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STRUCTURAL STUDIES OF A NEUTRAL POLYMERIC FRACTION FROM THE LIPOPOLYSACCHARIDE OF *Serratia marcescens* C.D.C. 1783-57 (O14:H9)

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ABSTRACT

A "neutral" polymer of glucose, galactose, and 2-acetamido-2-deoxyglucose (molar ratios 1:1:2) has been isolated from the lipopolysaccharide of *Serratia marcescens* strain C.D.C. 1783-57 (O14:H9). Degradative and spectroscopic studies esablished that the polysaccharide has a branched tetrasaccharide repeating-unit of the structure shown. The polymer was absent from other strains of serogroup O14 studied, but a polymer differing only in the configuration of the glucose residue has previously been isolated from a strain of *S. marcescens* O8. The polymer from strain C.D.C. 1783-57 also shares structural features with the *Escherichia coli* O18 antigen, which is known to be serologically related to the *S. marcescens* O8 antigen.

$$\begin{array}{c} \beta\text{-D-Glc}p\text{NAc} \\ 1 \\ \downarrow \\ 3 \\ \rightarrow 6)\text{-}\alpha\text{-D-Glc}p\text{-}(1\rightarrow 4)\text{-}\alpha\text{-D-Gal}p\text{-}(1\rightarrow 3)\text{-}\beta\text{-D-Glc}p\text{NAc-}(1\rightarrow \end{array}$$

INTRODUCTION

During a study of lipopolysaccharide preparations from three strains of *Serratia marcescens* belonging to the common serogroup O14, three different polysaccharides were detected[1]. One of the polymers, with a disaccharide repeating-unit of D-ribose and 2-acetamido-2-deoxy-D-galactose, was present[1] in the products from two strains of serovar O14:H12, and was subsequently isolated from other strains belonging to serogroup O12 (ref. 2) and O13 (ref. 3). A second polymer, characterised as a partially acetylated, acidic glucomannan[4], was common to all three O14 strains studied[1], as well as a strain of the related O6 serogroup[4], and may be the antigen which defines this complex. A third polymer, found only in strain C.D.C. 1783-57 (serovar O14:H9), contained 2-acetamido-2-deoxyglucose, galactose, and glucose (molar ratios 2:1:1), as also reported[5] for the O-specific

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polymer from a strain *S. marcescens* O8. We now describe the results of structural studies of the polymer from strain C.D.C. 1783-57.

RESULTS

As reported elsewhere[4], mild acid hydrolysis (aqueous 1% acetic acid, 100°, 2.25 h) of the lipopolysaccharide from *S. marcescens* C.D.C. 1783-57 gave a polymeric fraction consisting of approximately equal amounts of an acidic glucomannan and an essentially neutral polymer. The two polymers were cleanly separated by chromatography on DEAE-Sepharose CL-6B.

Analysis of the "neutral" polymer for neutral sugars by g.l.c. of the alditol acetates showed the presence of glucose (19.8%) and galactose (19.5%). The results of enzymic assays (19.1 and 19.0%, respectively) showed both hexoses to have the D configuration. The polymer also contained 2-amino-2-deoxyglucose (35.7% by autoanalysis), identified as the D isomer by polarimetry: $[\alpha]_D$ for the hydrochloride, +75.6° (*c* 0.3, water). The occurrence of the hexosamine as its *N*-acetyl derivative was shown by bands in the i.r. spectrum at 1640 and 1550 cm^{-1}, methyl singlets at δ 2.03 and 2.08 in the ^{1}H-n.m.r. spectrum, and signals at δ 174.6, 174.3, 22.6, and 22.5 in the ^{13}C-n.m.r. spectrum of the polymer. There was no evidence for an *O*-acyl substituent.

The impression of a tetrasaccharide repeating-unit, given by the molar proportions of the monosaccharides, was confirmed by the n.m.r. spectra. The anomeric region of the ^{1}H-n.m.r. spectrum contained four one-proton signals: δ 5.42 ($J_{1,2}$ ~4 Hz), 4.98 ($J_{1,2}$ ~3 Hz), 4.69 ($J_{1,2}$ ~8 Hz), and 4.60 ($J_{1,2}$ ~8 Hz). The corresponding signals in the ^{13}C-n.m.r. spectrum were at δ 103.62 ($^1J_{CH}$ 166 Hz), 101.49 ($^1J_{CH}$ 166 Hz), 99.15 ($^1J_{CH}$ 169 Hz), and 99.03 ($^1J_{CH}$ 174 Hz). The expected total of twenty-eight discrete signals in the ^{13}C-n.m.r. spectrum also included two for C-2 of the hexosamine residues at δ 55.93 and 54.30, and three near δ 60 (61.14, 60.69, and 59.59). The spectroscopic data indicate the presence in the repeating unit of two α-linked and two β-linked pyranosyl residues, and of only one residue substituted at position 6. On treatment of the polymer with sodium periodate, all of the glucose and half of the 2-acetamido-2-deoxyglucose were destroyed; the galactose was unaffected: analysis of the oxidised polymer gave galactose (20.8%) and 2-amino-2-deoxyglucose (17.7%).

Five monosaccharide derivatives were detected on methylation analysis of the polymer, monitored by g.l.c., g.l.c.–m.s., and t.l.c. of the products. The most volatile product (a trace only) had the retention time in g.l.c. of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol and gave the appropriate mass spectra: major fragment at *m/z* 264 (M − 59) in the chemical-ionisation spectrum with methane as the reagent gas, and primary fragments at *m/z* 205, 162, 161, and 118 in the electron-impact spectrum of the *1-d*-labelled product. The most volatile, major product was similarly shown to be derived from a 6-substituted hexopyranose residue. From the periodate-oxidation data, the parent hexose could be inferred to

be glucose, and this was confirmed by the retention time of the derivative and by deacetylation, isolation of the tri-*O*-methylhexitol by t.l.c., followed by demethylation and conversion into glucitol hexa-acetate. By using the same combination of methods, the third product of methylation analysis was identified as 1,3,4,5-tetra-*O*-acetyl-2,6-di-*O*-methylgalactitol-*1-d*, clearly derived from a branching point in the repeating unit. The remaining products were both derived from 2-acetamido-2-deoxyglucose residues, one unsubstituted (primary fragments in the electron-impact mass spectrum at *m/z* 205, 203, 161, and 159) and the other probably substituted at O-3 (primary fragments at *m/z* 275, 161, and 159, although only the last was prominent). Substitution at O-3 was confirmed by studies of Compound *Y* (see below).

After Smith degradation of the polymer, only oligomeric products were detected, showing that the 6-substituted glucopyranose residue occurred in the main chain. The major product (*X*) had R_{Glycerol} 0.45 (p.c., solvent *A*) and contained both galactose and 2-amino-2-deoxyglucose, but did not react with aniline hydrogenoxalate. A minor product with R_{Glycerol} 0.54 was detected but not studied further. Treatment of Compound *X* with β-D-galactosidase had no effect, but galactose was released on treatment with α-D-galactosidases from either *Aspergillus niger* or green coffee beans (hydrolysis was incomplete even after several treatments under various conditions). The destruction of galactose (but not 2-amino-2-deoxyglucose) on periodate oxidation of Compound *X* confirmed that it was the non-reducing terminal residue. When Compound *X* was subjected to a Smith degradation (or treated with an α-D-galactosidase) and subsequently incubated with a β-*N*-acetylglucosaminidase, free 2-acetamido-2-deoxyglucose was detected both by p.c. and by the Morgan–Elson reaction. These results indicated that Compound *X* had structure **1**, and suggested the partial structure **2** for the repeating unit of the parent polymer.

α-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→1)-L-Glycerol

1

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

2

In order to locate the substituents of the galactose residue, *N*-deacetylation–deamination of the polymer was attempted. Complete *N*-deacetylation proved to be difficult (typical of 3-substituted *N*-acetylhexosamine residues); even after alkaline treatment in aqueous Me_2SO for 16 h at 80°, about half of the product

remained polymeric on subsequent deamination. Chromatography on Sephadex G-10 of the reduced ($NaBD_4$) deamination products gave 2,5-anhydromannitol (identified by p.c. and by g.l.c. of its tetra-acetate) and a compound (*Y*) eluted slightly later than stachyose. Compound *Y* had R_{Glc} 0.65 (solvent *A*), reacted with the periodate–Schiff reagents but not with aniline hydrogenoxalate, and on acid hydrolysis gave equimolar amounts of glucose, galactose, and 2,5-anhydromannitol. Glucose was released on treatment of Compound *Y* with α-D-glucosidase but not with β-D-glucosidase, and was confirmed as the non-reducing terminal residue by methylation analysis. The other products identified by g.l.c.–m.s. were 3-*O*-acetyl-2,5-anhydro-1,4,6-tri-*O*-methylmannitol-*1-d* (ref. 6) and 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylgalactitol-*1-d* (*m/z* 233 and 118). From these results, structure **3** can be assigned to Compound *Y*, and structure **4** to the repeating unit of the polymer. From the detection of a small proportion of unsubstituted glucose on methylation analysis of the polymer, it seems likely that structure **4** represents the biological as well as the chemical repeating-unit.

α-D-Glc*p*-(1→4)-α-D-Gal*p*-(1→3)-2,5-Anhydro-D-mannitol

3

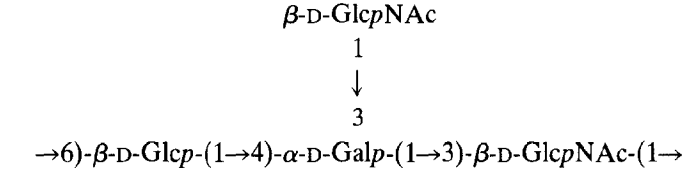
β-D-Glc*p*NAc
1
↓
3
→6)-α-D-Glc*p*-(1→4)-α-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→

4

DISCUSSION

Out of six O14 strains of *S. marcescens* examined in these laboratories, only one gave a lipopolysaccharide preparation containing the polymer with repeating-unit **4**, and the serological significance of the polymer (if any) is therefore unclear. However, there is a striking similarity between the structure proposed here and that reported[5] for the O-specific polymer from a strain of *S. marcescens* O8. The repeating-unit **5** proposed for the latter differs only in the anomeric configuration of the glucose residue. It has also been reported[7] that *S. marcescens* O8 shows serological cross-reactions with *Escherichia coli* O18. Structure **6** has recently[8] been established for the repeating unit of the O-specific polymer from a strain of serovar O18ac, and it has been shown that some monoclonal antibodies against *E. coli* O18 antigen also bind lipopolysaccharide from *S. marcescens* O8 (G. Pluschke, personal communication). One of these antibodies also binds weakly to the crude lipopolysaccharide of *S. marcescens* O14:H9 (C.D.C. 1783-57) "contaminated" by

an acetylated, acidic glucomannan[4] (G. Pluschke, personal communication), although it has not yet been possible to demonstrate binding to the purified "neutral" polymer characterised in the present study. It has yet to be determined which of the two polymers is an integral part of the lipopolysaccharide, and why only part of the "neutral" polymer could be eluted from DEAE-Sepharose CL-6B with water alone[4] (the n.m.r. spectra of the fractions eluted with water and with 0.1M NaCl were indistinguishable). Weak binding through a carboxyl group of a residue of 3-deoxy-D-*manno*-octulosonic acid in a terminal core oligosaccharide is a possible explanation.



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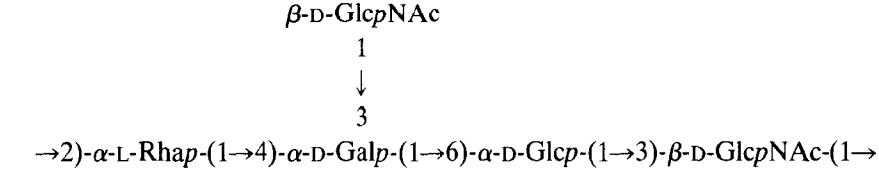

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EXPERIMENTAL

Growth of bacteria, and isolation and fractionation of the lipopolysaccharide. — *S. marcescens* strain C.D.C. 1783-57 was grown and processed as described previously[4]. Fractions of "neutral" polymeric material were eluted from a column of DEAE-Sepharose CL-6B with water or 0.1M NaCl.

General methods. — I.r. spectra were recorded with a Unicam SP-200 spectrophotometer for samples dispersed in KCl. Specific rotations were determined with a Bendix polarimeter (Model 143A). N.m.r. spectra (^{13}C and ^{1}H) were recorded for solutions in D_2O with a Bruker WH-400 spectrometer; ^{13}C spectra (with complete proton-decoupling or with gated decoupling) were recorded at ~50° with tetramethylsilane as the external standard; ^{1}H spectra were recorded at ~85° with sodium 4,4-dimethyl-4-silapentane-1-sulphonate as the internal standard. G.l.c. was carried out with a Pye Unicam 104 chromatograph and a column (1.6 m × 2 mm) of 3% of Silar 10C on Gas Chrom Q. G.l.c.–m.s. was carried out with a Finnigan model 1020B instrument fitted with a fused-silica capillary column (30 m) of SE-54. Mass spectra were obtained either by the electron-impact method or by

chemical ionisation with methane as the reagent gas[9]. Solvents, buffers, and detection reagents used for p.c., t.l.c., and paper electrophoresis were as listed previously: solvent *A* referred to in this paper was ethyl acetate–pyridine–water (13:5:4).

Determination of monosaccharide composition. — Methods used for the release, identification, and estimation of monosaccharides were those described elsewhere[1,4]. In general, glucose and galactose were determined by g.l.c. of the alditol acetates, and 2-amino-2-deoxyglucose by autoanalysis. Free 2-acetamido-2-deoxyglucose was determined by the Morgan–Elson reaction[10], D-glucose by using D-glucose oxidase (EC 1.1.3.4), and D-galactose by using D-galactose oxidase (EC 1.1.3.9) with peroxidase (EC 1.11.1.7).

Methylation analysis. — As in previous studies[1,4], standard procedures were used for methylation and the preparation of methylated alditol acetates. Products were identified by g.l.c., g.l.c.–m.s., and by *O*-demethylation of partially methylated alditols isolated by t.l.c.

Periodate oxidation and Smith degradation. — Samples of polysaccharide were oxidised with 50mM sodium periodate at 4°. The consumption of periodate (~4.2 μmol.mg^{-1}) was complete[11] within 48 h. After successive treatments with ethylene glycol, $NaBH_4$, and acetic acid, the mixture was freeze-dried and the polymeric product was desalted by chromatography on Sephadex G-50. After Smith hydrolysis (M trifluoroacetic acid, room temperature, 16 h), p.c. (solvent *A*) showed one major product (Compound *X*) with $R_{Glycerol}$ 0.45, and a minor product with $R_{Glycerol}$ 0.54. Compound *X* reacted rapidly with the periodate–Schiff reagents, initially giving a purple colour (diagnostic for the production of HCHO) which turned blue on standing; little or no reaction occurred with alkaline $AgNO_3$ or aniline hydrogenoxalate. Compound *X* contained both galactose and 2-acetamido-2-deoxyglucose; only the former was oxidised by periodate.

N-*Deacetylation and deamination.* — Samples of reduced ($NaBH_4$) polysaccharide were *N*-deacetylated[12] at 80° for 2.25 or 16 h. Subsequent deamination[13] indicated that deacetylation was incomplete even after 16 h, but a compound (*Y*) and 2,5-anhydromannitol were obtained by chromatography of the reduced deamination products on Sephadex G-10. Compound *Y* had $R_{Glycerol}$ 0.65 in p.c. (solvent *A*), and gave a blue colour with the periodate–Schiff reagents (the leading edge of the spot was yellow, indicating the presence of a minor by-product, possibly resulting from an alternative degradative pathway[14]), and its elution volume from Sephadex G-10 indicated that it was a trisaccharide-alditol.

Enzymic hydrolyses. — Compound *X* from the Smith degradation was treated with β-D-galactosidase (EC 3.2.1.23) from *E. coli* in 0.1M phosphate buffer (pH 7.3) containing 2mM $MgSO_4$. Other samples were treated with α-D-galactosidase (EC 3.2.1.22) from *Aspergillus niger* (using 0.05M acetate buffer, pH 5.5) or from green coffee beans (using 0.1M phosphate buffer, pH 6.5). The products from α-D-galactosidase treatment or Smith degradation of Compound *X* were also treated with β-*N*-acetylglucosaminidase (EC 3.2.1.30) from jack beans (using 0.1M

citrate buffer, pH 5.0). The release of 2-acetamido-2-deoxyglucose was monitored by p.c. (solvent *A*) and the Morgan–Elson reaction.

Samples of Compound *Y* from the *N*-deacetylation–deamination sequence were treated with α-D-glucosidase (EC 3.2.1.20) from yeast (using 0.1M acetate buffer, pH 6.6) or β-D-glucosidase (EC 3.2.1.21) from sweet almonds (using water alone). The release of glucose was checked by the D-glucose oxidase method.

All enzymes used were obtained from the Boehringer Corporation (London) Ltd. or the Sigma Chemical Co. Ltd.

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