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STRUCTURES OF NEUTRAL GLYCANS ISOLATED FROM THE LIPOPOLYSACCHARIDES OF REFERENCE STRAINS FOR *Serratia marcescens* SEROGROUPS O16 AND O20

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ABSTRACT

Neutral polymers have been isolated from the lipopolysaccharides of the reference strains for *Serratia marcescens* O16 and O20, serogroups which exhibit significant cross-reactivity. Both organisms produce a galactan with the disaccharide repeating-unit shown, and which apparently accounts for the serological observations. The same galactan has also been reported as the O4-specific polysaccharide of *Pasteurella haemolytica*. In *S. marcescens* O16, the galactan is apparently accompanied by a polymer of 2-substituted β-D-ribofuranosyl residues.

→3)-α-D-Gal*p*-(1→3)-β-D-Gal*f*-(1→

INTRODUCTION

The importance of *Serratia marcescens* as an opportunistic pathogen, and the problems encountered in using its heat-stable antigens as epidemiological markers[1], have encouraged us to undertake a systematic study of the surface polysaccharides produced by the organism. So far, we have concentrated on products from reference strains for the fifteen O serogroups defined by Edwards and Ewing[2], and most of the glycans isolated from lipopolysaccharide extracts have been characterized (ref. 3 and the preceding studies cited). A further five serogroups (O16 to O20) were described by Le Minor and Pigache[4], and confirmed by Traub[5]. As found for the original O serogroups[1], relationships between some of the antigens (*e.g.*, O16 and O20) can cause troublesome cross-reactions[4–7]. Structural studies of polymeric fractions from the lipopolysaccharides of the O16 and O20 reference strains have revealed the basis of the cross-reaction in this case.

RESULTS

Lipopolysaccharide was obtained from isolated cell walls of the O20 reference strain in 33% yield. Sugar analysis of the lipopolysaccharide showed D-galactose as the major component, and glucose, aldoheptoses, and 2-amino-2-

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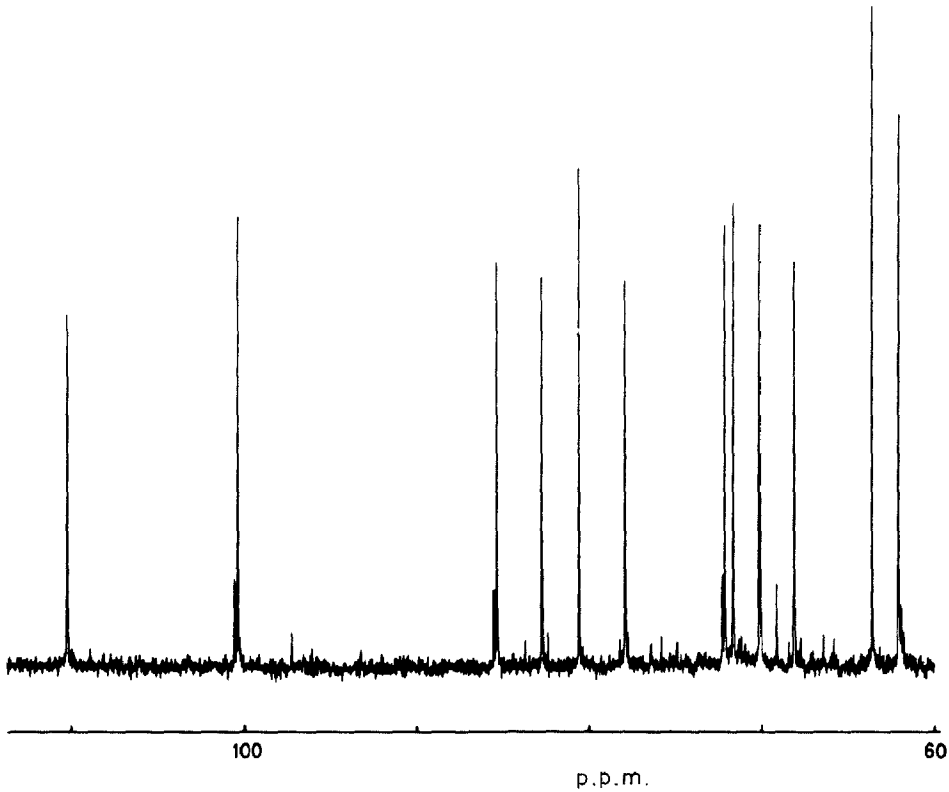


Fig. 1. ^{13}C-N.m.r. spectrum of the O20 galactan. The spectrum for a solution of the sample in D_2O was obtained at 100.62 MHz and 26°, with complete proton-decoupling and 1,4-dioxane as the internal reference (δ 67.40 relative to tetramethylsilane).

deoxyglucose (configurations not determined) as minor components. Mild acid hydrolysis of the lipopolysaccharide gave a single, neutral polymer, most of which (68%) was eluted from DEAE-Sepharose CL-6B with water, and the remainder with 0.1M NaCl. Both fractions had the same monosaccharide composition (essentially galactose, with traces of the other sugars identified above) and gave identical n.m.r. spectra. Structural studies were therefore confined to the major fraction. Methylation analysis of the polymer showed the presence of 3-substituted galactopyranosyl and 3-substituted galactofuranosyl residues (ratio of peak areas in g.l.c. of the methylated alditol acetates, 1.07:1.00). The n.m.r. spectra suggested that these residues comprised a disaccharide repeating-unit. The ^{1}H-n.m.r. spectrum contained two anomeric signals (each 1 H) at δ 5.27 (unresolved) and 5.12 ($J_{1,2}$ 3.4 Hz). The ^{13}C-n.m.r. spectrum (Fig. 1) contained twelve discrete signals, including anomeric signals at δ 110.19 and 100.30. There was no evidence for an *O*-acyl substituent. The spectral data can be interpreted (Table I) in favour of structure **1** for the repeating unit of the polymer.

→3)-α-D-Gal*p*-(1→3)-β-D-Gal*f*-(1→

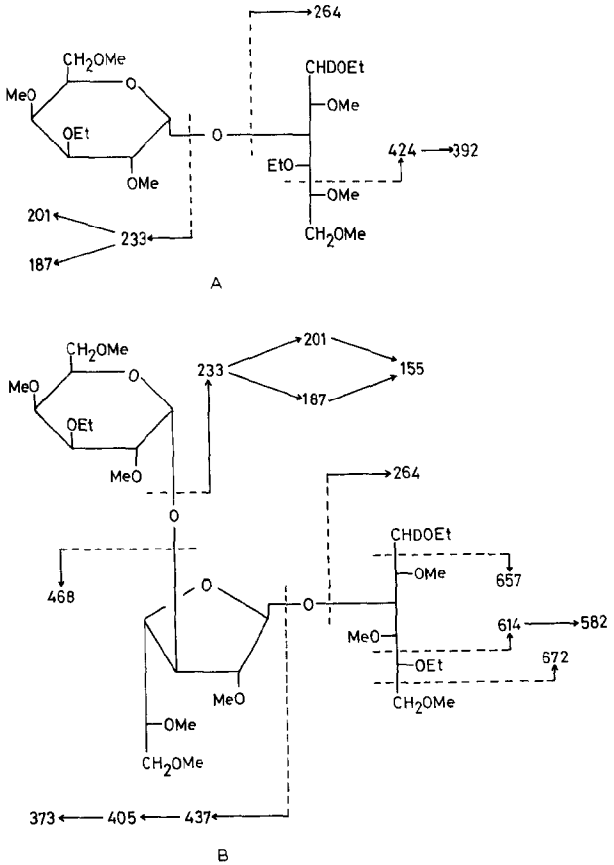
1

TABLE I

^{13}C-N.M.R. DATA FOR THE O20 GALACTAN

Carbon atom	*Chemical shift (p.p.m.)*[a]	
	→3)-α-D-Galp-(1→	*→3)-β-D-Galf-(1→*
C-1	100.30	110.19
C-2	68.03	80.53
C-3	77.88	85.30
C-4	70.07	82.70
C-5	71.60	72.12
C-6	62.01	63.57

[a]The spectrum was recorded at 26° with 1,4-dioxane as the internal reference. The provisional assignments were made with the aid of literature data[8].



Scheme 1. Fragmentation in e.i.-m.s. of the *O*-alkylated oligosaccharide-alditols PH1 (A) and PH2 (B) from the O20 galactan.

Supporting evidence for this structure was obtained by partial acid hydrolysis of the methylated polymer, followed by reduction ($NaBD_4$), ethylation, and fractionation of the products by h.p.l.c. The major product (PH1) and one of the minor products (PH2) were isolated.

G.l.c. indicated that PH1 was derived from a disaccharide-alditol, and the e.i.-m.s. fragmentation pattern (Scheme 1, A) was consistent with the structure shown. The structure was confirmed by the conversion of PH1 into alkylated alditol acetates through acid hydrolysis, reduction ($NaBD_4$), and acetylation, followed by mass spectrometry. The more volatile product was identified as 3-*O*-acetyl-1,4-di-*O*-ethyl-2,5,6-tri-*O*-methylgalactitol-*1-d*-(primary fragments with *m/z* 278, 263, 234, 89, 60, and 45) and the less volatile product as 1,5-di-*O*-acetyl-3-*O*-ethyl-2,4,6-tri-*O*-methylgalactitol-*1-d* (primary fragments with *m/z* 219, 176, 161, and 118). Thus, PH1 is the expected product corresponding to the disaccharide repeating-unit and is formed in high yield by selective hydrolysis of the furanosidic linkages in the methylated polymer.

The minor product PH2 was not rigorously identified because of the low yield, but g.l.c. indicated that it was derived from a trisaccharide-alditol. Three products were obtained when PH2 was converted into alkylated alditol acetates. The most volatile, corresponding to the alditol residue in PH2, had a retention time in g.l.c. slightly (but significantly) different from the analogous product from PH1 and gave a different mass spectrum (with the major high-mass peak at *m/z* 220). The second product from PH2 had the same retention time in g.l.c. and gave the same mass-spectral fragments as the second product from PH1 (*i.e.* it was also derived from a non-reducing-terminal pyranosyl residue). The least volatile product was similarly shown to be derived from a 3-substituted galactofuranosyl residue. These data and the m.s. fragmentation pattern for PH2 itself (Scheme 1, B) all support the structure shown, although the peak at *m/z* 155 for PH2 was surprisingly strong compared with that for PH1. Thus, it is tentatively inferred that PH2 arises through loss of a methylated galactofuranose residue from the reducing end of a tetrasaccharide precursor. This precursor (a "dimer" of PH1) and the alternative methylated trisaccharide were presumably among the minor products not isolated.

The lipopolysaccharide of the O16 reference strain was obtained from isolated cell walls in 32% yield. The monosaccharide components detected were galactose and ribose (both D and major), glucose, aldoheptoses, and 2-amino-2-deoxyglucose (all minor). After mild acid hydrolysis, 44% of the lipopolysaccharide was recovered as water-soluble, polymeric products. Most of the material (59%, 16F1) was eluted from DEAE-Sepharose CL-6B with water, and the remainder (16F2) with 0.1M NaCl. Both fractions contained galactose and ribose with traces of other monosaccharides, but the proportions of the major components differed markedly. Whereas fraction 16F1 contained similar amounts of galactose and ribose (peak areas in g.l.c. of the alditol acetates, 1.9:1.0; uncorrected for selective loss of the pentose during acid hydrolysis), the proportion of ribose in fraction 16F2 was much less (peak areas in g.l.c. of the alditol acetates, 7.8:1.0).

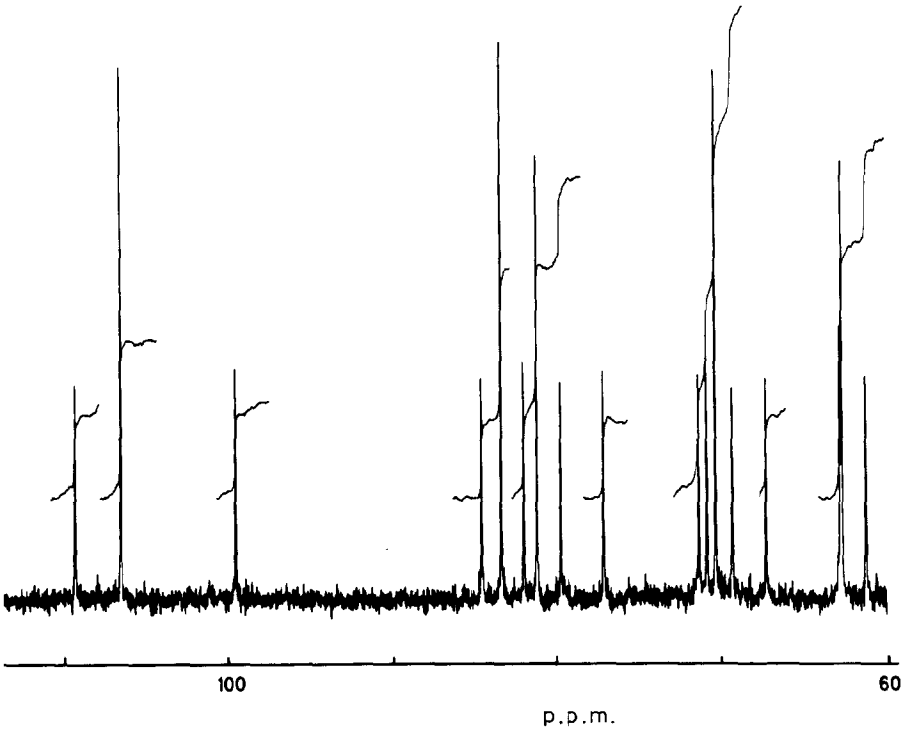


Fig. 2. ^{13}C-N.m.r. spectrum of the mixed glycan fraction 16F1. The spectrum for a solution of the sample in D_2O was obtained at 100.62 MHz and 50°, with complete proton-decoupling and tetramethylsilane as the external standard.

A comparison of the n.m.r. spectra of fractions 16F1 and 16F2 indicated that each was a mixture of two polysaccharides in different proportions. For fraction 16F1, the ^{1}H-n.m.r. spectrum contained anomeric signals at δ 5.32 (1 H, unresolved), 5.19 (0.6 H, unresolved), and 5.04 (0.6 H, $J_{1,2}$ ~3 Hz). Anomeric signals in the ^{13}C-n.m.r. spectrum were at δ 109.36, 106.59, and 99.51. Of the seventeen signals in the latter spectrum (Fig. 2), five were of relatively high intensity, and the remaining twelve matched those obtained for the O20 galactan (Fig. 1), allowing for a systematic discrepancy of ~0.8 p.p.m. attributable to the different operating parameters. Thus, fraction 16F1 appeared to consist of the O20 galactan and a ribose polymer in the molar ratio ~0.6:1.0. The n.m.r. spectra for fraction 16F2 pointed to the presence of the same two polymers in the inverse proportion (molar ratio ~2.0:1.0).

Methylation analysis of fraction 16F1 showed the presence of 2-substituted ribofuranosyl, 3-substituted galactopyranosyl, and 3-substituted galactofuranosyl residues (ratios of peak areas in g.l.c. of the methylated alditol acetates, 0.65:1.26:1.00). The identification of ribose as the D isomer, and assignments for the prominent signals in the ^{13}C-n.m.r. spectrum of fraction 16F1 (Fig. 2), indicated that the ribose-containing polymer was a homoglycan with the monosaccharide

repeating-unit **2**. Assignments were made with the aid of literature data[8]: δ 106.59 (C-1), 83.34 (C-4), 81.15 (C-2), 70.34 (C-3), and 62.70 (C-5).

$\rightarrow$2)-β-D-Rib*f*-(1$\rightarrow$

2

Attempts to separate the two O16 polymers by column chromatography in the presence of borate, using Dowex 1 resin or DEAE-Sepharose, were unsuccessful. However, partial success was achieved by ion-exchange chromatography on DEAE-Sephacel after successive treatment of fraction 16F1 with $NaIO_4$ and NaOI. Each fraction obtained by stepwise elution (water to M NaCl) contained carbohydrate. Early fractions of the eluate appeared to be enriched in ribose, and the ^{1}H-n.m.r. spectrum of the major fraction (eluted with M NaCl) confirmed that the ribose content had been reduced by ~50% compared with the parent material. Evidence that L-arabinofuranosyluronic acid residues had been produced in the galactan was also provided by the ^{1}H-n.m.r. spectrum. The major anomeric signals had shifted downfield to δ 5.32 and 5.21 (from 5.19 and 5.04, respectively), and a new signal at δ 4.54 (d, J ~5 Hz) had appeared (probably attributable to H-4 of the uronic acid residue).

DISCUSSION

The production of the galactan based on repeating unit **1** by both the O16 and the O20 reference strains of *S. marcescens* seems to account for the serological cross-reactions observed[4–7]. Thus, cells of the O16 reference strain are agglutinated by the O20 antiserum[4–6], lipopolysaccharides from the two strains give the same ladder pattern in sodium dodecyl sulphate–polyacrylamide gel electrophoresis[7], and the ladder pattern for O16 can be detected by immunoblotting with O20 antiserum[7]. Interestingly, the galactan from these strains is also the O-specific polysaccharide for *Pasteurella haemolytica* serotype 4 (ref. 9).

Serological studies[4–6] have also indicated that the O16 reference strain contains an additional antigen, which may give a "smeared" track on polyacrylamide gel electrophoresis, with either silver staining or immunoblotting[7]. The ribose polymer with repeating unit **2** may account for these results. If so, the galactan could correspond to the antigen factor 16a common to the two reference strains[6,10], and the riban to factor 16b. It is also interesting to note that a riban with the same structure occurs in the T1 lipopolysaccharides of *Salmonella* species, accompanied by a galactofuranose polymer[11,12].

EXPERIMENTAL

Growth of bacteria, and isolation and fractionation of lipopolysaccharides. —

The reference strains[4,5] for serogroups O16 and O20 were grown for 16 h at 30° in Nutrient Broth No. 2 (Oxoid) as 20-L batch cultures aerated at 20 L.min^{-1}, to give 128 g and 143 g, respectively, of wet cells. The cell walls (3.64 g and 3.55 g, respectively) prepared by mechanical disintegration of the cells were used for the extraction of lipopolysaccharides[3] (yields, 1.18 g and 1.17 g, respectively). Polymeric fractions (yields, 44% and 45%, respectively) were obtained by chromatography on Sephadex G-50 of the water-soluble products from hydrolysis of the lipopolysaccharides (aqueous 1% acetic acid, 2.25 h, 100°). The polymeric fractions were also examined by stepwise elution from DEAE-Sepharose CL-6B.

General methods. — Methods used for p.c., g.l.c., g.l.c.–m.s., and high-voltage paper electrophoresis were essentially those described previously[3,13,14]. N.m.r. spectra (^{13}C and ^{1}H) of solutions of samples in D_2O were recorded with a Bruker WH-400 or JEOL JNM-GX270 spectrometer. ^{1}H-N.m.r. spectra were recorded at 60° or 80° with sodium 3-trimethylsilylpropanoate-d_4 as the external reference; the corresponding parameters for ^{13}C-n.m.r. spectra were 26° or 50° with 1,4-dioxane or tetramethylsilane. Monosaccharides were identified and determined as in related studies[3]. Absolute configurations were determined by conversion of the monosaccharides into but-2-yl glycosides[15], followed by g.l.c. of the acetates on a fused-silica capillary column of BP20 (SGE). Methylation analyses were carried out by standard procedures[3].

Preparation of methylated oligosaccharide-alditols. — A sample (~3 mg) of methylated O20 polymer was hydrolysed (aqueous 90% formic acid, 1 h, 70°). After rotary evaporation of the hydrolysate, the products were reduced by treatment with $NaBD_4$ in D_2O–ethanol (1:1) overnight at 4°. After a conventional work-up followed by ethylation, two significant products were detected by g.l.c. (BP1), ratio of peak areas ~10:1. Under the g.l.c. conditions used (25-m column; carrier gas, ~44 cm.sec^{-1}; isothermal for 3 min at 220°, then programmed to 280° at 15°.min^{-1}), the major component was eluted in 3 min 52 s (*cf.* permethylated maltitol, 3 min 19 s) and the minor component in 9 min 32 s (*cf.* permethylated maltotri-itol, ~9.5 min). The products were isolated by h.p.l.c. on a column (25 cm × 4.6 mm) of Spherisorb S5 ODS2 eluted with methanol–water (3:1) at 0.5 mL.min^{-1}. The retention times were 19.5 min (the disaccharide derivative PH1) and 25 min (the trisaccharide derivative PH2). E.i.-m.s. was carried out by direct insertion. Significant fragment ions for PH1 included the following (relative intensities in brackets and some assignments[16] in square brackets, ALD is used to signify fission within the alditol residue): *m/z* 125(100), 149(52), 155(10) [aA_1 – MeOH – EtOH], 187(31) [aA_1 – EtOH], 201(27) [aA_1 – MeOH], 233(39) [aA_1], 238(22), 264(25) [bA_1], 323(8), 338(30) [abJ_1], 392(3) [baALD – MeOH], 424(7) [baALD]. Similar fragment ions for PH2 included the following: *m/z* 155(100) [aA_1 – MeOH – EtOH], 187(75) [aA_1 – EtOH], 201(27) [aA_1 – MeOH], 233(45) [aA_1], 264(12) [cA_1], 373(7) [baA_3], 405(3) [baA_2], 437(6) [baA_1], 468(9) [cbA_1], 542(6) [$abcJ_1$], 614(5) [cabALD], 657(0.3) [cabALD], 672(0.2) [cabALD], 685(0.1) [M – MeOH].

Oxidation and fractionation of the O16 polymers. — A sample (27 mg) of the mixed polymers in fraction 16F1 was treated with 50mM $NaIO_4$ (3 mL) for 4 days at 4°. After reduction of the excess of oxidant with ethylene glycol, the mixture was dialysed and freeze-dried. On the addition of water (0.2 mL), the product initially gave a gel but appeared to dissolve after storage overnight at 4°. To the solution were added 0.1M I_2–KI (3 mL) and 0.1M NaOH (3 mL), followed by further alkali (1.5 mL) after 10 min. After 30 min at room temperature, 0.1M HCl (5.25 mL) was added, most of the I_2 was removed by extraction with $CHCl_3$ (3 × 2 mL), and the aqueous residue was dialysed. The product (now readily soluble in water) was applied to a column (1 × 20 cm) of DEAE-Sephacel (chloride form) and eluted with 20-mL volumes of water and aqueous NaCl (0.1, 0.2, 0.3, and 1.0M). Individual fractions recovered after dialysis were examined for monosaccharide composition, and the ^{1}H-n.m.r. spectrum of the major fraction (eluted with 1M NaCl) was recorded.

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