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STRUCTURAL STUDIES OF AN ACIDIC GALACTOMANNAN FROM THE REFERENCE STRAIN FOR *Serratia marcescens* SEROGROUP O4*

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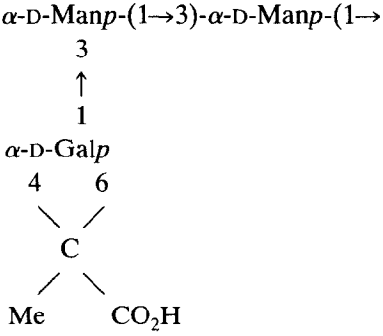
ABSTRACT

An acidic, partially acetylated galactomannan has been isolated from the lipopolysaccharide of the reference strain (C.D.C. 864-57) for *Serratia marcescens* serogroup O4. From the results of methylation analysis, Smith degradations, and n.m.r. spectroscopic studies of the *O*-deacetylated polymer, it was concluded that the repeating unit has the structure shown, in which the acetal-linked pyruvic acid has the *R* configuration. The polymer is believed to confer O specificity on the organism, but not to constitute the side chain of the lipopolysaccharide.



INTRODUCTION

Systematic studies of the lipopolysaccharides from the reference strains for the different O serogroups of the bacterium *Serratia marcescens* are in progress in this laboratory. During the course of this work, it was found that both neutral and acidic glycans could be isolated after mild acid hydrolysis of many of the products, including those from the strains representing serogroups O1 (ref. 1), O4 (ref. 2), O5 (ref. 3), O6 (ref. 4), O7 (ref. 2), O12 (ref. 5), and O14 (ref. 4). Partly because some neutral polymers were found in strains of more than one serogroup, and all

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strains of the same serogroup did not necessarily contain the same neutral polymer, it could be inferred that the acidic glycans were immunodominant and O-specific in organisms where both types co-existed.

The neutral glycan in the O4 reference strain has recently been identified as a partially acetylated glucorhamnan[2]. We now report on the structure of the acidic glycan, which preliminary studies[2] showed to be a galactomannan substituted by pyruvic acid.

RESULTS

The acidic galactomannan was the major polymeric product obtained on mild acid hydrolysis of the lipopolysaccharide (yield ~30% of the lipopolysaccharide). The monosaccharide components were D-galactose (45.8%) and D-mannose (45.2%): no sugar acid was detected. Although n.m.r. spectra for the native polymer were poorly resolved, signals at δ 2.18 (^{1}H) and 20.70 (^{13}C) indicated partial *O*-acetylation, and other signals at δ ~1.5 (^{1}H) and 25.36 (^{13}C) suggested the presence of acetal-linked pyruvic acid. Although heterogeneity was still apparent in the *O*-deacetylated polymer, its n.m.r. spectra pointed to a tetrasaccharide repeating-unit. In the ^{1}H-n.m.r. spectrum, there were four major anomeric signals at δ 5.36 (~0.8 H, $J_{1,2}$ 3.8 Hz), 5.25 (1 H, unresolved), 5.04 (1 H, unresolved), and

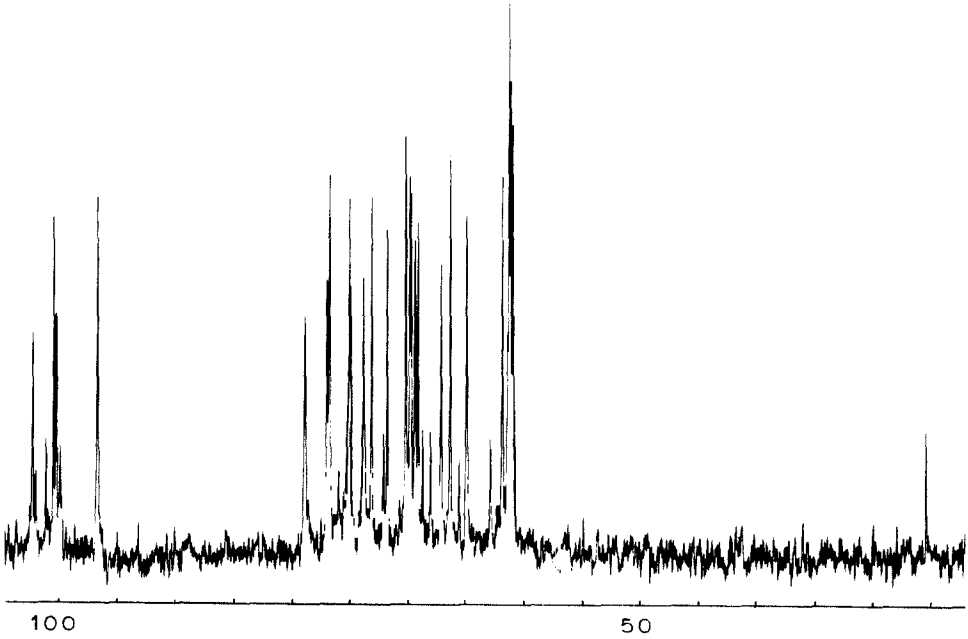

Fig. 1. ^{13}C-N.m.r. spectrum of the *O*-deacetylated, acidic galactomannan. The spectrum for the sample in D_2O was obtained at 100.61 MHz and 50° with complete proton-decoupling and Me_4Si as external reference. The signal for the carbonyl carbon of the pyruvic acid residue was not recorded.

4.51 (0.8 H, $J_{1,2}$ 7.3 Hz), and two minor signals at δ 5.40 (~0.2 H, $J_{1,2}$ 3.6 Hz) and 4.43 (~0.2 H, $J_{1,2}$ ~8 Hz). Similarly, in the ^{13}C-n.m.r. spectrum (Fig. 1), there were 24 discrete, major signals, including anomeric signals at δ 102.21, 100.40, 100.17, and 96.66, as well as various minor signals (including one at δ 25.29 attributable to the pyruvate methyl group). From these data, it could be inferred that one of the galactose residues was α-linked and the other β-linked, that both mannose residues were probably α-linked, and that the residual heterogeneity in the *O*-deacetylated polymer was due to partial (~20%) substitution of galactose by pyruvic acid.

Methylation analysis of the polymer (Table I, column A) showed that all sugar residues were pyranoid, that one residue each of galactose and mannose was 3-substituted, that the second mannose occurred mainly at a branching point (2,3-disubstituted), and that the second galactose was mainly unsubstituted and terminal. The minor products from the methylation analysis indicated that the lateral substituent was at the 3-position of the branching-point mannose, that the substituent was absent from ~10% of the repeating units, and that the pyruvate was attached at positions 4 and 6 of some of the terminal galactose residues.

After Smith degradation of the *O*-deacetylated galactomannan, all of the phenol–H_2SO_4-reactive material was recovered in polymeric form (product SD1). Pyruvic acid was detected after acid hydrolysis of the low-molecular-mass products of oxidation: the yield corresponded to a pyruvate content of 1.8% for the original polymer. Methylation analysis confirmed that SD1 was a linear polymer with a trisaccharide repeating-unit of 3-substituted galactopyranose, 3-substituted mannopyranose, and 2-substituted mannopyranose residues (Table I, column B). The ^{1}H-n.m.r. spectrum of SD1 contained three one-proton signals at δ 5.26 (unresolved), 5.02 (unresolved), and 4.50 ($J_{1,2}$ 7.6 Hz). The ^{13}C-n.m.r. spectrum (Fig. 2) con-

TABLE I

METHYLATION ANALYSIS OF THE ACIDIC GALACTOMANNAN AND THE PRODUCTS FROM SUCCESSIVE SMITH-DEGRADATIONS[a]

Methylation product[b]	*Relative peak area (g.l.c.)*		
	A	B	C
2,3,4,6-Gal	0.53		
2,3,4,6-Man			1.03
2,4,6-Gal	1.00	1.00	1.00
2,4,6-Man	1.02	1.10	
3,4,6-Man	0.12	1.16	
4,6-Man	1.07		
2,3-Gal	0.12		

[a]Key: *A*, native polymer; *B*, product from the first Smith-degradation of the *O*-deacetylated polymer (SD1); *C*, product from the second Smith-degradation (SD2). [b]2,3,4,6-Gal = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol, etc.

tained 17 signals (one of double intensity), including anomeric signals at δ 102.29 ($^1J_{CH}$ ~163 Hz), 100.43 ($^1J_{CH}$ 173 Hz), and 96.65 ($^1J_{CH}$ 171 Hz). No signals attributable to pyruvic acid residues were present in either spectrum. These results confirm that the lateral substituent in the parent polymer is a single α-galactopyranose residue which, in a minority of repeating units, carries a pyruvate residue. The data also confirm that the main chain contains two α-mannopyranosyl residues, one of which carries the lateral substituent at the 3 position.

To determine the sequence of residues in the main chain, SD1 was subjected to a second Smith-degradation. After the hydrolysis step, the products were treated with $NaBH_4$ and fractionated on Sephadex G-15. Depolymerisation was incomplete, but the material of low molecular mass (SD2) contained equimolecular amounts of galactose and mannose, and on methylation analysis gave the results shown in Table I (column C). Structure **1** can therefore be assigned to the trisaccharide repeating-unit of the main chain of the acidic galactomannan.

→3)-β-D-Gal*p*-(1→2)-α-D-Man*p*-(1→3)-α-D-Man*p*-(1→

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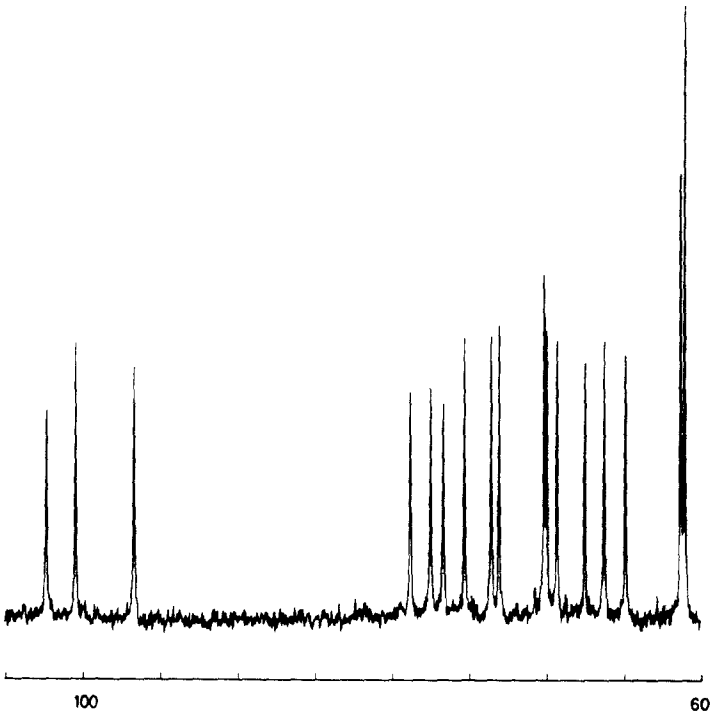
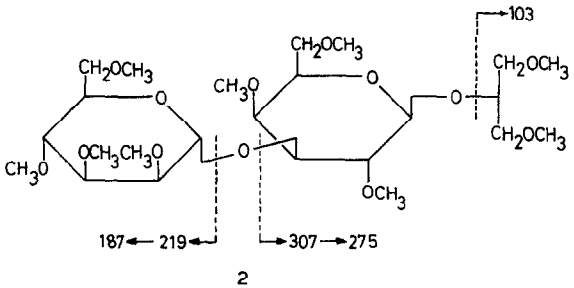


Fig. 2. ^{13}C-N.m.r. spectrum of the first Smith-degradation product (SD1) of the *O*-deacetylated polymer. The spectrum was obtained as for the parent polymer (Fig. 1).

Further study of fraction SD2 showed that it was heterogeneous. The ^{1}H-n.m.r. spectrum contained four signals in the anomeric region at δ 5.13 (~0.4 H, $J_{1,2}$ 3.6 Hz), 5.07 (~1.0 H, $J_{1,2}$ 1.4 Hz), 4.70 (~0.4 H, $J_{1,2}$ 7.8 Hz), and 4.61 (~0.5 H, $J_{1,2}$ 7.8 Hz). Similarly, the ^{13}C-n.m.r. spectrum contained twelve major signals (including δ 103.07 and 96.52) and eight minor signals (including δ 102.64). Neither a further treatment of SD2 with $NaBH_4$, nor repetition of the Smith hydrolysis step followed by $NaBH_4$ reduction, produced any change in the spectra. These results eliminated the possibility that SD2 was a reducing oligosaccharide or that it contained an acid-labile fragment from an incomplete degradation. The absence from the methylation products (Table I) of 3,4,6-tri-*O*-methylmannose showed that the heterogeneity was not due to incomplete oxidation, and the quantitative formation of 2,3,4,6-tetra-*O*-methylmannose showed that cleavage of the bond between the oxidised and unoxidised mannose residues was also complete.

Two major components were detected by g.l.c. of methylated fraction SD2. The later peak (41% of the total peak area) appeared to correspond to the product expected from Smith degradation of a polymer with structure **1**. Structure **2** indicates some of the diagnostic ions obtained by g.l.c.–m.s. The identity of the prod-



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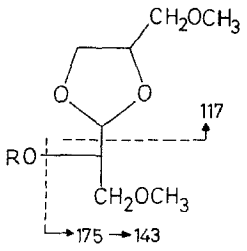
uct which gave the earlier peak in g.l.c. (59% of the total peak area) is more problematic. The methylation data for the mixture SD2 indicated that both components contained 3-substituted galactose and unsubstituted mannose residues, and peaks at *m/z* 219 and 187 in the mass spectrum of the more volatile methylated component confirmed the presence of a hexose at the non-reducing end. Other peaks in the mass spectrum at *m/z* 175 and 117 point to the possible presence of a 1,3-dioxolane residue at the reducing end (structure **3**). Such a residue could be produced by cyclisation of the ring-opened mannose during the hydrolytic step of the Smith degradation, with the participation of the primary hydroxyl group at either C-4 or C-6. Although not widely recognised, similar acid-catalysed (trans)-acetalations have been observed in other Smith-degradations[6]. However, further studies would be necessary to prove the identity of the non-classical Smith-degradation product in fraction SD2.

From the results obtained, structure **4** can be assigned to the repeating unit of the acidic galactomannan. Although the isolated polymer contained only a low



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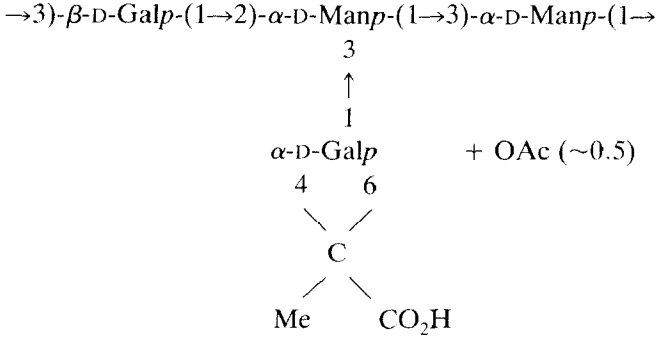
proportion of pyruvate residues, extensive loss of this acid-labile substituent must have occurred during the treatment with aqueous 1% acetic acid used for preparation of the polymer. From the n.m.r. data (particularly the methyl signal at δ_C ~25), the *R* configuration can be assigned[7] to the acetal carbon of the pyruvate residue in the 1,3-dioxane ring. Although the location(s) of *O*-acetyl substituents in the native polymer were not established, the ^{1}H-n.m.r. spectrum contained several signals in the range δ 5.34 to 5.46 (indicating heterogeneity of substitution), and the integrated intensity of the signal at δ 2.18 indicated the presence of ~0.5 acetyl per repeating unit.



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DISCUSSION

The partially acetylated, acidic galactomannan with the repeating-unit **4** is the only polymer so far isolated from "lipopolysaccharides" of *S. marcescens* in which acidity is conferred by acetal-linked pyruvate rather than by a hexuronic acid residue. On the other hand, pyruvate-containing polymers occur commonly as bacterial, capsular products. Although conclusive evidence has yet to be obtained, we expect (by analogy with other strains[4,5,8]) the acidic galactomannan to constitute the O4 antigen but to be microcapsular, rather than an integral component of the lipopolysaccharide. The side chain of the latter is expected to be the partially

acetylated glucorhamnan described elsewhere[2]. The neutral polymer is likely to be responsible for the serological cross-reactions described[9] between group O4 and other groups (O6 and O7) from which similar acetylated glucorhamnans have been isolated[2]. However, the absence of heterologous reactions for O4 antiserum has recently been reported[10] in tests using passive haemagglutination and alkali-treated extracts from heated cells of the reference strain (C.D.C. 864–57) used in the present studies. The absence of cross-reactions may be attributable to the methods used to obtain, treat, or test the extract (*e.g.*, loss of the potentially important *O*-acetyl group), or to the fact that the acidic galactomannan is absent from the other reference strains (O1 to O15) so far examined in these laboratories.

EXPERIMENTAL

Growth of bacteria, and isolation and fractionation of the lipopolysaccharide. — *S. marcescens* strain C.D.C. 864-57 (O4:H4) was grown and the cells were processed as described[2].

General methods. — N.m.r. spectra (^{13}C and ^{1}H) were recorded, for samples dissolved in D_2O, with a Bruker WH-400 spectrometer. ^{1}H-N.m.r. spectra were recorded at 20° or 60° with sodium 3-trimethylsilylpropanoate-d_4 as the external reference. ^{13}C-N.m.r. spectra (with complete proton-decoupling or with gated decoupling) were recorded at 50° with tetramethylsilane as the external reference. G.l.c. separations were carried out with a Carlo Erba Mega 5160 chromatograph fitted with fused-silica capillary columns (25 m) of BP1 (for alditol acetates, oct-2-yl glycoside acetates, and methylated oligosaccharide-alditols) or BP10 (for methylated alditol acetates). G.l.c.–m.s. was carried out with a Finnigan 1020B instrument.

Component analysis. — Monosaccharide components were identified and quantified as described[2]. The configurations of galactose and mannose were determined by g.l.c. of the oct-2-yl glycoside acetates[11]. Pyruvic acid was determined by using lactate dehydrogenase (EC 1.1.1.27) and by n.m.r. spectroscopy.

Degradative methods. — The native galactomannan was *O*-deacetylated by treatment with 0.1M NaOH for 16 h at room temperature, followed by neutralisation with Dowex 50 (H^+) resin. Methylation analysis was carried out as in previous studies[1–5]. The conditions for periodate oxidation and Smith degradation were also those described previously. After two such degradations of the *O*-deacetylated galactomannan and methylation of the product (SD2), two components were detected by g.l.c. (BP1; H_2 flow rate ~44 cm.sec^{-1}; temperature programme: 220° for 4 min, →270° at 15°.min^{-1}, then isothermal). The more volatile product (retention time, 7.77 min) gave a mass spectrum which showed, *inter alia*, signals at (relative intensities in brackets and some assignments[12] in square brackets): *m/z* 219(5)[aA_1], 187(14)[aA_2], 155(5)[aA_3], 72(100), 117(25), 143(8), and 175(3). The mass spectrum of the less volatile product (retention time, 11.85 min) showed, *inter alia*, signals at: *m/z* 219(26)[aA_1], 187(40)[aA_2], 155(9)[aA_3], 46(100), 103(11)[$aldA_1$], 127(20), 159(14)[bC_2^2], 215(2), 233(1), 307(0.3)[$baldA_1$], 275(0.5)[$baldA_2$], and 367(0.6)[$abaldJ_1$].

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