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STRUCTURAL STUDIES OF THE O-SPECIFIC SIDE-CHAIN OF THE LIPO-POLYSACCHARIDE FROM *Escherichia coli* O 55

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ABSTRACT

The structure of the O-specific side-chains of the lipopolysaccharide from *Escherichia coli* O 55 has been investigated, methylation analysis, specific degradations, and n.m.r. spectroscopy being the principal methods used. It is concluded that the O-specific side-chains are composed of pentasaccharide repeating-units having the following structure [where Col stands for colitose (3,6-dideoxy-L-*xylo*-hexose)].

→3)-α-D-Gal*p*-(1→3)-β-D-Gal*p*NAc-(1→6)-β-D-Glc*p*NAc(1→
3
↑
1
α-Col*p*-(1→2)-β-D-Gal*p*

INTRODUCTION

Colitose (3,6-dideoxy-L-*xylo*-hexose) is an immunodominant component of the O-antigen in different bacteria belonging to *Enterobacteriaceae*, *e.g.*[1], *E. coli* O 55 and O 111. *E. coli* O 55 also contains 2-amino-2-deoxy-D-galactose as a component of its O-antigen. The division into chemotypes was based upon qualitative sugar analysis and it could not be decided if any of the sugars known to occur in the core of the lipopolysaccharide were also components of its O-antigen. We now report on structural studies of the O-antigen from *E. coli* O 55.

RESULTS AND DISCUSSION

The polysaccharide (PS), $[\alpha]_{578}$ +59°, was prepared from the lipopolysaccharide by mild hydrolysis with acid[2]. Subsequent studies revealed that part of the colitose was also split off during this treatment. Hydrolysis of the PS and analysis of the sugars (as the alditol acetates, by g.l.c.) showed colitose, D-galactose, 2-amino-

2-deoxy-D-glucose, and 2-amino-2-deoxy-D-galactose in the molar proportions 18:50:17:15. Traces of D-glucose were also detected. Although response factors for the amino sugars were used in this analysis, it is not very accurate as *N*-deacetylation during the acid hydrolysis obscures the result. Only traces of L-*glycero*-D-*manno*-heptose, which is a component of the core, were detected, indicating that the O-specific side-chains make up the main part of the PS.

The amino sugars were further identified by deamination followed by borohydride reduction, which converted them into 2,5-anhydro-D-mannitol and 2,5-anhydro-D-talitol[3], respectively, which were indistinguishable from authentic samples in g.l.c. The D configuration of the galactose and the two *N*-acetylated amino sugars was proved by treating them with (+)-2-octanol and an acid catalyst, acetylation, and g.l.c. of the resulting mixture of acetylated glycosides[4,5].

The ^{1}H-n.m.r. spectrum of the PS showed, *inter alia*, signals at δ 1.13 ($J_{5,6}$ ~6 Hz), assigned to the protons on C-6 of colitose, and at δ 2.00–2.02, assigned to the protons of *N*-acetyl groups and to H-3,3′ of colitose. The signals for the anomeric protons at δ 4.4–5.2 were not well resolved. The relative intensities of the signals indicate that colitose and the two *N*-acetylamino sugars occur in nearly equimolecular proportions.

Methylation analysis of the PS yielded the sugars listed in Table I, analysed by g.l.c.–m.s. of the alditol acetates[6]. The colitose derivatives are volatile and were partially lost. The response of the amino sugar derivatives, which is lower than that of the neutral sugar derivatives, was not determined and no percentages can be given. However, the two amino sugar derivatives were obtained in comparable amounts. The assignment of the *galacto* configuration to the 4,6-di-*O*-methyl derivative and the *gluco* configuration to the 4-*O*-methyl derivative is based on evidence discussed below.

The O-specific side-chains of lipopolysaccharides have a regular structure and are composed of oligosaccharide repeating-units. The above results indicate that the

TABLE I

METHYLATION ANALYSIS OF THE O-ANTIGEN FROM *E. coli* O 55

Sugar[a]	T[b]	*Mol* %
2,4-Col	0.38	20
2,3,4,6-Gal	1.19	3
2,4,6-Gal	2.03	38
3,4,6-Gal	2.15	39
4,6-GalNAc		+
4-GlcNAc		+

[a]2,4-Col = 2,4-di-*O*-methylcolitose, *etc.* The main part of both amino sugar derivatives was *N*-methylated. [b]Retention time of the derived alditol acetate on an OV-225 column, relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol.

O-antigen from *E. coli* O 55 is composed of pentasaccharide repeating-units that contain two D-galactopyranosyl residues and one pyranosidic residue each of colitose, 2-acetamido-2-deoxy-D-glucose, and 2-acetamido-2-deoxy-D-galactose.

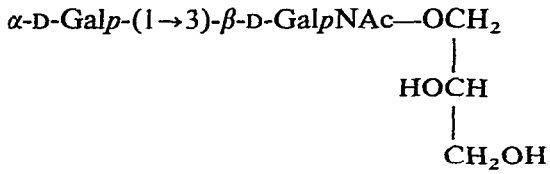
The glycosidic linkages of the colitosyl groups are more sensitive to acid hydrolysis than other glycosidic linkages in the PS and it was possible to selectively split off a high percentage of these groups. In the methylation analysis of this product, 2,4-di-*O*-methylcolitose and 3,4,6-tri-*O*-methyl-D-galactose had almost disappeared and the latter sugar was replaced by 2,3,4,6-tetra-*O*-methyl-D-galactose. The hydrolysis of the PS was monitored by polarimetry, and the initial increase in optical rotation indicated that the colitosyl groups are α-linked. These results therefore demonstrate the presence of partial structure **1** in the PS.

α-Col*p*-(1→2)-D-Gal*p*-(1→
1

The anomeric nature of the other sugar residues was determined by chromium trioxide oxidation of the fully acetylated PS, followed by sugar analysis, using *myo*-inositol hexa-acetate as internal reference[7]. β-Linked galactose, 2-amino-2-deoxy-glucose, and 2-amino-2-deoxygalactose residues should be oxidised under these conditions, but the corresponding α-linked residues should be resistant. Colitose will be destroyed independently of its anomeric configuration. Approximately 35% of the galactose survived and the other sugars were degraded, indicating that one of the two D-galactopyranosyl residues is α-linked and that the other, and the two amino sugar residues, are β-linked.

The PS was subjected to Smith-degradation[8] (sequential periodate oxidation, borohydride reduction, and mild hydrolysis with acid), during which the acetal linkages of the modified sugar residues should be selectively hydrolysed. Sugar analysis of the polyalcohol yielded colitose, D-galactose, 2-amino-2-deoxy-D-glucose, and 2-amino-2-deoxy-D-galactose in the molar proportions 14:20:31:35. Methylation analysis showed, as expected, that the 3,4,6-tri-*O*-methyl-D-galactose had disappeared. The polymeric product obtained after the hydrolysis had $[\alpha]_{578}$ +53°. Methylation analysis gave 2,4,6-tri-*O*-methyl-D-galactose, 2-deoxy-3,4-di-*O*-methyl-2-*N*-methylacetamido-D-glucose and 2-deoxy-4,6-di-*O*-methyl-2-*N*-methylacetamido-D-galactose. The assignment of *gluco* and *galacto* configurations, respectively, to the amino sugar derivatives rests on evidence discussed below. The alditol acetates of the amino sugars were not separated in g.l.c., but were identified from the mass spectrum of the mixture.

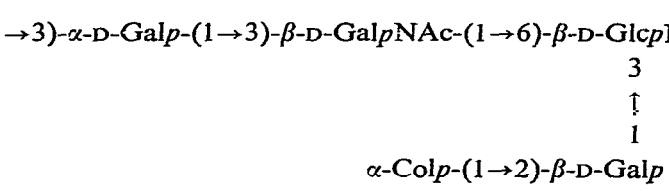
The ^{1}H-n.m.r. spectrum of the degraded product showed, *inter alia*, signals for anomeric protons at δ 5.06 ($J_{1,2}$ 3.7 Hz), 4.65 ($J_{1,2}$ ~9 Hz), and 4.55 ($J_{1,2}$ ~9 Hz), and for two *N*-acetyl groups at δ 1.99 and 2.03. As the amino sugars are known to be β-linked, this product consequently contains the α-linked D-galactopyranosyl residue. The product is linear, demonstrating that the disaccharide residue **1** is linked to O-3 of one of the two amino sugars.



2

The product of the first Smith-degradation was subjected to a second Smith-degradation, which yielded trisaccharide **2**, identified by the following evidence. Sugar analysis yielded equimolecular amounts of D-galactose and 2-acetamido-2-deoxy-D-galactose. Methylation analysis demonstrated that galactose occupied a terminal position and that the amino sugar was linked through O-3. The 2-deoxy-4,6-di-*O*-methyl-2-*N*-methylacetamidohexose in the methylation analysis of the original PS and the product from the first Smith-degradation are consequently the *galacto* derivative. The ^{1}H-n.m.r. spectrum contained, *inter alia*, signals for anomeric protons at δ 5.09 ($J_{1,2}$ 2.0 Hz) and 4.57 ($J_{1,2}$ 9.0 Hz) and for an *N*-acetyl group at δ 2.02. On chromium trioxide oxidation of fully acetylated **2**, the 2-acetamido-2-deoxy-D-galactosyl residue was completely oxidised and the D-galactopyranosyl group was resistant, showing that the former is β-linked and the latter α-linked.

The structure of the pentasaccharide repeating-unit of the *E. coli* O 55 antigen is thereby determined as **3**.



3

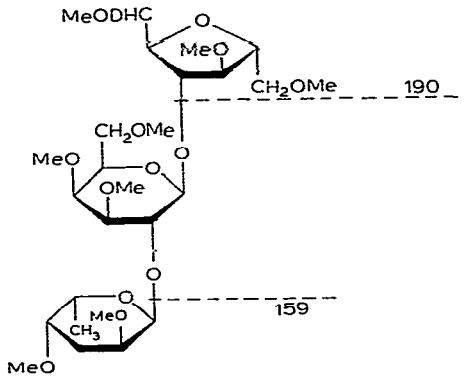
The PS was *N*-deacetylated by treatment with sodium hydroxide in methyl sulfoxide–water, deaminated by treatment with nitrous acid[10], and reduced with sodium borodeuteride, and the product fractionated by gel filtration. A trisaccharide was obtained, and identified as **4** by the following evidence.

α-Col*p*-(1→2)-β-D-Gal*p*-(1→3)-2,5-Anhydro-D-mannitol-*1-d*

4

Acid hydrolysis yielded colitose, D-galactose, and 2,5-anhydro-D-mannitol-*1-d* in equimolecular proportions. Methylation analysis demonstrated that colitose was terminal, D-galactose linked through O-2, and 2,5-anhydro-D-mannitol linked through O-3. Due to the deuterium labelling, it was possible to distinguish between positions 3 and 4 in the 2,5-anhydro-D-mannitol derivative by mass spectrometry[10]. The ^{1}H-n.m.r. spectrum showed, *inter alia*, signals for anomeric protons at δ 5.17 ($J_{1,2}$ 3 Hz) and 4.51 ($J_{1,2}$ 7 Hz), and for H-3,3′ and H-6 of the colitosyl group at δ 1.95 (m, 2 H) and 1.11 (3 H, $J_{5,6}$ 6 Hz). The mass spectrum[11] of the fully methylated trisaccharide

(5) was also in agreement with the postulated structure. The origins of some fragments are indicated in the formula. In addition, a strong cJ_1 fragment at m/z 250 was observed. The results therefore confirm the structure of the side chain in 3 and that it is linked to O-3 of the 2-acetamido-2-deoxy-D-glucosyl residue.



5

The second fragment expected after *N*-deacetylation and deamination, namely, 2,5-anhydro-3-*O*-α-D-galactopyranosyl-D-talitol-*1-d*, was not isolated and was probably hidden in the large salt fraction obtained on gel filtration of the reaction product.

TABLE II

PERTINENT ^{13}C-N.M.R. CHEMICAL SHIFTS[a] FOR THE *E. coli* O 55 ANTIGEN (A), THE POLYSACCHARIDE FROM THE SECOND SMITH-DEGRADATION (B), AND THE TRISACCHARIDE FROM THE SECOND SMITH-DEGRADATION (C)

Sugar unit	*Carbon atom*	*A*	*B*	*C*
α-Col*p*-(1→	C-1	100.6		
	C-3	34.9		
	C-6	17.4		
→2)-β-D-Gal*p*-(1→	C-1	102.4		
→6)-β-D-Glc*p*NAc-(1→	C-1	103.3	104.4	
↑3	C-2	56.6	57.5	
→3)-α-D-Gal*p*-(1→	C-1	97.1	97.1	97.2
→3)-β-D-Gal*p*-NAc-(1→	C-1	103.7	103.3	103.4
	C-2	52.4	52.4	52.5
β-D-Gal*p*-(1→	C-1	105.3[b]		
→6)-β-D-Glc*p*-NAc-(1→	C-1	104.1[b]		
↑3	C-2	56.4[b]		

[a]In D_2O relative to external tetramethylsilane. [b]Peaks derived from part of antigen preparation which has lost colitosyl groups during preparation.

By comparing ^{13}C-n.m.r. signals of the original PS, the polysaccharide from the first Smith-degradation, and the trisaccharide from the second Smith-degradation, it was possible to identify tentatively the signals given by five different anomeric carbons and the C-2 atoms of the two amino sugars (Table II). The chemical shifts are in good agreement with the postulated structure (3) and this lends further support to this structure.

EXPERIMENTAL

General methods. — General methods and methods for sugar[3] and methylation analysis[6] have been described previously. For g.l.c., Perkin–Elmer 990, Perkin–Elmer 3920, or Hewlett–Packard 5830A instruments equipped with flame-ionisation detectors were used. Separations were performed on glass columns [180 × 0.15 cm, with Gas Chrom Q (100–120 mesh) as support material] containing *A*, 3% of OV-225 (for alditol acetates and partially methylated alditol acetates); *B*, 3% of OV-17 (for amino sugar derivatives); or *C*, 3% of OV-1 (for oligosaccharide derivatives). Acetylated octyl glycosides were separated on an SP-1000 W.C.O.T. column (25 m × 0.25 mm) at 220°. G.l.c.–m.s. was performed with a Varian MAT 311-SS 100 m.s.–computer system fitted with appropriate g.l.c. columns. Spectra were recorded at 70 eV, with an ionisation current of 1000 μA. For n.m.r. spectroscopy, a JEOL FX-100 instrument operated in the PFT-mode was used. The spectra were recorded for solutions in D_2O with tetramethylsilane as external standard (^{13}C-n.m.r.) or sodium 1,1,2,2,3,3-hexadeuterio-4,4-dimethyl-4-silapentane-1-sulfonate as internal standard (^{1}H-n.m.r.).

Material. — The lipopolysaccharide (LPS) was isolated from *E. coli* O 55 strain Su 3912/41, as described earlier[12]. For preparation of PS, the LPS (600 mg) was treated with aqueous acetic acid (175 mL) of pH 3.1 for 2 h at 100°. The solution was cooled and centrifuged, and the supernatant was freeze-dried. The freeze-dried material was purified on a column (100 × 4.0 cm) of Sephadex G-50. The material eluted in the void volume was collected and freeze-dried (yield: 160 mg). The PS had $[\alpha]_{578}^{23}$ +59° (*c* 0.8, water).

Partial, acid hydrolysis. — A sample (12 mg) of the PS was dissolved in 0.06M trifluoroacetic acid (1 mL) and kept in a polarimeter tube at 80°; $[\alpha]_{578}^{80}$ +52 (5 min) → +77° (3 h).

Chromium trioxide oxidation. — A sample (7 mg) of the polysaccharide was dissolved in formamide (5 mL), and treated with acetic anhydride (1 mL) and pyridine (1 mL) overnight at room temperature. The acetylated material (10 mg) was recovered by dialysis and freeze-drying, and dissolved in acetic acid (3 mL); *myo*-inositol hexaacetate (2 mg) was added as the internal standard. Part (2/3) of the acetic acid solution was treated with chromium trioxide (50 mg) on an ultrasonic bath at 50° for 2 h. The material was recovered by partition between chloroform and water. The remaining portion was used as reference. Sugar analysis of the reference material showed *myo*-inositol, colitose, D-galactose, 2-acetamido-2-deoxy-D-glucose, and 2-acetamido-2-

deoxy-D-galactose in the proportions 1.0:0.13:0.9:1.0:1.0; the corresponding figures for the oxidised material were 1.0:0.02:0.3:0:0.

Smith-degradation of the polysaccharide. — The PS (26 mg) was dissolved in water (25 mL), sodium metaperiodate (214 mg) was added, and the solution was kept for 5 days at 4°. The excess of periodate was reduced with ethylene glycol (2 mL), and the mixture was dialysed. The solution was concentrated to ~30 mL, sodium borohydride (200 mg) was added, and the mixture was stirred overnight at room temperature. The excess of borohydride was destroyed by addition of 50% acetic acid, and the polyalcohol (23 mg) was recovered by dialysis and freeze-drying. A sample (1 mg) of this material was subjected to sugar analysis and another (2 mg) was subjected to methylation analysis. The remaining material was treated with 0.25M trifluoroacetic acid at room temperature for 18 h and concentrated to dryness. The product was purified by gel filtration on a column (40 × 2.5 cm) of Bio-Gel P2 eluted with water. The polymeric material was collected and freeze-dried (yield: 14 mg). A sample (1 mg) was subjected to sugar analysis and another (2 mg) to methylation analysis.

The remaining material was subjected to a second Smith-degradation, using essentially the same conditions as described above. Gel filtration of the degraded material on a column (40 × 2.5 cm) of Sephadex G-15 gave compound **2** (6.8 mg). A sample (~1 mg) of **2** was subjected to sugar analysis and another (~1 mg) to methylation analysis. A sample (~1 mg) of **2** and *myo*-inositol (0.25 mg) were acetylated by treatment with acetic anhydride (1 mL) and pyridine (1 mL) for 1 h at 100°. After work-up, the product was subjected to chromium trioxide oxidation, as described above. Sugar analysis of the reference sample showed *myo*-inositol, D-galactose, and 2-acetamido-2-deoxy-D-galactose in the proportions 1.0:1.0:0.7; the corresponding figures for the oxidised material were 1.0:0.7:0.

N-Deacetylation and deamination of the polysaccharide. — The PS (15 mg) was dissolved in water (0.5 mL) and methyl sulfoxide (2.5 mL), and sodium hydroxide (200 mg) and thiophenol (1 drop) were added. The solution, in a serum vial, was stirred for 15 h at 80°, neutralised with 2M hydrochloric acid, dialysed, and centrifuged. The PS (8 mg) was recovered from the supernatant solution by freeze-drying. The ^{1}H-n.m.r. spectrum of this material showed no signal for *N*-acetyl groups.

The *N*-deacetylated PS (8 mg) in water (0.5 mL) was treated with a mixture of 33% aqueous acetic acid (1 mL) and 5% aqueous sodium nitrite (1 mL) for 1 h at room temperature. The solution was diluted with water (3 mL) and freeze-dried. The product was dissolved in water (2 mL) and reduced with sodium borodeuteride (50 mg) for 2 h. The reaction mixture was acidified with acetic acid and evaporated to dryness, and methanol (3 × 5 mL) was distilled from the residue, which was then dissolved in water and applied to a column (40 × 2.5 cm) of Bio-Gel P2. Compound **4** (0.7 mg), which was eluted in the trisaccharide region, was recovered by freeze-drying. Samples (~0.2 mg) were subjected to sugar and methylation analysis. Another sample (~0.2 mg) was methylated, and analysed by g.l.c. using an OV-1 column. Compound **5** had T_{Mel} 5.8 (retention time relative to permethylated melibiitol). The

mass spectrum showed, *inter alia*, signals at (relative intensities in brackets and some assignments[11] in square brackets): *m/z* 95(15)[aA_3], 127(15)[aA_2], 158(28)[cA_2], 159(44)[aA_1], 190(19)[cA_1], and 250(20)[cJ_1].

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