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STRUCTURAL STUDIES OF GLUCORHAMNANS ISOLATED FROM THE LIPOPOLYSACCHARIDES OF REFERENCE STRAINS FOR *Serratia marcescens* SEROGROUPS O4 AND O7, AND OF AN O14 STRAIN

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ABSTRACT

Partially acetylated glucorhamnans have been isolated from the lipopolysaccharides of three strains of *Serratia marcescens*. The polymer from the reference strain (C.D.C. 864-57) for serogroup O4 has the disaccharide repeating-unit shown below, in which acetylation at position 2 of the rhamnosyl residue is ~90% complete. Similar glucorhamnans from the reference strain (C.D.C. 843-57) for serogroup O7 and from a pigmented strain (NM) of serogroup O14 differ only in the configuration of the L-rhamnopyranosyl residue (β) and the extent of *O*-acetylation (O7, almost stoichiometric; NM, 80–90%). Glucorhamnans of the second type have been isolated previously from the lipopolysaccharides of other strains of *S. marcescens*, including the reference strain for serogroup O6 and another pigmented O14 strain (N.C.T.C. 1377). In all cases, the lipopolysaccharide extracts also contained acidic glycans, but the glucorhamnans are believed to constitute the integral side-chains.

→4)-α-D-Glc*p*-(1→3)-α-L-Rha*p*-(1→

INTRODUCTION

Glucorhamnans have been described in several studies of polysaccharides produced by the bacterium *Serratia marcescens*. In studies[1,2] of strain N.R.C. S-29, such polymers were isolated as cellular and extracellular products, and the presence of 3-substituted rhamnopyranose and 4-substituted glucopyranose residues was determined. In the case of strain A.T.C.C. 264, the O-specific side-chain of the lipopolysaccharide was found to be a linear glucorhamnan, for which the disaccharide repeating-unit **1** was proposed[3]. Subsequent studies[4] revealed that the repeating-unit of this polymer, and of the corresponding product from strain N.C.T.C. 1377, actually had the structure **2**. The extent of *O*-acetylation was 80–

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90% for strain N.C.T.C. 1377, but only 20–30% for strain A.T.C.C. 264. Subsequently[5], the same polymer with ~50% *O*-acetylation was isolated from the lipopolysaccharide of the reference strain (C.D.C. 862-57) for *S. marcescens* serogroup O6.

→6)-β-D-Glc*p*-(1→2)-β-L-Rha*p*-(1→

1

OAc
|
2

→4)-α-D-Glc*p*-(1→3)-β-L-Rha*p*-(1→

2

We now describe the results of structural studies of other glucorhamnans from the O4 and O7 reference strains, and from a pigmented isolate (NM) belonging to serogroup O14.

RESULTS

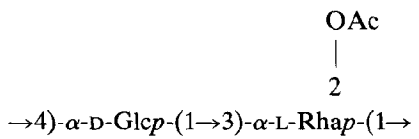
Lipopolysaccharide from the O4 reference strain. — The yield of lipopolysaccharide from this organism (strain C.D.C. 864-57) was ~26% of the whole cell wall. Data for the neutral sugar composition (expressed as percentages of the total peak area in g.l.c. of the alditol acetates) were glucose (21.7%), galactose (28.6%), mannose (27.1%), rhamnose (13.5%), D-*glycero*-D-*manno*-heptose (1.8%), and L-*glycero*-D-*manno*-heptose (7.3%). The product also contained 2-amino-2-deoxyglucose, but no hexuronic acid was detected. On polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate, a "ladder" typical of S-form lipopolysaccharides was produced in addition to a fast-moving band attributable to R-form lipopolysaccharide lacking the O-specific chain.

After mild acid hydrolysis of the lipopolysaccharide, 60% of the material was recovered as water-soluble, polymeric products (Sephadex G-50). These were fractionated by chromatography on DEAE-Sepharose CL-6B to give a neutral polymer (22%, eluted with water), an acidic polymer (46%, eluted with 0.1M and 0.2M NaCl), and a mixed fraction (21%, overlapping peaks in the early 0.1M NaCl eluate). Structural studies of the acidic polymer (a galactomannan containing pyruvic acid residues) will be reported elsewhere.

Sugar analysis of the neutral polymer revealed L-rhamnose (34.1%), D-glucose (38.9%), and a trace of heptose. The presence of an *O*-acetyl group was indicated by signals in the n.m.r. spectra at δ 2.11 (^{1}H, three-proton singlet) and δ 173.38 and 20.51 (^{13}C). The ^{1}H-n.m.r. spectrum contained three major signals

(each ~1 H) in the anomeric region at δ 5.27 (unresolved), 4.97 ($J_{1,2}$ 3.9 Hz), and 4.91 (unresolved), consistent with the presence in the polymer of a disaccharide repeating-unit containing an α-D-glucopyranosyl residue and an acetyl group on O-2 of an L-rhamnopyranosyl residue. The ^{13}C-n.m.r. spectrum included major anomeric signals at δ 98.01 and 94.94, and a signal for C-6 of glucose at δ 60.20, showing that this position was unsubstituted. Methylation analysis of the polymer, monitored by g.l.c.–m.s., showed that the repeating-unit was constructed from 3-substituted rhamnopyranose and 4-substituted glucopyranose residues. This linkage pattern was confirmed by periodate oxidation of the *O*-deacetylated polymer: after borohydride reduction and acid hydrolysis, rhamnose and erythritol (but no glucose) were produced in equimolecular amounts.

The structure determination was completed by comparing the n.m.r. spectra of the native and *O*-deacetylated polymer. As expected, the ^{1}H-n.m.r. spectrum of the latter contained only two signals in the anomeric region at δ 5.07 ($J_{1,2}$ 3.8 Hz) and 4.94 ($J_{1,2}$ 1.4 Hz), assigned to glucose and rhamnose residues, respectively. The ^{13}C-n.m.r. spectrum (Fig. 1) again contained two anomeric signals, at δ 100.40 ($^{1}J_{CH}$ 169.9 Hz) and 95.45 ($^{1}J_{CH}$ 169.6 Hz), showing that both sugars were present as α-pyranose residues. The signal at δ 100.40, assigned to C-1 of rhamnose, was 2.39 p.p.m. downfield of the corresponding signal for the native polymer, as expected from the removal of an acetyl group from O-2. A similar difference (2.64 p.p.m.) was observed for the values of the chemical shift for C-3 of rhamnose in the two polymers (Table I), whereas an upfield shift of 1.52 p.p.m. for the signal for C-2 occurred on *O*-deacetylation (Table I). From these results, structure **3** can be proposed for the disaccharide repeating-unit of the glucorhamnan from strain C.D.C. 864-57. Acetylation at O-2 of the rhamnosyl residue was largely complete (~90%), and minor signals in the n.m.r. spectra of the native polymer could mostly be explained by the presence of units lacking the substituent.



3

Lipopolysaccharide from the O7 reference strain. — The lipopolysaccharide isolated from this organism (C.D.C. 843-57) represented ~22% of the whole cell wall. The neutral monosaccharide composition (expressed as percentages of the total peak area on g.l.c. of the alditol acetates) was glucose (32.4%), galactose (12.2%), mannose (20.7%), rhamnose (24.8%), and heptoses (9.9%). Glucuronic acid and 2-amino-2-deoxyglucose were also detected. The behaviour of the material on polyacrylamide gel electrophoresis was similar to that of the product from the O4 strain.

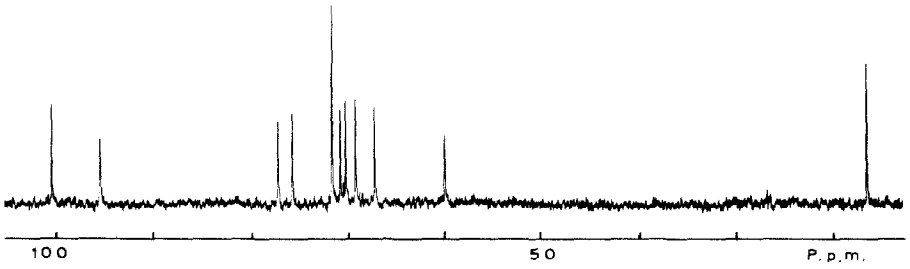


Fig. 1. ^{13}C-N.m.r. spectrum of the *O*-deacetylated glucorhamnan from *S. marcescens* C.D.C. 864-57 (O4). The spectrum for the sample in D_2O was obtained at 100.62 MHz and 50° with complete proton-decoupling and Me_4Si as the external reference.

Only 44% of the weight of the lipopolysaccharide was recovered as water-soluble, polymeric products after mild acid hydrolysis and chromatography on Sephadex G-50. Further chromatography on DEAE-Sepharose CL-6B gave a neutral polymer (yield 22%, eluted with 0.1M NaCl) and an acidic polymer (62%, eluted with 0.2M and 0.3M NaCl). The structure of the latter polymer has yet to be determined.

Sugar analysis of the neutral polymer gave L-rhamnose (35.8%) and D-glucose (37.5%), and the presence of an *O*-acetyl group was indicated by signals in the n.m.r. spectra at δ 2.23 (^1H) and δ 173.74 and 20.52 (^{13}C). From the integration and the simplicity of the spectra, it could be inferred that mono-*O*-acetylation was essentially stoichiometric and regiospecific.

Although the products obtained on methylation analysis were identical with those for the O4 glucorhamnan of repeating-unit **3**, it was apparent from the n.m.r.

TABLE I

ASSIGNMENTS OF SIGNALS IN THE ^{13}C-N.M.R. SPECTRA OF THE NATIVE AND DEACETYLATED GLUCORHAMNAN FROM STRAIN C.D.C. 864-57 (O4)[a]

Carbon atom	*Native polymer*[b]		*Deacetylated polymer*	
		OAc | *2*		
	→4-α-Glc-1→	*→3-α-Rha-1→*	*→4-α-Glc-1→*	*→3-α-Rha-1→*
C-1	94.94	98.01	95.45	100.40
C-2	71.53[c]	68.69	71.62[d]	67.17
C-3	71.66[c]	73.05	71.62[d]	75.69
C-4	77.42	70.74[c]	77.22	70.22
C-5	71.03[c]	69.22	70.77[d]	69.21
C-6	60.20	16.76	59.93	16.63
$-OC(O)CH_3$		173.38		
$-OC(O)CH_3$		20.51		

[a]Spectra were recorded at 50° with Me_4Si as the external reference. [b]Minor signals attributable to the presence of non-acetylated residues are not listed. [c,d]Sets of signals for which assignments are tentative.

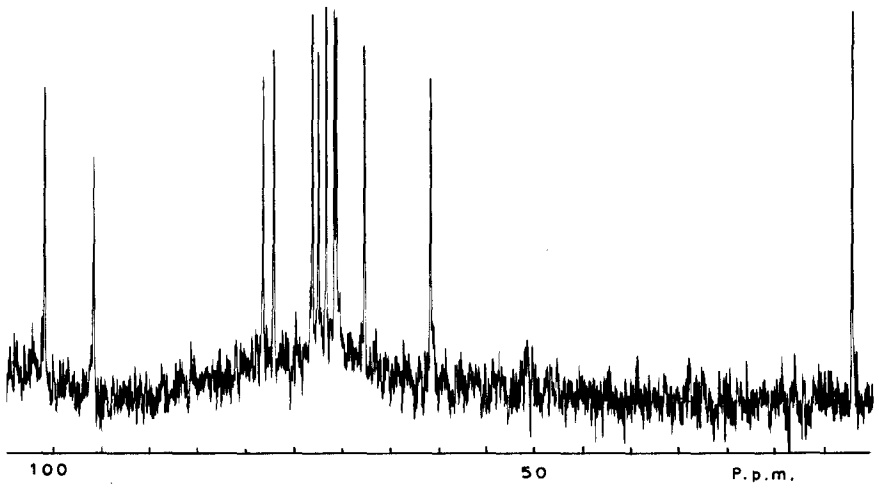


Fig. 2. ^{13}C-N.m.r. spectrum of the *O*-deacetylated glucorhamnan from *S. marcescens* C.D.C. 843-57 (O7). The spectrum was obtained as for the O4 polymer (Fig. 1).

spectra that the two polymers had different structures. The anomeric region of the ^{1}H-n.m.r. spectrum of the native polymer contained two unresolved signals at δ 5.7 (1 H) and 5.1 (2 H). On *O*-deacetylation of the polymer, these were replaced by 1-proton signals at δ 5.1 ($J_{1,2}$ 3.8 Hz) and 4.9 (unresolved). The ^{13}C-n.m.r. spectrum of the native polymer contained anomeric signals at δ 99.40 ($^{1}J_{CH}$ 163 Hz) and 94.67 ($^{1}J_{CH}$ 170 Hz). The corresponding signals in the spectrum for the *O*-deacetylated polymer (Fig. 2) were at δ 100.82 and 95.72. From these data, it can be concluded that the O7 polymer has a disaccharide repeating-unit containing α-glucopyranosyl and β-rhamnopyranosyl residues. In fact, the ^{13}C-n.m.r. spectra for both the native and the *O*-deacetylated polymer were indistinguishable from those obtained for the products from strain N.C.T.C. 1377 (ref. 4) and strain C.D.C. 862-57 (ref. 5). Thus, the repeating-unit for the O7 polymer also has the structure **2**, though with essentially complete *O*-acetylation at position 2 of the rhamnosyl residue.

Lipopolysaccharide from strain NM. — Extraction of whole cell walls of this pigmented strain typed as O14 gave lipopolysaccharide in 20% yield. By using the methods applied to the preceding lipopolysaccharides, similar amounts of a neutral glucorhamnan and an acidic glucomannan were isolated. The latter polymer had a glucose–mannose ratio of ~2:1, contained an *O*-acetyl substituent, and was clearly heterogeneous. The ^{1}H- and ^{13}C-n.m.r. spectra of this polymer, before and after *O*-deacetylation, showed sets of signals present in the spectra of similar products isolated[6] from other O14 strains and the O6 reference strain. Although quantitative variations between the polymers are apparent, these acidic glucomannans seem to define the O6/O14 serocomplex[6]. By means of sugar analysis, methylation analysis, and n.m.r. spectroscopy, it was shown that the glucorhamnan from strain NM had the repeating-unit of structure **2**, with *O*-acetylation 80–90% complete.

DISCUSSION

The results of this study support the view that glucorhamnans are relatively common in strains of *S. marcescens*. The polymer with the repeating-unit **2** has been found in the non-pigmented reference strains for serogroups O6 (ref. 5) and O7 (this work) and in the pigmented O14 strains N.C.T.C. 1377 (ref. 4) and NM (this work), as well as in strain A.T.C.C. 264 of unknown serotype[4]. The variations in extent of *O*-acetylation from 20–30% up to ~100% are presumably natural, as the same conditions of hydrolysis were used for the release of each of the polymers, and glycosyl substitution at the adjacent 3-position of the rhamnose residue prohibits acetyl migration. The finding of the same glucorhamnan in both the O6 and O7 reference strains presumably accounts for the known serological cross-reaction[7]. The polymer may also contribute to cross-reactions between groups O6 and O14, although the common antigen for strains in these groups seems to be an acidic glucomannan.

The neutral polymer isolated from the O4 reference strain is closely related to the glucorhamnans discussed above. The only structural difference in the repeating-unit **3** is the anomeric configuration of the rhamnosyl residue (α instead of β). Consistent with this similarity, serological cross-reactions between groups O4, O6, O7 have been described by some workers[7]. Apart from the serogroups discussed here, glucorhamnans have not been detected[8] in studies of the lipopolysaccharides of other serogroups up to O15.

Because each of the glucorhamnans described here was accompanied by an acidic glycan, their molecular origins and antigenic properties can only be assumed. However, the results of other studies[6,9] of lipopolysaccharides from *S. marcescens*, from which both neutral and acidic polymers have been obtained, suggest that the former are integral components of the lipopolysaccharides and that the acidic polymers have a microcapsular origin. The spacing of the steps in the ladders observed on polyacrylamide gel electrophoresis of the glucorhamnan-containing lipopolysaccharides was the same in each case and consistent with a disaccharide increment, thus supporting the inferences above.

EXPERIMENTAL

Growth of bacteria, and isolation and fractionation of the lipopolysaccharides. — *S. marcescens* strains C.D.C. 864-57 (O4), C.D.C. 843-57 (O7), and NM (O14) were grown and processed as in related studies[4–6,9]. From 20-L batch cultures, the respective yields of wet cells, dry cell walls, and lipopolysaccharide were as follows. C.D.C. 864-57: 146 g, 2.46 g, 0.64 g; C.D.C. 843-57: 126 g, 3.08 g, 0.68 g; NM: 52 g, 0.96 g, 0.19 g. After acid hydrolysis of the lipopolysaccharides (aqueous 1% acetic acid, 2.25 h, 100°), the water-soluble products were fractionated by successive chromatography on Sephadex G-50 and DEAE-Sepharose CL-6B.

General methods. — N.m.r. spectra (^{13}C and ^{1}H) were recorded for solutions

in D_2O with Bruker WH-400 and JEOL JNM-GX270 spectrometers. With the former instrument (results cited), ^{13}C spectra (with complete proton-decoupling or with gated decoupling) 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. G.l.c. was carried out with a Pye 104 chromatograph fitted with packed columns of OV-275 (alditol acetates) or OV-17 (methylated alditol acetates), or with a Carlo Erba Mega 5160 chromatograph fitted with fused-silica capillary columns of BP1 (alditol acetates and acetylated oct-2-yl glycosides) or BP10 (methylated alditol acetates). Other chromatographic and electrophoretic methods and equipment for g.l.c.–m.s. were those used in previous studies[4–6,9].

Identification of monosaccharide and other components. — Neutral sugars and polyols were identified by p.c., and by g.l.c. of the alditol acetates (providing quantitative data). Configurations of glucose and rhamnose were determined by the oct-2-yl glycoside acetate method[10] or, in the case of D-glucose, by using hexokinase (EC 2.7.1.1) and D-glucose 6-phosphate dehydrogenase (EC 1.1.1.49)[6]. Glucuronic acid was identified by p.c. and by high-voltage electrophoresis at pH 2.7, and amino sugars were identified by p.c., autoanalysis, and electrophoresis at pH 5.3. Pyruvic acid was detected by using lactate dehydrogenase (EC 1.1.1.27) and by n.m.r. spectroscopy.

Degradative methods. — Methods used for *O*-deacetylation of glucorhamnans, methylation analysis of the polymers, and periodate oxidation have been summarised in earlier studies[4–6,9].

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