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Structure of an acidic glycan present in the lipopolysaccharide extract from the reference strain for *Serratia marcescens* serogroup O18

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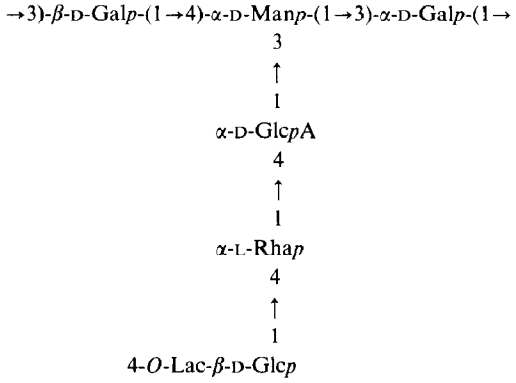
ABSTRACT

The lipopolysaccharide extract from the cell wall of the reference strain for *Serratia marcescens* serogroup O18 contained, in addition to a neutral glycan characterised previously, an acidic glycan. Acidity was contributed both by D-glucuronic acid and by 4-*O*-[(*R*)-1-carboxyethyl]-D-glucose (4-*O*-Lac-D-Glc). By using n.m.r. spectroscopy, methylation analysis, and chemical degradations, the repeating unit of the acidic glycan was identified as a branched hexasaccharide having the structure shown; an *O*-acetyl group also present was not located. The glycan is believed to define the O18 serogroup, but is probably not an integral component of the lipopolysaccharide.



INTRODUCTION

Although serological cross-reactions are common among the heat-stable antigens of *Serratia marcescens*, relatively few have been reported for serogroup O18. No relationship to other antigens was detected in one study[1], while cross-reactions found in other studies mainly involved O10 (refs. 2–4) and O22 (ref. 4). These cross-reactions seem to be based on structural similarities between the repeating units of neutral glycans from the O10 (**1**, ref. 5), O18 (**2**, ref. 6), and O22 (**3**, ref. 7) reference strains. Although the O10 glycan was the only polymer present in the lipopolysaccharide extract from the cell

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wall, both the O18 extract[6] and the O22 extract[7] also contained acidic glycans. We now report the structure of the repeating unit for the O18 acidic glycan.

α-D-Glc*p*
↓
4
→2)-α-L-Rha*p*-(1→2)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→3)-α-D-Glc*p*NAc-(1→

1

→2)-α-L-Rha*p*-(1→2)-α-L-Rha*p*-(1→2)-α-L-Rha*p*-(1→6)-α-D-Glc*p*NAc-(1→

2

→2)-α-L-Rha*p*-(1→2)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→3)-α-D-Glc*p*NAc-(1→

3

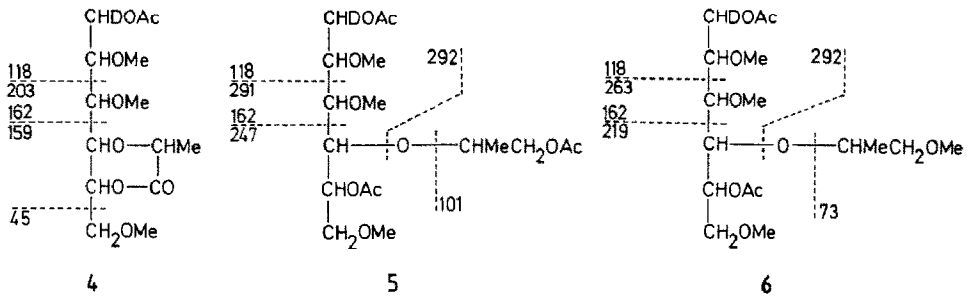
RESULTS

As described previously[6], an acidic glycan was isolated from the lipopolysaccharide of the O18 reference strain after mild acid hydrolysis, followed by ion-exchange chromatography of the polymeric products. This glycan constituted a minor fraction (18%) of the polymeric material and was eluted from DEAE-Sepharose CL-6B with M NaCl, but not with 0.3M NaCl. The neutral sugar components of the glycan were L-rhamnose, D-mannose, D-glucose, and D-galactose (relative peak areas in g.l.c. of the alditol acetates, 1.00:0.74:0.19:2.03). After carboxyl-reduction of the polymer, the proportions of glucose and mannose (relative to rhamnose = 1.00) increased to 0.92 and 1.01, respectively, showing the presence of glucuronic acid in the original polymer, probably linked to mannose. High-voltage paper electrophoresis of an acid hydrolysate confirmed the presence of glucuronic acid, and revealed two other acidic components that reacted with anilinehydrogen oxalate. The major component (**A**) had M_{GlcA} 0.48 and the minor component (**B**) had M_{GlcA} 0.80. Further studies of these products are described below.

The acidic glycan also carried *O*-acetyl groups (non-stoichiometric, less than one group per repeating unit), which complicated the n.m.r. spectra. However, the ^{1}H-n.m.r. spectrum of the *O*-deacetylated glycan contained six anomeric signals (each 1 H) at δ 5.28 ($J_{1,2}$ 3 Hz), 5.20 ($J_{1,2}$ 3 Hz), 5.07 (unresolved), 4.84 (unresolved), 4.72 ($J_{1,2}$ 8 Hz), and 4.56 ($J_{1,2}$ 8 Hz), as well as two doublets (each 3 H) at δ 1.48 (J 7 Hz) and 1.35 (J 6 Hz). The ^{13}C-n.m.r. spectrum also contained six anomeric signals at δ 103.9, 103.7, 101.7, 101.6, 96.8, and 96.1, as well as two signals for CH_3 at δ 19.2 and 17.6, and two signals for C=O

at δ 178.0 and 173.9. These data pointed to a hexasaccharide repeating-unit for the polymer, constructed from rhamnose (1), mannose (1), galactose (2), and glucuronic acid (1) residues, together with an unidentified acidic sugar (1) that contained an ethylidene group.

Four products were detected, and were characterised by g.l.c. and m.s., on methylation analysis of the acidic glycan. Three of these products were identified as the derivatives of 4-substituted rhamnopyranosyl, 3-substituted galactopyranosyl, and 3,4-disubstituted mannopyranosyl residues (relative peak areas in g.l.c. of the methylated alditol acetates, 0.85:2.00:1.11). The mass spectrum of the fourth product (relative peak area, 0.82) indicated[8] that it had the structure **4**, which corresponds to the lactonised derivative of an unsubstituted 4-*O*-(1-carboxyethyl)hexopyranosyl residue. A signal for the quasimolecular ion at m/z 322 $[M + H]^+$ was observed in c.i.-m.s. of the derivative with ammonia as the reagent gas. When the methylated glycan was reduced ($LiAlH_4$) before hydrolysis, the product **4** was replaced by the related product **5** as indicated by the mass-spectral fragmentation[8], and an additional product derived from a 4-substituted glucopyranosyluronic acid residue was detected. As expected, methylation analysis of the carboxyl-reduced glycan gave the derivatives from 4-substituted rhamnopyranosyl, 3-substituted galactopyranosyl, 3,4-disubstituted mannopyranosyl, and 4-substituted glucopyranosyl residues (relative peak areas in g.l.c., 0.74:2.00:1.33:0.95), as well as the product **6** (relative peak area, 0.93) with the mass-spectral fragmentation pattern shown.



4 **5** **6**

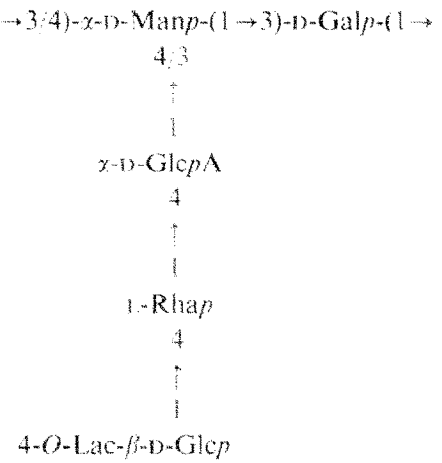
In order to identify the acidic sugar (a lactylic acid) that gave rise to compounds **4**, **5**, and **6**, partial hydrolysis (2M trifluoroacetic acid, 3.25 h, 105°) of the parent polymer was carried out. The acidic products **A** and **B** (see above) were isolated by h.p.l.c. Compound **B** had the same retention time and the same mobility in paper electrophoresis as the aldobiouronic acid **7** isolated[9] from an acidic glycan produced by the O23 reference strain of *S. marcescens*. The ^{1}H-n.m.r. spectrum of compound **B** contained anomeric signals at δ 5.35 (1 H, $J_{1,2}$ 4 Hz), 5.22 (0.6 H, $J_{1,2}$ <2 Hz), and 4.96 (0.4 H, unresolved), consistent with structure **7** (or the isomeric aldobiouronic acid that contains 4-substituted mannose).

α-D-Glc*p*A-(1→3)-D-Man

7

The ^{1}H-n.m.r. spectrum of compound **A** contained anomeric signals at δ 5.27 (0.4 H, $J_{1,2}$ 4 Hz) and 4.68 (0.6 H, $J_{1,2}$ 8 Hz), as well as methyl doublets at δ 1.50 (1.2 H, J 7 Hz) and 1.49 (1.8 H, J 7 Hz), indicating that the compound was the lactylic acid. Removal of the lactic acid residue, catalysed by BBr_3, produced D-glucose, thereby identifying compound **A** as a 4-*O*-(1-carboxyethyl)-D-glucose. The mass spectrum of the alditol acetate from the related 2-hydroxy-1-methylethyl sugar (released on acid hydrolysis of the carboxyl-reduced polymer) contained the diagnostic peaks with *m/z* 375, 347, 275, and 101. G.l.c. comparisons with the corresponding alditol acetates from glucolactylic acids in other bacterial polysaccharides permitted the *R* configuration to be assigned to the lactyl group in the sugar from *S. marcescens* O18.

Smith degradation of the carboxyl-reduced, *O*-deacetylated glycan gave a polymeric product (SDI) that, unexpectedly, contained glucose as well as mannose and galactose (molar ratios, 0.77:0.87:2.00). Methylation analysis gave the derivatives of unsubstituted glucopyranosyl, 3-substituted galactopyranosyl, and 3,4-disubstituted mannopyranosyl residues (relative peak areas, 1.20:2.00:1.25). The ^{1}H-n.m.r. spectrum of SDI contained four anomeric signals at δ 5.20 ($J_{1,2}$ 4 Hz), 5.18 ($J_{1,2}$ 4 Hz), 5.05 ($J_{1,2}$ < 2 Hz), and 4.54 ($J_{1,2}$ 8 Hz). Compared with the ^{1}H-n.m.r. spectrum of the original *O*-deacetylated glycan, the anomeric signals at δ 4.84 (H-1 of Rha) and 4.72 (H-1 of the lactylic acid), together with the related methyl doublets, had disappeared. These results for SDI show that the backbone of the O18 acidic glycan is a galactomannan with a trisaccharide repeating-unit, and confirm that glucuronic acid is attached to the mannose residue at the branch point. Taken with other data presented, the results permit a partial structure (**8**) to be assigned to the repeating unit of the original, *O*-deacetylated polymer.



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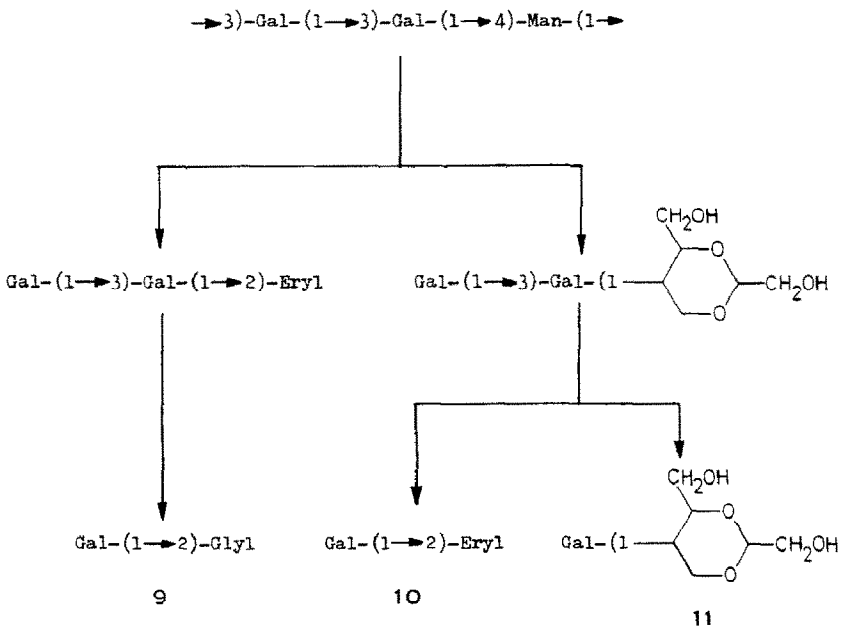
When SD1 was subjected to a further Smith degradation, and when the original *O*-deacetylated glycan was subjected to two successive oxidation–reduction cycles before mild hydrolysis, a new polymeric product (SD2) was obtained. This result indicates that the unexpected survival of the 4-substituted glucopyranosyl residue in the degradation leading to SD1 was due to hemiacetal formation, involving its 3-hydroxyl group and a carbonyl group of the adjacent oxidised rhamnose residue. The product SD2 contained only mannose and galactose (molar ratio, 0.88:2.00), and on methylation analysis gave the derivatives from 4-substituted mannopyranosyl and 3-substituted galactopyranosyl residues (relative peak areas, 1.16:2.00). The ^{1}H-n.m.r spectrum for SD2 included three anomeric signals (each 1 H) at δ 5.18 ($J_{1,2}$ 4 Hz), 5.08 ($J_{1,2}$ < 2 Hz), and 4.53 ($J_{1,2}$ 8 Hz).

The ^{1}H-n.m.r. data for SD1 and SD2 show that one of the galactose residues is α-linked and the other β. In order to assign these configurations, SD2 was subjected to two further Smith degradations. The final product (SD3) had a retention time in h.p.l.c. consistent with that of the expected disaccharide-alditol (a galactosylglycerol). However, g.l.c. of permethylated SD3 showed the presence of three components, for which the relative peak areas (given in order of increasing retention time) were 5.84:2.56:1.00. E.i.-m.s. showed that the major component was the galactosylglycerol (**9**) predicted

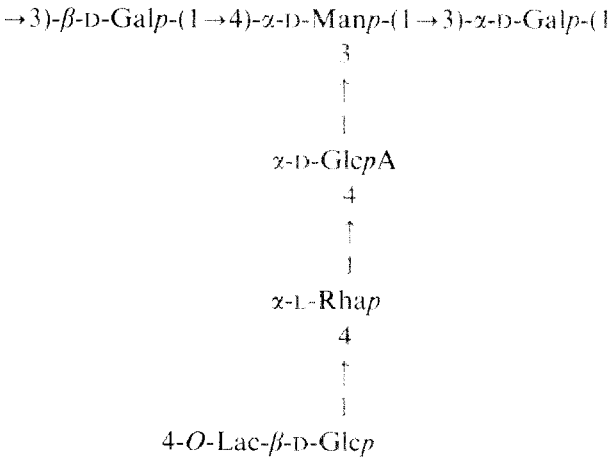


Eryl = Erythritol; Glyl = Glycerol.

The aglycon in **11** and its precursor could be an isomeric 1,3-dioxolane[10].

Scheme 1. Reaction pathways leading to the mixture of products (SD3) on two consecutive Smith-degradations of the linear galactomannan SD2 (see text).

from classical Smith degradations. The second component (**10**) was a galactosyl derivative of a tetritol (expected to be erythritol, from the oxidised mannose residue), and the final component (**11**) was probably a similar derivative of a 1,3-dioxane[10]. Scheme 1 shows the reaction pathways leading to the three components of SD3. In the ^{1}H-n.m.r. spectrum of SD3, anomeric signals were detected only for the two most abundant components (**9** and **10**), at δ 4.54 (1 H, $J_{1,2}$ 8 Hz) and 4.50 (0.4 H, $J_{1,2}$ 8 Hz). Thus, the glycan contains the sequence →3)-β-D-Gal*p*-(1→4)-α-D-Man*p*-(1→, the α configuration for mannose being assigned from the chemical shift (δ 5.07) for its anomeric signal in the *O*-deacetylated glycan (and similar values for SD1 and SD2). The only remaining problem was the anomeric configuration of the rhamnosyl residue. Although the chemical shift (δ 4.84) for its anomeric proton could suggest the β configuration, the chemical shift for the H-5 resonance at $\delta \sim 4.1$ (determined from a COSY spectrum of the glycan) is diagnostic for the α configuration[11]. Thus, the structure of the repeating unit for the *O*-deacetylated glycan can be finalised as **12**.



12

DISCUSSION

Compared with other acidic glycans isolated from lipopolysaccharide extracts of *S. marcescens*, the polymer from the O18 reference strain is unusual in several respects. In the glycans already characterised, the repeating unit is either linear or monoglycosylated, whereas both the backbone and the branch in **12** are trisaccharide residues. In other polymers, acidity is usually conferred by a hexuronic acid, but in one case[12] by pyruvic acid. In only one other polymer[9] (from the O23 reference strain) is there an additional source of acidity, which arises from partial amidation of D-glucuronic acid by L-glutamic acid. Like the O18 glycan, the modified O23 glycan is strongly bound by DEAE-Sepharose CL-6B.

Lactyl ethers of various monosaccharides are components of bacterial lipopolysaccharides and exopolysaccharides. The 4-(*R*)-lactyl ether of D-glucose, present in the O18 acidic glycan, also occurs in the lipopolysaccharides of *Escherichia coli* O124 (ref. 13) and *Shigella dysenteriae* type 3 (ref. 8), as well as in the capsular polysaccharide of *Klebsiella* type 66 (ref. 14). The corresponding *S* isomer is present in an exopolysaccharide from *Aerococcus viridans*[15], and the 4-(*S*)-lactyl ether of D-glucuronic acid is found in the capsular polysaccharides of *Klebsiella* types K22 (ref. 16) and K37 (ref. 17). Other examples[18,19] of this unusual class of acidic sugar include ethers of D-galactose, D-mannose, and L-rhamnose.

The acidic glycan based on the repeating unit **12** probably defines the O18 serogroup of *S. marcescens*. However, the absence of core sugars (such as heptoses) from the polymer, and comparisons with other strains which produce both neutral and acidic glycans[20], indicate that the latter are of (micro)capsular origin. Apparently, microheterogeneity of the O18 acidic glycan is introduced by partial *O*-acetylation and may also involve the absence of lactyl groups from some non-reducing termini (one possible explanation for the small amount of "glucose" present in acid hydrolysates, although borohydride reduction of some glucuronolactone is more likely).

EXPERIMENTAL

Growth of bacteria, and isolation and fractionation of lipopolysaccharide. — These operations were carried out by standard methods, and the yields of products have been recorded[6].

General methods. — Neutral sugars and glucuronic acid were identified, and their absolute configurations were determined, as in related studies[9]. The parent sugar of the lactylic acid was identified as D-glucose by using D-glucose oxidase (EC 1.1.3.4) and by g.l.c. of the alditol acetate, following *O*-dealkylation[21] of the peracetylated compound with BBr_3 in dichloromethane. The *R* configuration of the lactyl group was established by g.l.c. of the acetylated 2-hydroxy-1-methylethyl ether obtained from the carboxyl-reduced polymer by acid hydrolysis and reduction ($NaBH_4$) of the products. The relevant product from the O18 glycan had a retention time in g.l.c. [using a fused-silica capillary column (25 m) of BP1 at 200°] which was the same as that of the corresponding product from the *Klebsiella* K66 polysaccharide, and different from that of the diastereoisomeric product from either *Klebsiella* K37 or *Aerococcus viridans* polysaccharides.

N.m.r. spectra (^1H and ^{13}C) of samples in D_2O were recorded with a Bruker WH-400 spectrometer (for *O*-deacetylated glycan) or a JEOL JNM-GX270 spectrometer (for other products). The ^{13}C-n.m.r. spectrum of the *O*-deacetylated glycan was recorded at 28° with 1,4-dioxane as the internal reference (δ 67.4), and the corresponding ^1H-n.m.r. spectrum at 61.6° with sodium 3-trimethylsilylpropanoate-d_4 as the external reference (δ 0.00). Other ^1H-n.m.r. spectra were obtained at 70° with sodium 4,4-dimethyl-4-silapentane-1-sulphonate as the external reference. The 2D COSY-45 spectrum for the *O*-deacetylated glycan was obtained with a standard pulse sequence, and the polymer as the ammonium salt.

Methods and conditions used for *O*-deacetylation and carboxyl-reduction of the acidic glycan, reduction ($LiAlH_4$) of ester groups in the permethylated polymer, Smith degradations, and methylation analyses were essentially those used in related studies[5,9]. E.i.-m.s. was carried out with a Finnigan 1020B spectrometer, and c.i.-m.s. with a Nicolet FTMS-2000 spectrometer and ammonia as the reagent gas[22].

Partial acid hydrolysis. — In order to obtain an aldobiouronic acid, the O18 glycan (2 mg) was hydrolysed with 2M trifluoroacetic acid for 3.25 h at 105°. After being rotary-dried, the hydrolysate was fractionated by h.p.l.c. using a TSKgel G-Oligo-PW column (Anachem) eluted with 0.2M sodium acetate (1 mL.min^{-1}), followed by treatment of the eluate with Dowex 50 resin (H^+ form). The aldobiouronic acid (**B**) had a retention time of 17 min, and that of glucolactylic acid (**A**) was 19 min.

Characterisation of product SD3. — By means of Smith degradations, the *O*-deacetylated glycan was degraded to a linear polymer (SD2). Two further degradations produced a mixture (SD3) of three components (**9**–**11**), the major ones (**9** and **10**) being disaccharide-alditols. Components **9**–**11** were studied by ^{1}H-n.m.r. spectroscopy, by g.l.c. of their per-*O*-methyl derivatives on the BP1 column (isothermal at 170° for 3 min, then programmed to 290° at 15° min^{-1}; flow rate ~44 cm.s^{-1}), and by e.i.-m.s. (significant fragment ions, with relative intensities in brackets and assignments[23] in square brackets, are indicated below). The major component (retention time in g.l.c., 4.47 min) was the derivative of 2-*O*-β-D-galactosylglycerol (**9**): *m/z* 219(0.3) [aA_1], 187(3) [aA_2], 163(52) [abJ_1], and 103(33) [bA_1]. The second component (retention time, 6.08 min) was the corresponding derivative of a galactosylerythritol (**10**): *m/z* 219(0.3) [aA_1], 207(15) [abJ_1], 187(3) [aA_2], 147(8) [bA_1], and 115(30) [bA_2]. The third component (retention time, 7.98 min) was probably derived from a 1,3-dioxolane (**11**): *m/z* 235(5) [abJ_1], 219(0.8) [aA_1], 187(3) [aA_2], and 175(10) [bA_1].

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