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STRUCTURAL STUDIES OF THE O-SPECIFIC SIDE-CHAINS OF THE CELL-WALL LIPOPOLYSACCHARIDE FROM *Escherichia coli* O 69

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ABSTRACT

The structure of the O-specific side-chains of the cell-wall lipopolysaccharide from *Escherichia coli* O 69 has been investigated. Methylation analysis, n.m.r. spectroscopy, and *N*-deacetylation–deamination, followed by analysis of the product, were the principal methods used. These studies demonstrate that the O-antigen is composed of tetrasaccharide repeating-units having the following structure:

→3)-β-D-GlcNAc*p*-(1→2)-α-L-Rha*p*-(1→2)-α-L-Rha*p*-(1→2)-α-D-Gal*p*-(1→

INTRODUCTION

Escherichia coli O 69 is an organism that may cause infections of the urinary tract in man. Qualitative sugar analysis of its lipopolysaccharide[1] (LPS) demonstrated the presence of L-rhamnose, in addition to D-glucose, D-galactose, and 2-acetamido-2-deoxy-D-glucose, sugars that are known to be components of the core region. We now report structural studies on the O-specific side-chains of this LPS.

RESULTS AND DISCUSSION

The LPS was isolated from *E. coli* O 69:K ?:H 38, strain P 96, by extraction of whole cells with phenol–water[2]. The polysaccharide (PS) was prepared from the LPS by mild hydrolysis with acid[3], followed by purification on a column of Sephadex G-50. The PS, $[\alpha]_{578}$ +67°, on acid hydrolysis followed by deamination[4], yielded 2,5-anhydro-D-mannose, L-rhamnose, D-galactose, D-glucose, and L-*glycero*-D-*manno*-heptose in the relative proportions 0.7:2:1.2:0.46:0.28. The sugars were analysed by g.l.c. of their alditol acetates, in the presence of an internal standard, and together accounted for ~77% of the PS.

The ^{1}H-n.m.r. spectrum of the PS showed, *inter alia*, signals for methyl protons of the L-rhamnose residues (δ 1.20–1.38, 6 H), for acetyl protons (δ 2.05, 3 H)

(proved by i.r. spectroscopy to be *N*-acetyl), and for anomeric protons [δ 4.64 ($J_{1,2}$ 8 Hz, 1 H), 5.16 (2 H), 5.46 (1 H)].

O-Specific side-chains of LPS are generally composed of oligosaccharide repeating-units, and the results presented so far suggest that the O-69 antigen is composed of tetrasaccharide repeating-units containing D-galactose, 2-acetamido-2-deoxy-D-glucose, and two L-rhamnose residues. The D-glucose and L-*glycero*-D-*manno*-heptose found in the sugar analysis most probably derive from the core region of the LPS.

In the n.m.r. spectrum, the signal at δ 4.64 ($J_{1,2}$ 8 Hz) probably derives from the anomeric proton of a β-D-galactopyranosyl or 2-acetamido-2-deoxy-β-D-glucopyranosyl residue. The chemical shifts of the other signals suggest that the three other sugar residues are α-linked. The anomeric region of the n.m.r. spectrum also contained some minor peaks, which may derive from the core sugars.

Methylation analysis of the PS gave the sugar derivatives listed in Table I, column *A*; they were analysed by g.l.c.–m.s. of their alditol acetates[5,6]. In addition, 2-deoxy-4,6-di-*O*-methyl-2-*N*-methylacetamido-D-glucose was found, but the analysis of this sugar was qualitative only, as the response factor of its alditol acetate was not known. The results support the suggestion of a tetrasaccharide repeating-unit. They also demonstrate that the sugars are pyranoid, that the 2-acetamido-2-deoxy-D-glucose residue is linked through O-3, and that the other sugar residues of the repeating unit are linked through O-2.

TABLE I

METHYLATION ANALYSIS OF ORIGINAL AND MODIFIED POLYSACCHARIDE FROM *E. coli* 0 69

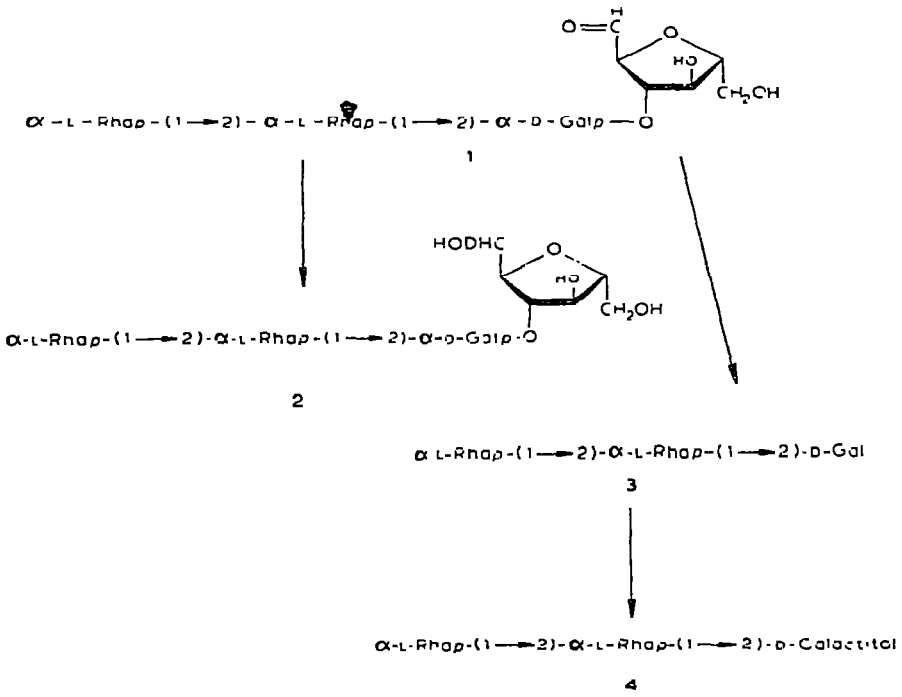
Methylated sugar[a]	*T*[b]	*Detector response* (%)[c]			
		A	B	C	D
1,3,4,5,6-Galactitol	0.21				38
2,3,4-Rha	0.48			21	35
1,4,6-2,5-Anhydro-Man	0.38			28	
3,4-Rha	0.88	62	40	29	27
2,3,4,6-Gal	1.14	3	4		
3,4,6-Glc	1.68	2	4		
3,4,6-Gal	1.96	32	49	22	
2,4-Glc	3.5	1	2		

[a]2,3,4-Rha = 2,3,4-tri-*O*-methyl-L-rhamnose, etc. [b]Retention time for the corresponding alditol acetate, relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol. [c]*A*, original PS; *B*, *N*-deacetylated PS; *C*, tetrasaccharide **2**; *D*, trisaccharide **4**.

The PS was *N*-deacetylated by treatment with sodium hydroxide–sodium thiophenolate in aqueous methyl sulfoxide[7]. The product, $[\alpha]_{578}$ +40°, yielded L-rhamnose, D-galactose, and D-glucose in the relative proportions 1:1.3:0.3 on acid hydrolysis. Since 2-amino-2-deoxyglucopyranosidic linkages are hydrolysed only with difficulty, these results, in conjunction with the sugar analysis of the original PS, show

that this sugar is linked to L-rhamnose. This linkage should also be resistant to acid hydrolysis during the methylation analysis (Table I, column *B*) of the *N*-deacetylated material, and a considerable decrease in 3,4-di-*O*-methyl-L-rhamnose was in fact observed. The n.m.r. spectrum of the *N*-deacetylated PS confirmed complete removal of *N*-acetyl groups and showed signals for anomeric protons at δ 4.67 ($J_{1,2}$ 8 Hz, 1 H), 5.18 (2 H), and 5.50 (1 H).

The *N*-deacetylated PS was deaminated by treatment with sodium nitrite in aqueous acetic acid. Part of the product (which according to results presented below has structure **1**) was reduced with sodium borodeuteride, and fractionated on a column of Sephadex G-25. A tetrasaccharide (**2**), $[\alpha]_{578}$ +15°, was obtained; on hydrolysis, **2** yielded L-rhamnose, D-galactose, and 2,5-anhydro-D-mannitol in the relative proportions 2:1:1. The n.m.r. spectrum of **2** showed signals for anomeric



protons at δ 5.02 (1 H) and 5.15–5.26 (2 H). The disappearance of the high-field signal having a large coupling constant, observed in the n.m.r. spectrum of the original PS, demonstrates that the 2-acetamido-2-deoxy-D-glucopyranosyl residue in that material is β-linked and consequently that the D-galactopyranosyl residue is α-linked. Methylation analysis of **2** (Table I, column *C*) showed that one of the L-rhamnopyranosyl residues was terminal, thus confirming that the 2-acetamido-2-deoxy-D-glucopyranosyl residue is linked to L-rhamnose in the original PS. The analysis of **2**, however, does not give any information on the mutual order of the two

remaining sugar residues. The substitution pattern of the 3-*O*-acetyl-2,5-anhydro-1,4,6-tri-*O*-methyl-D-mannitol-*1-d* was evident from its mass spectrum, the interpretation of which has been reported elsewhere[8].

In order to determine the sequence of the sugar residues, the oligosaccharide obtained by deamination was treated with base under mild conditions, when the oligosaccharide linked to O-3 of the 2,5-anhydro-D-mannose residue was liberated by β-elimination. The resulting trisaccharide (**3**), $[\alpha]_{578}$ −3°, yielded L-rhamnose and D-galactose in the ratio 2:1 on acid hydrolysis. Its n.m.r. spectrum showed signals in the anomeric region [δ 4.76 (1 H) and 5.08 (1 H)] which could be assigned to two L-rhamnopyranosyl residues. This region also contained signals at δ 4.64 ($J_{1,2}$ 8 Hz), 5.23 ($J_{1,2}$ 2 Hz), and 5.31 ($J_{1,2}$ 3 Hz), totalling 1 H, which were assigned to different forms of a terminal D-galactose residue.

On reduction of **3** to its alditol (**4**), $[\alpha]_{578}$ −34°, using sodium borodeuteride, the signals in the n.m.r. spectrum assigned to the terminal D-galactose residue disappeared. The mass spectrum of the fully methylated trisaccharide alditol[9] showed, *inter alia*, peaks at *m/e* 189 and 331, assigned to the aA_1 and the baA_2 fragments, respectively, and at *m/e* 236, assigned to the alditol moiety (cA_1). The sequence of the sugar residues in **4** is thereby established, and it is further confirmed by the methylation analysis (Table I, column *D*).

The n.m.r. spectra and optical rotations of the oligosaccharides **2**, **3**, and **4** indicate that the 2-acetamido-2-deoxy-D-glucopyranosyl residue in the tetrasaccharide repeating-unit is β-linked and that the three other residues are α-linked. From the combined evidence, structure **5** for the repeating unit in the O-specific side-chains of the *E. coli* O 69 LPS is proposed.

→3)-β-D-GlcNAc*p*-(1→2)-α-L-Rha*p*-(1→2)-α-L-Rha*p*-(1→2)-α-D-Gal*p*-(1→

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EXPERIMENTAL

General methods. — Concentrations were performed under reduced pressure at bath temperatures not exceeding 40°. For g.l.c., a Perkin–Elmer 990 or Hewlett–Packard 5830 A instrument fitted with flame-ionisation detectors was used. Separations of alditol acetates were performed at 210° on glass columns (180 × 0.15 cm) containing 3% of OV-225 on Gas Chrom Q, and of partially methylated alditol acetates at 220° on a glass-capillary column wall-coated with SP 1000 (25 m × 0.25 mm). G.l.c.–m.s. was performed with a Varian MAT 311-SS 100 m.s.–computer system. N.m.r. spectra for solutions in D_2O were recorded on a Varian XL-100 instrument at 80°. High-speed liquid chromatography of free sugars was performed with two Micro-Bondapac Carbohydrate (Waters) (30 × 0.5 cm) columns and with acetonitrile–water (9:1) as eluant.

Preparation of PS. — *E. coli* O 69:K ?:H 38, strain P 96, was used. Acetone-dried bacteria were extracted with phenol–water[2], and a sample of the resulting LPS

(220 mg) was treated with 1% acetic acid (50 ml) at 100° for 2 h. The hydrolysate was centrifuged, and the supernatant was washed with ethyl ether (3 × 15 ml) and freeze-dried. Chromatography of the product on a column (86 × 1.6 cm) of Sephadex G-50 yielded the PS (50 mg), $[\alpha]_{578}^{25}$ +67° (*c* 0.5, water), eluted with the void volume. In the i.r. spectrum of the PS, absorption typical for amides (~1650 cm^{-1}), but not for esters (~1750 cm^{-1}), was observed.

Sugar analysis[4]. — A sample of the PS (3 mg) was treated with 2M hydrochloric acid at 100° for 4 h, and the hydrolysate was concentrated to dryness. A solution of the residue in water (0.3 ml) was treated with 33% acetic acid (0.5 ml) followed by 5% aqueous sodium nitrite (0.5 ml) and kept for 40 min at room temperature. The solution was deionised with Dowex 50(H^+) resin and freeze-dried. The sugars, including 2,5-anhydro-D-mannose, were converted into alditol acetates, and analysed by g.l.c.–m.s.

The following sugars from an acid hydrolysate of the PS were also isolated by using high-speed liquid chromatography: L-rhamnose, $[\alpha]_{578}^{25}$ +12°; D-galactose, $[\alpha]_{578}^{25}$ +53°; 2-amino-2-deoxy-D-glucose hydrochloride, $[\alpha]_{578}^{25}$ +70° (all optical rotations in water, *c* 0.2).

Methylation analyses. — These were performed as previously described[5,6]. Polymeric methylated products were isolated by dialysis, and oligomeric products by partition between chloroform and water. The identification of the partially methylated sugars, by g.l.c.–m.s. of their alditol acetates, was unambiguous and will not be discussed.

N-*Deacetylation*[7]. — A solution of PS (20 mg), sodium hydroxide (400 mg), and thiophenol (400 mg) in water (1 ml) and methyl sulfoxide (5 ml) was kept at 100° for 15 h, neutralised with acetic acid, filtered, dialysed, and freeze-dried. The modified PS (13 mg) showed $[\alpha]_{578}^{25}$ +40° (*c* 0.7, water).

Deamination. — *N*-Deacetylated PS (40 mg) was dissolved in water (0.6 ml); 33% aqueous acetic acid (1 ml) and 5% aqueous sodium nitrite (1 ml) were added, and the solution was kept at 25° for 40 min, passed through a column of Dowex 50(H^+) resin, and freeze-dried.

A solution of half of the product in water (1 ml) was treated with sodium borodeuteride (20 mg), neutralised with Dowex 50(H^+) resin, filtered, and concentrated. Boric acid was removed from the residue by codistillation with methanol (3 × 3 ml). Fractionation of this product on a column (86 × 1.6 cm) of Sephadex G-25 yielded oligosaccharide **2** (3.3 mg), $[\alpha]_{578}^{25}$ +15° (*c* 0.3, water), which was eluted in the tri- to tetra-saccharide region.

The other half of the deaminated product was treated with 0.05M aqueous sodium hydroxide (1 ml) at 37° for 30 min. The solution was made neutral with M hydrochloric acid and then fractionated on a column (80 × 0.8 cm) of Sephadex G-15. Pure trisaccharide **3** (2.5 mg), $[\alpha]_{578}^{25}$ −3° (*c* 0.1, water), was obtained.

Reduction of **3** with sodium borodeuteride yielded **4**, $[\alpha]_{578}^{25}$ −34° (*c* 0.1, water). The n.m.r. spectrum of **4** showed, *inter alia*, signals at δ 1.16–1.26 (6 H), 4.95 (1 H), and 5.03 (1 H). The mass spectrum of fully methylated **3** showed, *inter alia*, the

following pertinent fragments (relative intensities in brackets): *m/e* 45 (27), 46 (7), 59 (45), 88 (100), 89 (28), 101 (64), 125 (11), 145 (23), 157 (29), 189 (70), 203 (4), 236 (18), 295 (1), 331 (2), and 377 (2).

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