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Structure of an acidic glycan from the reference strain for *Serratia marcescens* serogroup O22

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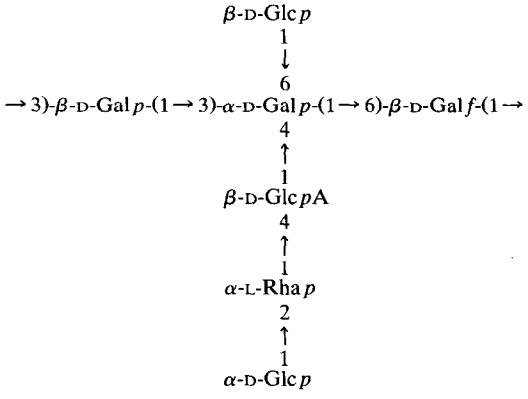
ABSTRACT

In addition to a neutral glycan, lipopolysaccharide extracts from the reference strain for *Serratia marcescens* serogroup O22 contain an acidic polymer which probably defines the serogroup and is of microcapsular origin. The polymer is doubly branched with a heptasaccharide repeating unit and a galactan backbone. By means of spectroscopic and degradative studies, the structure of the repeating unit was established as that shown.



INTRODUCTION

Many of the reference strains for the different O serogroups of *Serratia marcescens* produce both a neutral and an acidic surface polysaccharide[1]. The former type of polymer appears to be the true O-specific side chain of the

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lipopolysaccharide, but does not define the serogroup in those strains which also produce an acidic polymer. The dominant antigen is the K-type acidic polymer, which is believed to be of microcapsular origin, even though it seems to be an integral component of the cell envelope and is present in "lipopolysaccharide" extracts from isolated envelopes[1]. A previous study[2] has shown the presence of both types of polymer in the O22 reference strain and characterised the repeating unit of the neutral glycan as structure **1**. We now report the results of structural studies of the accompanying acidic glycan.

→ 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

RESULTS AND DISCUSSION

The acidic glycan (71% of the polymeric water-soluble products obtained[2] by mild acid hydrolysis of the "lipopolysaccharide") was eluted from DEAE-Sepharose CL-6B partly (F1) as a broad, low peak (starting near the end of the 0.1 M NaCl eluate, and extending into the following 0.3 M NaCl eluate) and partly (F2) as a sharp peak in the 0.3 M NaCl eluate. Both fractions had almost identical monosaccharide compositions and ^{1}H-NMR spectra, so only fraction F2 was used in further work.

The monosaccharide components of the polymer were rhamnose, glucose, galactose (relative peak areas in GLC of the alditol acetates, 1.00 : 2.82 : 3.18), and glucuronic acid: all sugars apart from rhamnose (L) belonged to the D series. Although the quantitative data were ambiguous the NMR spectra clearly pointed to a heptasaccharide repeating-unit. Thus, the ^{13}C-NMR spectrum of the glycan (Fig. 1) was characteristic of a regular polymer and contained 35 discrete signals, seven of which apparently corresponded to two carbons each. There were seven

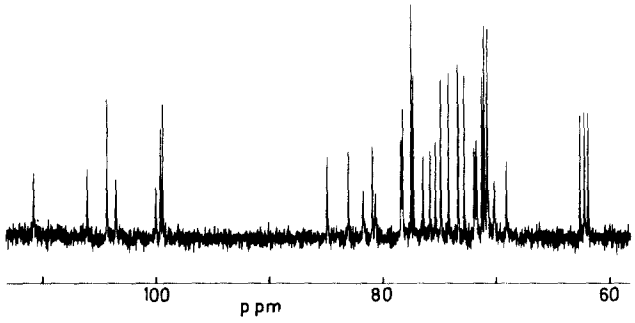


Fig. 1. ^{13}C-NMR spectrum of the O22 acidic glycan. The spectrum for the sample in D_2O was recorded at 100.62 MHz and 27° with 1,4-dioxane (δ 67.40) as the internal reference. In addition to the signals shown, the spectrum contained others at δ 176.01 and 17.22.

TABLE I

NMR data [a] for the O22 acidic glycan

Atom	Residue						
	a (β-Gal*f*)	*b* (α-Gal*p*)	*c* (α-Glc*p*)	*d* (α-Rha*p*)	*e* (β-Glc*p*A)	*f* (β-Gal*p*)	*g* (β-Glc*p*)
H-1	5.24 (< 1)	5.02 (2.7)	4.94 (3.8)	4.89 (< 1)	4.86 (7.9)	4.65 (t)	4.48 (7.9)
C-1	110.09 (173)	99.27 (~ 170)	97.68 (171)	98.87 (167)	102.83 (161)	105.35 (~ 160)	103.81 (161)
H-2	4.21 (< 1)	4.13 (–)	3.55 (10.0)	3.96 (3.4)	3.37 (8.5)	3.73 (–)	3.28 (9.3)
H-3	4.10 (< 1)	4.11 (–)	3.74 (~ 10)	3.86 (9.5)	3.59 (9.3)	~ 3.73 (–)	3.50 (9.0)
H-4	4.03 (~ 4)	4.43 (< 4)	3.43 (9.8)	3.48 (9.5)	3.65 (9.0)	4.08 (–)	3.40 (~ 10)
H-5			3.99 (–)	4.00 (6.3)	3.72 (–)		
H-6				1.27			

[a] Values for chemical shifts relative to acetone (δ_H 2.22) or 1,4-dioxane (δ_C 67.40); values for coupling constants are given in parentheses (t indicates a triplet). Dashes indicate that no data were obtained.

signals in the anomeric region (Table I), as well as signals for one carbonyl group (δ 176.01), three unsubstituted hydroxymethyl groups (δ 61.94, 61.55, and 61.23), and one methyl group (δ 17.22). The ^{1}H-NMR spectrum (Fig. 2, Table I) also contained seven major anomeric signals (each ~ 1 H) labelled *a* to *g* (in order of decreasing chemical shift), as well as a minor anomeric signal (~ 0.1 H) at δ 4.59 ($J_{1,2}$ 7.6 Hz) and a methyl doublet at δ 1.27 ($J_{5,6}$ 6.3 Hz). The C-1 signals of residues *a* to *g* were identified from a 2D C–H correlation NMR spectrum, and most ring-proton resonances were assigned from COSY, relayed COSY, and double-relayed COSY ^{1}H-NMR spectra. Residue *a* was identified as a β-furanosyl residue from the large chemical shift (δ 110.09) for C-1. Residue *b* was clearly an α-pyranosyl residue and, although the values for $J_{2,3}$ and $J_{3,4}$ could not be determined because of the close proximity of the signals for H-2 and H-3, the signal for H-4 (δ 4.43, with low *J* values) was characteristic of a galactopyranosyl residue. The large values (Table I) of $J_{2,3}$, $J_{3,4}$, and $J_{4,5}$ for residues *c*, *e*, and *g* indicated that these all had the glucopyranosyl configuration, with the first being α-linked and the others β-linked. Furthermore, the absence of any 5,6-coupling showed that residue *e* corresponded to the glucuronic acid. The connectivities for residue *d* could be traced from H-1 to H-6 and clearly identified it as a rhamnopyranosyl residue. The anomeric configuration could not be determined from the chemical shift for H-1 or C-1, nor from the $J_{C\text{-}1,H\text{-}1}$ value, but the relatively

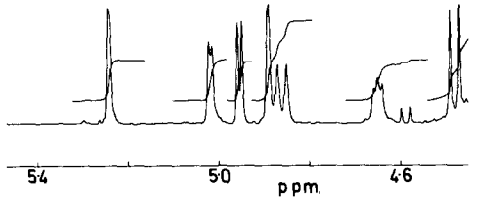


Fig. 2. Anomeric region of the ^{1}H-NMR spectrum of the O22 acidic glycan. The spectrum for the sample in D_2O was recorded at 400.13 MHz and 70° with acetone (δ 2.22) as the internal reference.

low-field locations of the signals for H-3 and H-5 (Table I) showed that the rhamnose was α-linked. Despite the triplet appearance of the signal at δ 4.65, the correlation with a ^{13}C signal at δ 105.35 clearly showed that it was derived from an anomeric proton. Furthermore, the values of the chemical shifts and of $J_{C\text{-}1,H\text{-}1}$ were all indicative of a β-pyranosyl residue. It seems that the triplet appearance of the H-1 resonance is a second-order effect caused by superposition of the signals for H-2 and H-3.

As the polysaccharide contained an acid-labile furanosyl linkage, it seemed possible that the minor anomeric signal at δ 4.59 arose as a consequence of hydrolysis during the mild acid treatment of the parent "lipopolysaccharide". This possibility was explored by further hydrolysis of the acidic glycan with 50 mM trifluoroacetic acid at 100° for different periods (10–40 min). The ^1H-NMR spectra of the reduced ($NaBH_4$) products showed a progressive decrease in the relative intensities of the signal for H-1*a* and the triplet H-1*f*, together with a corresponding increase in intensity of the minor anomeric signal. The products of hydrolysis for 40 min were fractionated by HPLC, whereby a major acidic oligosaccharide was obtained and was reduced ($NaBH_4$) to give the oligosaccharide-alditol (PH1). FABMS of PH1 and its per-*O*-methyl derivative (both positive- and negative-ion spectra) showed that they corresponded to the heptasaccharide repeating unit of the glycan. The ^1H-NMR spectrum of PH1 showed the disappearance of the signals for H-1*a* and H-1*f*, together with a proportionate enhancement of the signal at δ 4.59. These results can be explained by assuming that the glycan contains the disaccharide sequence $a \rightarrow f$, and that when this linkage is hydrolysed, the resonances for H-2*f* and H-3*f* shift sufficiently to produce a first-order subspectrum in which H-1*f* gives the expected doublet.

The results described above indicated that the repeating unit of the acidic glycan was constructed from residues of galactose (3), glucose (2), glucuronic acid (1), and rhamnose (1). As with monosaccharide analysis, the results from methylation analysis of the polymer (Table II, column A) were quantitatively misleading, but indicated that *both* glucopyranosyl residues were unsubstituted and showed the presence of 2-substituted rhamnopyranosyl, 3-substituted galactopyranosyl, 6-substituted galactofuranosyl, and 3,4,6-trisubstituted galactopyranosyl residues. Consistent with the NMR data and the inferences from partial hydrolysis of the glycan, an unsubstituted galactopyranosyl group was detected as a trace component. Similar analysis of the heptasaccharide-alditol PH1 confirmed this picture (Table II, column C) and showed that the disaccharide sequence $a \rightarrow f$ involved the 6-substituted β-galactofuranosyl residue (*a*) and the 3-substituted galactopyranosyl residue (*f*). Apart from the 6-substituted galactofuranosyl residue, for which a low relative peak area was again obtained, improved quantitative data were obtained on methylation analysis of the carboxyl-reduced glycan (Table II, column B), which also showed that the glucosyluronic acid residue was 4-substituted.

Smith degradation of the carboxyl-reduced glycan resulted in complete depolymerisation, giving materials that were eluted in about the trisaccharide region in

TABLE II

Methylation analyses of the O22 acidic glycan, its derivatives and degradation products [a]

Methylation product [b]	Relative peak area (GLC)												
	A	B	C	D	E	F	G	H	I	J	K	L	M
1,2,3-Thr						+ [c]	+ [c]	+ [c]	+ [c]				
1,2,3,4,5-Gal			0.3								0.2	0.2	0.3
3,4-Rha	0.7	0.9	1.2										
2,3,4,6-Glc	1.3	1.8	2.4					1.4 [d]		1.1	1.1	1.0	
2,3,4,6-Gal	Tr	Tr	1.0	2.1	1.1	1.0 [e]	1.0 [e]	1.0 [e]	1.0 [e]	0.3	1.0		1.0
2,3,6-Glc		0.9											
2,4,6-Gal	1.0	1.0		1.0	1.0	1.2 [d]				1.0			1.2
2,3,5-Gal	0.3	0.5								0.4			
2,3,4-Gal												0.9	
2,6-Gal								1.4 [f]	1.6 [f]				
2,4-Gal										1.0	1.5		
2-Gal	1.0	1.1	1.4				1.5						

[a] Key: A, acidic glycan; B, carboxyl-reduced glycan; C, heptasaccharide-alditol PH1; D, SD1a; E, SD1b; F, SD2a; G, SD2b; H, SD2c; I, SD2d; J, LD1; K, LD1a; L, LD1b; M, LD1c; Tr, trace; +, present but not quantified. [b] 1,2,3-Thr = 4-*O*-acetyl-1,2,3-tri-*O*-methylthreitol; 3,4-Rha = 1,2,5-tri-*O*-acetyl-3,4-di-*O*-methylrhamnitol; etc. [c] 2-OCD_3. [d] 4,6-di-OCD_3. [e] 3-OCD_3. [f] 6-OCD_3.

size-exclusion HPLC. Two products (SD1a and SD1b) were isolated by further chromatography (relative peak areas, 1.8 : 1.0), and were shown to be pure by GLC of their per-*O*-methyl derivatives. Both products contained galactose and threitol, and both gave derivatives of unsubstituted and 3-substituted galactopyranosyl residues on methylation analysis (Table II, columns D and E). FABMS of the per-*O*-methylated SD1a showed pseudomolecular ions (Table III) consistent with a dihexosyltetritol structure. The ^{1}H-NMR spectrum of SD1a contained two anomeric signals (each 1 H) at δ 5.00 (broad singlet) and 4.62 ($J_{1,2}$ 7 Hz), indicating that both α- and β-galactopyranosyl residues were present. Thus, SD1a is the classical product of a Smith degradation, and is derived from a trigalactose

TABLE III

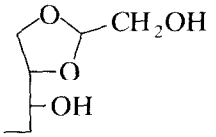
FABMS data for per-*O*-methylated degradation products

Product	Pseudomolecular ion		
	$(M+H)^+$	$(M+NH_4)^+$	$(M+Na)^+$
SD1a	587	604	609
SD1b			637
SD2a	599	616	621
SD2b	621	638	643
SD2c	806	823	828
SD2d	820	837	842
LD1a	879	896	901
LD1b	675	692	697
LD1c	675	692	697

sequence in the glycan. It can be assigned the partial structure **2** (in which the threitol residue is derived from the 6-substituted galactofuranosyl residue *a*).

α/β-Gal*p*-(1 → 3)-β/α-Gal*p*-(1 → 1)-Thr-ol

2



α/β-Gal*p*-(1 → 3)-β/α-Gal*p*-(1 → O

3

The ^{1}H-NMR spectrum of the minor Smith-degradation product SD1b contained three signals (each 1 H) in the anomeric region, at δ 5.24 (t, $^3J_{H,H}$ 3 Hz), 5.02 (broad singlet), and 4.62 ($J_{1,2}$ 7 Hz). FABMS of the per-*O*-methylated derivative showed an intense pseudomolecular ion at m/z 637 $(M + Na)^+$. These data point to SD1b having a modified (dioxolane) residue at the reducing terminus, with a structure such as **3**, as found in several previous studies (e.g., ref. 3). The fact that the anomeric signal for the β-galactosyl residue had the same chemical shift for both SD1a and SD1b suggested that this residue was at the non-reducing end in each oligosaccharide-alditol. This conclusion was confirmed by the ^{1}H-NMR spectrum of the product from further oxidation ($NaIO_4$) and reduction ($NaBH_4$) of SD1a, which contained only one anomeric signal (δ 4.98, $J_{1,2}$ 3.5 Hz).

Further evidence for the structure about the branching region of the glycan was obtained by a modified Smith degradation, in which the native polymer (as the ammonium salt) was oxidised, reduced, and then methylated before mild acid hydrolysis, followed by further reduction ($NaBH_4$) and trideuteriomethylation to give the product SD2. Because prior methylation prevents the formation by transacetalation of a compound corresponding to SD1b during hydrolysis of the oxidised polymer, it was expected that the only product would be per-*O*-methylated SD1a. However, GLC of SD2 revealed four major components (SD2a–d), which were separated by reverse-phase HPLC. The results obtained by methylation analysis, FABMS and ^{1}H-NMR spectroscopy of the four component are summarised in Tables II, III, and IV, respectively. Component SD2a is clearly the expected product, having structure **4** and the same retention time in GLC as permethylated SD1a. Fractions SD2c and SD2d both contained products of incomplete periodate oxidation. Comparison of their ^{1}H-NMR spectra with that of SD2a showed that both contained an additional β-linked sugar residue. Methylation analysis showed that the additional residue was attached at O-4 of the internal galactose in each structure, and that the additional residue in SD2c was derived from carboxyl-reduced, 4-substituted glucuronic acid, leading to the proposed structure **5**. No additional residue was detected in SD2d, but it seemed likely that this component was the unreduced counterpart of SD2c with structure **6**. These inferences were supported by the FABMS data (Table III). The reason

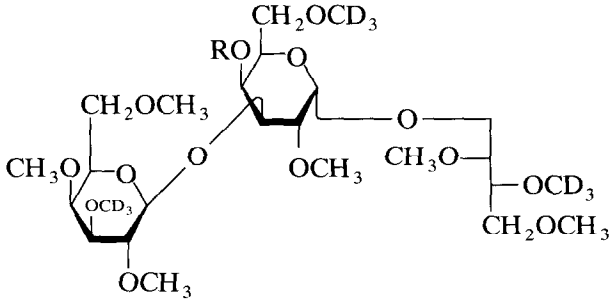
TABLE IV

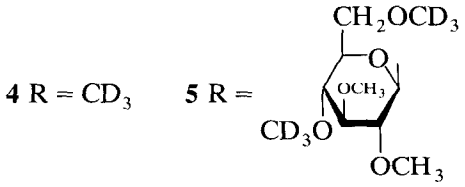
^{1}H-NMR data [a] for (methylated) oligosaccharide-alditols

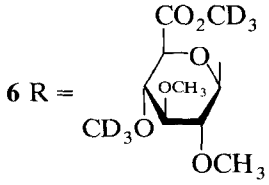
Product	Anomeric signals			Number of methoxyl groups
SD2a	4.98 (4)		4.59 (8)	6
SD2b	5.07 (4)	4.73 (4,6)	4.56 (8)	7
SD2c	5.00 (4)	4.77 (8)	4.56 (8)	8
SD2d	5.00 (4)	4.92 (8)	4.55 (8)	8
LD1a	5.03 (4)	4.63 (7)	4.52 (8)	
LD1b	5.01 (4)		4.52 (8)	
LD1c	5.03 (3)	4.63 (7)		

[a] Values for chemical shift for SD2a–d relative to internal tetramethylsilane (δ 0.00) and for LD1a–c relative to external tetramethylsilane; values of coupling constants are given in parentheses.

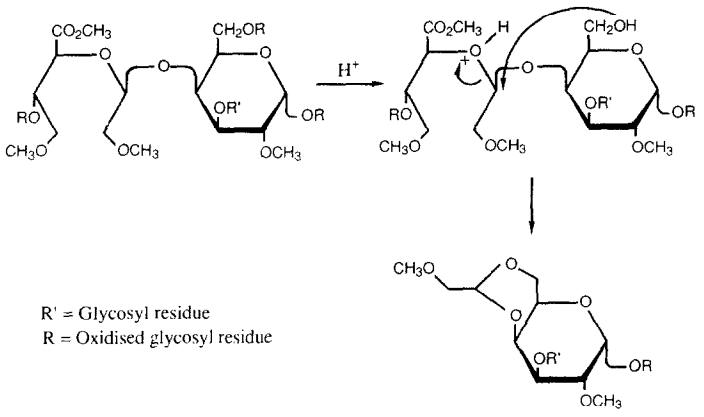
for the incomplete oxidation of the glucuronic acid residue (giving rise to **5** and **6**) is not clear. One possibility is protection by hemiacetal formation involving HO-2 or -3 and a carbonyl group of an adjacent oxidised residue. However, this seems unlikely, as similar oxidation of the carboxyl-reduced glycan seemed to be complete. An alternative explanation is lactonisation during oxidation, even though the glycan had been pretreated with base.





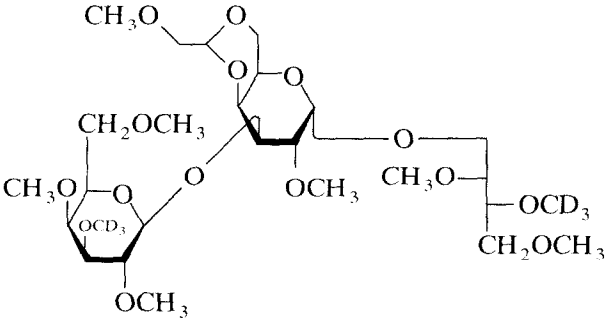


Component SD2b had a molecular mass 22 Da greater than that of SD2a (Table III) and contained an extra methoxyl group. An additional signal (1 H) in the anomeric region of the ^{1}H-NMR spectrum (δ 4.73, dd) could only have arisen from an acetal proton with an adjacent methylene group. Methylation analysis showed



Scheme 1.

that the acetal involved the 4- and 6-positions of the internal galactose residue, thus pointing to structure **7** for SD2b. The C_2 fragment producing the acetal could have originated from the oxidised glucuronic acid attached to O-4 of the branch-point galactose as shown in Scheme 1, or from oxidised glucose, if such a substituent were present at position 6 of the same galactose.



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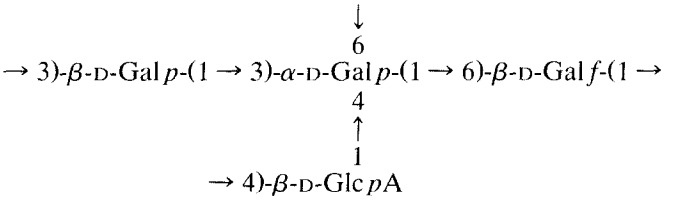
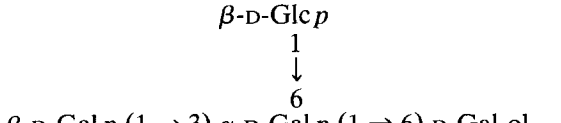


8

From the combined results of the two types of Smith degradation, it can be inferred that the repeating unit of the glycan contains the partial structure **8**. In order to complete the structure determination, the acidic glycan was subjected to degradation by lithium in ethylenediamine[4]. Fractionation of the neutral products

by size-exclusion HPLC gave a polymeric fraction (LD1, excluded from the TSKgelG-Oligo-PW column) and a disaccharide fraction (LD2), from which two components (LD2a and LD2b) were isolated by further HPLC. After per-*O*-methylation, LD2a and LD2b each gave a single peak in GLC, and EIMS indicated that each was the derivative of a hexosyl-6-deoxyhexitol. By methylation analysis, both products were shown to contain an unsubstituted glucopyranosyl group, but the expected 6-deoxyhexitol derivative (2-substituted) was only detected for LD2b, probably because of the high volatility of such derivatives. The ^{1}H-NMR spectrum of LD2a contained one anomeric signal (1 H) at δ 5.18 ($J_{1,2}$ 4 Hz) and a methyl doublet at δ 1.25 ($J_{5,6}$ 6 Hz). The corresponding signals in the very similar spectrum for LD2b were at δ 5.12 ($J_{1,2}$ 4 Hz) and δ 1.25 ($J_{5,6}$ 6 Hz). On acid hydrolysis, LD2a gave glucose and rhamnitol, whereas LD2b gave glucose and a different 6-deoxyhexitol, the alditol acetate of which co-chromatographed with quinovitol penta-acetate in GLC. Thus, it seems likely that LD2b was produced via epimerisation of the rhamnose residue under the basic conditions of the degradation, although such a change does not seem to have been reported previously.

The polymeric fraction (LD1) from the degradation contained only glucose and galactose (molar ratio, 1.0:2.7), and a comparison of the ^{1}H- and ^{13}C-NMR spectra with those of the native glycan showed that the anomeric signals assigned to the α-glucopyranosyl, α-rhamnopyranosyl, and β-glucopyranosyluronic acid residues had been lost. The results of methylation analysis of LD1 (Table II, column J) were essentially in accord with a repeating unit constructed from a linear trigalactose backbone and monoglucosylation at O-6 of the branch-point residue, although there was evidence for some cleavage of the main chain. Partial acid hydrolysis of LD1 yielded one major tetrasaccharide and two minor trisaccharides, which were reduced to give the oligosaccharide-alditols LD1a, LD1b, and LD1c, respectively. The per-*O*-methyl derivatives each gave a single peak in GLC and gave the expected pseudomolecular ions in FABMS (Table III). The relevant data from methylation analyses and ^{1}H-NMR spectra of LD1a–c are summarised in Tables II and IV, respectively. Taken with other results, these data indicate structure **9** for LD1a, **10** for LD1b, **11** for LD1c, and **12** for the parent polymer LD1. Thus, the repeating unit for the native O22 acidic glycan can be finalised as structure **13**.



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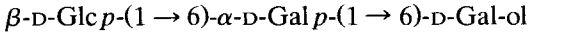

10

β-D-Gal*p*-(1 → 3)-α-D-Gal*p*-(1 → 6)-D-Gal-ol

11

β-D-Glc*p*
1
↓
6
→ 3)-β-D-Gal*p*-(1 → 3)-α-D-Gal*p*-(1 → 6)-β-D-Gal*f*-(1 →

12

β-D-Glc*p*
1
↓
6
→ 3)-β-D-Gal*p*-(1 → 3)-α-D-Gal*p*-(1 → 6)-β-D-Gal*f*-(1 →
4
↑
1
β-D-Glc*p*A
4
↑
1
α-L-Rha*p*
2
↑
1
α-D-Glc*p*

13

The acidic glycan from the O22 reference strain of *S. marcescens* has the largest and most complex repeating unit of any such polymers so far characterised, but does not contain any unusual components. Nor, in contrast to many other acidic glycans from this species[1], does the polymer contain *O*-acetyl substituents. On the other hand, the polymer shows some similarity in composition and architecture to the corresponding acidic glycan from the O18 reference strain[5]. The latter glycan also has a repeating, main-chain trisaccharide (containing one mannose and two galactose residues) and a trisaccharide branch [in which the monosaccharide components are again glucose (as the 4-lactyl ether), glucuronic acid, and rhamnose, in the same sequence as for the O22 polymer but differing in structural details].

EXPERIMENTAL

Growth of bacteria, and isolation and fractionation of the lipopolysaccharide.— The methods used and the yields of polymeric fractions have been reported[2].

General methods.—NMR spectra of the native polysaccharide in D_2O were recorded with a Bruker WH-400 spectrometer. ^{1}H-NMR spectra were obtained at 70° with acetone (δ 2.22) as the internal reference, and ^{13}C spectra at 27° with 1,4-dioxane (δ 67.40) as the internal reference. 2D-NMR spectra (C–H correla-

tion, COSY, relayed COSY, and double-relayed COSY) were obtained at 70° by using standard pulse sequences. All other NMR spectra were recorded with a JEOL JNM-GX270 spectrometer with tetramethylsilane (δ 0.00) as the external reference for samples in D_2O (most samples) or as internal reference for samples in $CDCl_3$ (products SD2a–d). The ^{1}H-NMR spectra were obtained at 60° (PH1, LD1a–c), 70° (LD1, SD1a,b), or 21° (SD2a–d), and the ^{13}C spectrum at 21° (LD1). FABMS was carried out with a VG ZAB-E instrument and samples in thioglycerol.

Methods used to determine monosaccharide composition and configuration were those used in related studies[2,6]. Carboxyl reduction of glucuronic acid residues in the acidic glycan was carried out by the carbodi-imide method[7]. For the conversion of the native polymer into a uniform salt form, it was treated with dilute alkali[6] and the solution passed through a column of Dowex 50 (NH_4^+) resin. Per-*O*-methylation of polymers and their degradation products, and the preparation and analysis of methylated alditol acetates (by GLC and MS) followed standard procedures[7–9].

Graded hydrolysis of the acidic glycan.—Samples of the polymer were treated with 50 mM trifluoroacetic acid at 100° for periods between 10 and 40 min. The products of the 40-min treatment were fractionated by HPLC[5], using a TSKgelG-Oligo-PW column (Anachem). The major acidic component was reduced ($NaBH_4$) to give the oligosaccharide-alditol PH1.

Smith degradations.—Degradation of the carboxyl-reduced glycan was carried out under standard conditions[2]. After the hydrolysis step, the products were reduced ($NaBH_4$) and fractionated by size-exclusion HPLC and chromatography on HPX-87P (Bio-Rad) to give the oligosaccharide-alditols SD1a and SD1b. In a modified degradation carried out on the acidic glycan (NH_4^+ salt), the routine steps of oxidation, reduction, and work-up were followed by per-*O*-methylation (using CH_3I) and hydrolysis with aqueous 90% formic acid at 40° for 1 h. The products were reduced with $NaBH_4$ in 50% aqueous ethanol at 4° for 16 h, then trideuteriomethylated. GLC (BP1) showed four major peaks, and the corresponding materials were isolated by HPLC on a column of Spherisorb S5 ODS2 eluted with aqueous methanol[10].

Lithium–ethylenediamine degradation.—The acidic glycan was suspended (4 $mg \cdot mL^{-1}$) in ethylenediamine and sonicated for 1 h to give a clear solution. The solution was treated with an excess of lithium for 1.25 h, followed by the usual work-up[4] and reduction ($NaBH_4$). The products were applied to a column of Dowex 1 (acetate form) resin, the neutral components were eluted with water, then fractionated by size-exclusion HPLC to give a polymer (LD1) and a disaccharide fraction (LD2). The individual disaccharide-alditols (LD2a,b) present in the latter fraction were separated by HPLC on a column of HPX-87P. The polymer LD1 was hydrolysed with 50 mM trifluoroacetic acid at 100° for 40 min. Size-exclusion HPLC of the hydrolysate gave a major fraction eluting in the tri- or tetra-saccharide region, and further HPLC (HPX-87P) of the reduced ($NaBH_4$) products gave the components LD1a–c.

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