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STRUCTURAL STUDIES OF AN ACIDIC GALACTORHAMNAN FROM THE O2 REFERENCE STRAIN (C.D.C. 868-57) OF *Serratia marcescens*

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ABSTRACT

A partially acetylated acidic galactorhamnan has been isolated from the lipopolysaccharide of the O2 reference strain (C.D.C. 868-57) of *Serratia marcescens*. By means of n.m.r. spectroscopy and methylation analysis of the polymer and the products of Smith degradations, the repeating unit of the polymer was found to have the structure shown. The location of *O*-acetyl groups was not established.

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

INTRODUCTION

During the course of a study aimed at determining the chemical basis for the O-serotyping scheme of *Serratia marcescens*, the lipopolysaccharides from a number of strains have been examined. After mild acid hydrolysis, most of the lipopolysaccharides produced both neutral and acidic polysaccharides (*e.g.*, ref. 1). In two cases, only neutral polysaccharides were produced[2,3], and in one case only an acidic polysaccharide was produced[1]. It is thought that the acidic polymers are microcapsular in origin and confer O-specificity when present, while the neutral polymers are thought to be the lipopolysaccharide side-chains and only represent the O-antigens in strains which do not produce acidic polymers.

We now report the structure of an acidic galactorhamnan isolated from the lipopolysaccharide of the O2 reference strain.

RESULTS

The material extracted from the cell walls of *Serratia marcescens* C.D.C. 868-57 did not give the "ladder" pattern typical of S-type lipopolysaccharides on examination by sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS-PAGE). A very intense fast-moving band, corresponding to R-type lipopolysaccharide, and 2 or 3 much fainter fast-moving bands were observed.

The major sugar components of the lipopolysaccharide were rhamnose,

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galactose, and galacturonic acid. Minor components (originating from the core oligosaccharide or lipid A) were glucose, 2-amino-2-deoxyglucose, L-*glycero*-D-*manno*-heptose, and D-*glycero*-D-*manno*-heptose, as found in similar studies[1-3] of other lipopolysaccharides from *S. marcescens*.

Fractionation of the water-soluble products of mild acid hydrolysis of the lipopolysaccharide yielded a polymeric product, which was eluted from a column of DEAE-Sepharose CL-6B with 0.2M NaCl. Analysis of the polymer showed that it contained D-rhamnose (40.1%), D-galactose (20.6%), and D-galacturonic acid (24.7%). Methylation analyses of the polymer, with and without carboxyl-reduction of the uronic acid (Table I, columns A and B), indicated that it had a linear tetrasaccharide repeating-unit of 3-substituted rhamnose (2 parts), 3-substituted galactose (1 part), and 4-substituted galacturonic acid (1 part).

The presence of *O*-acetyl substituents was apparent from signals at δ 20.58 and 21.05 (^{13}C), and at δ 2.17 and 2.22 (^{1}H) in the n.m.r. spectra of the polymer. Both spectra were complicated by the non-stoichiometric amount (~0.6 OAc per repeating unit) and probable variability of the location of *O*-acetyl groups (possibly involving more than one sugar residue), and were greatly simplified by *O*-deacetylation. The ^{1}H-n.m.r. spectrum of the *O*-deacetylated polymer contained 4 signals in the anomeric region at δ 5.29 ($J_{1,2}$ ~4 Hz), 4.94 ($J_{1,2}$ 1–2 Hz), 4.91 ($J_{1,2}$ 1–2 Hz), and 4.60 ($J_{1,2}$ 7.5 Hz). Thus, one of the sugars with the *galacto* configuration had an α linkage, the other had a β linkage, and the two rhamnose residues were probably α-linked. The ^{13}C-n.m.r. spectrum of the *O*-deacetylated polymer (Fig. 1) contained 20 signals, four of which were of double intensity. Three signals were present in the anomeric region at δ 101.19 (1 C, $^{1}J_{CH}$ 174 Hz), 100.78 (2 C, $^{1}J_{CH}$ apparently 172 Hz), and 96.70 (1 C, $^{1}J_{CH}$ 170 Hz). The values for the C–H coupling constants are all indicative of α-pyranosyl residues, although the ^{1}H-n.m.r. spectrum clearly showed the presence of one β-pyranosyl residue. Thus, the two-carbon signal at δ 100.78 must contain the C-1 resonances of both α- and β-pyranosyl residues. This inference was subsequently confirmed.

TABLE I

METHYLATION ANALYSES[a]

Methylation product[b]	*Relative peak area (g.l.c.)*			
	A	*B*	*C*	*D*
2,3,4-Rha			0.82	1.70
2,4-Rha	2.00	2.00	1.00	1.00
2,4,6-Gal	1.07	1.11	0.91	1.15
2,3-Gal		0.88		

[a] Key: A, native polymer; B, native polymer with reduction of the uronic acid methyl ester; C, SDA; D, SDN. [b] 2,3,4-Rha = 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylrhamnitol, *etc.*

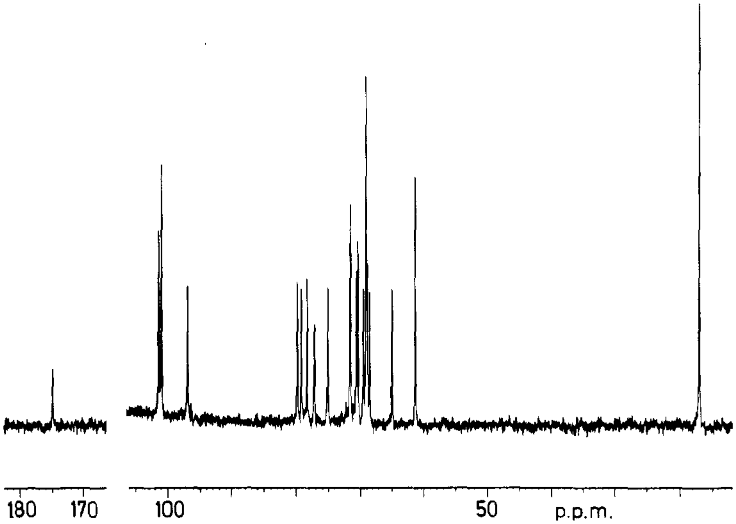

Fig. 1. ^{13}C-N.m.r. spectrum of the *O*-deacetylated acidic galactorhamnan. The spectrum for the sample in D_2O was obtained at 100.61 MHz and 50°, with complete proton-decoupling and tetramethylsilane as the external reference.

Only the galacturonic acid was oxidised when the *O*-deacetylated polymer was treated with aqueous sodium periodate. Smith degradation of the product yielded an oligomeric fraction, although the depolymerisation was incomplete. Paper electrophoresis (pH 2.7) revealed that this fraction contained an acidic component with M_{GalA} 0.42, and a neutral component. The two products, SDA and SDN, respectively, were separated by ion-exchange chromatography on Dowex 1 (AcO^-) resin. Both products contained rhamnose and galactose in the molar ratio 2:1. The results of methylation analyses on SDA and SDN are shown in Table I, columns C and D. SDN was treated with sodium borodeuteride prior to methylation because of the possibility that it may have been a reducing oligosaccharide. However, the failure to detect any mono-*O*-acetyl derivatives indicated that this was not the case. The n.m.r. spectra of SDA were consistent with its being a triglycosyl derivative of threonic acid, which was the expected product of Smith degradation of the polymer. The ^{13}C-n.m.r. spectrum contained 18 signals, three of which were of double intensity. These included anomeric signals at δ 100.99 (2 C) and 96.61 (1 C). The expected signal for the carboxyl group was not observed due to the high level of background noise. The ^{1}H-n.m.r. spectrum contained three one-proton signals in the anomeric region at δ 4.98 (unresolved), 4.90 (unresolved), and 4.58 ($J_{1,2}$ 7.6 Hz), as well as two signals for H-6 of rhamnosyl residues. The galactosyl residue was therefore β-linked and, consequently, the galacturonosyl residue in the native polymer was α-linked. The n.m.r. spectra of SDN were more complex, indicating that it was heterogeneous. This situation was presumably due, at least in part, to inter- or intra-molecular esterification of the carboxyl group.

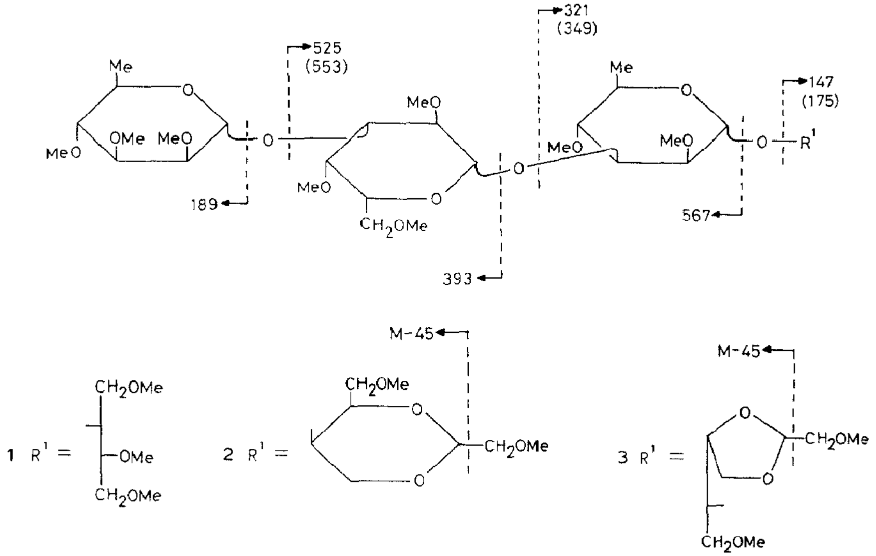

Fig. 2. A_1 fragment ions for the per-*O*-methylated oligosaccharides **1**–**3**. The figures in parentheses show the ions from **2** and **3**, where they differ from those from **1**.

SDA was subjected to a second Smith-degradation. Methylation analysis of the product showed two major components which were derived from 3-substituted rhamnopyranosyl and 3-substituted galactopyranosyl residues, and a minor component derived from a terminal galactopyranosyl group. The absence of a terminal rhamnopyranosyl derivative shows that this residue had been completely oxidised by the periodate treatment, but most of the oxidised rhamnose was apparently not removed by the subsequent mild treatment with acid. Since it was thought that this failure may have been due to the presence of the carboxyl group, a portion of the *O*-deacetylated O2 polymer was carboxyl-reduced and subjected to a Smith degradation. The reduced polymer was completely depolymerised by this procedure, giving a product, SD1, which contained rhamnose, galactose, and threitol (molar ratios 2.00:1.03:1.00). Two components were detected by g.l.c. of per-*O*-methylated SD1, and were separated by preparative t.l.c. From the e.i.-mass spectra (Table II), it was concluded that the major component was the classical Smith-degradation product with structure **1**, while the minor component had either structure **2** or structure **3** (Fig. 2). Such products could be formed during the hydrolysis step of the Smith degradation by cyclisation of the oxidised galactose involving the primary hydroxyl group on C-3 or C-6 (ref. 4).

Smith degradation of SD1 gave a product (SD2) which also contained two oligosaccharides. From the e.i.-mass spectra of the per-*O*-methylated derivatives (Table II), it could be inferred that the major component had structure **4** and that the minor component had either structure **5** or structure **6** (Fig. 3).

The mass spectra of all four per-*O*-methylated oligosaccharides contained

TABLE II

DIAGNOSTIC E.I.-M.S. IONS[a] FOR PER-*O*-METHYLATED OLIGOSACCHARIDES

Per-O-methylated oligosaccharide	*R.t.*[b]	*E.i.-m.s. fragment ions*[c]																		
		aA_1	aA_2	baA_1	baA_2	$cabA_1$	$cabA_2$	dA_1	dA_2	cdA_1	cdA_2	$bcdA_1$	$bcdA_2$	$abcdJ_1$	cdJ_0	$bcdJ_0$	cdL_1	$bcdM_1$	$bcdL_1$	$M - 44$
1	17.87	189 (100)	157 (30.3)	393 (20)	361 (7.3)	567 (0.9)	535 (1.0)	147 (6.6)	115 (38.7)	321 (0.6)	289 (1.1)	525 (0.9)	493 (1.6)	585 (3.0)	193 (0.9)	367 (8.4)	277 (20)	451 (0.7)	433 (2.1)	
2/3	26.23	189 (100)	157 (33.2)	393 (17.3)	361 (5.7)	567 (1.5)	535 (2.3)	175 (7.8)	143 (7.0)	349 (0.7)	317 (0.7)	553 (0.6)		613 (1.6)	221 (0.6)	395 (2.1)	305 (20.5)	479 (0.9)	461 (0.8)	714[d] (1.1)
		aA_1	aA_2	baA_1	baA_2	cA_1	bcA_1	bcA_2	$abcJ_1$	bcJ_0	bcL_1	$M - 45$								
4	7.77	219 (34.7)	187 (55)	393 (0.6)	361 (1.3)	103 (45)	277 (4.4)	245 (3.5)	337 (21.3)	149 (4.9)	233 (100)									
5/6	11.85	219 (48.4)	187 (62.3)	393 (1.3)	361 (4.4)	175 (18.1)			409 (7.3)	221 (5.1)	305 (96.4)	539 (2.0)								

[a] In the designation of the ions, lower case letters represent sugar residues, the first letter being the residue undergoing cleavage, and upper case letters denote the type of ion. For a detailed explanation, see ref. 6 (for A_1, A_2, and J_1), ref. 7 (for J_0), and text (for L_1 and M_1). [b] Retention time in g.l.c. under the conditions described in the Experimental. [c] Figures in parentheses show the intensity of the peak relative to that of the base peak (100). [d] The expected ion at *m/z* 713 (M − 45) was not observed. The ion at *m/z* 714 may have been produced from this ion by the addition of a hydrogen atom.

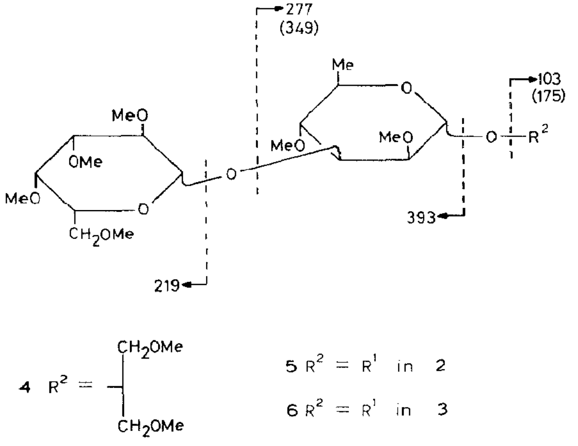

Fig. 3. A_1 fragment ions for the per-*O*-methylated oligosaccharides **4**–**6**. The figures in parentheses show the ions from **5** and **6**, where they differ from those from **4**.

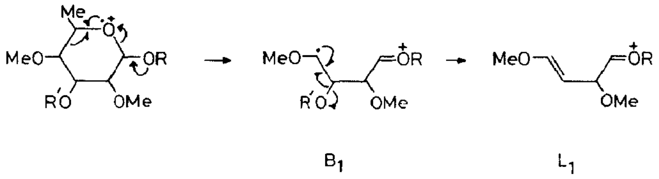

Fig. 4. A possible mechanism for the formation of the L_1 fragment ion.

ions analogous to those described by Kärkkäinen[5] for trisaccharide-alditols containing 3-substituted pyranosyl residues. These ions, designated L_1 and M_1, both contain the aglycon moiety but not the 3-substituent of the residue undergoing cleavage. As can be seen from Table II, cleavage of the internal rhamnopyranosyl residues gives rise to L_1 but not M_1 ions, whereas cleavage of the internal galactopyranosyl residues gives both types of ion. A possible mechanism for the formation of the L_1 ion is shown in Fig. 4.

The n.m.r. spectra of SD2 were in agreement with the above results. The ^{1}H-n.m.r. spectrum contained two major anomeric signals at δ 5.03 (1 H, $J_{1,2}$ 1.8 Hz) and 4.53 (1 H, $J_{1,2}$ 7.6 Hz), attributable to α-rhamnopyranosyl and β-galactopyranosyl residues, respectively. Three minor anomeric signals were also present at δ 5.11 (0.3 H, $J_{1,2}$ 1.8 Hz), 5.07 (0.3 H, triplet, $J_{H,H}$ 3.4 Hz), and 4.52 (0.3 H, $J_{1,2}$ 7.5 Hz). The signal at δ 5.07 can be assigned to the acetal proton of the aglycon in the minor component. The ^{13}C-n.m.r. spectrum (Table III) contained 14 major signals (one of double intensity), including two for anomeric carbons at δ 101.13 ($^1J_{CH}$ 162 Hz) and 99.28 ($^1J_{CH}$ 170 Hz), three for hydroxymethyl groups, and one for C-6 of the rhamnosyl residue. Several minor signals were also apparent. These included two in the anomeric region at δ 103.52 and 100.83.

TABLE III

^{13}C-N.M.R. DATA FOR SD2[a]

Carbon atom	*Chemical shift*[b] *(p.p.m.)*		
	Gal	*Rha*	*Glycerol*
C-1[c]	101.13	99.28	61.58
C-2	70.97	68.89	78.52
C-3	72.92	78.27	60.48
C-4	68.89	70.63	
C-5	75.44	68.35	
C-6	61.21	16.89	

[a] Only the major signals are listed. [b] Chemical shifts are given in p.p.m. downfield from external tetramethylsilane. [c] Assignments of signals with similar chemical shifts may be interchanged.

From the combined results, structure **7** can be assigned to the repeating unit of the O2 polysaccharide. No attempt was made to locate the *O*-acetyl substituents which were present.

→3)-α-D-Rha*p*-(1→3)-β-D-Gal*p*-(1→3)-α-D-Rha*p*-(1→4)-α-D-Gal*p*A-(1→

7

DISCUSSION

The isolation of the partially acetylated, acidic galactorhamnan with structure **7** as the only polymeric product from the "lipopolysaccharide" from *S. marcescens* strain C.D.C. 868-57 must mean that it is the O2 group antigen. However, it cannot represent the lipopolysaccharide side-chain, since analysis by SDS-PAGE showed the absence of high-molecular-weight bands. This polysaccharide is therefore probably of microcapsular origin, as has been suggested for other acidic polysaccharides isolated from *S. marcescens* "lipopolysaccharides".

It is interesting to note the similarity between the polymer described here and an extracellular polymer produced by a strain of *Serratia piscatorum* (IFO 12527), for which structure **8** has been proposed[8]. Some strains of *S. piscatorum* have since been reclassified as *S. marcescens*.

→3)-L-Rha*p*-(1→3)-D-Gal*p*-(1→3)-L-Rha*p*-(1→4)-D-Gal*p*A-(1→

8

A further point of interest is the occurrence in the O2 polymer of the rare sugar D-rhamnose. This has previously been reported as a component of only a few bacterial polysaccharides, *e.g.*, from *Pseudomonas cepacia*[9] and *Pseudomonas syringae*[10].

EXPERIMENTAL

Growth of bacteria, and isolation and fractionation of the lipopolysaccharides. — *S. marcescens* O2 (C.D.C. 868-57) was grown and processed as described previously[2]. The water-soluble products of mild acid hydrolysis (1% acetic acid, 2.25 h, 100°) of the lipopolysaccharide were fractionated on Sephadex G-50 and DEAE-Sepharose CL-6B.

General methods. — T.l.c. of per-*O*-methylated oligosaccharides was performed on Silica Gel $60F_{254}$ (Merck) with benzene–methanol (10:1). Other chromatographic and electrophoretic methods were those used in previous studies[1-3]. G.l.c. was performed on fused-silica capillary columns (25 m) of BP1 (for alditol acetates, acetylated oct-2-yl glycosides, and per-*O*-methylated oligosaccharides) and BP10 (for methylated alditol acetates) in a Carlo Erba Mega 5160 chromatograph. For the analysis of per-*O*-methylated oligosaccharides, the column temperature was held for 4 min at 220°, increased at $15°.min^{-1}$, then held at 270° The carrier gas flow-rate was $44\ cm.sec^{-1}$. G.l.c.–m.s. was performed on a Finnigan model 1020B instrument fitted with the above columns and scanning from *m/z* 50 to 800. 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 standard. ^1H-Spectra were recorded at 60° or 80° with sodium 3-trimethylsilylpropanoate-d_4 as the external standard.

Determination of monosaccharide composition. — In general, methods used for the release, identification, and determination of monosaccharides were those used previously[1-3]. All the sugars were shown to have the D configuration by g.l.c. of the acetylated oct-2-yl glycoside derivatives[11]. For galactose and rhamnose, these results were confirmed by enzymic assay and by the c.d. spectrum[12] of the alditol acetate, respectively.

Degradative methods. —Smith degradation and methylation analysis were done as described previously[1-3]. The products of Smith degradation of the *O*-deacetylated O2 polysaccharide were applied to a column of Dowex 1 (AcO^-) resin. The neutral component (SDN) was eluted with water, and the acidic component (SDA) with 2M acetic acid. Carboxyl-reduction of the *O*-deacetylated O2 polysaccharide was achieved by treatment with 1-cyclohexyl-3-(2-morpholinoethyl)carbodi-imide metho-*p*-toluenesulphonate and sodium borohydride[13].

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