Carbohydrate Research, 186 (1989) 287–293
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

STRUCTURE OF THE O-SPECIFIC POLYSACCHARIDE FROM *Enterobacter cloacae* STRAIN N.C.T.C. 11579 (SEROGROUP O10)

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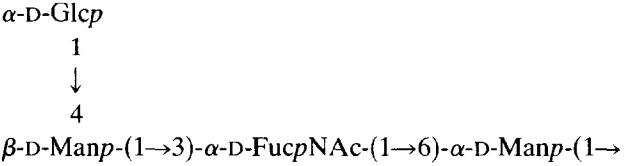
(Received August 1st, 1988; accepted for publication, September 26th, 1988)

ABSTRACT

The O-specific polysaccharide from the reference strain (N.C.T.C. 11579) for *Enterobacter cloacae* serogroup O10 has been isolated and characterised. By means of n.m.r. spectroscopy and methylation analysis, and by studies of the products obtained by Smith degradation or by *N*-deacetylation–deamination, the repeating unit of the polysaccharide could be allocated the structure shown. The polysaccharides from two cross-reacting serogroups (O9 and O11) have the same monosaccharide composition.



INTRODUCTION

Enterobacter cloacae is one of a number of Gram-negative bacteria of which antibiotic-resistant strains are increasingly common[1–4] and of growing concern as opportunistic pathogens. Reliable, economical, and discriminatory methods for epidemiological studies of the species are highly desirable. Schemes of O serotypes[5,6] and H serotypes[5] have been drawn up, and schemes for bacteriocin[7,8] and bacteriophage[9] typing have also been developed. An attempt has been made[10] to establish the chemical basis for serological differentiation between 14 reference strains for one O-serotyping scheme[6] by analysis and fractionation of their lipopolysaccharides (the presumed O antigens). Three lipopolysaccharides (from strains representing the cross-reacting O9, O10, and O11 serotypes) all gave rise to polymers containing glucose, mannose, and fucosamine (2-amino-2,6-di-deoxygalactose) in the same proportions. We now report the results of a structural study of the O10 polymer.

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RESULTS AND DISCUSSION

The O-specific polysaccharide (yield, 48% of the parent lipopolysaccharide) contained D-mannose (60.1%), D-glucose (18.6%), and D-fucosamine (~15%, uncorrected). The presence of an *N*-acetyl group (and the absence of *O*-acetyl substituents) were apparent from the i.r. spectrum (bands at 1645 and 1560 cm^{-1}) and the n.m.r. spectra [signals at δ 2.05 (^1H), and at δ 173.99 and 22.30 (^{13}C)]. Failure of the polysaccharide to adsorb onto DEAE-Sepharose CL-6B showed the absence of an acidic sugar.

A pentasaccharide repeating-unit of mannose (3), glucose (1), and 2-acetamido-2-deoxyfucose (1) residues was clear from the n.m.r. spectra of the polymer. In the ^1H-n.m.r. spectrum, unresolved anomeric signals (each 1 H) at δ 5.34, 5.08, and 4.82 could be assigned to mannopyranosyl residues, and doublets (each 1 H) at δ 5.28 ($J_{1,2}$ ~4 Hz) and 4.84 ($J_{1,2}$ ~4 Hz) to α-pyranosyl residues of the other sugars. The spectrum also contained a methyl doublet at δ 1.24 ($J_{5,6}$ 6.5 Hz) from the 2-acetamido-2-deoxyfucose residue. Anomeric signals in the ^{13}C-n.m.r. spectrum (Fig. 1) at δ 102.62 ($^1J_{CH}$ 173 Hz), 101.16 ($^1J_{CH}$ 160 Hz), 100.26 ($^1J_{CH}$ 172 Hz), 99.33 ($^1J_{CH}$ 176 Hz), and 97.52 ($^1J_{CH}$ 171 Hz) showed that only one of the three mannose residues was β-linked. In support of this inference, the mannose–glucose ratio changed from 3:1 to 2:1 on oxidation of the peracetylated polymer with CrO_3. Other signals of interest in the ^{13}C-n.m.r. spectrum were those at δ 15.52 (C-6 of fucosamine), 48.90 (C-2 of fucosamine, diagnostic[11] for α), and three signals for hydroxymethyl carbons at δ 60.74, 60.93, and 61.15 (showing that position 6 was substituted in one hexose residue).

The results of methylation analysis of the polysaccharide (Table I, column A) showed that the repeating unit was branched, with 2,4-disubstituted mannose at

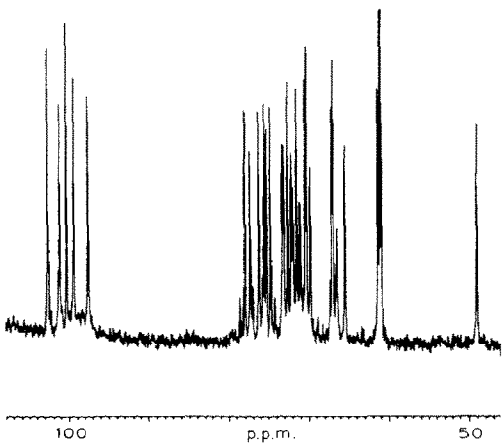

Fig. 1. ^{13}C-N.m.r. spectrum of the O-specific polysaccharide. In addition to the signals shown, the spectrum contained signals at δ 173.99, 22.29, and 15.52. The spectrum for the sample in D_2O was obtained at 100.61 MHz and 50°, with complete proton-decoupling and tetramethylsilane as the external reference.

the branch point. The terminal (unsubstituted) hexose was identified as glucose by h.p.l.c. of the 2,3,4,6-tetra-*O*-methylhexitol[12] and by g.l.c. of the 2,3,4,6-tetra-*O*-methylhexose 1-acetates[13]. Consistent with the results of methylation analysis, the glucose and two of the mannose residues were destroyed on periodate oxidation of the polymer, and the consumption of $NaIO_4$ (5.94 μmol.mg^{-1}) was in good agreement with the value (5.99 μmol.mg^{-1}) expected. After reduction ($NaBH_4$) and Smith hydrolysis of the oxidised polymer, a single product, eluted from Sephadex G-15 in about the position of a trisaccharide, was obtained. The product also gave a single spot in p.c. and (as the methylated oligosaccharide-alditol) one major peak in g.l.c.

The ^{1}H-n.m.r. spectrum of the Smith-degradation product contained anomeric signals (each 1 H) at δ 4.84 ($J_{1,2}$ 3.7 Hz) and 4.74 (unresolved), and methyl signals at δ 2.05 (singlet) and 1.25 ($J_{5,6}$ 6.6 Hz). The ^{13}C-n.m.r. spectrum contained 17 discrete signals, including anomeric signals at δ 101.50 ($^1J_{CH}$ 158 Hz) and 97.50 ($^1J_{CH}$ 171 Hz) and two signals for hydroxymethyl carbons at δ 62.64 and 61.09. These data show that the periodate-resistant (branch point) mannose residue is β-linked, that it is adjacent to the 2-acetamido-2-deoxyfucose residue, and that the aglycon end of the Smith-degradation product is 1-substituted glycerol (from the original 6-substituted mannose) rather than 2-substituted glyceraldehyde (from the original 2-substituted mannose). The sequence of residues in the oligosaccharide-alditol was established by m.s. of its methylation product, giving fragment ions of *m/z* 219 and 187, diagnostic for terminal hexose, and of *m/z* 318, diagnostic for a 2-acetamido-2-deoxyfucosylglycerol residue, *inter alia* (Fig. 2). Confirmation was provided by the production of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylmannitol-*1-d* on methylation analysis of the oligosaccharide-alditol. Thus, structure **1** can be assigned to the Smith-degradation product, and structure **2** to the corresponding trisaccharide sequence in the parent polymer. An interpretation of the ^{13}C-n.m.r. spectrum for the Smith-degradation product is given in Table II.

TABLE I

METHYLATION ANALYSES[a]

Methylation product[b]	*Relative peak area (g.l.c.)*	
	A	*B*
1,4-AHdTal		+
2,3,4,6-Glc	1.03	1.50
2,3,4,6-Man		
3,4,6-Man	1.00	1.00
2,3,4-Man	0.86	Trace
3,6-Man	0.76	0.85
2,4-FucNAc	+	

[a]Key: A, native polymer; B, oligosaccharide-alditol from deamination reaction sequence. [b]1,4-AHdTal = 3-*O*-acetyl-2,5-anhydro-6-deoxy-1,4-di-*O*-methyltalitol; 2,3,4,6-Glc = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol, *etc.*

β-D-Man*p*-(1→3)-α-D-Fuc*p*NAc-(1→1)-Glycerol
1

↓
4
→2)-β-D-Man*p*-(1→3)-α-D-Fuc*p*NAc-(1→6)-α-D-Man*p*-(1→
2

In order to locate the 2-substituted mannose residue and the lateral substituent at the branch point, the polymer was subjected to *N*-deacetylation and then deamination. Most of the product was eluted from Sephadex G-15 in about the position of the tetrasaccharide stachyose, depolymerisation confirming that 2-acetamido-2-deoxyfucose was a component of the main chain in the polymer. Methylation analysis, monitored by g.l.c.–m.s. of the methylated alditol acetates (Table I, column B) and by g.l.c. of the methylated aldose acetates, showed that the deamination product contained non-reducing terminal residues of both glucose and mannose, the latter being derived from the residue originally 6-substituted. When the deamination product was treated with α-D-mannosidase or α-D-glucosidase, the appropriate monosaccharide was released. Methylation analysis of the α-D-glucosidase-treated material showed that the 2,4-disubstituted mannose residue had been lost, with a corresponding increase in abundance of 2-substituted mannose (but no 4-substituted mannose). From this result, it can be concluded that the original 2-substituted mannose residue was in the main chain, and that the terminal glucose residue was attached directly to the branch-point mannose residue at position 4. Thus, structure **3** can be assigned to the repeating unit of the O10 polymer.

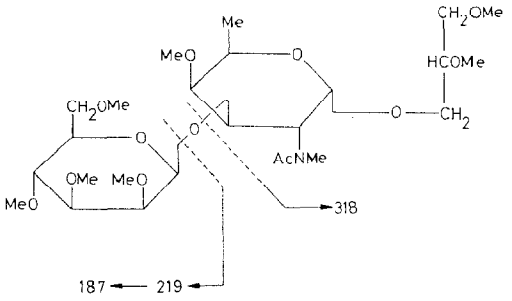

Fig. 2. Diagnostic fragment ions in the electron-impact mass spectrum of the per-*O*-methylated Smith-degradation product from the O-specific polysaccharide.

TABLE II

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

Carbon atom	*Chemical shift (p.p.m.)*[a]		
	β-D-Manp-(1→	*→3)-α-D-FucpNAc-(1→*	*→1)-Glycerol*
C-1	101.50	97.50	68.67[b]
C-2	70.55	48.45	71.30
C-3	73.02	77.39	62.64[b]
C-4	66.86	70.91	
C-5	76.32	66.56	
C-6	61.09[b]	15.46	
-NHC(O)CH_3		174.49	
-NHC(O)CH_3		22.04	

[a]The spectrum was recorded at 40° with tetramethylsilane as the external reference. Assignments of some signals with similar chemical shifts may be interchanged. The chemical shifts recorded were ~1.3 p.p.m. lower than the literature data[14–18] used in making the assignments. [b]Signals which gave a triplet in the proton-coupled spectrum.

α-D-Glc*p*
1
↓
4
→2)-α-D-Man*p*-(1→2)-β-D-Man*p*-(1→3)-α-D-Fuc*p*NAc-(1→6)-α-D-Man*p*-(1→

3

The O10 antigen described above is one member of a group (O8 to O12, inclusive) of related antigens, three of which are known to be constructed from glucose, mannose, and fucosamine[10]. There are, presumably, structural differences between these related antigens, as monospecific O antisera can be prepared by absorption with appropriate boiled cells[6]. The presence of an *O*-acyl substituent in the O11 antigen may contribute to the specificity of the antigen[10].

EXPERIMENTAL

Growth of bacteria, and isolation and fractionation of the lipopolysaccharide. — *E. cloacae* N.C.T.C. 11579 (the reference strain for the O10 serogroup[6]) was grown for 16 h at 37° in Nutrient Broth No. 2 (Oxoid) with aeration. From 123 g of wet cells, 3.6 g of cell walls were obtained, as in related studies[19], and used for the extraction of lipopolysaccharide (1.4 g). The water-soluble products of mild acid hydrolysis (1% acetic acid, 1.5 h, 100°) were fractionated on Sephadex G-50 and DEAE-Sepharose CL-6B (ref. 19).

General methods. — I.r. spectra were recorded with a Unicam SP-200

spectrophotometer for samples dispersed in KBr. Optical rotation was determined with a Bendix polarimeter (Model 143A). N.m.r. spectra were recorded for solutions in D_2O with Bruker WH-400 spectrometers. ^{13}C-N.m.r. spectra (with complete proton-decoupling, with gated decoupling, or with the INEPT pulse sequence) were recorded at 40° or 50° with tetramethylsilane as the external reference. ^1H-N.m.r. spectra were recorded at 80° or 85° with sodium 3-trimethylsilylpropanoate-d_4 as the internal reference. G.l.c. was carried out either with a packed column of OV-275 in a Pye Unicam 104 chromatograph, or with fused-silica capillary columns of BP1 or BP10 in a Carlo Erba Mega 5160 chromatograph. Reverse-phase h.p.l.c. of 2,3,4,6-tetra-*O*-methylhexitols was carried out with aqueous acetonitrile[12]. G.l.c.–m.s. was carried out with a Finnigan model 1020B instrument, either in the e.i. mode or by c.i. with methane as the reagent gas[20]. The solvent systems and detection reagents used in p.c. were those described previously[19]. High-voltage paper electrophoresis was done at pH 5.3 with the buffer system pyridine–acetic acid–water (5:2:43).

Determination of monosaccharide composition. — For neutral sugars, samples were hydrolysed with 2M HCl for 2 h at 105°. After neutralisation of the acid and deionisation, hexoses in the hydrolysates were identified and determined by p.c., by enzymic assay[19], and by g.l.c. of the alditol acetates.

For amino sugars, samples were hydrolysed with 4M HCl for 6 h at 105° under nitrogen. Fucosamine was identified by p.c. of the compound and its *N*-acetyl derivative, by high-voltage electrophoresis and autoanalysis, by a positive Elson–Morgan reaction[21], and by g.l.c.–m.s. of the derived 2,5-anhydro-6-deoxytalitol triacetate[10]. It was assigned the D configuration from the positive optical rotation of the hydrochloride.

Degradative methods. — Standard procedures were used for the preparation[22], purification[23], and hydrolysis[24] of methylated poly- and oligo-saccharides. The products were analysed as methylated alditol acetates (by g.l.c.–m.s.), methylated aldose acetates (by g.l.c.[13]), methylated alditols (by h.p.l.c.[12]), or the methylated oligosaccharide-alditol (by m.s.). Periodate oxidation of the O-specific polysaccharide (28 mg) was carried out with 50mM $NaIO_4$ for 5 days at 4°. The consumption of $NaIO_4$ was monitored by the method of Avigad[25]. After a conventional work-up and reduction ($NaBH_4$), the oxidised polymer was hydrolysed[19] and the Smith-degradation product isolated by chromatography on Sephadex G-15. The *N*-deacetylation of reduced ($NaBH_4$) polysaccharide (40 mg) with alkali in aqueous Me_2SO[26] was carried out for 16 h at 95°, and the purified product was deaminated[27], then reduced ($NaBH_4$), and chromatographed on Sephadex G-15. Enzymic hydrolyses[19] of the deamination product were carried out with α-D-mannosidase (EC 3.2.1.24, from jack bean, Sigma) or α-D-glucosidase (EC 3.2.1.20, from rice, Sigma), and were monitored by p.c. and by enzymic assay of the monosaccharides released. Peracetylation and CrO_3 oxidation (1 h, 50°) of the O-specific polysaccharide were done by standard methods[27]. *O*-Acetyl groups were removed by treatment with methanolic 0.2M KOH for 2.5 h at room temperature.

ACKNOWLEDGMENTS

We thank the S.E.R.C. for access to the high-field n.m.r. services at the Universities of Sheffield and Warwick, the staff of these services for their help, Mr. A. D. Roberts (for g.l.c.–m.s.), Mr. C. Casey (for i.r. and polarimetry), Dr. M. J. Moore (for h.p.l.c.), and Dr. M. A. Gaston (for the culture of *E. cloacae*, and his interest in this project).

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