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STRUCTURAL STUDIES OF AN ACIDIC GALACTOGLUCOMANNAN FROM THE O3 REFERENCE STRAIN (C.D.C. 863-57) OF *Serratia marcescens*

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ABSTRACT

A partially acetylated acidic galactoglucomannan has been isolated from the lipopolysaccharide of the O3 reference strain (C.D.C. 863-57) of *Serratia marcescens*. By means of n.m.r. spectroscopy, methylation analysis, and degradative studies, the polymer was found to have the branched pentasaccharide repeating-unit shown. The position(s) of partial acetylation were not determined. Although the polymer is believed to confer O specificity on the parent organism, it is probably not an integral component of the lipopolysaccharide.

$$\begin{array}{c} \beta\text{-D-Man}p \\ 1 \\ \downarrow \\ 4 \\ \rightarrow 3)\text{-}\alpha\text{-D-Man}p\text{-}(1\rightarrow 3)\text{-}\alpha\text{-D-Gal}p\text{-}(1\rightarrow 2)\text{-}\alpha\text{-D-Glc}p\text{A-}(1\rightarrow 3)\text{-}\alpha\text{-D-Man}p\text{-}(1\rightarrow \end{array}$$

INTRODUCTION

Both neutral and acidic polymers (singly or together) have been isolated during fractionation of the "lipopolysaccharides" from reference strains for different O serogroups of *S. marcescens* (ref. 1 and the preceding studies cited). When present, the acidic polymers seem to be the dominant antigens, whereas the neutral polymers seem to constitute the lipopolysaccharide side-chains. Four of the "lipopolysaccharides" from reference strains (representing serogroups O2, O3, O11, and O13 in the scheme of Edwards and Ewing[2]) yield only acidic polymers on mild acid hydrolysis. The O2 polymer has been characterised as a galactorhamnan[3] and the O13 product as a galactoglucomannan[4]. We now report the structure of the acidic polymer from a third member (O3) of this group of strains.

RESULTS

Like other "lipopolysaccharides" which fail to yield a neutral, polymeric fraction on mild acid hydrolysis, the O3 product did not give the "ladder" pattern

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typical of S-type lipopolysaccharides on sodium dodecyl sulphate–polyacrylamide gel electrophoresis. Nevertheless, the "lipopolysaccharide" reacted specifically with the homologous antiserum[5], indicating the presence of the O3 group antigen.

The monosaccharide components of the polymeric fraction isolated after mild acid hydrolysis of the "lipopolysaccharide" were D-mannose (45.0%), D-galactose (15.5%), and D-glucuronic acid (13.3%). The presence of *O*-acetyl substituent(s) was indicated by signals in the n.m.r. spectra at δ 20.39 and 20.32 (^{13}C), and between δ 2.20 and 2.10 (^{1}H). The n.m.r. spectra were greatly simplified by *O*-deacetylation of the polymer. The ^{1}H-n.m.r. spectrum of the product contained five anomeric signals of equal integral at δ 5.43 ($J_{1,2}$ ~3 Hz), 5.27 ($J_{1,2}$ ~3 Hz), 5.14 (unresolved), 5.05 (unresolved), and 4.68 (unresolved). These data showed that both galactose and glucuronic acid were present as α-pyranosyl residues, and suggested that two of the three mannosyl residues were α-linked and one β-linked (each as a pyranoside). The ^{13}C-n.m.r. spectrum (Fig. 1) contained 27 discrete signals, including anomeric signals at δ 100.28 ($^{1}J_{CH}$ 174 Hz), 99.87 ($^{1}J_{CH}$ 161 Hz), 97.79, 96.07, and 95.68 ($^{1}J_{CH}$ 171 Hz). Coupling constants for the anomeric signals at δ 97.79 and 96.07 could not be determined from the INEPT spectrum, as the positive line of one doublet coincided with the negative line of the other. However, the combined value of 342 Hz, calculated from the residual outer signals, was consistent with an attribution to α-pyranosyl residues. In the proton-decoupled spectrum (Fig. 1), several signals (including that at δ 99.87) had relatively high intensities and low line-widths, indicating that the nuclei giving rise to these signals had relatively long spin–lattice relaxation times and were located in a flexible region of the polymer (*e.g.*, in a terminal residue). Indeed, all of the high-intensity signals could be assigned to a terminal β-mannopyranosyl residue[6].

Methylation analysis of the native polymer (Table I, column A) did not give the results expected for a polymer with a branched pentasaccharide repeating-unit. The recovery of the product from the terminal mannosyl group was reproducibly low. A possible explanation for this result was loss of the group by base-catalysed elimination from an esterified glucuronic acid residue in the first stage of the

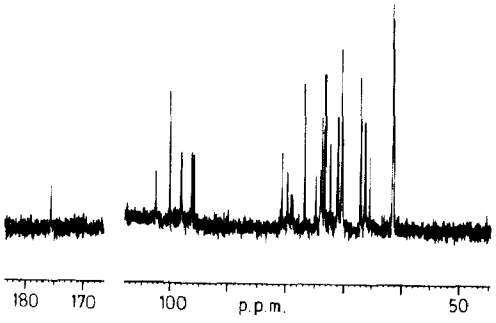

Fig. 1. ^{13}C-N.m.r. spectrum of the *O*-deacetylated acidic galactoglucomannan. The spectrum for the sample in D_2O was obtained at 100.61 MHz and 50°, with complete proton-decoupling and tetramethylsilane as the external reference.

TABLE I

METHYLATION ANALYSIS OF THE ACIDIC GALACTOGLUCOMANNAN AND DERIVED PRODUCTS[a]

Methylation product[b]	*Relative peak area (g.l.c.)*				
	A	*B*	*C*	*D*	*E*
2,3,4,6-Man	0.22	0.98	0.77	1.48[c]	
2,4,6-Man	2.00	2.00	2.00	1.00	2.00
2,4,6-Gal	0.92	0.99	0.85	0.51	0.92
3-Glc			0.81		

[a]Key: A, native polymer; B, *O*-deacetylated polymer; C, *O*-deacetylated polymer with carboxyl reduction; D, product from β-elimination and trideuteriomethylation; E, Smith-degradation product (O3SD). [b]2,3,4,6-Man = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylmannitol, *etc.* [c]Trideuteriomethylated at O-3 (~80%).

methylation. Support for this hypothesis was provided by the results obtained on methylation analysis of the *O*-deacetylated polymer (Table I, column B), which confirmed that the galactose and two of the mannose residues were 3-substituted, and that the third mannose residue was terminal. When the methylated polymer was reduced before hydrolysis, an additional product derived from a 2,4-disubstituted residue of glucopyranosyluronic acid was detected (Table I, column C).

In order to identify the sugar residue substituted by the uronic acid, a β-elimination experiment was carried out on the methylated, *O*-deacetylated polymer, and the products were remethylated using trideuteriomethyl iodide so as to distinguish between the original unsubstituted mannopyranosyl group and any similar group produced by the β-elimination. Analysis of the products (Table I, column D) showed the presence of unsubstituted mannose, 3-substituted mannose, and 3-substituted galactose. The relative intensities of the members of pairs of fragment ions with *m/z* 145 and 148, 162 and 165, and 205 and 208 in the mass spectrum of the first product showed that trideuteriomethylation at O-3 was ~80% complete. Therefore, this product was derived mostly from a residue in the main chain of the polymer substituted at O-3 by the uronic acid, and not from the lateral mannopyranosyl group which would have been eliminated from position 4 of the uronic acid. These results allow the partial structure **1** to be assigned to the unit at the branching point of the polymer. The fact that the three products (Table I) of the β-elimination were not obtained in similar proportions can probably be explained as a consequence of further elimination of the 2-substituent from the 4,5-unsaturated residue initially formed from the uronate ester, followed by alkaline "peeling" of 3-substituted hexoses from the reducing end of the oligosaccharide[7]. Thus, the relatively low yield of 3-substituted galactose (Table I, column D) suggests that this is the residue exposed first, *i.e.*, that galactose is the unidentified hexose in structure **1**, and hence that the linear tetrasaccharide of the repeating unit has structure **2**.

$$\begin{array}{c}\beta\text{-D-Man}p\\ 1\\ \downarrow\\ 4\end{array}$$

$$\rightarrow 3)\text{-Hex}p\text{-}(1\rightarrow 2)\text{-}\alpha\text{-D-Glc}p\text{A-}(1\rightarrow 3)\text{-}\alpha\text{-D-Man}p\text{-}(1\rightarrow$$

1

$$\rightarrow 3)\text{-}\alpha\text{-D-Man}p\text{-}(1\rightarrow 3)\text{-}\alpha\text{-D-Man}p\text{-}(1\rightarrow 3)\text{-}\alpha\text{-D-Gal}p\text{-}(1\rightarrow 2)\text{-}\alpha\text{-D-Glc}p\text{A-}(1\rightarrow$$

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In order to confirm the structural inferences above, the *O*-deacetylated polymer was subjected to a Smith degradation, which gave a polymeric product (O3SD) containing mannose (38.6%), galactose (20.2%), and glucuronic acid (17.9%). Methylation analysis of O3SD (Table I, column E) showed that the lateral mannosyl substituent had been removed. The ^{1}H-n.m.r. spectrum of O3SD contained four one-proton anomeric signals at δ 5.50 ($J_{1,2}$ 3 Hz), 5.22 ($J_{1,2}$ 3 Hz), 5.15 (unresolved), and 5.06 (unresolved). Compared with the spectrum of the parent *O*-deacetylated polymer, the spectrum lacked the unresolved signal at δ 4.68. Likewise, the ^{13}C-n.m.r. spectrum lacked the signal at δ 99.87 attributable to the lateral β-mannopyranosyl group.

After carboxyl reduction, acid hydrolysis of O3SD gave mannose, galactose, and glucose in the molar ratios 2.00:1.03:0.60, corresponding to ~60% reduction of the glucuronic acid residues. Methylation analysis of the carboxyl-reduced O3SD gave the derivative of a 2-substituted hexopyranose in addition to the products listed in Table I, column E, confirming that the lateral mannosyl substituent was at position 4 of the uronic acid residue in the native polymer.

Confirmation of galactosyl substitution of the uronic acid was obtained by lithium–ethylenediamine degradation of the *O*-deacetylated polymer[8]. Despite prolonged sonication, the polymer did not dissolve completely in the ethylene-

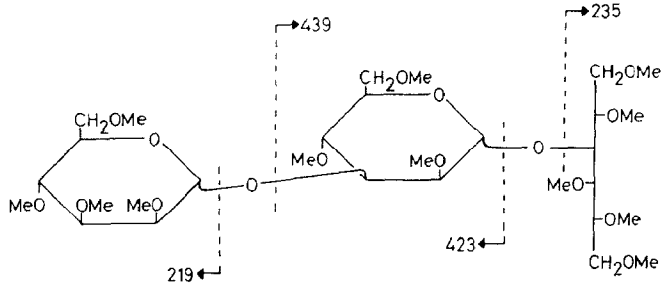
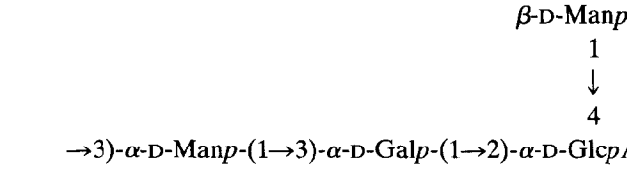


Fig. 2. Diagnostic fragment ions in the electron-impact mass spectrum of the per-*O*-methylated trisaccharide-alditol (O3LD) produced by lithium–ethylenediamine degradation of the *O*-deacetylated acidic galactoglucomannan.

diamine, and the subsequent degradation was therefore incomplete. Fractionation of the products by gel-permeation h.p.l.c. gave a major component (O3LD) eluted in the trisaccharide region. Permethylated O3LD gave a single peak in g.l.c. with a retention time characteristic of the derivative from a trisaccharide-alditol, and the expected fragment ions were present in the mass spectrum of the derivative (Fig. 2). Acid hydrolysis of O3LD gave only mannose and galactitol, consistent with the structure shown (Fig. 2). Thus, the structure of the repeating unit in the *O*-deacetylated O3 polymer is established as **3**. Although the *O*-acetyl groups present in the native polymer have not been located, the ^{1}H-n.m.r. spectrum indicated the presence of ~2 groups per repeating unit, and, probably, heterogeneity in their location.



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DISCUSSION

Like other acidic polymers from *S. marcescens* so far characterised[1,3,4,9,10], the O3 polymer has a relatively large and complex repeating-unit compared with the neutral polymers studied, many of which have a disaccharide repeating-unit[11–16]. Branching occurs in the acidic polymers from the O4 (ref. 10), O6/O14 (ref. 9), O13 (ref. 4), and O15 (ref. 1) reference strains, and partial *O*-acetylation of the polymer is found for O2 (ref. 3), O4 (ref. 10), and O6/O14 (ref. 9). In each of the acidic polymers except that from the O4 strain (ref. 10), acidity is conferred by a hexuronic acid (in O4 by pyruvic acid). The evidence from the results of methylation analysis (Table I, columns A and B) that the glucuronic acid in the O3 polymer has an esterified (lactonised?) carboxyl group is novel for this group of polymers, although heterogeneity in the O6/O14 (ref. 9) and O13 (ref. 4) polymers also involves the uronic acid residue.

The acidic polymer which most closely resembles the O3 product is that from the O15 strain[1], which has the branched pentasaccharide repeating-unit **4**. However, there is no problem in differentiation of the respective O serogroups[2,17], and the O15 strain produces a neutral as well as an acidic polymer[16]. Serological cross-reactions between groups O2 and O3 have been reported[2,17], although the structure of the repeating unit (**5**) for the O2 polymer[3] is not strikingly similar to that (**3**) for the O3 product. No neutral polymer was isolated from either reference strain (suggesting that the lipopolysaccharides were of the R-type, as also indicated by their electrophoretic profiles). Thus, the cross-reactions observed may be due to core-based interactions or to other surface components.

$$\begin{array}{c} \alpha\text{-D-Man}p \\ 1 \\ \downarrow \\ 4 \\ \rightarrow 3)\text{-}\beta\text{-D-Man}p\text{-}(1\rightarrow 3)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow 2)\text{-}\alpha\text{-D-Gal}p\text{A-}(1\rightarrow 3)\text{-}\alpha\text{-D-Man}p\text{-}(1\rightarrow \end{array}$$

4

$$\rightarrow 3)\text{-}\alpha\text{-D-Rha}p\text{-}(1\rightarrow 3)\text{-}\beta\text{-D-Gal}p\text{-}(1\rightarrow 3)\text{-}\alpha\text{-D-Rha}p\text{-}(1\rightarrow 4)\text{-}\alpha\text{-D-Gal}p\text{A-}(1\rightarrow$$

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EXPERIMENTAL

Growth of bacteria, and isolation and fractionation of lipopolysaccharide. — *S. marcescens* O3 (C.D.C. 863-57) was grown for 16 or 24 h at 30° in Nutrient Broth No. 2 (Oxoid) in a 20-L batch fermenter. The lipopolysaccharide (0.20 g) was extracted from cell walls (3.72 g) prepared by mechanical disintegration of the cells (122 g wet-weight), as in previous studies[4]. The acidic galactoglucomannan was obtained (yield, 53% of the lipopolysaccharide) by mild acid hydrolysis (1% acetic acid, 2.25 h, 100°), followed by chromatography of the water-soluble products on Sephadex G-50 and DEAE-Sepharose CL-6B (ref. 9).

General methods. — Methods used for p.c., g.l.c., g.l.c.–m.s., and high-voltage paper electrophoresis were those described in previous studies[3,4,10,14–16]. H.p.l.c. was carried out with Gilson equipment and a TSKgel G-Oligo-PW column (Anachem) eluted with water (1 mL.min^{-1}) at room temperature. N.m.r. spectra were recorded for solutions in D_2O with a Bruker WH-400 spectrometer. ^{13}C-Spectra were recorded at 50° with tetramethylsilane as the external reference, and ^1H-spectra at 80° with sodium 3-trimethylsilylpropanoate-d_4 as the external reference. Electrophoretic analysis of the lipopolysaccharide[18] in the presence of sodium dodecyl sulphate was carried out with gels containing 15% (w/v) of polyacrylamide.

Determination of monosaccharide composition. — Methods used to release, identify, and determine monosaccharides have been described previously[1,3,4]. Quantitative data for neutral monosaccharides were obtained by g.l.c. of the alditol acetates, and for glucuronic acid by a colorimetric method[19]. The D configuration for each monosaccharide was established by enzymic assay, using D-galactose oxidase (EC 1.1.3.9), D-glucose oxidase (EC 1.1.3.4; after reduction[20] of the glucuronic acid), or hexokinase (EC 2.7.1.1) in combination with D-glucose 6-phosphate dehydrogenase (EC 1.1.1.49), D-glucose phosphate isomerase (EC 5.3.1.9), and D-mannose phosphate isomerase (EC 5.3.1.8), as appropriate.

Degradative methods. — Methods used for *O*-deacetylation, methylation analysis, periodate oxidation, Smith degradation, β-elimination, lithium–

ethylenediamine degradation, and reduction of glucuronic acid residues (either by the carbodi-imide method[20] or by hydride reduction of the permethylated polymer) were those used in related studies[1,3,4,10]. The trisaccharide-alditol (O3LD) obtained by the lithium–ethylenediamine degradation[8] of the *O*-deacetylated acidic galactoglucomannan gave a permethylated derivative with a g.l.c. retention time (BP1) similar to that of permethylated maltotri-itol. The mass spectrum of the derivative contained the following signals *inter alia* (relative intensities in brackets and some assignments[21] in square brackets): *m/z* 219(64) [aA_1], 187(99) [aA_2], 155(31) [aA_3], 235(34) [$aldA_1$], 423(21) [baA_1], 439(6) [$baldA_1$], 281(4) [$baldJ_1$], 365(11) [$baldL_1$], 347(2) [$baldM_1$], 585(5), and 629(1).

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