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STRUCTURAL STUDIES OF ACIDIC GLUCOMANNANS FROM STRAINS OF *Serratia marcescens* O14 AND O6*

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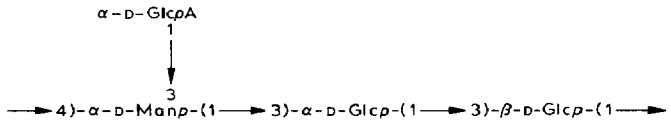
ABSTRACT

Partially acetylated acidic glucomannans have been isolated from three strains of *Serratia marcescens* serogroup O14 and one strain of the cross-reacting serogroup O6. Degradative and spectroscopic studies established that the polysaccharides have branched tetrasaccharide repeating-units of the structure shown. Individual polymers may vary in the extent or location of *O*-acetylation, and in the extent of undefined heterogeneity apparently associated with the glucosyluronic acid residues. Although the polymers were obtained from lipopolysaccharide extracts, there are indications of a (micro)capsular origin. The acidic glucomannans may constitute a common antigen which defines the O14–O6 complex of *S. marcescens*.



INTRODUCTION

Gram-negative bacteria can produce a variety of heat-stable antigens, usually associated with surface carbohydrates, although there seems to be a need for more caution in making this assumption[1]. Best known and frequently dominant are the lipopolysaccharides which characterise and contribute unique properties to the outer membrane of the cell envelope[2]. Where present (in complete, S-form lipopolysaccharides), the polymeric side-chain confers O-specificity on the cell. The same polymer may also occur as a hapten, associated with the envelope but not part of the lipopolysaccharide[3]. Another polysaccharide which occurs in both immunogenic and haptenic forms is the Enterobacterial Common Antigen[4–6] found in almost all members of the family Enterobacteriaceae[7]. Many organisms in this family (notably strains of *Escherichia coli* and *Klebsiella*) also produce clinically important capsular (K) antigens.

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Even in terms of this scenario, the results of early studies[8,9] have created the impression that *Serratia marcescens* (another enterobacterial species) produces an exceptionally complex range of surface carbohydrates. Although twenty-one O-serogroups have been defined[10], the chemical basis for serological distinction and cross-reactions has yet to be established. During studies of the lipopolysaccharides from three strains of the common O14 serogroup, three different polysaccharides were encountered[11]. Only one of these polymers (a glucomannan) was present in all three strains, and was therefore a candidate as the O-antigen. We now report on the results of structural studies of this polymer from one strain (C.D.C. 1783-57) of serovar O14:H9, and make comparisons with the corresponding products from two strains of serovar O14:H12 and another of the closely related[10] serovar O6:H3.

RESULTS

As reported[11], chromatography on Sephadex G-50 or G-75 of the polymeric material produced (36% yield) on mild, acid hydrolysis (aqueous 1% acetic acid, 100°, 2.25 h) of the lipopolysaccharide from *S. marcescens* C.D.C. 1783-57 did not give discrete fractions. However, analyses of fractions of the eluate from the more

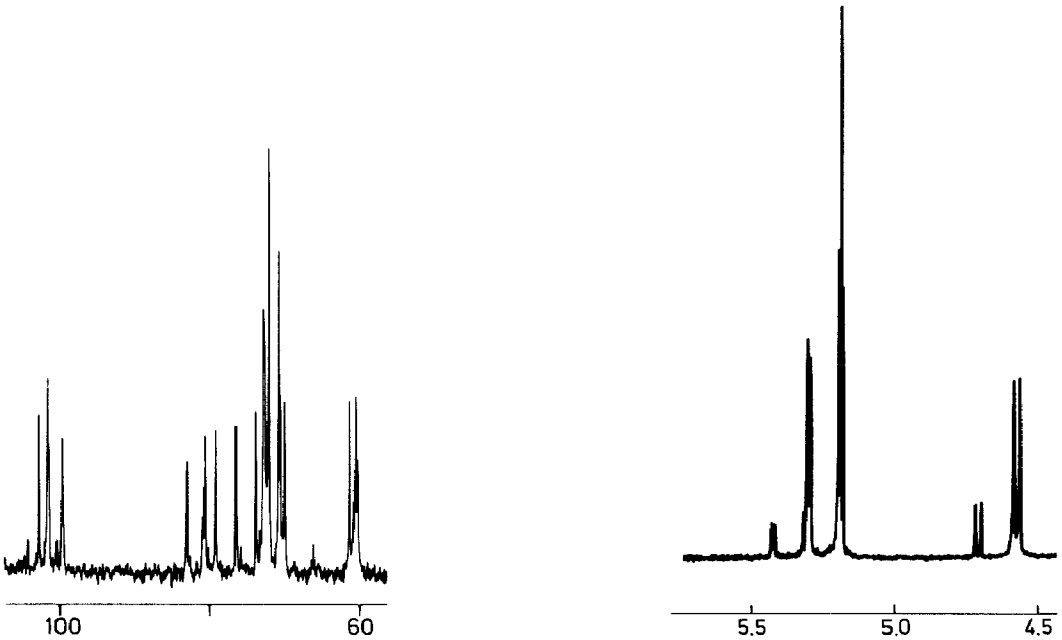


Fig. 1. ^{13}C-N.m.r. spectrum of *O*-deacetylated acidic glucomannan from *S. marcescens* C.D.C. 1783-57. The spectrum for the sample in D_2O was obtained at 100.57 MHz and 50° with complete proton-decoupling. In addition to the signals shown, the spectrum contained a signal for a carbonyl carbon at δ 176.0 with reference to external tetramethylsilane.

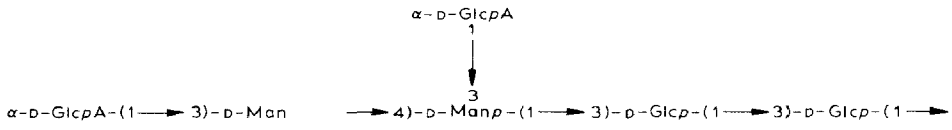
Fig. 2. Anomeric region of the ^{1}H-n.m.r. spectrum of *O*-deacetylated glucomannan from *S. marcescens* C.D.C. 1783-57. The spectrum for the sample in D_2O was obtained at 400.13 MHz and 85°, with sodium 4,4-dimethyl-4-silapentane-1-sulphonate as internal reference.

porous gel for neutral and amino sugars indicated the presence of two polymers. Early fractions of the eluate were rich in glucose and mannose, and later ones rich in glucose, galactose, and 2-amino-2-deoxyglucose. The two polymers were cleanly separated by chromatography on DEAE-Sepharose CL-6B with stepwise elution. The polymer containing 2-acetamido-2-deoxy-D-glucose, D-glucose, and D-galactose (molar ratios 2:1:1) was eluted partly with water (20% yield) and partly with 0.1M NaCl (32% yield). Structural studies of this polymer will be described elsewhere. The second polymer was also obtained as two fractions identical in monosaccharide composition (D-glucose, D-mannose, and D-glucuronic acid in the molar ratios ~2:1:1) by elution with 0.2M NaCl (15% yield, broad, low elution profile) and 0.3M NaCl (33% yield, sharp peak). Although the two fractions were identical in other respects, there was an unexplained difference in specific rotation: $[\alpha]_D$ +104° (*c* 0.7, water) for the first fraction and +42° (*c* 1.9, water) for the second.

The i.r. spectra of the fractions of acidic glucomannan contained, *inter alia*, peaks at 1720 and 1250 cm^{-1}, suggesting the presence of an *O*-acetyl substituent. This was confirmed by the n.m.r. spectra: signals at δ 2.19, 2.17, and 2.13 in the ^{1}H-n.m.r. spectrum (total peak area equivalent to about two-thirds of an acetyl group per tetrasaccharide repeating-unit), and signals at δ ~21 and ~175 (complex) in the ^{13}C-n.m.r. spectrum. In order to eliminate this source of heterogeneity, the polymer was *O*-deacetylated for structural studies.

Analyses of the alkali-treated polymer gave the composition D-glucose (44%), D-mannose (20%), and D-glucuronic acid (22%). Major anomeric signals at δ 102.58 ($^1J_{CH}$ 166 Hz), 101.43, 101.26, and 99.64 ($^1J_{CH}$ values not accurately determinable) in the ^{13}C-n.m.r. spectrum (Fig. 1) confirmed a tetrasaccharide repeating-unit with at least one β-linked residue. The signals at δ 61.18, 60.45, and 60.14 showed that all three hexose residues were unsubstituted at C-6. In the ^{1}H-n.m.r. spectrum (Fig. 2), the major anomeric signal at δ 4.57 ($J_{1,2}$ ~8 Hz) showed that one sugar with the *gluco* configuration was β-linked. However, the presence of minor signals in both n.m.r. spectra pointed to residual heterogeneity or contamination.

Two significant peaks (relative areas 2.3:1) for methylated alditol acetates were obtained in g.l.c. of the products from methylation analysis of the polymer. Mass spectrometry showed that the major and more volatile product was derived from a 3-substituted hexopyranose residue: major fragment at *m/z* 292 (M − 59) in the chemical-ionisation spectrum with methane as the reagent gas[12], and primary fragments at *m/z* 277, 234, 161, and 118 in the electron-impact spectrum of the *1-d*-labelled product. The product had the g.l.c. retention time of 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylglucitol and, after isolation by t.l.c., gave glucitol on *O*-deacetylation and demethylation. By using the same methods, the less-volatile product of methylation analysis was identified as 1,3,4,5-tetra-*O*-acetyl-2,6-di-*O*-methylmannitol-*1-d*, showing that the mannosyl residue was present at a branching point. When the methylated polymer was reduced ($LiAlH_4$) and re-methylated before hydrolysis, 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol-*1-d* was an addi-



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tional product, showing that the glucuronic acid occurred in the polymer as an unsubstituted pyranosyl residue. In agreement with the above data, the uronic acid (but not the hexoses) was destroyed on treatment of the polymer with sodium periodate.

A direct linkage between the uronic acid and mannosyl residues was proved by the isolation and characterisation of the aldobiouronic acid (M_{GlcA} 0.77 at pH 2.7), which gave only these sugars on acid hydrolysis. The ^{1}H-n.m.r. spectrum of the reduced ($NaBD_4$) aldobiouronic acid contained only one anomeric signal, δ 5.13 ($J_{1,2}$ 3.8 Hz), indicating an α linkage. Methylation analysis of the reduction product gave 3-*O*-acetyl-1,2,4,5,6-penta-*O*-methylmannitol-*1-d*. Thus, the aldobiouronic acid had the structure **1**, and the partial structure **2** could be assigned to the repeating unit of the polymer.

In order to confirm the structure of the repeating unit, the deacetylated polymer was subjected to two successive Smith-degradations. As expected, the product from the first degradation remained polymeric, and methylation analysis confirmed that the 3,4-disubstituted mannosyl residue in the original polymer had been replaced by a 4-substituted mannosyl residue (primary fragments at *m/z* 233 and 118 in the electron-impact mass spectrum of the *1-d*-labelled methylated alditol acetate). In contrast to the parent polymer, the degradation product was homogeneous, as judged by its n.m.r. spectra. The ^{13}C-n.m.r. spectrum (Fig. 3) contained seventeen signals, of which one (δ 69.98) was of double intensity. The anomeric signals at δ 102.80 ($^1J_{CH}$ 163 Hz), 100.80 ($^1J_{CH}$ 173 Hz), and 99.39 ($^1J_{CH}$ 173 Hz) showed that only one of the residues in the main chain was β-linked, and this could be diagnosed as a glucosyl residue from the ^{1}H-n.m.r. data for the original polymer (see above). The inference was supported by the data for anomeric signals in the ^{1}H-n.m.r. spectrum (Fig. 4) of the Smith-degradation product: one-proton signals at δ 5.33 ($J_{1,2}$ 3.4 Hz), 5.25 (unresolved), and 4.55 ($J_{1,2}$ 7.8 Hz).

The sequence of the two glucosyl residues was determined by a Smith degradation of the linear glucomannan, during which the mannose was oxidised and a diglucosylerythritol was produced. The latter had $R_{Mannitol}$ 0.67 (p.c. in solvent *A*), and was unaffected by β-D-glucosidase. Half of the glucose in the compound was released on treatment with α-D-glucosidase, along with a monoglucosylerythritol ($R_{Mannitol}$ 0.96), which was itself hydrolysed by β-D-glucosidase. Structure **3** could therefore be assigned to the product of the second Smith-degradation, and structure **4** to the repeating unit of the original deacetylated polymer. Because *O*-acetylation of the parent polymer was not stoichiometric and was apparently variable in position (perhaps as a result of hydrolysis or migration during the treatment with hot

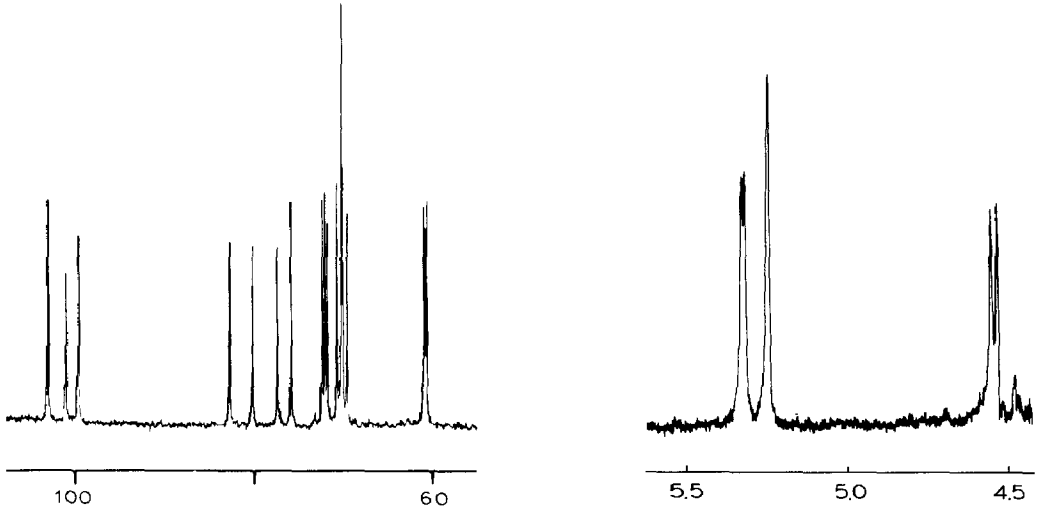

Fig. 3. ^{13}C-N.m.r. spectrum of the neutral glucomannan obtained after Smith degradation of the acidic polymer from *S. marcescens* C.D.C. 1783-57. The spectrum for the sample in D_2O was obtained at 100.61 MHz and 50° with complete proton-decoupling and tetramethylsilane as external reference.

Fig. 4. Anomeric region of the ^{1}H-n.m.r. spectrum of the neutral glucomannan obtained after Smith degradation of the acidic polymer from *S. marcescens* C.D.C. 1783-57. The spectrum for the sample in D_2O was obtained at 400.14 MHz and 82°, with sodium 4,4-dimethyl-4-silapentane-1-sulphonate as internal reference.

α-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→2)-Erythritol

3

α-D-Glc*p*A
1
↓
3
→4)-α-D-Man*p*-(1→3)-α-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→

4

aqueous 1% acetic acid), no attempt was made to locate the acetyl group(s). The absence from the polymer of sugars other than glucose, mannose, and glucuronic acid, taken with the homogeneity of the first Smith-degradation product, suggested that the complexity of the deacetylated polymer indicated by its n.m.r. spectra could be due to incomplete (or variable) substitution of the main chain by the glucosyluronic acid group (other possibilities are noted below).

Because the acetylated acidic glucomannan was a candidate as the O14 antigen of *S. marcescens*, the polymeric fractions from the lipopolysaccharides of two other O14 strains (both O14:H12) were also examined. From strain C.D.C. 4444-60, a fraction of composition D-glucose (43%), D-mannose (20%), and D-glucuronic

acid (25%) was isolated in 26% yield by the methods described for strain C.D.C. 1783–57. Methylation analysis of this polymer gave the glucose and mannose derivatives described above, and an aldobiouronic acid with an electrophoretic mobility identical to that of **1** was obtained on acid hydrolysis of the polymer. The i.r. spectrum indicated a relatively low degree of *O*-acetylation, confirmed by the ^{1}H-n.m.r. spectrum to be variable (signals at δ 2.20, 2.18, and 2.14) and to amount to about one-third of an acetyl group per tetrasaccharide repeating-unit. In consequence, the ^{13}C-n.m.r. spectrum closely resembled that of the deacetylated polymer from strain C.D.C. 1783–57 (Fig. 1). Also, the anomeric region of the ^{1}H-n.m.r. spectrum contained the major signals shown in Fig. 2 for the latter product, but only traces of the minor signals, indicating structural consistency in the repeating units.

The acidic glucomannan from the second O14:H12 strain (C.D.C. 874-57) was studied more briefly. The product was actually that described previously[11] as fraction Fla, obtained by chromatography on Sephadex G-75. The glucose–mannose ratio was 2:1, glucuronic acid and the aldobiouronic acid were released on acid hydrolysis, and the ^{13}C-n.m.r. spectrum of the polymer resembled that of the polymer from the first O14:H12 strain. Thus, it may be concluded that the acidic glucomannans from all three O14 strains have the same architecture, but may differ in the extent (or location) of *O*-acetylation and of substitution of the main chain by glucuronic acid residues.

Because cross-reactions between serogroups O14 and O6 of *S. marcescens* are so extensive[13–15] that the value of the distinction is questionable[10], structural studies were also carried out on the acidic glucomannan isolated from a reference strain (C.D.C. 862-57) of serovar O6:H3. This glycan was obtained in 43% yield by chromatography of the polymeric fraction from the lipopolysaccharide on DEAE-Sepharose. Its monosaccharide composition of D-glucose (44%), D-mannose (20%), and D-glucuronic acid (23%) agreed with those of the acidic glucomannans already described. The extent of *O*-acetylation (rather less than one-third of an acetyl group per repeating unit) was similar to that of the product from strain C.D.C. 4444-60, but slightly more complex (four signals in the ^{1}H-n.m.r. spectrum between δ 2.14 and 2.20). Methylation analysis and Smith degradation of the deacetylated polymer (n.m.r. spectra of the glucomannan were identical to those shown in Figs. 3 and 4) confirmed that the backbone was the same as that in the polymers from the O14 strains. However, the minor signals noted in the n.m.r. spectra for the deacetylated polymer from the O14:H9 strain (Figs. 1 and 2) were much more pronounced for the polymer from the O6:H3 strain. Thus, whereas the minor β-anomeric signal at δ 4.69 had an area only 15% of the major (related?) one at δ 4.57 in the case of the former strain (Fig. 2), the value was 30% in the case of the latter strain. As the monosaccharide compositions and other data for all four polymers are in close agreement, the spectral evidence for heterogeneity may have an explanation different from those put forward above. One possibility would be a variation in the ionic versus covalent state of the uronic acid, *e.g.*, as a result of intra- or inter-residue esterification.

DISCUSSION

This study has revealed the presence in strains of *S. marcescens* O14 and O6 of acidic glucomannans with a common architecture. Structural variations may include the extent or location of *O*-acetylation, and heterogeneity associated with substitution of the glucomannan by the glucosyluronic acid group. Although serological confirmation is required, these results suggest that the acidic glucomannans are antigens common to serogroups O14 and O6, and may contribute the O-antigenic factor 6_2 recognised[14] to be present in both the O14 strain C.D.C. 4444-60 and the O6 strain C.D.C. 862-57 studied here. By contrast, two different neutral polymers have been isolated[11] from the three O14 strains and cannot therefore confer O14 specificity, while a third neutral polymer (a glucorhamnan) is present in the O6 strain[16]. Preliminary studies of polymeric fractions from the lipopolysaccharides of reference strains for two other serogroups (O1 and O12) involved in cross-reactions with O14 strains have again revealed the presence of both neutral and acidic components. Although these acidic polymers also contain D-glucose and D-mannose, and have branched repeating-units, they differ[16,17] in composition and structure from the acidic glucomannans characterised in the present study. It is also interesting to note that acidic glucomannans have been described[8,9] as extracellular, capsular, and cellular products of *S. marcescens* N.R.C. S-29. Although detailed comparisons with our products are not possible, many structural features seem to be shared (*e.g.*, a glucomannan backbone containing a nigerose unit; the aldobiouronic acid **1**).

In view of the work of Adams and his colleagues[8,9], the molecular and cellular origins of our acidic glucomannans are of some interest. None of the strains used were visibly encapsulated, and the use of isolated cell envelopes (prepared by an abrasive mechanical method) as the starting material should have eliminated both extracellular and intracellular polysaccharides from the products. Although the glucomannans were obtained from "lipopolysaccharides" extracted from the cell envelopes by the hot, aqueous phenol method, there are reasons to doubt that they are integral components of the true lipopolysaccharides. Thus, aldoheptoses and other core-specific components can be detected in the neutral polymers obtained from mild acid hydrolysates of the "lipopolysaccharides", but not in the acidic glucomannans[11,18]. Also, when aqueous-phenol extracts of whole cells of *S. marcescens* C.D.C. 4444-60 were subjected to ultracentrifugation, the mannose-containing polymer remained in the supernatant fluid, whereas the neutral polymer sedimented in the "lipopolysaccharide" pellet. No contamination by acidic glucomannans was noted in studies of two other lipopolysaccharides from *S. marcescens* where ultracentrifugation was used in the process of purification[19,20]. It is therefore suggested that the acidic glucomannans may be (micro)capsular polymers associated with the cell envelope in some unknown way. If confirmed, this leads to the conclusion that the current scheme of O-serogroups for *S. marcescens* is exceptional in not being based (entirely) on the chemistry of the organism's lipopolysaccharides.

EXPERIMENTAL

Growth of bacteria, and isolation and fractionation of lipopolysaccharides. — Methods used for the growth of *S. marcescens* strains C.D.C. 1783-57, 4444-60, 874-57, and 862-57, for the preparation of cell envelopes, and for the extraction of lipopolysaccharides have been described[11]. The only modification was the use of a Dyno-Mill KDL (W. A. Bachofen AG, Basel) for the continuous disintegration of suspended cells. After hydrolysis (aqueous 1% acetic acid, 100°, 2.25 h), the water-soluble products were fractionated[11] on Sephadex G-50. Polymeric fractions were then eluted from a column (40 × 2.2 cm) of DEAE-Sepharose CL-6B, using water, 0.1M, 0.2M, and 0.3M NaCl (300 mL each), collecting fractions (5 mL) at a flow rate of 15 mL.h^{-1}. Fractions from both columns were analysed for carbohydrate[21], and combined fractions from the ion-exchange column were dialysed and freeze-dried.

General methods. — I.r. spectra were recorded with a Unicam SP-200 spectrophotometer for samples dispersed in KCl. Specific rotations were determined with a Bendix polarimeter (Model 143A). N.m.r. spectra (^{13}C and ^{1}H) were recorded for solutions in D_2O with a Bruker WH-400 spectrometer; ^{13}C spectra (with complete proton-decoupling or with gated decoupling) were recorded at ~50° with tetramethylsilane as the external standard; ^{1}H spectra were recorded at ~80° with sodium 4,4-dimethyl-4-silapentane-1-sulphonate as the internal standard. G.l.c. was performed with a Pye Unicam 104 instrument and a column (1.6 m × 2 mm) of 3% of Silar 10c on Gas Chrom Q. G.l.c.–m.s. was carried out with a Finnigan model 1020B instrument fitted with a fused-silica capillary column (30 m) of SE-54. Mass spectra were obtained either by the electron-impact method or by chemical ionisation with methane as the reagent gas[12]. P.c. was carried out with Whatman No. 1 paper and *A*, ethyl acetate–pyridine–water (13:5:4); *B*, ethyl acetate–pyridine–acetic acid–water (5:5:1:3). Paper electrophoresis[22] was performed with a pyridine–acetic acid buffer (pH 2.7). The detection reagents used were aniline hydrogenoxalate, alkaline silver nitrate, and the periodate–Schiff reagents[11]. T.l.c. of partially methylated hexitols was carried out on Silica Gel 60 F_{254} (Merck), with butanone saturated with 3% ammonia. The separated components were detected with iodine vapour and eluted using methanol.

Determination of monosaccharide composition. — For the estimation and identification of neutral sugars, samples (and appropriate standards) were treated with 2M HCl at 105° for 2 h. After neutralisation with Dowex 1 (HCO_3^-) resin and deionisation, the hydrolysates were analysed by p.c. (solvent *A*), g.l.c. of the alditol acetates (with inositol hexa-acetate as the internal standard), and enzymic assays. Glucose was determined either by using D-glucose oxidase (EC 1.1.3.4) or by using the combination of hexokinase (EC 2.7.1.1) and D-glucose 6-phosphate dehydrogenase (EC 1.1.1.49). Mannose was determined by using the latter combination together with D-glucose phosphate isomerase (EC 5.3.1.9) and D-mannose phosphate isomerase (EC 5.3.1.8). All enzymes were obtained from Boehringer und Soehne G.m.b.H. In all cases, the results of g.l.c. and enzyme assays were in excellent agreement.

Glucuronic acid (and an aldobiouronic acid) were released by hydrolysis of samples with 0.5M H_2SO_4 at 105° for 4 h. After neutralisation of the hydrolysate ($BaCO_3$), the uronic acid was identified by p.c. (solvent *B*) and paper electrophoresis. The uronic acid was also converted into D-glucose by treatment with 1% methanolic HCl at 75° for 10 h, followed by reduction with $NaBH_4$, and acid hydrolysis (2M HCl, 105°, 2 h). The product was identified by p.c., g.l.c. of the alditol acetate, and enzymic assay. Total glucuronic acid was determined by a colorimetric assay[23].

The aldobiouronic acid had R_{GlcA} 0.89 in p.c. (solvent *B*) and M_{GlcA} 0.77 in paper electrophoresis (pH 2.7). On further hydrolysis (0.5M H_2SO_4, 105°, 16 h), it gave only mannose (p.c. and g.l.c.) and glucuronic acid (p.c. and paper electrophoresis).

O-*Deacetylation*. — Polymers were treated overnight at room temperature with 0.1M NaOH. After neutralisation (HCl) and freeze-drying, the product was desalted on a column of Sephadex G-50, re-dried, and converted into the ammonium salt by using Dowex 50 (NH_4^+) resin.

Methylation analysis. — Standard methods[11] were used for methylation and the preparation of methylated alditol acetates. The products were identified by g.l.c. and g.l.c.–m.s. For confirmation of the identity of the parent sugars, products were deacetylated by treatment with 0.2M methanolic KOH at room temperature for 2.5 h and the partially methylated alditols separated by t.l.c. After *O*-demethylation[24], the alditols were identified by g.l.c. of the peracetates. Uronic acid residues in the methylated acidic glucomannan from strain C.D.C. 1783-57 were reduced by treatment with $LiAlH_4$ in tetrahydrofuran under reflux for 4 h, and the product was re-methylated before acid hydrolysis.

Periodate oxidation and Smith degradation. — Oxidation of polymers was carried out for 5 days at 4° with 50mM sodium periodate. After the addition (successive) of ethylene glycol, $NaBH_4$, and acetic acid, the mixture was freeze-dried and the product was desalted using Sephadex G-50. Selective Smith hydrolysis was carried out overnight at room temperature with M trifluoroacetic acid, and the products were fractionated on suitable grades of Sephadex (G-50 when the main product was polymeric, and G-10 when it was an oligosaccharide-alditol).

Enzymic hydrolyses. — The oligosaccharide-alditol obtained after two consecutive Smith-degradations of the deacetylated acidic glucomannan from strain C.D.C. 1783-57 ($R_{Mannitol}$ 0.67, solvent *A*) was treated with α-D-glucosidase (EC 3.2.1.20, Boehringer or Sigma), β-D-glucosidase (EC 3.2.1.21), or a combination of both enzymes in 0.1M acetate buffer (pH 6.6) at 37° for 4 h (or overnight under toluene). The release of glucose was followed by paper chromatography (solvent *A*) and by enzymic assay.

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