*-methyl-L-rhamnose and 2,4-di-*O*-methyl-L-rhamnose, and that the second peak consisted of 2-*O*-methyl-L-rhamnose. The relative proportions of the di-*O*-methyl-rhamnoses were found by g.l.c. of the methyl glycosides obtained by methanolysis of the methylated polysaccharides under standard conditions[1]. Four well-separated glycoside peaks were obtained on ECNSS-M (column *A*, 125°) and XF-1150+EGS (column *B*, 125°). M.s. analysis[19] of the peaks identified the first as methyl 2,4-di-*O*-methyl-L-rhamnoside (*m/e* 78 > *m/e* 88) and the second as methyl 2,3-di-*O*-methyl-L-rhamnoside (*m/e* 78 < *m/e* 88); each of the polysaccharides from the three strains contained these isomers in equal proportions (Table II). The third and fourth glycoside peaks (*T* 1.8 and 2.6, respectively) were identified as anomers of methyl (methyl 2,3,4-tri-*O*-methyl-D-glucosid)uronate[20]. In addition, the fourth peak contained methyl 2-*O*-methyl-L-rhamnoside.

Ethylation and butylation analysis of the 71a native polysaccharide. — Samples (4 mg) of 71a polysaccharide were alkylated in dimethyl sulphoxide with sodium methylsulphinylmethylide and with ethyl iodide and with butyl iodide, respectively[21]. I.r.-spectroscopic analysis of the isolated products showed that the ethylated polysaccharide was free of hydroxyl groups but that the butylated product had not been completely substituted. Analysis of the alditol acetate derivatives[32] by g.l.c. on ECNSS-M (column *A*, 185°) and m.s. showed that the two diethylrhamnose derivatives could be partially separated, sufficiently to determine their relative amounts

(Table III). The butylated derivatives gave a similar pattern of peaks, but they were not identified because their m.s. results were complex.

Structural analysis of carboxyl-reduced 71a polysaccharide. — The polysaccharide (30 mg) was dissolved in water (9 ml) and buffered with 2-(*N*-morpholino)-ethanesulphonic acid (1.25M, pH 4.75, 1 ml)[33]; sodium hydrogencarbonate (5% solution) was added dropwise to adjust the solution to pH 4.75 and 1-ethyl-3-(3-diethylaminopropyl)carbodiimide (EDC)[22] (190 mg) was added. The pH of the solution was maintained at 4.75 by addition of hydrochloric acid (0.1M) and more EDC (50 mg) was added one h later. After a further h, sodium borohydride (1.9 g in 25 ml of water) was added dropwise with stirring during one h, during which time hydrochloric acid was also added dropwise to maintain the solution at pH 7.0. The solution was kept overnight to allow evolution of hydrogen to cease, dialysed exhaustively, deionised with Zeokarb 225(H^+) resin and lyophilised; yield, 26 mg. The reduced polysaccharide was hydrolysed and examined by p.c. (solvent *A*); 4-*O*-methyl-D-glucuronic acid was found to be absent and was replaced by a new component (R_{Glc} 1.90). Methanolysis and analysis of the methyl glycoside Me_3Si derivatives by g.l.c. (column *E*, 125°) confirmed the absence of 4-*O*-methyl-D-glucuronic acid from the reduced polysaccharide. Analysis of the alditol acetate derivatives by g.l.c. and m.s. confirmed the presence of 4-*O*-methylglucose (*T* 0.84 relative to the alditol acetate of glucose; column *B*, 195°). In addition, another peak of significant size, equivalent to about 10% of the 4-*O*-methylglucose peak and with a very short retention-time (*T* 0.05 relative to the alditol acetate of glucose) was found in this alditol acetate preparation of the reduced polysaccharide. The m.s. of this component showed the following main fragments (relative intensities in parentheses): *m/e* 43 (81), 67 (100), 85 (14), 109 (28), 124 (16), 148 (9), 157 (28), and 166 (69). These data are identical with the published spectrum[23] of 3-acetyl-2,6-dimethyl-pyran-4-one and with the m.s. of a sample of this compound. Feathery, needle-like crystals of the component were isolated from the alditol acetate mixture by preparative g.l.c., and its m.p. (56–57°) and u.v. absorption spectrum (maximum 248 nm) were identical with those of the authentic pyrone[34].

The reduced 71a polysaccharide (10 mg) was ethylated as already described and the ethylated sugar residues were analysed as the alditol acetate derivatives by g.l.c. and m.s. (Table III). 3-Acetyl-2,6-dimethyl-pyran-4-one was present also in this preparation.

Sequential degradation of 71a polysaccharide. — Methylated 71a polysaccharide (10 mg) and toluene-*p*-sulphonic acid (trace) were dissolved in dimethyl sulphoxide (2 ml) containing 5% of 2,2-dimethoxypropane, in an atmosphere of nitrogen, and sodium methylsulphinylmethylide (2.16M, 1 ml) was added[24]. The solution was stirred for 1 h, kept overnight at room temperature, acidified with acetic acid, and extracted into chloroform. The product was remethylated by using deuteriomethyl iodide. Analysis of the alditol acetates of this material by g.l.c. and m.s. showed the presence of derivatives of only 2,3-di-*O*-methyl-L-rhamnose and 2,4-di-*O*-methyl-L-rhamnose; m.s. detected CD_3 groups (indicated by the fragments *m/e* 164 and 206)

only at O-3 of 2,3-di-*O*-methyl-L-rhamnose, showing that the 4-*O*-methyl-D-glucuronic acid was attached to O-3 of **3**. The similarity in intensities of the deuterated ions and the non-deuterated ions (*m/e* 161 and 203) indicated that 2,3-di-*O*-methyl- and 2-*O*-methyl-3-*O*-deuteriomethyl-L-rhamnoses were present in equal amounts. The relative amounts of 2,3- and 2,4-di-*O*-methyl-L-rhamnose in the degraded polysaccharide were determined by methanolysis and g.l.c.; the peak of the 2,3-di-*O*-methyl isomer was now twice as large as that of 2,4-di-*O*-methyl-L-rhamnose.

Oxidation of the alcohol group liberated by the removal of 4-*O*-methyl-D-glucuronic acid just described was performed with chlorine[25]. A solution (1.4M) of dry chlorine in dichloromethane was freshly prepared and 5 ml was added to dimethyl sulphoxide (3 ml) under anhydrous conditions at −45° (Dry Ice–acetonitrile mixture). Degraded, methylated 71a polysaccharide, prepared as already described, was dissolved in dichloromethane, added to the oxidation mixture, and stirred for 6 h at −45°. Triethylamine (2.5 ml) was added to the cold mixture, the solution was warmed to room temperature and dialysed exhaustively. The dialysed product was recovered and subjected to oxidation again by the same procedure. The product was recovered as a yellow residue from the dialysis sac, dried, dissolved in 3:1 chloroform–acetone and applied to a column (0.9 × 30 cm) of Sephadex LH-20 (Pharmacia) prepared in the same solvent. The column was eluted with the chloroform acetone mixture at 1 ml/min and 1-ml fractions were collected. The polysaccharide-containing fractions were detected by the anthrone reaction and combined. The solvent was evaporated, the degraded oxidised polysaccharide dissolved in dichloromethane (2 ml), and sodium ethoxide (0.8M, 2.5 ml) added. After 1 h at room temperature, the solution was neutralised with 90% aqueous acetic acid and evaporated to dryness. Aqueous acetic acid (50%, 10 ml) was added to the residue and the solution was heated for 1 h at 100°. The hydrolysed mixture was again evaporated to dryness, dissolved in water, and extracted into chloroform. The chloroform solution was evaporated and the now degraded, oxidised, and partially hydrolysed polysaccharide residue was dried over phosphorus pentaoxide before being ethylated. The ethylated product was hydrolysed, and the resulting sugars were analysed as the alditol acetates by g.l.c. and m.s. G.l.c. on OV-225 (column *E*, 165°) revealed three peaks of approximately equal size; the first (*T* 0.32, relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-glucitol) had the m.s. of a sugar degradation product, the second (*T* 0.44) was the alditol acetate of 2,4-di-*O*-methyl-3-*O*-ethyl-L-rhamnose, and the third (*T* 0.90) was 2,3-di-*O*-methyl-L-rhamnose.

Periodate oxidation of 71a polysaccharide. — Preliminary experiments showed that sodium periodate at pH 4.6 or pH 7 failed to oxidise all of the 4-*O*-methyl-D-glucuronic acid residues in the polysaccharide, although all of the susceptible rhamnose residues (**1**) appeared to be oxidised. The periodate oxidation was repeated successfully in the presence of sodium perchlorate[14]. The 71a polysaccharide (50 mg) was dissolved in a solution (50 ml) of sodium periodate (0.05M) and sodium perchlorate (0.2M), and the mixture was kept in the dark at 4°. Ethylene glycol (2 ml) was added after 48 h, followed 2 h later by sodium borohydride (250 mg), and the

solution was then kept overnight. It was acidified with acetic acid, dialysed exhaustively, deionised with Zeokarb 225(H^+) resin, and an aliquot was examined by methanolysis, conversion into Me_3Si derivatives, and g.l.c. (column *E*, programmed from 100° at 3°/min). The oxidised polysaccharide contained 95% of rhamnose and 5% of 4-*O*-methyl-D-glucuronic acid. The polysaccharide solution was made 0.5M with trifluoroacetic acid and kept for 24 h at room temperature, evaporated to dryness, and reduced with sodium borohydride (200 mg) for 18 h. An excess of acetic acid was added and the borate was removed by evaporation with methanol[15]. The residue was dissolved in water (3 ml) and applied to a column (95 × 1.5 cm) of Bio-Gel P-2 (100–200 mesh) (Bio-Rad) operated at 65° (ref. 35). The column was eluted with water at 36 ml/h and the emergence of products monitored by the anthrone reaction. A small amount of polymeric material near the void volume was followed by the major product, which was eluted as a single, symmetrical peak in the volume at which raffinose was eluted during calibration of the column. This material was collected, deionised with Zeokarb 225 (H^+) and Deacidite FF (acetate) resins, and dried *in vacuo* over phosphorus pentaoxide and sodium hydroxide; yield, 13 mg; $[\alpha]_D$ +13.6° (*c* 0.86, water). The n.m.r. spectrum (270 MHz) included anomeric-proton signals at δ 4.99 (1 H, $J_{1,2}$ 1.5 Hz) and 4.70 (1 H, $J_{1,2}$ 1 Hz) from α- and β-linked rhamnose residues, respectively, a signal at δ 1.30 (6 H, $J_{5,6}$ 7 Hz) from the CH_3 group of two rhamnose residues, and a signal at δ 1.19 (3 H, $J_{1,2}$ 6 Hz) from the CH_3 group of 1-deoxyerythritol[36], indicating structure **6**.

The non-reducing, terminal rhamnose residue of this oligosaccharide was subjected to another periodate oxidation similar to that already described, except that perchlorate was omitted from the mixture; yield, 4 mg; $[\alpha]_D$ +18° (*c* 0.38, water). The n.m.r. spectrum (270 MHz) showed the following significant signals: δ 4.70 (1 H, $J_{1,2}$ 1 Hz), 1.29 (3 H, $J_{5,6}$ 6 Hz), and 1.17 (3 H, $J_{1,2}$ 7 Hz), consistent with the product being β-L-rhamnosyl-1-deoxyerythritol (**7**).

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