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STRUCTURAL STUDIES OF THE *Escherichia coli* O-ANTIGEN 6

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ABSTRACT

The structure of the *Escherichia coli* O-antigen 6 has been investigated using n.m.r. spectroscopy, methylation analysis, and various specific degradations. It is concluded that the O-antigen is composed of pentasaccharide repeating-units having the following structure.

→3)-β-D-Man*p*-(1→4)-β-D-Man*p*-(1→3)-α-D-Glc*p*NAc-(1→4)-α-D-Gal*p*NAc-(1→
2
↑
1
β-D-Glc*p*

INTRODUCTION

Although *E. coli* is always found in the normal intestinal bacterial flora of man, particular strains (clones) of this organism are pathogenic. Thus, some *E. coli* clones cause infantile diarrhoea and others cause urinary tract infections. The most severe type of urinary tract infection, namely pyelonephritis, has recently been shown to be caused[1] by *p*-fimbriated *E. coli* clones of serotypes O1, O2, O4, O6, O7, O8, O9, O18, O25, and O75. The structures of the corresponding O-antigens 7[2], 8, 9, 25, and 75[3] have been determined, and we now report on structural studies of the O-antigen 6.

RESULTS AND DISCUSSION

The lipopolysaccharide (LPS) was obtained from *E. coli* O6 (strain 8316/41) by extraction with phenol–water[4]. Delipidation of the LPS with aqueous acetic acid yielded the polysaccharide (PS), which had $[\alpha]^{20}_{578}$ +81° (water). Sugar analysis of

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the PS revealed D-mannose, D-glucose, 2-amino-2-deoxy-D-glucose, and 2-amino-2-deoxy-D-galactose in the relative proportions 2:1:1:1. That each of these sugars has the D configuration was demonstrated by the method of Gerwig *et al.*[5]. The percentages of galactose and heptose were low, indicating that the major part of the PS consisted of O-specific side-chains.

The ^{1}H-n.m.r. spectrum of the PS showed, *inter alia*, signals for two *N*-acetyl groups at δ 1.96 and 2.01, and for five anomeric protons at δ 4.69 ($J_{1,2}$ 7.8 Hz), 4.79 (not resolved, 2 H), 4.93 ($J_{1,2}$ 3.9 Hz), and 5.31 ($J_{1,2}$ 3.9 Hz). In agreement with these results, the ^{13}C-n.m.r. spectrum showed, *inter alia*, signals for two *N*-acetyl groups at δ 23.7. 23.8, 175.7, and 176.2, for two C-2 of aminodeoxy sugars at δ 51.8 and 54.6, and for five anomeric carbons at δ 99.6, 100.8, 102.1, 102.2, and 105.1. Some minor peaks, assigned to core sugars, confirmed that the core constituted only a small part of the PS.

The above results indicate that the O-antigen is composed of pentasaccharide repeating-units containing two D-mannopyranosyl residues, and one D-glucopyranosyl, one 2-acetamido-2-deoxy-D-glucopyranosyl, and one 2-acetamido-2-deoxy-D-galactopyranosyl residue. From the chemical shifts of the signals for C-2 in the last two sugars, each should be α-linked. The signal at δ 4.69 ($J_{1,2}$ 7.8 Hz) in the ^{1}H-n.m.r. spectrum therefore demonstrates that the D-glucopyranosyl residue is β-linked. The signals at δ 4.79, which were not resolved, could be assigned to the two D-mannopyranosyl residues, the value for the shifts indicating that both these residues are β-linked.

Methylation analysis of the PS, with analysis of the methylated sugars as the

TABLE I

METHYLATION ANALYSES OF THE *E. coli* O6 ANTIGEN AND OF DEGRADATION PRODUCTS[a]

Sugar[b]	T[c]	T[d]	*Mole %*			
			A	B	C	D
2,5-Anhydro-1,4,6-[1-^{2}H]mannitol	0.24			10		
1,2,3,5,6-[1-^{2}H]Mannitol	0.35				16	
2,3,4,6-Glc	1.00	0.27	26	35	39	8
3,4,6-Man	1.82			30	45	
2,4,6-Man	1.90					58
2,3,6-Man	2.03	0.39	32	26		
4,6-Man	2.92	0.51	22			8
2,3,4,6-GlcNAc		0.85				11
2,4,6-GlcNAc		1.36	10			
2,3,6-GalNAc		1.20	10			15

[a]Key: *A*, methylated O6; *B*, methylated tetrasaccharide obtained after deamination; *C*, methylated trisaccharide obtained after base-catalysed degradation and reduction with $NaBD_4$ of the preceding tetrasaccharide; *D*, methylated trisaccharide-glycoside obtained after Smith degradation. [b]2,3,4,6-Glc = 2,3,4,6-tetra-*O*-methyl-D-glucose, *etc.* [c]Retention time of the corresponding alditol acetate relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol on an OV-225 column at 170°. [d]As in *c*, but relative to glucitol hexa-acetate on an SE-30 column at 180°.

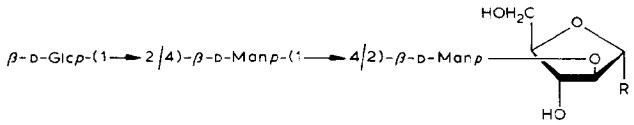
alditol acetates by g.l.c.–m.s., gave the sugars listed in Table I, column A, together with traces of other sugars, derived from the core region of the LPS. The analysis confirms that the PS is composed of pentasaccharide repeating-units. D-Glucose occurs as a terminal group, one D-mannose is linked through O-2 and O-3, the other through O-4, 2-acetamido-2-deoxy-D-glucose is linked through O-3 and 2-acetamido-2-deoxy-D-galactose through O-4. The methylation patterns of the two amino sugars were evident from the mass spectra, but the full identification was based on results of a specific degradation, as discussed below.

A sample of the PS was *N*-deacetylated by treatment with sodium hydroxide and thiophenol in aqueous dimethyl sulfoxide[6]. The ^{1}H-and ^{13}C-n.m.r. spectra revealed that *N*-deacetylation was essentially complete. The ^{13}C-n.m.r. spectrum showed, *inter alia,* signals for C-2 of two amino sugars at δ 53.1 and 56.0, and for five anomeric carbons at δ 99.7, 101.5, 102.1 (2 C), and 105.1.

Deamination of the *N*-deacetylated PS was performed by treatment with sodium nitrite in aqueous acetic acid[7]. Part of the product was reduced with sodium borodeuteride and fractionated on Sephadex G-15. The main component (eluted in the tetrasaccharide region), on sugar analysis, gave 2,5-anhydro-D-[1-^{2}H]mannitol, D-mannose, and D-glucose in the proportions ~1:2:1. The 2,5-anhydro-D-[1-^{2}H]mannitol, which was derived from the 2-amino-2-deoxy-D-glucopyranosyl residue and was identified by g.l.c.–m.s. of its acetate, was well separated from the corresponding D-talitol derivative, which should be formed from the 2-amino-2-deoxy-D-galactopyranosyl residue. The ^{1}H-n.m.r. of the tetrasaccharide showed, *inter alia,* signals for the anomeric protons at δ 4.55 ($J_{1,2}$ 7 Hz), 4.66 (not resolved), and 4.73 (not resolved). A coupled ^{13}C-n.m.r. spectrum showed, *inter alia,* signals for anomeric carbon atoms at δ 101.4 ($^{1}J_{C,H}$ 161 Hz), 102.1 ($^{1}J_{C,H}$ 162 Hz), and 104.9 ($^{1}J_{C,H}$ 166 Hz). The values for the coupling constants demonstrate that the three sugar residues are β-linked[8]. The coupling constant for the latter peak is somewhat high, but the chemical shift appears in the region for β-linked hexopyranosides having the *gluco* or *galacto* configuration. Methylation analysis of the oligosaccharide gave the sugars listed in Table I, column B. Because of the deuterium labelling at C-1, the 2,5-anhydro-1,4,6-tri-*O*-methyl-D-mannitol was distinguishable from the corresponding 1,3,6-tri-*O*-methyl derivative[7]. The result therefore demonstrates that the 2,5-anhydro-D-mannitol, and consequently also the 2-acetamido-2-deoxy-D-glucose, is linked through O-3 and that the 4,6-di-*O*-methylated amino sugar obtained in the methylation analysis of the PS is the *gluco* derivative.

The tetrasaccharide therefore has structure **1**, in which the mutual order of the two β-D-mannopyranosyl residues was not determined.

In order to determine this order, the deamination of the *N*-deacetylated PS was repeated and the tetrasaccharide (**2**) containing the 2,5-anhydro-D-mannose residue was isolated by gel-permeation chromatography. On treatment of **2** with base under mild conditions, the trisaccharide **3**, linked β to the aldehyde group in **2**, was eliminated. This trisaccharide was isolated and gave signals for anomeric



1 R = CHDOH
2 R = CHO

protons in the ^{1}H-n.m.r. spectrum at δ 4.53 ($J_{1,2}$ 7.3 Hz), 4.57 ($J_{1,2}$ 6.8 Hz; together 1 H, β-D-Glc*p*), 4.76 (not resolved; 1 H, β-D-Man*p*), 4.86 ($J_{1,2}$ 1 Hz) and 5.14 ($J_{1,2}$ 1 Hz; together 1 H, β- and α-D-Man*p*, respectively). Part of the trisaccharide was reduced with sodium borodeuteride and subjected to methylation analysis (Table I, column C), which showed that the trisaccharide has structure **3**. Part of the volatile 1,2,3,5,6-penta-*O*-methyl-D-mannitol and its acetate were lost during concentrations, and the low percentage of this component was therefore not unexpected.

β-D-Glc*p*-(1→2)-β-D-Man*p*-(1→4)-D-Man

3

From the results discussed above, it is concluded that the pentasaccharide repeating-unit of the *E. coli* O-antigen 6 has structure **4**.

→3)-β-D-Man*p*-(1→4)-β-D-Man*p*-(1→3)-α-D-Glc*p*NAc-(1→4)-α-D-Gal*p*NAc-(1→
2
↑
1
β-D-Glc*p*

4

For confirmation of this structure, the PS was subjected to a Smith degradation[9] (periodate oxidation, borohydride reduction, and hydrolysis with acid under mild conditions). For some unknown reason, not all of the terminal β-D-glucopyranosyl groups were oxidised, even when the periodate oxidation–borohydride reduction sequence was repeated. The product from the hydrolysis was purified on Sephadex G-15. Methylation analysis (Table I, column D) indicated that it had structure **5**, expected for a polysaccharide composed of the repeating-unit **4**. However, some of the β-D-glucopyranosyl groups were still linked to O-2 of the D-mannopyranosyl residue.

α-D-Glc*p*NAc-(1→4)-α-D-Gal*p*NAc-(1→3)-β-D-Man*p*-(1→2)-D-Erythritol

5

EXPERIMENTAL

General methods. — Concentrations were performed under diminished pres-

sure at bath temperatures not exceeding 40°. Optical rotations were measured with a Perkin–Elmer 241 polarimeter. For g.l.c., Perkin–Elmer 990 and Hewlett–Packard 5830 A instruments equipped with flame-ionisation detectors were used. Separations were performed on glass columns (100 × 0.15 cm, with 100/120 mesh Gas Chrom Q as support material) containing 3% of OV-225 (alditol acetates and partially methylated alditol acetates) and on SE-30 W.C.O.T. columns (partially methylated alditol acetates and trimethylsilylated 2-butyl glycosides).

G.l.c.–m.s. was performed with a Varian MAT 311 instrument equipped with the appropriate g.l.c. columns. For n.m.r. spectroscopy, JEOL FX-100 and GX-400 instruments were used. Spectra of solutions in D_2O were recorded at 85°. Chemical shifts are given in p.p.m. relative to external tetramethylsilane (^{13}C) or internal sodium 1,1,2,2,3,3-hexadeuterio-4,4-dimethyl-4-silapentane-1-sulfonate (^1H).

Methylations were performed by the method of Hakomori[10,11] with sodium methylsulfinylmethanide/methyl iodide in dimethyl sulfoxide. Methylated products were recovered by dialysis against water, followed by freeze-drying. For materials of low molecular weight, or when partial depolymerisation was suspected, dimethyl sulfoxide was removed by vacuum distillation and the product isolated by partition between chloroform and water. Determination of absolute configurations was performed by the method of Gerwig *et al.*[5].

Isolation and purification of the E. coli *O6 O-polysaccharide.* — The lipopolysaccharide was isolated from *E. coli* (O:6, K:15) strain F8316/41, kindly provided by Drs. F. and I. Ørskov (Statens Seruminstitut, Copenhagen, Denmark).

The bacteria were propagated in submerged culture to late log phase, and the cells were harvested and washed twice by low-speed centrifugations. The lipopolysaccharide was extracted with phenol–water as described[4]. Isolation of the O-polysaccharide from the lipopolysaccharide was achieved by mild hydrolysis with acid (HOAc 1%, 100°, 1 h) and subsequent work-up as described previously[4]. The polysaccharide had $[\alpha]_{578}$ +81° (*c* 1, water).

Hydrolysis of native and methylated materials. — In order to increase the yields of the amino sugars, solvolyses were performed with hydrogen fluoride in a specially designed apparatus made of Teflon and Kel-F (Peninsula Laboratories Inc., San Carlos, CA). The hydrogen fluoride was dried by distillation over cobalt trifluoride. The product (2 mg, thoroughly dried) was treated with anhydrous hydrogen fluoride at room temperature for 2 h. The hydrogen fluoride was then distilled off at diminished pressure and the reaction vessel was thoroughly evacuated. The product was dissolved in aqueous 50% acetic acid and allowed to stand at room temperature for 8 h.

N-*Deacetylation*[6]. — The polysaccharide (60 mg), sodium hydroxide (450 mg), and thiophenol (120 μL) were dissolved in water (1.2 mL). Dimethyl sulfoxide (9 mL) was added, and the stirred mixture was heated in a serum vial at 80° for 5 h. The solution was then cooled, neutralised with 4M hydrochloric acid, con-

centrated, dialysed against tap water for 24 h, centrifuged to remove insoluble contaminants, and purified by chromatography on Sephadex G-25 by irrigation with 0.5M aqueous pyridine.

Deamination[7]. — *N*-Deacetylated PS (35 mg) was dissolved in water (3 mL), and aqueous 33% acetic acid (4.5 mL) and aqueous 5% sodium nitrite (4.5 mL) were added. The solution was kept at 25° for 1 h, and then freeze-dried.

One half of the product was dissolved in water (5 mL) and reduced with sodium borodeuteride (90 mg), the solution was neutralised with 4M hydrochloric acid and concentrated, and methanol (3 × 3 mL) was distilled from the residue. Fractionation of the product on a column of Sephadex G-15 by irrigation with water gave one main fraction, eluted in the tetrasaccharide region. On hydrolysis, 2,5-anhydro-D-[1-^{2}H]mannitol, D-mannose, and D-glucose were obtained in the proportions 19:56:25. Methylation analysis of the reduced oligosaccharide fraction gave the sugars listed in Table I, column B.

The other half of the deaminated material was treated with 0.05M sodium hydroxide (1 mL) at 37° for 30 min. The solution was neutralised with 0.5M hydrochloric acid, and the product was fractionated on a column of Sephadex G-15. A major component was obtained in the trisaccharide region. A hydrolysate of the reduced trisaccharide contained D-mannose and D-glucose in the ratio 2:1. Methylation analysis of the reduced trisaccharide yielded the sugars listed in Table I, column C.

Smith degradation. — The polysaccharide (30 mg) was dissolved in sodium acetate buffer (pH 3.9, 0.1M, 6 mL), 15mM sodium metaperiodate in the same buffer (6 mL) was added, and the mixture was kept in the dark at 4° for 120 h. Excess of periodate was reduced with ethylene glycol (3 mL), and the mixture was dialysed overnight against tap water. The solution was concentrated to 10 mL and sodium borohydride (400 mg) was added. The oxidation–reduction procedure was repeated once. Sugar analysis gave the expected sugars and, in addition, D-glucose (8%).

The remaining part was hydrolysed with 0.5M trifluoroacetic acid for 16 h at room temperature, the solution was freeze-dried, and the residue was purified by gel filtration on Sephadex G-25. Part of the material was subjected to methylation analysis (Table I, column D).

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