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Chemical and immunochemical studies of the O-antigen from enteropathogenic *Escherichia coli* O158 lipopolysaccharide

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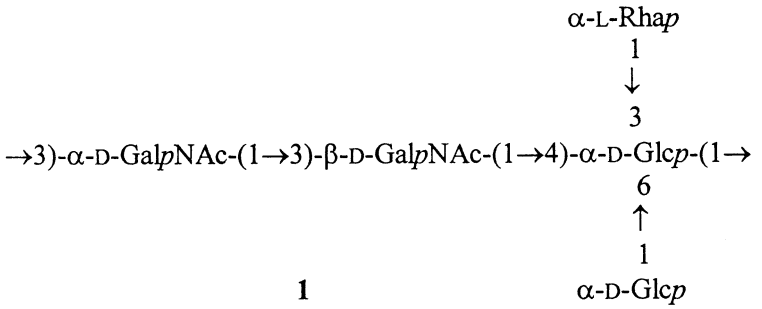
Abstract

The O-specific polysaccharide isolated from *Escherichia coli* O158 smooth lipopolysaccharide contains L-rhamnose, D-glucose and 2-acetamido-2-deoxy-D-galactose in the molar ratios 1:2:2. Studies on composition, methylation analysis and specific degradations together with a ^{1}H and ^{13}C NMR spectral study established that the O-antigen is built up from a pentasaccharide repeating unit having the following structure:



The most effective inhibitory part of the oligosaccharide from *E. coli* O158 lipopolysaccharide has been serologically characterized by an ELISA-inhibition study using different sugars. The results showed that methyl α- and β-D-Gal*p*NAc are the most effective inhibitors among the monosaccharides tested, while the main antibody specificity lies on the main-chain trisaccharide repeating unit.

Keywords: *Escherichia coli* O158; O-Specific polysaccharide; Lipopolysaccharide; ELISA

1. Introduction

Escherichia coli is one of the most opportunistic pathogens causing intestinal and extraintestinal diseases both in humans and in animals [1]. The enteropathogenic *E. coli* (EPEC) strains are known to be associated with infantile diarrhoea, which is one of the major causes of illness and death among children in developing countries [2]. The surface of the Gram-negative bacteria, of which lipopolysaccharide (LPS) is a major component, mediates its interaction with the host [3].

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Knowledge of the structures of O-specific polysaccharide from *E. coli* is therefore important in order to understand the relationship between structure and immunochemical specificity of these antigens [4]. The structures of about 60 *E. coli* serovars have already been determined [5]. We now describe the structural and immunochemical studies of the O-PS from *E. coli* O158.

2. Results and discussion

The LPS was extracted from the *E. coli* O158 cells (10 g) by the hot phenol–water method [6] and then purified [7] to afford pure LPS (300 mg, 3%). Polyacrylamide gel electrophoresis (PAGE) and subsequent blotting and immunoenzymatic development of LPS by polyclonal homologous antibodies are shown in Fig. 1. Hydrolysis of the LPS with hot dilute acetic acid (1.5%, v/v) gave lipid-A (15%), as insoluble precipitate, and a water-soluble carbohydrate fraction containing the crude O-specific polysaccharide (O-PS). The latter fraction was passed through a Sephadex G-50 column to give a polysaccharide fraction (36 mg, 42%) having a distribution coefficient K_{av} 0.12, and $[\alpha]_D$ +85° (*c* 1.0, water) and a core oligosaccharide fraction (K_{av} 0.65, 14 mg, 16%) having $[\alpha]_D$ +191° (*c* 0.5, water). The molecular weight of the O-PS was determined by comparing with dextran markers using a Sephadex G-200 column and was found to be ~10,000. Complete hydrolysis of a portion of the O-PS gave a mixture of monosaccharides. Analysis of the mixture with GLC showed the presence of L-rhamnose, D-glucose and 2-acetamido-2-deoxy-D-galactose in the molar ratio 1:2:2 (Table 1). The observed specific rotations of the sugars isolated by preparative paper chromatography confirmed the assignments for the D-glucose ($[\alpha]_D$ +50°), 2-acetamido-2-deoxy-D-galactose ($[\alpha]_D$ +90°) and L-rhamnose ($[\alpha]_D$ +7°) residues (Table 1).

The ^{1}H NMR spectrum of the O-PS had signals at 5.09 (d, *J* 2.7 Hz), 4.96 (unresolved d), 4.85 (unresolved d), 4.67 (unresolved d), and 4.54 (d, *J* 8.1 Hz), representing five anomeric protons. A signal at δ 1.35 (*J* 6.3 Hz) is characteristic of the CH_3 of L-rhamnose; whereas, two three-proton singlets of equal intensity for $NHCOCH_3$ were present at δ 2.23 and 1.98, respectively. The positions of these signals and their coupling constants indi-

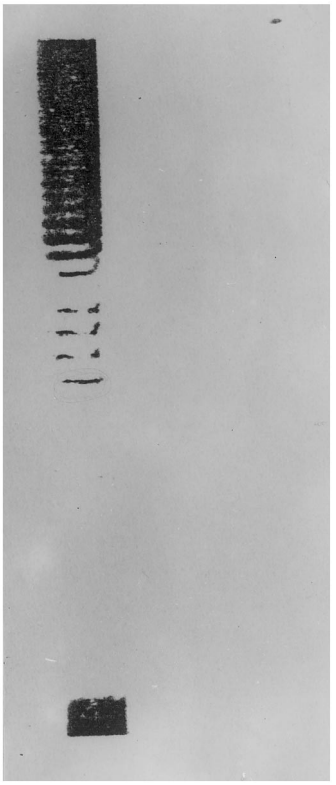

Fig. 1. Immunoblot analysis of *E. coli* O158 LPS with homologous polyclonal antibodies; 10 μg of LPS were loaded into the polyacrylamide gel and submitted to electrophoresis.

Table 1
Chemical composition of the *E. coli* O158 lipopolysaccharide

Component	Chemical composition (w/w)%		
	LPS	O-PS	$[\alpha]_D$
L-Rhamnose	13.05	19.07	+7.2° (*c* 1.0, H_2O)
D-Galactose	8.70		
D-Glucose	15.10	40.62	+51° (*c* 1.0, H_2O)
N-Acetyl-D-glucosamine	8.80		
N-Acetyl-D-galactosamine	23.80	39.56	+90° (*c* 1.0, H_2O)
L-Glycero-D-*manno*-heptose	8.70		
KDO	present		
C12:O	3.30		
C14:O	1.50		
3-OH C14:O	14.50		

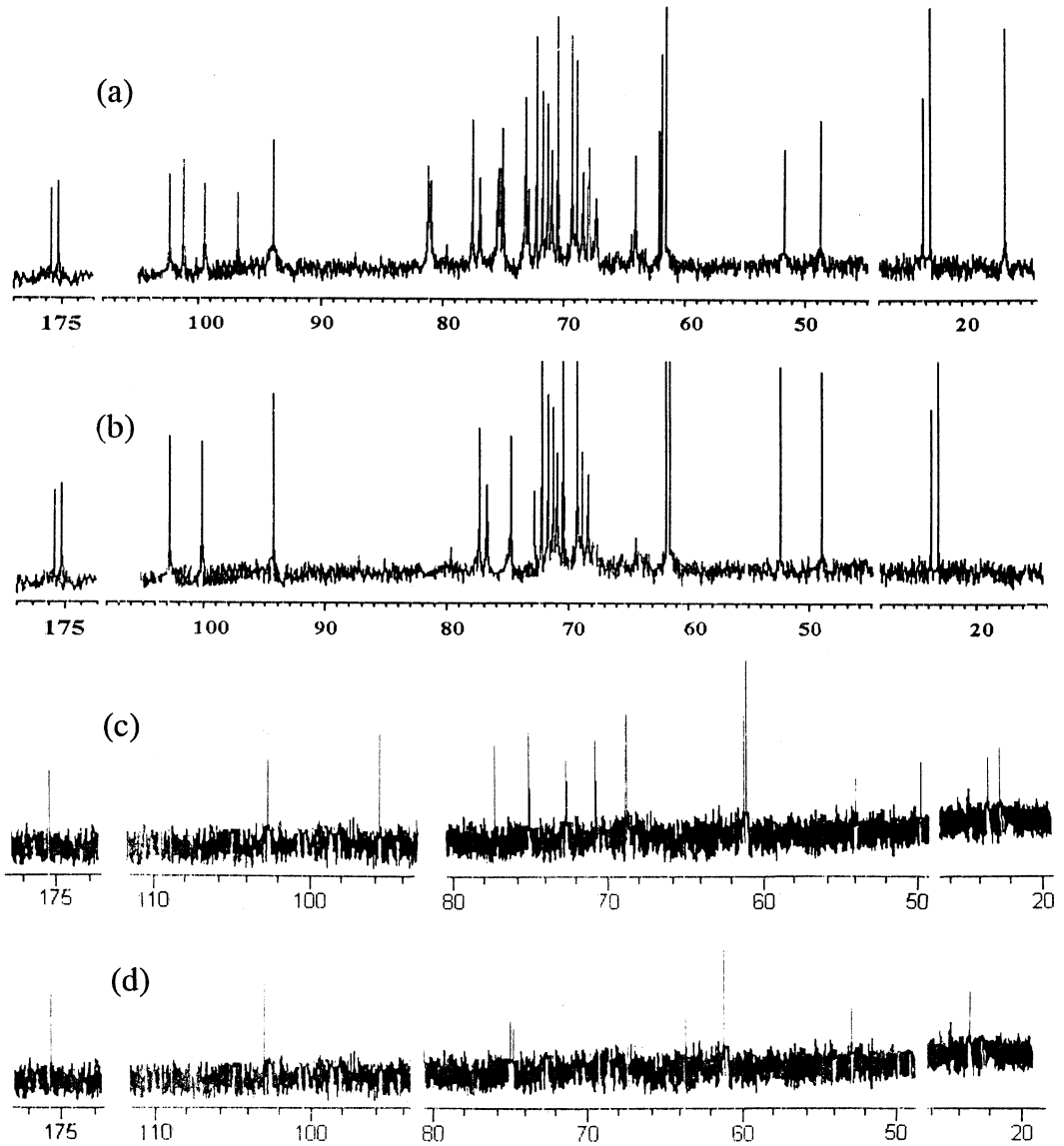

Fig. 2. (a) ^{13}C NMR spectrum of the O-PS of *E. coli* O158. (b) ^{13}C NMR spectrum of the Smith-degraded product **4**. (c) ^{13}C NMR spectrum of the second Smith-degraded product **6**. (d) ^{13}C NMR spectrum of the third Smith-degraded product **7**.

cated that the O-PS was composed of a pentasaccharide repeating unit and that there were four α-linked and one β-linked sugar moieties; the β-linkage was probably attributable to β-Gal*p*NAc or β-Glc*p*. The ^{13}C NMR spectrum (Fig. 2(a)) showed five anomeric carbon signals at δ 102.8, 101.3, 99.0, 97.1 and 94.2, respectively. The signal at δ 102.8 was probably due to a Gal*p*NAc or Glc*p* in its β configuration. The spectrum also showed two signals for the $NHCOCH_3$ groups at δ 23.3 and 22.9, one signal for the CH_3 of L-rhamnose at δ 16.5, two signals for the carbonyl groups at δ 175.8, 175.3 and two signals for the C-2 carbons of the amino sugars at δ 49.1 and 52.1, respectively. The signal in the ^{13}C NMR spectrum at δ 49.1 could be assigned to C-2 of a 2-acetamido-2-deoxy-α-D-galactopyranosyl residue and that at δ 52.1 to C-2 of a 2-acetamido-2-deoxy-β-D-galactopyranosyl residue [8–10]. Methylation analysis of the polysaccharide (Table 2) showed the presence of 2,3,4-tri-*O*-methylrhamnose, 2,3,4,6-tetra-*O*-methylglucose, 2-*O*-methylglucose, and 2-deoxy-4,6-di-*O*-methyl-2-(*N*-methyl-acetamido)-galactose in the molar ratio 1:1:1:2, demonstrating the presence of the following structural elements in the O-PS:

α-L-Rha*p*-(1→ →3)-α-D-Gal*p*NAc-(1→

D-Glc*p*-(1→ →3)-β-D-Gal*p*NAc-(1→

↓

4

→3)-D-Glc*p*-(1→

6

↑

The O-PS was treated with 0.25 M trifluoroacetic acid (TFA) at 85 °C and the hydrolysis was monitored by ^{1}H NMR spectroscopy at 15 min intervals. After 1 h, when the signal at δ 5.09 disappeared, the reaction was stopped and the degraded product was purified on a Sephadex G-50 column [11]. The signal at δ 5.09 probably represented the anomeric proton signal of the α-L-rhamnosyl moiety. This assignment was supported by the anomeric signals of α-L-rhamnopyranosyl residues reported previously [12]. Methylation analysis of the degraded product also revealed the absence of 2,3,4-tri-*O*-methylrhamnose and 2-*O*-methylglucose and appearance of 2,3-di-*O*-methylglucose (Table 2). The L-rhamnopyranosyl residue is consequently linked to the 3-position of the branching D-glucosyl residue in the probable structure **2** of the degraded product.

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

6

↑

1

2 α-D-Glc*p*

More drastic hydrolysis of the O-PS with 0.5 M TFA at 100 °C gave an oligosaccharide. NMR spectral analysis of the oligosaccharide gave signals at δ 4.94 (s), 4.84 (s) and 4.53 (d, J 7.8 Hz) representing the three anomeric protons and at δ 2.21 and 1.97 for two NHCOCH_3 signals, respectively. Composition analysis followed by methylation analysis of the oligomer revealed the presence of 2-deoxy-4,6-di-*O*-methyl-2-(*N*-methylacetamido)-D-galactose, 2-deoxy-3,4,6-tri-*O*-methyl-2-(*N*-methylacetamido)-D-galactose unit and 2,3,6-tri-*O*-methylglucose in equimolar ratio. The probable structure of the oligomer can therefore be written as **3**.

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

3

Smith degradation of the O-PS destroyed both the glucose and rhamnose residues. Composition studies and methylation analysis (Table 1) of the degraded polymeric product **4** showed that two terminal sugar residues in the O-PS were eliminated and that the 3- and 6-positions of the main-chain D-glucopyranosyl residues were exposed during the degradation. The terminal D-glucopyranosyl residue was consequently linked to O-6 of the D-glucopyranosyl residue of the main chain. These data gave information on the positions of the terminal sugar moieties and not on the structure of the main chain. The ^{13}C spectrum (Fig. 2(b)) of the Smith-degraded product contained signals at δ 103.0, 100.0 and 94.5 for three anomeric carbons, namely β-D-Gal*p*NAc, α-D-Glc*p* and α-D-Gal*p*NAc, respectively. Such assignments of the signals of β-D-Gal*p*NAc and α-D-Gal*p*NAc were reported earlier [13]. The absence of the anomeric signals at δ 101.3 and 97.1 in **4** confirmed that these two signals represent the anomeric signals of α-Rha*p* and one α-Glc*p* residue, respectively, in the origi-

Table 2
Methylation analysis of the *E. coli* O158 polysaccharide and the degraded sugars [a] (in molar ratios)

Methylated sugar	O-PS	**2**	**3**	**4**	**6**
2,3,4-Me$_3$-Rha*p*	1.0			[b]	
2,3,4,6-Me$_4$-Glc*p*	1.0	1.0			
2-Me-Glc*p*	1.0				
4,6-Me$_2$-Gal*p*N(Me)Ac	1.8	1.8	0.9	1.8	1.0
2,3-Me$_2$-Glc*p*		1.0			
2,3,6-Me$_3$-Glc*p*			1.0	1.0	
3,4,6-Me$_3$-Gal*p*N(Me)Ac			0.8		0.8

[a] 2,3,4-Me$_3$-Rha*p*, 2,3,4-tri-*O*-methyl-L-rhamnose; 2,3,4,6-Me$_4$-Glc*p*, 2,3,4,6-tetra-*O*-methyl-D-glucose, etc.
[b] Component absent.

nal O-PS. The ^{13}C NMR spectrum also contained two NHCOCH_3 signals at δ 23.7 and 23.1, two C-2 carbons of the amino sugars at δ 52.1 and 49.1, and two carbonyl groups at δ 176.9 and 175.3. The ^1H NMR spectrum of **4** gave signals at δ 5.0 (unresolved d), 4.9 (unresolved d) and 4.5 (d, J 8.1 Hz) for the anomeric protons, and at δ 2.1 and 2.0 for NHCOCH_3, respectively. The results of NMR and methylation data therefore demonstrated the presence of the trisaccharide moiety **5** in the original O-PS.

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

4

α-L-Rha*p*
1
↓
3
→4)-α-D-Glc*p*-(1→
6
↑
1
α-D-Glc*p*

5

In order to gain further knowledge on the sequence of sugar residues in the O-PS chain, the Smith-degraded material was subjected to a second Smith degradation, which, as expected, yielded the disaccharide glycoside of erythritol **6**.

α-D-Gal*p*NAc-(1→3)-β-D-Gal*p*NAc-(1→2)-erythritol

6

The ^{13}C NMR spectrum of **6** (Fig. 2(c)) altogether contained 20 signals; the easily recognisable ones being at δ 102.9 and 95.0 in the anomeric region, at δ 23.2 and 22.7 for two NHCOCH_3, at δ 54.0 and 49.8 for C-2 carbons of the amino sugars and at δ 175.7 and 175.4 for two carbonyl groups, respectively. The absence of the anomeric signal at δ 100.0 in the spectrum of **6**, when compared with that of **4**, therefore indicated that the anomeric signal at δ 100.0 in **4** was due to the α-Glc*p* residue in the main chain. The ^1H NMR spectrum of **6** contained signals at δ 5.14 (s) and 4.57 (d, J 8 Hz) for two anomeric protons and at δ 1.95 and 1.94 for two NHCOCH_3 signals, respectively. Methylation analysis of **6** gave peaks for 2-deoxy-3,4,6-tri-*O*-methyl-2-(*N*-methylacetamido)-D-galactose and one 2-deoxy-4,6-di-*O*-methyl-2-(*N*-methylacetamido)-D-galactose. The NMR data of **6** agreed well with the data reported previously for a methyl glycoside analog [14,15]. A third Smith degradation of **6** afforded **7**, whose ^1H and ^{13}C NMR spectra (Fig. 2(d)) showed characteristic anomeric signals at δ 4.56 (d, J 8.4 Hz) and δ 102.7, respectively, in agreement with the structure **7**.

β-D-Gal*p*NAc-(1→2)-glycerol

7

Based on all these data, the structure that could be assigned to the O-PS from *E. coli* O158 is represented by **1**:

α-L-Rha*p*
1
↓
3
→3)-α-D-Gal*p*NAc-(1→3)-β-D-Gal*p*NAc-(1→4)-α-D-Glc*p*-(1→
6
↑
1
α-D-Glc*p*

1

This structure **1** has some similarities to that of the O-PS from *E. coli* O25 [11], where two Gal*p*NAc and α-D-Glc*p* were replaced by α-L-Fuc*p*NAc, β-D-Glc*p*NAc and β-D-Glc*p*, respectively.

Immunochemical studies on the O-specific polysaccharide.—Antiserum against *E. coli* O158 was raised in rabbits against the heat-killed bacterial whole cells, suspended in 0.3% formalin in isotonic solution. In the passive haemagglutination test, all the antiserum containing antibodies against *E. coli* O158 showed high titre (1:8) against sensitised erythrocytes with alkali-treated LPS.

The results of ELISA-inhibition study of *E. coli* O158 LPS with homologous rabbit antiserum in the presence of different sugars are shown in Table 3. Both methyl α- and β-*N*-acetyl-D-galactosamine were found to have the higher potency, requiring only 1.35 and 1.97 μM, respectively for 50% inhibition (IC_{50}), while methyl α-D-glucopyranoside has a potency in between that of the two amino sugar moieties. Methyl α-L-rhamnopyranoside was found to have the least potency among the monosaccharide units present in the O-PS, requiring 23.70 μM for IC_{50}. The poor inhibition indicates that it contributes very little to the immunospecificity. These results were in good agreement with the proposed assignments of the anomeric configuration of the

Table 3
ELISA-inhibition assay of different sugars isolated from O-PS with LPS [a]

Inhibitor	IC_{50}
Meβ-D-Glc*p*	50.90
Me α-D-Glc*p*	1.64
Meβ-D-Gal*p*NAc	1.97
Me α-D-Gal*p*NAc	1.35
Meβ-L-Rha*p*	63.50
Me α-L-Rha*p*	23.70
→3)-α-D-Gal*p*NAc-(1→3)-β-D-Gal*p*NAc-(1→4)-α-D-Glc*p*-(1→ 6 ↑ 1 (2) α-D-Glc*p*	18.79
α-D-Gal*p*NAc-(1→3)-β-D-Gal*p*NAc-(1→4)-α-D-Glc*p* (**3**)	1.19
→3)-α-D-Gal*p*NAc-(1→3)-β-D-Gal*p*NAc-(1→4)-α-D-Glc*p*-(1→ (**4**)	1.71
α-D-Gal*p*NAc-(1→3)-β-D-Gal*p*NAc-(1→2)-erythritol (**6**)	2.29
β-D-Gal*p*NAc-(1→2)-glycerol (**7**)	2.55
O-PS (**1**)	71.54

[a] IC_{50}, concentration of the sugar required for 50% inhibition (50% inhibition value was obtained from the semilogarithmic curves).

constituent sugars in the O158 polysaccharide. Compounds **2**, **3**, **4**, **6** and **7** were isolated and their comparative inhibitory effects were assayed by ELISA-inhibition test (Table 3). The results showed that compounds **3**, **4**, **6** and **7** required only 1.19, 1.71, 2.29 and 2.55 μM, respectively for IC_{50} against LPS, whereas the polymeric compound **2** was found to have moderate potency, requiring 18.79 μM for IC_{50}. The O-PS itself was found to have very little potency against O158 LPS, requiring 71.54 μM for IC_{50}. It has been claimed that the immunogenic properties of the bacterial cell-surface polysaccharides can be expressed by carbohydrate structures smaller than the native polysaccharides [16]. In the case of the intact polysaccharide, the whole repeating unit is not immunodominant, probably because of its high molecular weight, and only a small part of it is responsible for the immunological specificity. In general, the combining site in the antibody was directed towards only a small part of a macromolecular antigen. The results indicated that the most effective inhibitor is the trisaccharide **3**. Thus **3**, and its constituent sugars, must therefore be involved in the biological repeating unit of the O158 O-PS. Figs. 3(a) and (b) demonstrates the effects of varying amounts of monosaccharides and oligosaccharides in the ELISA-inhibition study. These results thus provide information with regard to the selection of the target oligosaccharide that may be used, after conjugation with a suitable carrier molecule, for diagnostic or protective purposes.

3. Experimental

Bacterial cell culture.—The enteropathogenic strain *E. coli* O158 used in this study was obtained from the Central Research Institute, Kasauli, India. The bacteria were grown at 37 °C in beef extract (1%), peptone (1%), sodium chloride (0.5%) and agar (1.5%) in Roux bottles for 48 h. The cells were harvested at an early stationary phase by gently shaking the cultures with 0.85% saline solution and isolated by centrifugation. The bacterial cells were then thoroughly washed with water and freeze-dried.

Isolation of the LPS and preparation of the O-specific polysaccharide.—LPS was extracted from the dried cell (10 g) using the hot phe-

nol–water methodology [6]. The crude LPS (1 g) obtained from the water extract was dialysed and freeze-dried. Crude LPS was then dissolved in water and then repeatedly ultracentrifuged at 105,000*g* to remove RNA; the sediment was dispersed in water and freeze-dried to give pure LPS. LPS was then dissolved in water, followed by fractional precipitation with cetyl trimethyl ammonium bromide and centrifuged at 8,000 rpm for 30 min, the precipitate containing the nucleic acid was discarded and the supernatant dialysed and freeze-dried [7]. The resulting LPS was finally purified by passing through a column of Sephadex G-100 when 300 mg of pure LPS was obtained. The LPS (100 mg), dissolved in 1.5% (v/v) AcOH (20 mL), was heated for 2 h on a boiling water bath. The precipitated lipid-A (15 mg) was removed from the cooled solution by centrifugation and the supernatant was lyophilised. Gel permeation chromatography of the lyophilised product on a column of Sephadex G-50 gave the pure O-PS, a core oligosaccharide and a third fraction containing KDO and phosphate.

Polyacrylamide gel electrophoresis and immunoblotting.—Polyacrylamide gel electrophoresis was carried out using tris–glycine buffer, (pH 8.4), containing 0.25% sodium dodecylsulphate (SDS-PAGE) with 13% acrylamide running gel and 4% stacking gel [17]. After pre-electrophoresis at 25 mA, the samples were separated at 18 mA until the dye reached the bottom of the gel. The LPS gels were then electrotransferred to nitrocellulose sheets (Bio Rad, USA) with a Trans-blot apparatus for 18 h at 100 mA. Blocking of non-specific binding of the antibodies and staining were carried out by Sturm et al. [18].

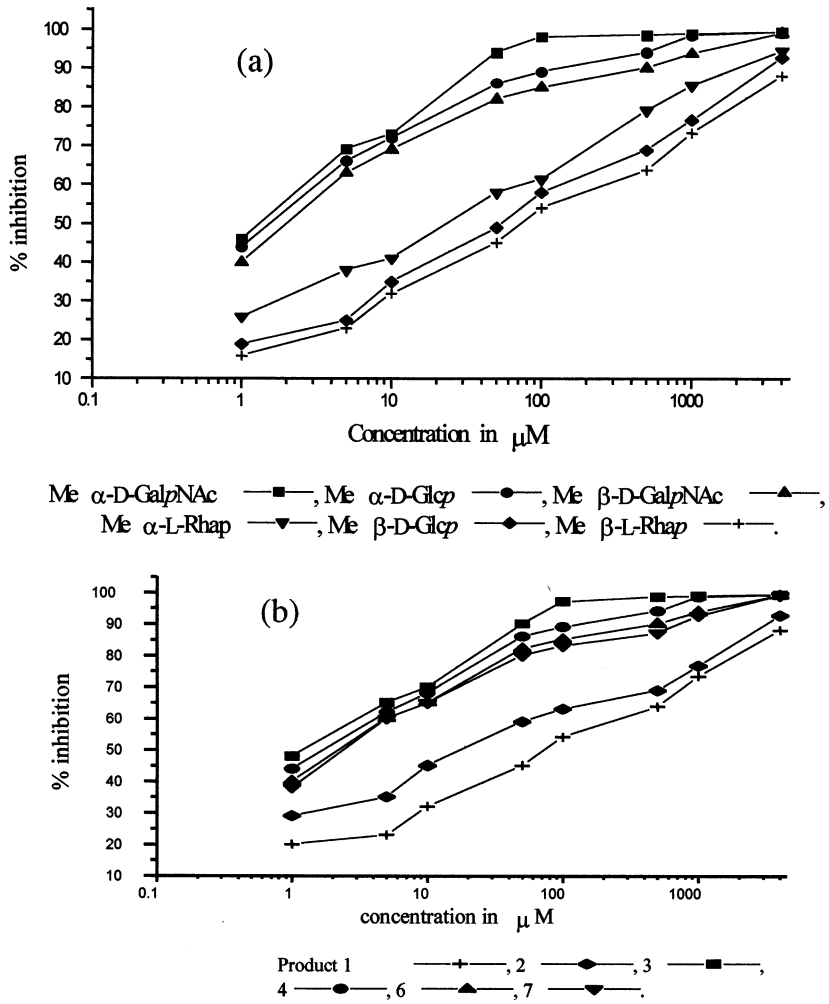

Fig. 3. ELISA-inhibition studies with (a) monosaccharides as methyl glycosides and (b) isolated products from *E. coli* O158 O-PS.

Analytical methods.—GLC was performed with a Hewlett–Packard 5890 Gas Chromatograph Series II equipped with a flame ionisation detector and an HP 3365 Series Chemstation. The sugars were resolved as their alditol acetates on an HP-1 fused silica column (0.32 mm × 30 m) at 220 °C. Partially methylated alditol acetates were characterised by GC–MS on a Fisons Instruments spectrometer fitted with a mass detector (MD 8000 series) on an HP-5 fused silica column (0.32 mm × 30 m) at 160 °C for 2 min followed by an increase of 2 °C/min to 280 °C. Paper chromatography was done on Whatman No. 1 and 3 MM paper using 10:3:3 butan-1-ol–pyridine–water. Gel filtrations were performed on Sephadex G-100 (70 × 2 cm), G-50 (70 × 2 cm) and G-15 (70 × 2 cm) columns. A Sephadex G-200 (85 × 1.5 cm) column was used for molecular-weight determination of the O-PS using different dextran markers having M_w values of 8800, 39,000, 70,000 and 503,000. Specific rotations were measured with a Perkin–Elmer 241 MC spectropolarimeter. The ^1H and ^{13}C NMR spectra were recorded with Bruker AMX 500 and DPX 300 spectrometers in D_2O.

Analysis of monosaccharide components.—The monosaccharides in the LPS or O-PS were liberated by hydrolysis with 2 M TFA at 120 °C for 2 h [19]. A portion of the hydrolysate was converted into the alditol acetates and analysed by gas liquid chromatography (GLC). Preparative paper chromatography of the rest of the hydrolysate enabled the isolation of the constituent monosaccharides.

Methylation analysis.—Samples (2–4 mg) were methylated according to the method of Ciucanu and Kerek [20]. The product in each case was isolated by partition between CH_2Cl_2 and water and was further purified by passage through a Sep-Pak C_{18} cartridge [21]. Permethylated samples were hydrolysed with 2 M TFA at 120 °C for 2 h, reduced with $NaBD_4$, acetylated with Ac_2O in pyridine, and analysed by GLC or GC–MS or both [22].

Partial acid hydrolysis of the O-PS.—The O-PS (15 mg) was treated with 0.25 M TFA at 85 °C. The hydrolysis was monitored by ^1H NMR. When the signal at δ 5.09 had disappeared, the solution was concentrated to dryness and the residue passed through a column of Sephadex G-50. The polymeric Fraction 2 (12 mg) was eluted in the void volume. In another experiment the O-PS (20 mg) was treated with 0.5 M TFA at 100 °C for 1 h, releasing **3** (10 mg), which was isolated by preparative paper chromatography on Whatman No. 3 MM paper, and purified by Sephadex G-15 column.

Periodate oxidation and Smith-degradation studies.—A solution of the O-PS (100 mg) and $NaIO_4$ (900 mg) in water (40 mL) was kept for 3 days in the dark at 25 °C. Excess periodate was reduced with ethylene glycol, and the solution was dialysed and freeze-dried. The residue was dissolved in water (20 mL), $NaBH_4$ (40 mg) was added, and the solution was kept overnight, neutralised with 50% aq AcOH, dialysed, and freeze-dried. A solution of the product (48 mg) in 0.5 M TFA (10 mL) was kept at 25 °C for 48 h, diluted with water (20 mL), and freeze-dried. The polymeric reaction product **4** (37 mg), $[\alpha]_D$ +209.5° (*c* 0.8, water) was isolated by chromatography on a column of Sephadex G-50. For the second Smith degradation, the product from the first oxidation (28 mg) was oxidised with periodate, reduced with $NaBH_4$, and hydrolysed as above. The product was fractionated on a column of Sephadex G-15, to give **6** (12 mg), $[\alpha]_D$ +179.5° (*c* 0.8, water). The third Smith degradation of **6** (11 mg) was carried out and purified on a column of Sephadex G-15 as above to give **7** (5 mg).

Preparation of antisera.—Antisera against *E. coli* O158 were raised in rabbits with heat-killed cells. The animal received four intravenous injections through the marginal ear vein with doses of 0.2, 0.5, 1.0 and 1.5 mL of bacterial suspension (10^{10} cfu mL^{-1}) at 4 day intervals. The optimum time for bleeding was 4 days after the last injection [23,24].

Enzyme-linked immunosorbent assay (ELISA).—ELISA was performed using flexible PVC microtitre plates. The plates were coated with 1 μg/50 μL of LPS in 0.01 M PBS (pH 7.4) per well and incubated overnight at 4 °C. The plates were washed three times with 0.01 M PBS containing 0.05% Tween 20 (T-PBS) and then incubated with 1% BSA in 0.01 M PBS for 1 h at 37 °C to prevent non-specific binding. Dilutions of the serum (50 μL) in wash buffer containing 1% BSA were added

to the wells. The plates were incubated for 3 h at 37 °C and washed three times with T-PBS. Horseradish peroxidase conjugated goat anti-rabbit IgG (Kirkegaard and Perry, Gaithersburg, MD) (50 μL), diluted 1:500 in 0.01 M PBS containing 1% BSA, was added to each well and incubated for 1 h at 37 °C. Excess conjugate was removed by washing the plates three times with T-PBS. Finally, 50 μL of the substrate solution (1 mg/mL *O*-phenylenediamine in citrate phosphate buffer, pH 5.0, containing 0.01% H_2O_2) was added to each well and incubated for 10 min at 37 °C in the dark. The plates were then read on a Titertek Multiscan Plus MKII photometer (Flow Laboratories, UK) at 492 nm.

ELISA-inhibitions studies.—For ELISA inhibition, microtiter plates coated with 1 μg/50 μL of LPS in PBS were treated identically as above except that 50 μL of the inhibitor–antibody mixture was used instead of the antibody alone. The inhibitor–antibody mixture consists of 50 μL of serial dilutions of inhibitor solution and 50 μL of diluted antibody (1:500) preincubated overnight at 4 °C in haemagglutination plates coated with PBS containing 1% BSA. The inhibitory effect of the sugars was calculated according to the formula:

$$A_{492}\ (\text{LPS–diluted serum in PBS}) - A_{492}\ (\text{LPS–diluted serum–inhibitor in PBS}) \times 100/A_{492}\ (\text{LPS–diluted serum in PBS})$$

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