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STRUCTURAL STUDIES OF A NEUTRAL POLYMER (THE PUTATIVE O10 ANTIGEN) ISOLATED FROM THE LIPOPOLYSACCHARIDE OF *Serratia marcescens* STRAIN C.D.C. 1287-54 (O10:H8)

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ABSTRACT

A neutral glucorhamnan has been isolated from the lipopolysaccharide of the O10 reference strain (C.D.C. 1287-54) of *Serratia marcescens*. By means of n.m.r. spectroscopy, methylation analysis, and degradative studies, the polymer (the putative O-specific antigen) was found to have the branched, pentasaccharide repeating-unit shown.

$$\begin{array}{c} \alpha\text{-D-Glc}p \\ 1 \\ \downarrow \\ 4 \\ \rightarrow 2)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow 2)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow 3)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow 3)\text{-}\alpha\text{-D-Glc}p\text{NAc-}(1\rightarrow \end{array}$$

INTRODUCTION

During studies of the surface polysaccharides produced by strains of *Serratia marcescens* representing different O serogroups in the scheme of Edwards and Ewing[1], we have encountered both neutral and acidic polymers[2–15]. Polymers of both types occur in over half of the reference strains (which creates doubt over the identity of the O antigen), and several polymers occur in strains of different O serogroups (which presumably contributes to serological cross-reactions). In only three of the reference strains (for serogroups O8, O9, and O10) does a single, neutral polymer (probably an integral component of the lipopolysaccharide) seem to be the O antigen, although the evidence for this is largely circumstantial. We have previously reported the structure of the repeating unit of the neutral polymer for O8 (ref. 8) and O9 (ref. 9), and now report on the repeating unit for O10.

RESULTS

Lipopolysaccharide was obtained from *S. marcescens* C.D.C. 1287-54 in a yield corresponding to 26% of the whole cell wall. The major monosaccharide com-

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ponents were glucose, rhamnose, and 2-amino-2-deoxyglucose; minor components detected by g.l.c. of the alditol acetates were L-*glycero*-D-*manno*-heptose and D-*glycero*-D-*manno*-heptose (or their enantiomers).

After mild acid hydrolysis (aqueous 1% acetic acid, 2.25 h, 100°) of the lipopolysaccharide, 53% of the material was recovered (Sephadex G-50) as a water-soluble polymer. Most of the polymer (73%) was eluted from DEAE-Sepharose CL-6B with water, and a minor fraction (16%) with 0.1M NaCl. Both fractions had the same monosaccharide composition. From the fact that the parent lipopolysaccharide gave a pattern typical of mixed S- and R-type products in polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate, it could be inferred that the neutral polymer corresponded to the O-specific side chain.

Monosaccharide analysis of the polymer gave the composition as L-rhamnose (40.5%), D-glucose (18.6%), 2-amino-2-deoxy-D-glucose (14.4%, uncorrected), and heptoses (traces). The results of methylation analysis (Table I, column A) suggested a pentasaccharide repeating-unit containing pyranose residues of unsubstituted glucose, 3-substituted 2-amino-2-deoxyglucose, 2-substituted rhamnose (two residues), and 3,4-disubstituted rhamnose (a branch point). The size of the repeating unit and the presence of three rhamnose residues were confirmed by the n.m.r. spectra. In the ^{13}C-n.m.r. spectrum, anomeric signals at δ 101.13 ($^1J_{CH}$ 165 Hz), 100.78 ($^1J_{CH}$ 172 Hz), 99.51 ($^1J_{CH}$ 170 Hz), 98.18 ($^1J_{CH}$ 172 Hz), and 96.05 ($^1J_{CH}$ 171 Hz) showed that at least four of the pyranose residues were α-linked. From the anomeric signals in the ^{1}H-n.m.r. spectrum at δ 5.20 ($J_{1,2}$ 3.5 Hz), 5.08 (unresolved), 5.07 (unresolved), 5.03 ($J_{1,2}$ 3.5 Hz), and 4.94 (unresolved), it could be concluded that both sugars with the *gluco* configuration were α-linked. Evidence for three rhamnose residues was provided by signals at δ 18.00, 17.14, and 16.93 (^{13}C), and at δ 1.40, 1.38, and 1.32 each with $J_{5,6}$ ~6 Hz (^{1}H). Signals diagnostic for a 2-acetamido group were recorded at δ ~174, 53.24, and 22.44 (^{13}C), and at δ 2.11 (^{1}H, 3 H). Other signals at δ 60.90 and 60.73 (^{13}C) confirmed that position 6 in both

TABLE I

METHYLATION ANALYSIS OF THE NEUTRAL O10 POLYMER AND SOME DEGRADATION PRODUCTS[a]

Methylation product[b]	*Relative peak area (g.l.c.)*		
	A	*B*	*C*
2,3,4-Rha		1.00	1.04
3,4-Rha	2.04		1.05
2,3,4,6-Glc	1.00		1.00
2-Rha	1.02		0.49
1,4,6-AHMan			0.55
2,4,6-GlcNAc	+	+	

[a]Key: *A*, native polymer; *B*, Smith-degradation product SD1; *C*, products from *N*-deacetylation–deamination (mainly DA1). [b]2,3,4-Rha = 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylrhamnitol; 1,4,6-AHMan = 3-*O*-acetyl-1,4,6-tri-*O*-methyl-2,5-anhydromannitol, *etc.*

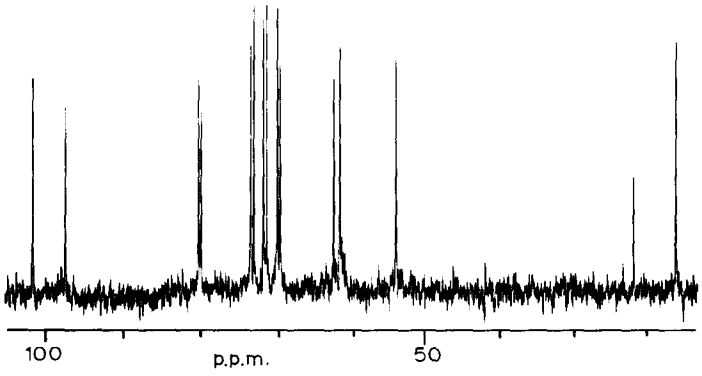


Fig. 1. ^{13}C-N.m.r. spectrum of the Smith-degradation product (SD1). The spectrum was recorded at 50° with tetramethylsilane as the external reference. In addition to the signals shown, the spectrum contained a signal at δ 174.30.

glucose and 2-acetamido-2-deoxyglucose was unsubstituted. Also consistent with the substitution pattern inferred from methylation analysis was the composition of the periodate-oxidised polymer: rhamnose (15.7%), 2-amino-2-deoxyglucose (11.9%, uncorrected), glucose (0%).

When the periodate-oxidised polymer was subjected to mild acid hydrolysis (Smith degradation) followed by reduction ($NaBD_4$), ~50% of the material remained polymeric. Repetition of the two treatments on the latter material resulted in a similar conversion into products of low molecular weight. Paper chromatography of the combined degradation products showed a major component (SD1) with $R_{Lactose}$ 1.45 and a minor component (too little for further study) with $R_{Lactose}$ 1.11. The n.m.r. spectra of product SD1 confirmed that it was a trisaccharide-alditol that contained rhamnose, 2-acetamido-2-deoxyglucose, and glycerol-*1-d* residues. The ^{1}H-n.m.r. spectrum contained anomeric signals at δ 5.07 ($J_{1,2}$ 3.8 Hz) and 4.92 ($J_{1,2}$ <2 Hz), each 1 H, and methyl signals at δ 2.09 (singlet) and 1.28 ($J_{5,6}$ 6.3 Hz). The ^{13}C-n.m.r. spectrum contained the expected sixteen signals (Fig. 1), including anomeric signals at δ 101.43 ($^1J_{CH}$ 166 Hz) and 97.17 ($^1J_{CH}$ 172 Hz). An interpretation of the spectrum (Table II) and the results of methylation analysis (Table I, column B) permit the assignment of structure **1** to the major product of Smith degradation.

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

1

Initially, the configuration of the rhamnosyl group in SD1 was in doubt because of the intermediate value (166 Hz) for $^1J_{CH}$ of the anomeric carbon (and of

TABLE II

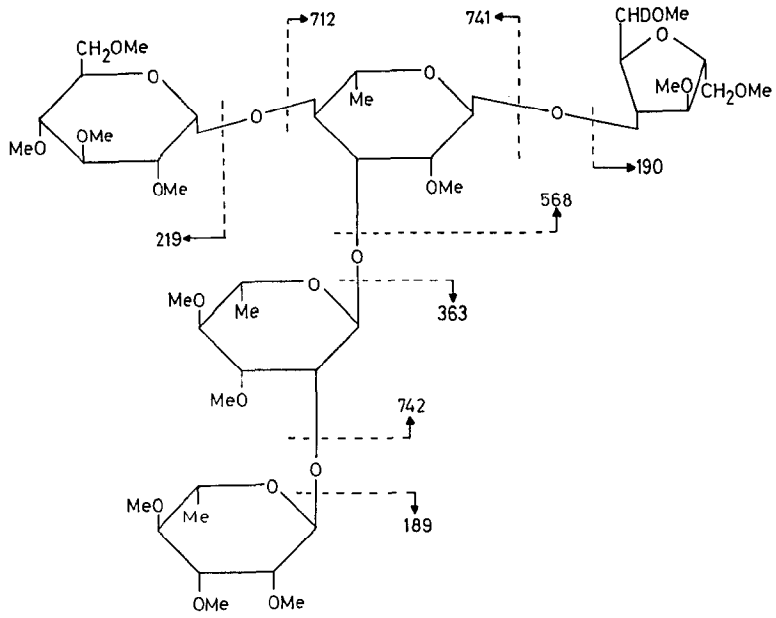
^{13}C-N.M.R. DATA FOR THE SMITH-DEGRADATION PRODUCT SD1 (**1**)

Carbon atom	*Chemical shift (p.p.m.)*[a]		
	α-L-*Rhap*-(*1*→	→*3*)-*α*-D-*Glc*p*NAc*-(*1*→	→*2*)-*Glycerol*-*1*-d
C-1	101.43	97.17	n.d.[b]
C-2	71.01	53.50	79.18
C-3	70.54	79.52	61.67
C-4	72.68	69.13	
C-5	68.79	72.23	
C-6	16.73	60.89	
$-NHC(O)CH_3$		174.30	
$-NHC(O)CH_3$		22.20	

[a]The spectrum was recorded at 50° with tetramethylsilane as the external reference. The assignments were made with the aid of literature data[16,17] and are provisional for signals with similar chemical shifts. [b]Not determined.

the similar value, 165 Hz, for the parent branch-point residue in the polymer), and because of evidence that the sugar was destroyed on oxidation of peracetylated SD1 with CrO_3, indicating the β configuration[18]. However, coupling constants as low as 164 Hz have been reported for some α-rhamnopyranosides[19,20], and misleading results can also be obtained by the CrO_3 method (*e.g.*, refs. 21 and 22). The ^{13}C-n.m.r. data for SD1 (Table II) could not be reconciled readily with the presence of a β-rhamnopyranosyl group. Specifically, the signals for C-3, C-4, and C-5 of a terminal β-rhamnopyranosyl group should cluster[16,17] in the range δ 73–74 (only two such signals were observed for SD1, allowing for an upfield displacement of ~1 p.p.m. for our data, compared with the literature), and the signal for C-5 of an α-rhamnopyranosyl group is characteristically[19,23,24] near δ 69. Furthermore, the ^{1}H-n.m.r. spectrum of SD1 did not contain a signal at δ ~3.4 expected[25,26] for H-5 of a β-rhamnopyranosyl group. The signal for the rhamnose H-5 in SD1, identified by double irradiation, had δ 4.01 ($J_{4,5}$ ~10 Hz), more consistent with the α configuration.

The characterization of SD1 showed that at least one of the 2-substituted rhamnopyranose residues (that giving rise to the glycerol residue in SD1) was present in the main chain of the polymer. The remaining problems were (*a*) the location of the second such residue, and (*b*) the identification of the substituents at positions 3 and 4 of the branch-point rhamnopyranose residue. The former problem was solved by *N*-deacetylation and deamination of the polymer. The major product was eluted from Sephadex G-50 in the position of the tetrasaccharide stachyose and had $R_{Lactose}$ 0.94 in paper chromatography. The hydrolysis products were identified as rhamnose, glucose, and 2,5-anhydromannitol by g.l.c. of the alditol acetates (relative peak areas 3.00:1.08:0.64). Methylation analysis of the material gave the results shown in Table I, column C, indicating cleavage, as expected, between the 2-amino-2-deoxyglucose and a 2-substituted rhamnose residue during deamination.



Scheme 1. Fragmentation in e.i.-m.s. of DA1 (see text).

However, the n.m.r. spectra indicated that the material was heterogeneous, and this conclusion was confirmed by g.l.c. of the methylated oligosaccharides. The major product (DA1) was isolated by preparative t.l.c. and examined by e.i.-m.s. In addition to fragment ions with *m/z* 189, 157, and 125 (the aA series[27]) diagnostic for rhamnose at a non-reducing terminus, and analogous ions with *m/z* 219, 187, and 155 (the eA series) for non-reducing-terminal glucose, the significant ions (Scheme 1) included the members of the baA series with *m/z* 363, 331, and 299. From these and other diagnostic ions, it was inferred that DA1 had the sequence of residues shown in Scheme 1 and that the second 2-substituted rhamnose residue was a component of the main chain, rather than interposed between the lateral glucosyl substituent and the branch-point rhamnose.

The two minor products of the deamination reaction sequence were obtained in similar yields (each ~25% of the total products). Their methylated derivatives had retention times in g.l.c. much shorter than that of DA1, which indicated the absence of at least one monosaccharide residue. However, like DA1, both clearly contained a rhamnobiose residue at the non-reducing terminus, and at least one also contained the terminal glucosyl group. Presumably, the minor products arose through alternative reaction pathways during deamination[28].

Attempts to remove the substituents from the branch-point rhamnose in DA1 by treatment with either α-D-glucosidase or α-L-rhamnosidase were unsuccessful. Therefore, partial acid hydrolysis of the parent polymer was used in order to determine the position of glucosylation. After hydrolysis with 0.1M trifluoroacetic

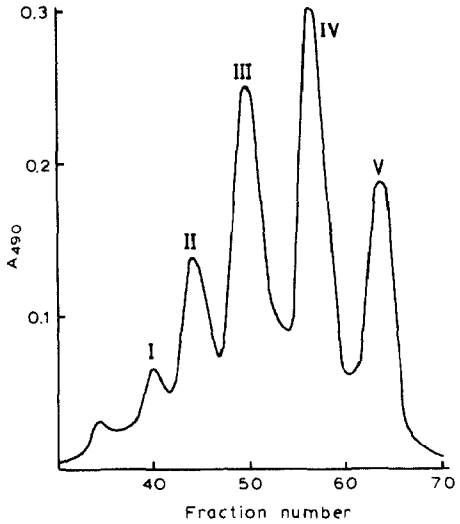


Fig. 2. Fractionation of products from partial hydrolysis of the O10 polymer. The polymer (18 mg) was hydrolysed with 0.1M trifluoroacetic acid for 1.5 h at 100°, and the products were fractionated on a column (72 × 1.5 cm) of Sephadex G-50 with pyridine–acetic acid buffer (pH 5.4) at a flow rate of 14 mL.h^{-1}. Fractions (2 mL) were analysed by the phenol–H_2SO_4 method.

acid for 1.5 h at 100°, followed by chromatography on Sephadex G-50, five distinct fractions were obtained (Fig. 2), which suggested relatively selective depolymerization. Fractions I, II, and III were virtually immobile in t.l.c. and paper chromatography, while fraction V consisted mainly of free glucose and rhamnose. Fraction IV, which was eluted in about the position of stachyose, showed a single spot with $R_{Lactose}$ 0.94 in paper chromatography, but two spots in t.l.c. with R_{Glc} values of 0.98 and 1.1. The components were isolated by preparative t.l.c. and converted into oligosaccharide-alditols ($NaBD_4$). The ^{1}H-n.m.r. spectra showed that neither product was completely pure, but that each contained one major component which incorporated 2-acetamido-2-deoxyglucose and three residues of rhamnose or rhamnitol. The anomeric region of the spectrum for the product (PH-F) of greater mobility in t.l.c. contained three one-proton signals at δ 5.15 ($J_{1,2}$ ~2 Hz), 5.10 ($J_{1,2}$ 3.7 Hz), and 5.07 ($J_{1,2}$ ~2 Hz). The spectrum for the other product (PH-S) contained an additional one-proton signal at δ 5.20 ($J_{1,2}$ 3.8 Hz), which could be attributed to the presence of a glucosyl group. This inference was confirmed by methylation analysis, which gave mainly the products from 2-substituted rhamnose and unsubstituted glucose residues. No derivative from a 3,4-disubstituted rhamnose residue was detected, but a significant minor product was identified as 3,4-di-*O*-acetyl-1,2,5-tri-*O*-methylrhamnitol-*1-d* (the low recovery being attributable to the volatility of the compound). Thus, it could be inferred that the major component in PH-S was a pentasaccharide-alditol produced by hydrolysis of the glycosidic linkage between the branch-point rhamnose and the 2-acetamido-2-deoxyglucose residues, followed by reduction of the oligosaccharide.

Methylation analysis of PH-F gave the derivative from 2-substituted rhamnose as the only major product, but a significant minor product was identified as 3-*O*-acetyl-1,2,4,5-tetra-*O*-methylrhamnitol-*1-d*. The origin of this product again must have been the rhamnose residue at the branch-point in the polymer, and its identification showed that the glucosyl substituent had been released from position 4. Thus, structures **2** and **3** can be assigned to the oligosaccharides leading to the major alditols in the products PH-F and PH-S, respectively, and structure **4** to the repeating unit in the original polymer.

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

2

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

3

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

4

DISCUSSION

The observations that the O10 lipopolysaccharide gave a banding pattern in polyacrylamide gel electrophoresis, and that it released a single polymer on mild acid hydrolysis indicated that the polymer was the O-specific side chain. Compared with other neutral polymers from *S. marcescens*, that from the O10 reference strain is relatively complex. Apart from the O8 polymer (which has a branched tetrasaccharide repeating-unit[7,8]), all other neutral polymers characterized so far have simple disaccharide repeating-units[2,3,5,6,9,10–13]. Although the O10 polymer differs markedly from other neutral polymers of *S. marcescens* which have been studied, the tetrasaccharide repeating-unit which forms the linear backbone is identical with that of the repeating unit for the O antigen of a strain of *Pseudomonas solanacearum*[29]. Similarities with some serotypes of *Shigella flexneri*[30] are also apparent, although the anomeric configuration of the 2-acetamido-2-deoxyglucose

residue is β in these cases. The structural similarities suggest the possibility of serological cross-reactions between the organisms, but we are unaware of any relevant studies.

EXPERIMENTAL

Growth of bacteria, and isolation and fractionation of the lipopolysaccharide. — *S. marcescens* O10 (C.D.C. 1287-54) was grown for 16 h at 30° in Nutrient Broth No. 2 (Oxoid) as a 20-L batch culture aerated at 20 L.min^{-1}. The cell walls (3.7 g) were prepared by mechanical disintegration of the cells (91 g, wet weight), and lipopolysaccharide (0.95 g) was extracted from the defatted walls by the hot, aqueous phenol method[4]. After mild acid hydrolysis of the lipopolysaccharide, the water-soluble products were fractionated[4] on Sephadex G-50 and DEAE-Sepharose CL-6B.

General methods. — N.m.r. spectra (^{13}C and ^{1}H) of samples in D_2O were recorded with a Bruker WH-400 spectrometer. ^{1}H-N.m.r. spectra were recorded at 60° or 80° 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. In general, chromatographic, electrophoretic, and mass-spectrometric methods were those described previously[8,9]. The solvent used for paper chromatography was ethyl acetate–pyridine–water (13:5:4). Oligosaccharides obtained by partial acid hydrolysis were separated by t.l.c. on Silica Gel $60F_{254}$ (Merck) with double development in acetone–propan-2-ol–M lactic acid (2:2:1). Bands were detected by using 1-naphthol–H_2SO_4 (ref. 31) or iodine vapour, the separated products were recovered using water, and the eluates were deionised. The solvent used for t.l.c. fractionation of per-*O*-methylated oligosaccharides was benzene–methanol (10:1), again with double development. Methods used to identify, determine, and assign configuration to monosaccharides were those used in previous studies[10].

Degradative methods. — Methylation analysis, periodate oxidation, and Smith degradation were carried out as described[8,9]. Oxidation of the per-*O*-acetylated Smith-degradation product (SD1) with CrO_3 (ref. 18) was done for 1 h at 50°. *N*-Deacetylation of the reduced ($NaBH_4$) O10 polymer was carried out for 16 h at 95°, followed by work-up, deamination, and fractionation of the products (Sephadex G-50) as previously[9]. Attempts to degrade the major deamination product (DA1, 51% of the total) were made using α-D-glucosidase (EC 3.2.1.20)[7] and α-L-rhamnosidase (EC 3.2.1.40)[32]. Partial hydrolysis of the O10 polymer was carried out by using 0.1M trifluoroacetic acid at 100°: checks by paper chromatography indicated that 1.5 h was the optimum time for the formation of useful oligosaccharides, which were fractionated on Sephadex G-50.

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