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STRUCTURAL STUDIES ON THE O-SPECIFIC SIDE-CHAINS OF THE CELL-WALL LIPOPOLYSACCHARIDE FROM *Escherichia coli* O 75

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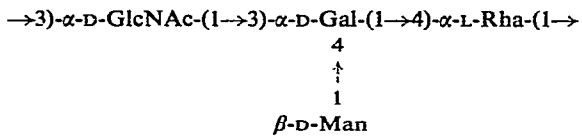
ABSTRACT

The structure of the O-specific side-chains of the cell-wall lipopolysaccharide of *Escherichia coli* O 75 has been investigated, using methylation analysis and Smith degradation as the principal methods. The O-specific side-chain was found to be composed of a tetrasaccharide repeating-unit of the following structure:



INTRODUCTION

The O-specific side chains of the cell-wall lipopolysaccharides (LPS) from *Escherichia coli* have been studied only to a limited extent by structural, chemical methods, and tentative structures have been suggested only for the O-specific side-chains of LPS from *E. coli* O 111:B4[1], *E. coli* O 86[2,3], and *E. coli* O 8[4]. Serologically, some 150 strains of *E. coli* have been recognized[5]. Sugar analyses have been carried out for many of these serotypes and enabled a classification into chemotypes[5]. We now report structural studies on the O-specific side-chains of the LPS from *E. coli* O 75, which is an organism known to cause urinary-tract infections in man.

RESULTS

The LPS was extracted from whole bacteria by the hot phenol–water method[6], and purified by ultracentrifugation and by treatment with ribonuclease. The purified

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LPS had $[\alpha]_D^{20}$ +52° (*c* 0.12, water), and did not contain any *O*-acyl groups. Acid hydrolysis of the LPS yielded D-glucose, heptose, D-galactose, D-mannose, L-rhamnose, and 2-acetamido-2-deoxy-D-glucose. The identity of the sugars was determined by gas–liquid chromatography[7] and mass spectrometry[8] of their corresponding alditol acetates. The absolute configuration of the sugars was determined from optical rotation data.

In order to release hexosamine residues quantitatively, a "three-step hydrolysis procedure" was adopted. Thus, the LPS was hydrolysed with 0.25M sulphuric acid for 6 h at 100° and re-*N*-acetylated with acetic anhydride–pyridine, and the procedure repeated twice; 85% of the material was released as D-glucose, heptose, D-galactose, D-mannose, L-rhamnose, and 2-acetamido-2-deoxy-D-glucose in the relative molar proportions 0.23:0.45:1.41:1.05:1.00:1.27.

The LPS was methylated with methylsulphinyl sodium–methyl iodide in methyl sulphoxide[9]. The methylated LPS was hydrolysed, and the mixture of partially methylated sugars was converted into alditol acetates and analysed by g.l.c.–m.s.[10] (Table I). Only the neutral sugars were eluted on the OV-225 column, and the alditol acetates of 2,3,4,6-tetra-*O*-methyl-D-mannose, 2,3-di-*O*-methyl-L-rhamnose, and

TABLE I

METHYL ETHERS FROM A HYDROLYSATE OF THE METHYLATED LPS

Sugars[a]	T[b]	*Rel. mol. %*[d]
2,3-Me_2-L-Rha		1.0
2,3,4,5-Me_4-D-Man		1.0
2,6-Me_2-D-Gal		1.1
4,6-Me_2-D-GlcN(Me)Ac	13.4[c]	+
4,6-Me_2-D-GlcNAc	12.5[c]	+

[a]4,6-Me_2-D-GlcN(Me)Ac = 2-deoxy-4,6-di-*O*-methyl-2-(*N*-methylacetamido)-D-glucose, etc. [b]Retention times of the corresponding alditol acetates on the OV-225 column relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol. [c]Retention times of the corresponding alditol acetates on the XE-60 column relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol. [d]The relative molar percentages for the amino sugar derivatives could not be determined due to lack of response factors.

2,6-di-*O*-methyl-D-galactose were found in equimolar proportions. Only traces (less than 1%) of other partially methylated sugars were present. Partially methylated 2-acetamido-2-deoxy sugars can be eluted by using the non-polar column XE-60, but the response factors between neutral partially methylated and partially methylated 2-acetamido-2-deoxy sugars are not known and therefore the latter can only be analysed qualitatively by this method. G.l.c. on an XE-60 column gave, *inter alia*, two peaks in the relative proportions 3:2. Mass spectrometry showed that these peaks consisted of the alditol acetates of 2-deoxy-4,6-di-*O*-methyl-2-(*N*-methylacetamido)-hexose and 2-acetamido-2-deoxy-4,6-di-*O*-methylhexose. Since 2-acetamido-2-deoxy-D-glucose is the only 2-acetamido-2-deoxy sugar present in the LPS, the two components are identified as 2-deoxy-4,6-di-*O*-methyl-2-(*N*-methylacetamido)-D-glucose

and 2-acetamido-2-deoxy-4,6-di-*O*-methyl-D-glucose. The primary mass-fragmentation patterns of these components are shown in Fig. 1.

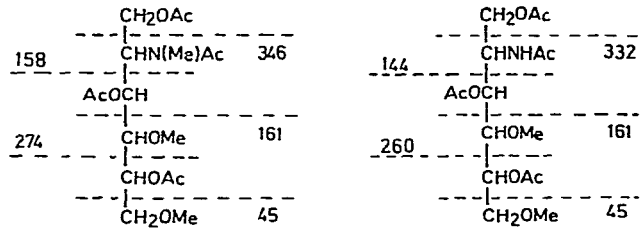

Fig. 1. Primary fragmentation pattern for 2-acetamido-1,3,5-tri-*O*-acetyl-2-deoxy-4,6-di-*O*-methylhexitol and 1,3,5-tri-*O*-acetyl-2-deoxy-4,6-di-*O*-methyl-2-(*N*-methylacetamido)hexitol.

In order to determine the sequence of sugar residues, the O-75 LPS was subjected to Smith degradation[11], *i.e.*, periodate oxidation, borohydride reduction, and mild hydrolysis with acid. The degraded material was fractionated on a Sephadex G-25 column, and a major peak was obtained in the trisaccharide region.

Sugar analysis of the material in this peak gave 2-acetamido-2-deoxy-D-glucose and D-galactose in the relative molar proportions 0.9:1.0, together with a small amount of a 4-deoxytetritol. The major portion of 4-deoxytetritol was lost during the concentration procedures due to its volatility. The 4-deoxytetritol must be formed by degradation of the L-rhamnose residue, and thus is 4-deoxy-L-erythritol. The oligosaccharide had $[\alpha]_D$ +116°, and methylation yielded a product which gave a single

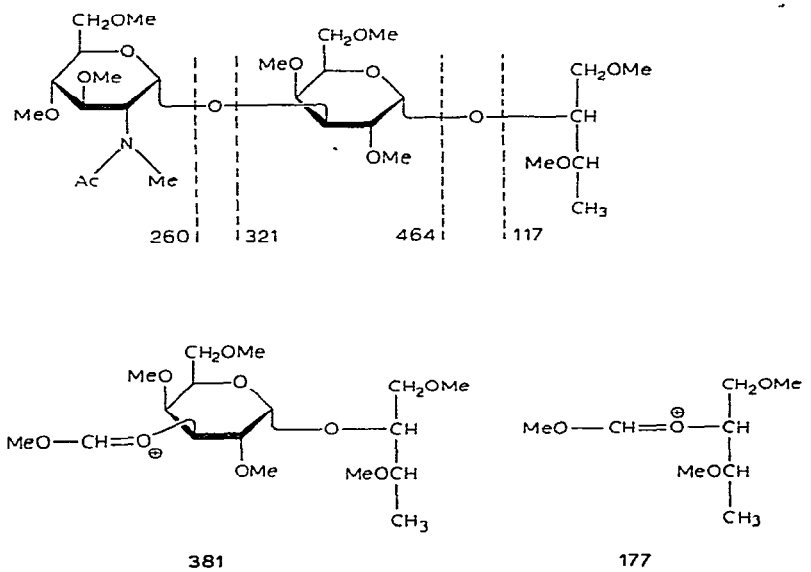

Fig. 2. Characteristic fragmentations for α-D-GlcNAc*p*-(1→3)-α-D-Gal*p*-(1→2)-4-deoxy-L-erythritol.

peak on g.l.c. Hydrolysis of the methylated oligosaccharide gave comparable amounts of 2-deoxy-3,4,6-tri-*O*-methyl-2-(*N*-methylacetamido)-D-glucose and 2,4,6-tri-*O*-methyl-D-galactose.

The mass spectrum of the methylated compound before hydrolysis showed peaks which identify it as a permethylated acetamidohexopyranosyl → hexopyranosyl → 4-deoxytetritol (Fig. 2).

DISCUSSION

Lipopolysaccharides from *Enterobacteriaceae* are generally composed of O-specific side-chains, a core part, and a lipid. The core contains D-glucose, D-galactose, 2-acetamido-2-deoxy-D-glucose, heptose, and 2-deoxy-2-keto-octulosonic acid (KDO)[5]. L-Rhamnose and D-mannose in the *E. coli* O 75 LPS are therefore derived from the O-specific side-chains (*cf.* Ref. 5). Part of the D-galactose and 2-acetamido-2-deoxy-D-glucose, and probably all of the D-glucose and the heptose are derived from the core. Methylation analysis of the LPS yielded 2,3-di-*O*-methyl-L-rhamnose, 2,3,4,6-tetra-*O*-methyl-D-mannose, and 2,6-di-*O*-methyl-D-galactose, in almost equal amounts, as the major neutral components. The methyl ethers from the 2-acetamido-2-deoxy-D-glucose residue could not be obtained in quantitative yield due to thermal degradation during g.l.c., but since 2-deoxy-4,6-di-*O*-methyl-2-(*N*-methylacetamido)-D-glucose (and its 2-acetamido analogue) was the only methylated derivative found, it can be safely assumed that it represents all the 2-acetamido-2-deoxy-D-glucose residues.

Smith degradation yielded an oligosaccharide having $[\alpha]_D^{20}$ +116°, which was eluted in the trisaccharide region from a column of Sephadex G-25. Sugar analysis of the oligosaccharide showed that it was composed of 2-acetamido-2-deoxy-D-glucose, D-galactose, and 4-deoxy-L-erythritol. Methylation analysis of the oligosaccharide demonstrated that 2-acetamido-2-deoxy-D-glucose was the non-reducing terminal, the D-galactose a (1→3)-linked chain residue, and the 4-deoxy-L-erythritol a 2-linked terminal. From the high, positive optical rotation, it may be inferred that all sugar residues are α-linked. The structure of the oligosaccharide thus is α-D-GlcNAc*p*-(1→3)-α-D-Gal*p*-(1→2)-4-deoxy-L-erythritol.

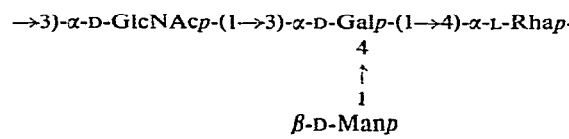

Fig. 3. Proposed structure for a repeating unit of the O-specific side-chains of the lipopolysaccharide from *E. coli* O 75.

If it is assumed that the O-specific side-chains are composed of repeating units, the evidence presented above allows us to postulate a structure for this repeating unit (Fig. 3), as the terminal D-mannose residue must be linked to the branched D-galactose residue.

The anomeric configuration of the terminal D-mannose residue was assigned β, as the LPS did not precipitate with concanavalin A. This lectin precipitates macromolecules having multiple, non-reducing, α-linked, but not β-linked, D-mannose residues[12]. From the specific optical rotation (+52°) of the LPS, it may be inferred, from calculation using Hudson's isorotation rules, that the L-rhamnose residue is α-linked.

EXPERIMENTAL

General methods. — Concentrations were performed under diminished pressure at bath temperatures not exceeding 40°. G.l.c. was performed on Perkin–Elmer 900 or 990 instruments fitted with flame-ionisation detectors. Separations were performed on (*a*) glass columns (180 × 0.15 cm) containing 3% of ECNSS-M on Gas Chrom Q (100/200 mesh) at 170° (partially methylated alditol acetates) or 190° (alditol acetates). (*b*) OV 225 S.C.O.T. columns (50 ft × 0.020 in.) at 190° (partially methylated alditol acetates), (*c*) glass columns (180 × 0.15 cm) containing 5% of XE-60 on Chromosorb W (80/100 mesh) at 190° (partially methylated 2-acetamido-2-deoxyalditol acetates), or (*d*) 5% of OV 1 on Chromosorb Q at 250° (methylated oligosaccharide alditols). For mass spectrometry, a MAT 311 instrument fitted with the appropriate column was used. Mass spectra were recorded at an ionisation potential of 70 eV, an ionisation current of 1000 μA, and an ion-source temperature of 100°. Optical rotations were recorded by using a 10-cm micro-cell and a Perkin–Elmer 141 instrument.

Isolation of LPS. — *E. coli* O 75, isolated from a patient having urinary-tract infection, was obtained from Dr. K. Lincoln, University of Gothenburg. The bacteria were grown for 18 h at 37° on a synthetic substrate containing sodium lactate as the carbon source. Extraction of LPS from bacteria, which had been washed twice with phosphate-buffered saline (pH 7.3), was performed by the hot phenol–water procedure of Westphal[6]. The LPS was further purified by ultracentrifugation and by treatment with ribonuclease from bovine pancreas (Type XII A, Sigma Chemical Co.).

Sugar analysis (3-step procedure). — A solution of the LPS (2.4 mg) and an internal standard of D-xylose (0.43 mg) in 0.25M sulphuric acid (1 ml) was heated at 100° for 6 h. The partial hydrolysate was neutralised with barium carbonate, filtered, and concentrated to dryness. The resulting mixture of sugars was acetylated with acetic anhydride (1 ml) and pyridine (1 ml) at 100° for 20 min. The mixture was concentrated to dryness, and the acetylated material was hydrolysed again in 0.25M sulphuric acid at 100° for 6 h. After neutralisation and acetylation as described above, the mixture was hydrolysed a third time for 6 h at 100° with 0.25M sulphuric acid. The resulting sugars were converted into alditol acetates as previously described[7], and analysed by g.l.c.[7]–m.s.[8].

Methylation analysis. — The LPS (5 mg) was methylated with methylsulphinyl sodium–methyl iodide in methyl sulphoxide[9], and recovered by dialysis against distilled water followed by concentration to dryness. The methylated material was hydrolysed with 90% aqueous formic acid (3 ml) for 5 h and, after concentration to

dryness, the hydrolysis was continued for 18 h with 0.25M sulphuric acid (3 ml). After neutralisation, the methylated sugars were converted into alditol acetates and analysed by g.l.c.–m.s.[10].

Smith degradation of the LPS. — To a solution of LPS (50 mg) in an acetate buffer (pH 3.9; 40 ml) was added a solution of 5 ml of 0.2M sodium periodate. The mixture was kept in the dark at 4° for 120 h. Ethylene glycol (1 ml) was added and, after 30 min, the solution was dialysed against distilled water. The dialysed solution was concentrated to 50 ml and sodium borohydride (500 mg) was added. After 9 h, the solution was adjusted to pH 3 by the addition of acetic acid, dialysed, and lyophilised. Sugar analysis gave D-galactose and 2-acetamido-2-deoxy-D-glucose in the proportions 1.0:0.9, together with only traces of L-rhamnose and D-mannose.

The lyophilised material was dissolved in 0.25M aqueous sulphuric acid (5 ml) and left at 22° for 60 h. The hydrolysate was neutralised with Dowex 3 (HO^-) resin, sodium borohydride (50 mg) was added, and after 10 h the solution was adjusted to pH 3 by the addition of acetic acid. After concentration to 3 ml, the solution was added to the top of a column (2.6 × 100 cm) of Sephadex G-25. The fractionation was monitored by differential refractometry. The fractions containing the major, second peak were combined and lyophilised. The yield of the oligosaccharide was 6.0 mg.

Part (2 mg) of the oligosaccharide was methylated with methylsulphinyl sodium–methyl iodide in methyl sulphoxide. The permethylated oligosaccharide was recovered by partioning between chloroform and water. It gave a single peak on g.l.c. (OV 1) with a mass spectrum containing peaks at *m/e* 45 (97), 56 (33), 59 (97), 71 (100), 72 (31), 73 (11), 74 (25), 75 (47), 85 (47), 87 (61), 88 (36), 101 (92), 111 (61), 115 (61), 117 (50), 127 (67), 129 (83), 142 (61), 147 (19), 154 (31), 155 (31), 159 (92), 163 (22), 177 (22), 182 (56), 186 (28), 187 (36), 228 (89), 229 (28), 247 (8), 260 (75), 289 (39), 290 (6), 321 (0.8), 359 (1.4), 381 (2.2), 423 (3.9), 432 (0.6), 447 (1.1), and 464 (4.2). The permethylated oligosaccharide (1 mg) was hydrolysed with 90% aqueous formic acid (1 ml) for 5 h, and after concentration to dryness the hydrolysis was continued for 18 h with 0.25M sulphuric acid (1 ml). The hydrolysate was neutralised with barium carbonate and filtered, and the product was reduced with sodium borohydride and then acetylated. The resulting, partially methylated alditol acetates were analysed by g.l.c.–m.s.

Precipitin analyses. — Quantitative precipitin analyses were performed by a microprecipitin technique[13] employing a final volume of 200 μl. The tubes were incubated at 4° for 1 week. Nitrogen, in the washed precipitates, was determined by the ninhydrin procedure[14]. To each tube, 30 μg of concanavalin A (Pharmacia Fine Chemicals, Uppsala, Sweden) and 0.5–100 μg of *E. coli* O 75 LPS were added.

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