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The structure of the O-antigen of *Escherichia coli* O116:K$^+$:H10

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Abstract

The primary structure of the O-antigen of *Escherichia coli* O116:K$^+$:H10 was shown by monosaccharide analysis, a partial hydrolysis study and by 1D and 2D ^{1}H and ^{13}C NMR spectroscopy to be composed of linear pentasaccharide repeating units with the structure:

→6)-α-D-Glc*p*NAc-(1→4)-α-D-Gal*p*NAc-(1→4)-α-D-Gal*p*A-(1→3)-β-D-Glc*p*NAc-(1→2)-β-D-Qui*p*4NAc-(1→

Keywords: *Escherichia coli*; Lipopolysaccharide; O116 antigen; Structure; NMR spectroscopy

1. Introduction

Lipopolysaccharides (LPSs) or O-antigens isolated from bacteria of the species *Escherichia coli* can be characterised according to their immune electrophoretic patterns and their chemical structures [1,2]. The majority of *E. coli* O-antigens are neutral polysaccharides, but a small number contain acidic sugars, resembling certain strains of *Shigella dysenteriae*, and are thus referred to as '*Shigella*-like'. In fact, many cross-reactions have been reported between O-antigens of *E. coli* and antigens of other pathogens such as *Shigella*, *Salmonella*, *Klebsiella* and *Vibrio cholerae* [3]. *E. coli* O116 strains have been implicated in avian colibacillosis, bovine septicaemia and verocytotoxin infections [3–6]. The O-antigen of *E. coli* O116:K$^+$:H10 is classed as a type 2a antigen on the basis of its immunoelectrophoretic behaviour and joins the group of '*Shigella*-like' antigens.

2. Results and discussion

Isolation and composition of the polysaccharide.—*E. coli* O116 bacteria were grown on Mueller–Hinton agar at 37 °C for 20 h, after which the cells were killed with phenol, isolated by centrifugation and washed. The LPS was extracted from the lysozyme and ribonuclease-treated cells using a modified version of the phenol extraction procedure [7]. The O-polysaccharide (PS) was cleaved from lipid A by mild-acid hydrolysis and was purified by GPC on Sephacryl S-200 SF.

The ^{1}H NMR spectrum of the PS (Fig. 1(a)), recorded in D_2O at 35 °C, contained three H-1 signals typical for α-linked hexapyranoses at δ 5.344 ($J_{1,2}$ 4.4 Hz), 5.009 ($J_{1,2}$ 4.4 Hz), and 4.945 ($J_{1,2}$ 3.0 Hz), and two H-1 signals typical for β-linked hexapyranoses

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at 4.890 ($J_{1,2}$ 7.9 Hz) and 4.460 ($J_{1,2}$ 6.4 Hz). In addition, signals were observed for the methyl protons of four NAc groups at δ 2.085 (6 H), 2.032 and 1.983, and a signal for H-6 of a deoxy sugar at δ 1.186 ($J_{5,6}$ 5.0 Hz). The ^{13}C NMR data complemented the ^{1}H NMR results and confirmed a pentasaccharide repeating unit for the PS, with signals at 102.69, 102.39, 101.12, 99.85 and 99.32 ppm in the anomeric region (95–105 ppm). Signals for carbonyl carbons occurred at 175.65, 175.49, 175.42, 175.14 and 175.04 ppm, indicating the presence of a uronic acid, while signals at 57.84, 55.25, 54.87 and 50.72 indicated the presence of four C–N bonds. The ^{13}C NMR spectrum (Fig. 1(b)) of the PS also contained a signal for the carbon of a methyl group of a 6-deoxy sugar at 17.47 ppm and signals for the methyl carbons of N-acetyl groups at 24.11, 23.23, 22.99 and 22.69 ppm.

Hydrolysis of the PS with 4 M CF_3CO_2H followed by GLC–MS examination of the derived alditol acetates showed the presence of GalN, GlcN and a 4-amino-4,6-dideoxyhexose. Methanolysis of the PS, followed by reduction of the methoxycarbonyl groups, hydrolysis and GLC–MS analysis of the derived alditol acetates revealed the presence of Gal in addition to the previously identified sugars, thus identifying galacturonic acid as the uronic acid.

Gal, GlcNAc and GalNAc were shown to have the D configuration by GLC analysis of the derived acetylated (−)-2-octyl glycosides [8]. A sample of the PS was partially hydrolysed with 0.5 M CF_3CO_2H and, after N-acetylation of the sample, the 4-acetamido-4,6-dideoxyhexose was isolated by column chromatography on Toyopearl TSK-40. The sugar was identified as Qui4NAc (4-acetamido-4,6-dideoxyglucose) by 1D and 2D ^{1}H and ^{13}C spectroscopy, using COSY [9] and HMQC [10] experiments (data presented in Table 1). The $[\alpha]_D$ was +43° (H_2O), indicat-

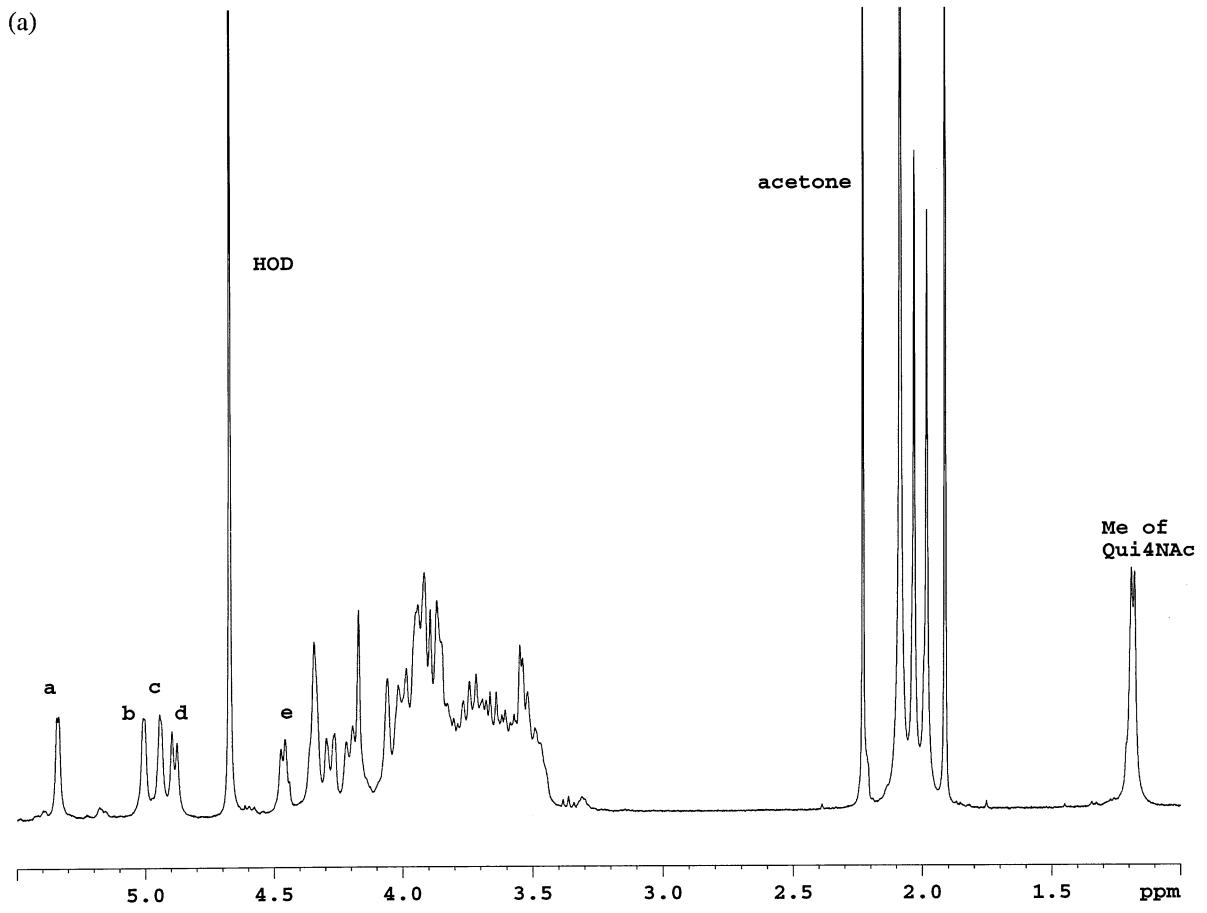

Fig. 1. (a) ^{1}H NMR spectrum of the PS in D_2O at 35 °C. For **a**, **b**, **c**, **d**, **e**, see text. (b) ^{13}C NMR spectrum of the PS in D_2O at 35 °C (see next page).

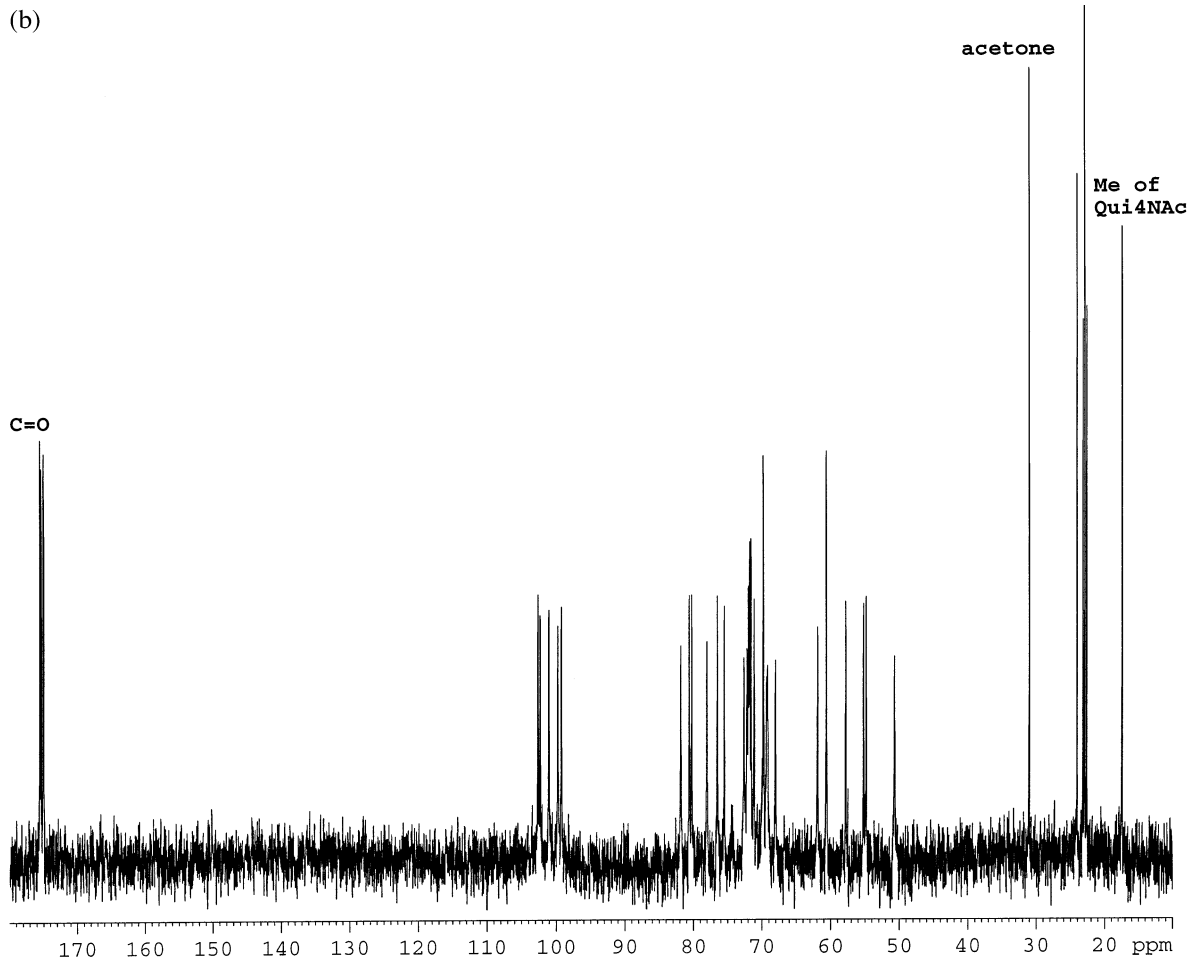

Fig. 1. (*Continued*)

ing that the sugar had the D configuration. A second fraction isolated from the partial hydrolysis proved to be a mixture of a pentasaccharide and a tetrasaccharide; this will be discussed at a later stage.

2D NMR spectroscopy of the PS.—The sequence and linkage positions of the residues in the repeating unit of the PS were established by 2D NMR experiments recorded on a solution of the PS at 35 °C. The residues were labelled **a**–**e** in order of the decreasing chemical shift of their anomeric protons. Carbon and proton resonances were established from COSY, HOHAHA [11], HMQC, HMQC–TOCSY (run with short and long mixing times) [12], HMBC [13] and NOESY [14] experiments. The NMR data for the PS are collected in Table 2 and the HMQC contour plot shown in Fig. 2.

Residue **a** *[→4)-α-*D*-Gal*p*A].*—^{1}H resonances for H-1,2 and H-5,4,3 of this residue were readily assigned from the COSY spectrum. Although the H-4/5 coupling constant was small, as expected for a Gal-type residue, the cross-peak was clearly visible in the COSY spectrum. The ^{1}H assignments were confirmed in the HOHAHA spectrum. In spite of some congestion in the HMQC spectrum, the ^{13}C resonances for C-1 to C-5 of this residue could be assigned by comparing ^{1}H assignments with the ^{1}H–^{13}C correlation data. The assignments for the C-2 and C-3 resonances were confirmed from the HMQC–TOCSY spectrum, which showed clear correlations from H-1 to C-1, C-2 and C-3. The C-6 resonance was assigned from the HMBC spectrum, which showed a clear correlation from H-5 to the carbonyl signal at 175.14 ppm.

Residue **b** *[→4)-α-*D*-Gal*p*NAc].*—The ^{1}H resonances for H-1,2,3,4 of this residue were assigned from the COSY and HOHAHA spectra. The corresponding carbon values

were assigned by comparing ^{1}H assignments with the ^{1}H–^{13}C correlation data from the HMQC spectrum, with confirmation from the HMQC–TOCSY spectrum. In particular, the C-2 track showed clear correlations to the H-1,2,3,4 signals. The H-5 resonance was assigned from the H-3/5 and H-4/5 cross-peaks in the NOESY spectrum. The H-6 resonances could then be assigned from the COSY spectrum and the corresponding ^{13}C resonances from the HMQC spectrum. These assignments were confirmed from the HMQC–TOCSY spectrum, which showed correlations between H-5 and C-6, and from the HMBC spectrum, which showed correlations between H-1 and C-5. The ^{13}C resonance of the NAc carbonyl signal was assigned from the correlation between H-2 of residue **b** and this carbon in the HMBC spectrum.

Residue **c** *[→6)-α-*D*-Glc*p*NAc].*—^{1}H resonances for H-1,2,3,4 were assigned from the cross-peaks in the COSY spectrum and were confirmed in the HOHAHA spectra. The H-6 resonances were assigned from the NOESY spectrum, which showed correlations from H-4 to H-6$_a$ and H-6$_b$. The H-5 resonance could now be traced in both the COSY and HOHAHA spectra. The corresponding ^{13}C resonances were assigned from the HMQC spectrum. The ^{13}C resonance of the NAc carbonyl signal was assigned from the correlation between H-2 of residue **c** and this carbon in the HMBC spectrum.

Residue **d** *[→3)-β-*D*-Glc*p*NAc].*—All the ^{1}H resonances for this residue were readily assigned from the COSY spectrum and confirmed from the HOHAHA spectrum. Magnetisation relayed well through the spin system, as expected for the β-*gluco* configuration, and all cross-peaks were clearly visible. The ^{13}C resonances were assigned from the HMQC data. The ^{13}C resonance of the NAc carbonyl signal was assigned from the correlation between H-2 of residue **d** and this carbon in the HMBC spectrum.

Residue **e** *[→2)–β-*D*-Qui*p*4NAc].*—^{1}H resonances for residue **e** were assigned from the COSY spectrum. H-1,2,3,4 were traced via their cross-peaks starting from H-1, while the H-5 resonance was assigned from the H-5/6 cross-peak. The values obtained showed that H-3 and H-5 were very close and that the H-4/5 cross-peak was obscured. These assignments were confirmed from the HOHAHA spectrum. ^{13}C resonances for C-1, C-2 and C-6 were obtained from the HMQC spectrum. Due to ^{1}H signal overlap, ^{13}C resonances for the other carbons were assigned with the help of the HMQC–TOCSY spectrum, run with a shorter mixing time to show fewer correlations. The HMQC–TOCSY spectrum showed correlations in the H-1 track to C-1,2,3 and in the H-6 track to C-4,5,6. This confirms the position of the NAc group as being at C-4. The ^{13}C resonance of the NAc carbonyl signal was assigned from the correlation between H-4 of residue **e** and this carbon in the HMBC spectrum.

Comparison of the chemical shift data for residues **a**–**e** with those reported for methyl glycosides [15–17] permitted identification of

Table 1
NMR data [a] for Qui4NAc

Residue	Proton or carbon							
		1	2	3	4	5	6	Me of NAc
α-Qui4NAc	H	5.216	3.603	3.707	3.575	3.965	1.154	2.050
	^3J [b]	3.9	9.5	9.5		6.4		
	C	92.68	72.80	71.36	57.45	67.32	17.62	22.83
β-Qui4NAc	H	4.613	3.299	3.494	3.572	3.570	1.193	2.041
	^3J	8.1	10.0	9.5		5.6		
	C	96.38	75.49	74.36	57.44	71.78	17.62	22.86

[a] Chemical shifts in ppm with acetone as internal standard, δ 2.23 and 31.07 ppm for ^{1}H and ^{13}C, respectively.
[b] Coupling constants in Hz.

Table 2
Chemical shift data [a] for the PS

Residue		Proton or carbon							
		1	2	3	4	5	6a	6b	NAcC=O
→4)-α-D-Gal*p*A (**a**)	H	5.344	3.844	3.926	4.338	4.174			
	C	101.12	69.22	69.83	**80.67** [b]	72.16	175.14		
→4)-α-D-Gal*p*NAc (**b**)	H	5.009	4.278	4.004	4.067	4.346	3.678	3.678	175.65
	C	99.85	50.72	68.07	**78.09**	72.68	60.66		
→6)-α-D-Glc*p*NAc (**c**)	H	4.945	3.954	3.885	4.208	3.926	4.012	3.902	175.04
	C	99.32	54.87	71.16	72.00	69.83	**69.36**		
→3)-β-D-Glc*p*NAc (**d**)	H	4.890	3.869	3.744	3.644	3.471	3.779	3.946	175.49
	C	102.39	55.25	**81.91**	71.82	76.55	61.93		
→2)-β-D-Qui*p*4NAc (**e**)	H	4.460	3.604	3.535	3.553	3.530	1.186		175.42
	C	102.69	**80.31**	75.63	57.84	71.53	17.47		

[a] Chemical shifts in ppm with acetone as internal standard, δ 2.23 and 31.07 ppm for ^{1}H and ^{13}C, respectively.
[b] Linkage carbons are indicated in bold.

residue **a** as 4-linked α-GalA, residue **b** as 4-linked α-GalNAc, residue **c** as 6-linked α-GlcNAc, residue **d** as 3-linked β-GlcNAc, and residue **e** as 2-linked β-Qui4NAc.

The sequence of the residues in the repeating unit was established from the HMBC spectrum, which showed clear correlations between H-1 of residue **a** and C-3 of residue **d**, between H-1 of residue **b** and C-4 of residue **a**, between H-1 of residue **c** and C-4 of residue **b**, between H-1 of residue **d** and C-2 of residue **e**, and between H-1 of residue **e** and C-6 of residue **c**. The NOESY experiment confirmed this sequence information, showing clear inter-residue NOEs between H-1 of residue **a** and H-3 of residue **d**, between H-1 of residue **b** and H-4 of residue **a**, between H-1 of residue **c** and H-4 of residue **b**, between H-1 of residue **d** and H-2 of residue **e**, and between H-1 of residue **e** and H-6_a and H-6_b of residue **c**. HMBC correlations are presented in Table 3 and NOE data in Table 4.

The combined chemical and NMR data permit the structure of the pentasaccharide repeating unit of the *E. coli* O116 O-specific polysaccharide to be written as:

c **b** **a** **d** **e**
→6)-α-D-Glc*p*NAc-(1→4)-α-D-Gal*p*NAc-(1→4)-α-D-Gal*p*A-(1→3)-β-D-Glc*p*NAc-(1→2)-β-D-Qui*p*4NAc-(1→

Partial hydrolysis studies of the PS.—The PS was partially hydrolysed with 0.5 M CF_3CO_2H and, after N-acetylation of the sample, both Qui4NAc and an oligosaccharide fraction were isolated from the hydrolysate by column chromatography. The oligosaccharide fraction proved to contain mainly tetrasaccharide (TS) with a small quantity of pentasaccharide. The structure of the TS was determined by 1D and 2D ^{1}H and ^{13}C NMR spectroscopy, using COSY, HOHAHA (data obtained with short and long mixing times), HMQC and NOESY experiments. The ^{1}H NMR spectrum of the oligosaccharide (Fig. 3) contained H-1 signals at δ 5.399 ($J_{1,2}$ 3.7 Hz), 5.354 ($J_{1,2}$ 4.0 Hz), 5.166 ($J_{1,2}$ 3.4 Hz), 5.012 ($J_{1,2}$ 3.6 Hz), 4.964 ($J_{1,2}$ 3.4 Hz) and 4.734 ($J_{1,2}$ 7.9 Hz). A partial H-1 signal was also visible at δ 4.445, corresponding to a small residual amount of Qui4NAc. The anomeric signals were labelled, in order of decreasing chemical shift, as **a**, **a′**, **c**, **b**, $\mathbf{d}_\alpha$ and $\mathbf{d}_\beta$, in order to relate them to the residues already identified in the PS. In addition, the ^{1}H NMR spectrum contained three signals for the methyl protons of

NAc groups at δ 2.093, 2.085 and 1.991. These results indicated that the main component of the oligosaccharide mixture was a tetrasaccharide. The NMR data of the TS are given in Table 5.

Residues **a** *and* **a**′ *[→4)-*D*-Gal*p*A].*—The ^{1}H resonances for these residues were easily traced via their cross-peaks in the COSY and HOHAHA spectra. ^{13}C resonances were assigned by comparing the ^{1}H assignments with the ^{1}H–^{13}C correlation data obtained from the HMQC experiment. Residues **a** and **a**′ appear as partial signals because they are linked to the reducing end of the TS, i.e. the β- and α-GlcNAc residues $\mathbf{d}_\beta$ and $\mathbf{d}_\alpha$, respectively.

Residue **b** *[→4)-α-*D*-Gal*p*NAc].*—The ^{1}H resonances for H-1,2,3,4 were assigned from the cross-peaks in the COSY and HOHAHA spectra, and did not differ greatly from their positions in the original polysaccharide. The H-5 resonance was assigned from the H-4/5 and H-3/5 cross-peaks in the NOESY spectrum. The H-5 and the H-6a and H-6b resonances were then obtained from the COSY spectrum. ^{13}C resonances were obtained from the HMQC spectrum by comparison with the ^{1}H assignments.

Residue **c** *[α-*D*-Glc*p*NAc].*—^{1}H resonances for residue **c** were readily traced in the COSY spectrum and were confirmed in the HOHAHA experiments. The corresponding ^{13}C resonances were assigned from the HMQC spectrum.

Residue $\mathbf{d}_\alpha$ *[→3)-α-*D*-Glc*p*NAcOH].*—The ^{1}H resonances for H-1,2,3,4 of this residue were assigned from the COSY spectrum while H-5 was obtained from the HOHAHA spectra. No assignments were possible for the H-6 resonances. The ^{13}C resonances were assigned from the HMQC spectrum.

Residue $\mathbf{d}_\beta$ *[→3)-β-*D*-Glc*p*NAcOH].*—In spite of considerable signal overlap, all the resonances for this residue were assigned. The

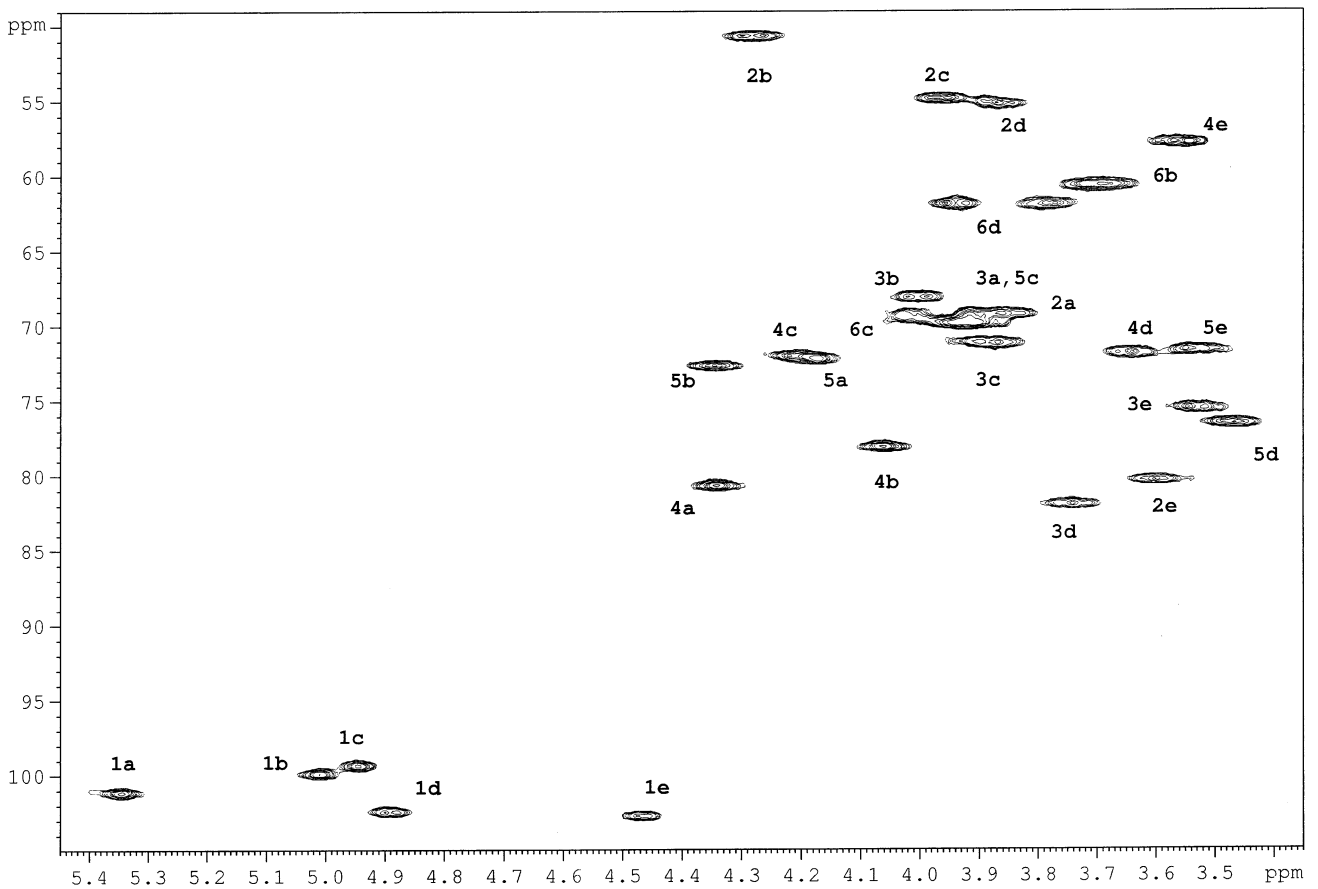

Fig. 2. Partial contour plot of the HMQC experiment on the PS. **1a** denotes the cross-peak observed between H-1 and C-1 of residue **a**, etc.

H-1,2 resonances and H-5,6_a,6_b resonances were traced in the COSY spectrum and the other resonances were obtained from the HOHAHA spectra. The ^{13}C resonances were assigned from the HMQC spectrum by comparison.

From the above assignments it was deduced that the major product of partial hydrolysis was a tetrasaccharide. The linkage positions of the residues in the tetrasaccharide were established as being C-4 of residues **a** and **a′**, C-4 of residue **b** and C-3 of residues $\mathbf{d}_\alpha$ and $\mathbf{d}_\beta$, by the significant deshielding of these C atoms.

The sequence of residues was established from the NOESY spectrum. Correlations were observed between H-1 of residue **a** and H-3 of residue $\mathbf{d}_\beta$, between H-1 of residue **a′** and H-3 of residue $\mathbf{d}_\alpha$, between H-1 of residue **c** and H-4 of residue **b**, and between H-1 of residue **b** and H-4 of residues **a** and **a′**.

These data, combined with those already obtained for the PS, permit the structure of the TS to be written as:

c **b** **a,a′** $\mathbf{d}_{\alpha,\beta}$

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

3. Conclusions

The *E. coli* O116 O-polysaccharide joins a small group of acidic O-antigens found within this bacterial species. Within this group, neuraminic acid and GlcA are the most commonly reported acidic moieties [18], while GalNAcA occurs in *E. coli* O121 [19]. The *E. coli* O116 O-antigen is the first of this group to contain GalA. Amino sugars are common amongst the *E. coli* O-antigens, but Qui4NAc is found in only one other *E. coli* O-antigen viz., *E. coli* O7 [20]. *E. coli* O121 [19] and O123 [21] contain Qui4N (4-amino-4,6-dideoxy-D-glucose) amidically linked to glycine and alanine, respectively. The *E. coli* O116 O-antigen has been reported to cross-react with the O123 antigen [1,2], but there areno structural similarities between the two polysaccharides other than the presence of the Qui4N component.

Table 3
Two- and three-bond ^1H–^{13}C correlations for the PS [a]

Residue	Proton	Correlation to
→4)-α-D-Gal*p*A (**a**)	H-1	**81.91** (**d**; C-3), 69.83 (**a**; C-3), 72.16 (**a**; C-5)
	H-4	**99.85** (**b**; C-1), 69.22 (**a**; C-2), 69.83 (**a**; C-3)
→4)-α-D-Gal*p*NAc (**b**)	H-1	**80.67** (**a**; C-4), 68.07 (**b**; C-3), 72.68 (**b**; C-5)
	H-4	**99.32** (**c**; C-1), 50.72 (**b**; C-2), 68.07 (**b**; C-3)
	H-5	78.09 (**b**; C-4), 60.66 (**b**; C-6)
→6)-α-D-Glc*p*NAc (**c**)	H-1	**78.09** (**b**; C-4), 71.16 (**c**; C-3)
	H-2	71.16 (**c**; C-3)
	H-3	54.87 (**c**; C-2), 69.83 (**c**; C-5)
→3)-β-D-Glc*p*NAc (**d**)	H-1	**80.31** (**e**; C-2)
	H-2	102.39 (**d**; C-1), 81.91 (**d**; C-3)
	H-3	**101.12** (**a**; C-1), 55.25 (**d**; C-2), 71.82 (**d**; C-4)
→2)-β-D-Qui*p*4NAc (**e**)	H-1	**69.36** (**c**; C-6)
	H-2	**102.39** (**d**; C-1), 75.63 (**e**; C-3)
	H-4	75.63 (**e**; C-3), 71.53 (**e**; C-5)

[a] Inter-residue correlations are in bold.

Table 4
NOE data for the PS [a]

Residue	Proton	NOE to
→4)-α-D-Gal*p*A (**a**)	H-1	3.844 (**a**; H-2), **3.744** (**d**; H-3)
	H-3	4.174 (**a**; H-5)
	H-4	3.844 (**a**; H-2)
→4)-α-D-Gal*p*NAc (**b**)	H-1	4.278 (**b**; H-2), **4.338** (**a**; H-4)
	H-3	4.346 (**b**; H-5)
	H-4	4.346 (**b**; H-5), 3.678 (**b**; H-6)
→6)-α-D-Glc*p*NAc (**c**)	H-1	3.954 (**c**; H-2), **4.067** (**b**; H-4)
	H-4	4.012 (**c**; H-6_a), 3.902 (**c**; H-6_b)
→3)-β-D-Glc*p*NAc (**d**)	H-1	**3.604** (**e**; H-2), 3.744 (**d**; H-3), 3.471 (**d**; H-5)
	H-3	3.471(**d**; H-5)
→2)-β-D-Qui*p*4NAc (**e**)	H-1	**4.012** (**c**; H-6_a), **3.902** (**c**; H-6_b), 3.535 (**e**; H-3)

[a] Inter-residue NOEs are in bold.

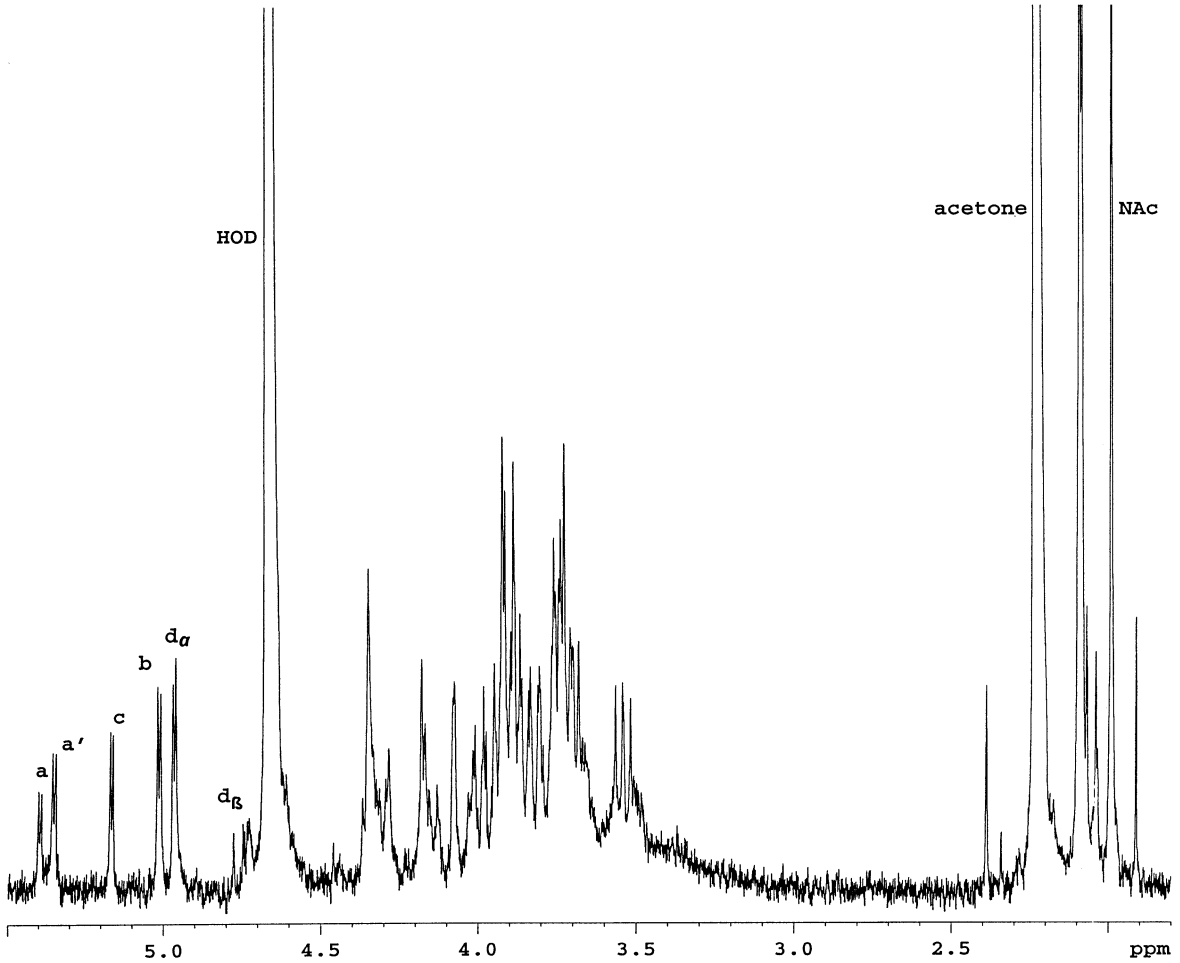

Fig. 3. ^{1}H NMR spectrum of the TS in D_2O at 35 °C. For **a**, **b**, **c**, **d**, see text.

4. Experimental

General methods.—Analytical GLC was performed on a Hewlett–Packard 5890A gas chromatograph fitted with flame-ionisation detectors and a 3392A recording integrator, with He as carrier gas. A J&W Scientific fused-silica DB-17 bonded-phase capillary column (30 m × 0.25 mm, film thickness 0.25 μm) was used for separating alditol acetates and partially methylated alditol acetates (programme I), and acetylated octyl glycosides (programme II). The temperature programmes used were: (1) 180 °C for 2 min, then 3 °C/min to 240 °C; (2) 180 °C for 2 min, then 2 °C/min to 240 °C. The identification of all derivatives was determined by comparison with authentic standards and confirmed by GLC–MS on a Hewlett–Packard 5988A instrument, using the appropriate column. Spectra were recorded at 70 eV and an ion-source temperature of 200 °C.

Polysaccharide samples were hydrolysed with 4 M CF_3CO_2H for 1 h at 125 °C. Alditol acetates were prepared by reduction of the products in aq solns of hydrolysates with $NaBH_4$ for 1 h followed by acetylation with 2:1 Ac_2O–pyridine for 1 h at 100 °C. Samples were methanolysed by refluxing with methanolic 3% HCl for 16 h, followed by treatment with $NaBH_4$ in dry MeOH to effect carboxyl reduction.

Preparation of the O116 polysaccharide.—An authentic culture of *E. coli* O116 (test strain 28w) was propagated on Mueller–Hinton agar (9 trays, 30 × 60 cm, each inoculated with 10 mL liquid culture) at 37 °C for 20 h. The bacterial cells were harvested and mixed

Table 5
Chemical shift data [a] for the TS

Residue		Proton or carbon						
		1	2	3	4	5	6a	6b
→4)-α-D-Gal*p*A (**a**)	H	5.399	3.864	3.926	4.347	4.168		
	C	100.91	71.27	~69.83	**80.73** [b]	72.35		
→4)-α-D-Gal*p*A (**a′**)	H	5.354	3.873	3.932	4.341	4.174		
	C	101.43	71.20	~69.83	**80.83**	72.94		
→4)-α-D-Gal*p*NAc (**b**)	H	5.012	4.301	3.998	4.076	4.350	3.673	3.732
	C	99.96	50.73	68.17	**78.20**	72.73	60.67	
→3)-α-D-Glc*p*NAcOH (**d_α**)	H	5.166	3.990	3.923	3.702	3.896		
	C	91.84	53.22	**80.37**	71.64	72.20		
α-D-Glc*p*NAc (**c**)	H	4.964	3.934	3.865	3.538	4.137	3.743	3.819
	C	99.28	54.80	72.06	70.56	72.85	61.15	
→3)-β-D-Glc*p*NAcOH (**d_β**)	H	4.734	3.748	3.745	3.686	3.499	3.746	3.904
	C	95.87	55.97	**81.97**	70.75	76.39		

[a] Chemical shifts in ppm with acetone as internal standard, δ 2.23 and 31.07 ppm for ^1H and ^{13}C, respectively.
[b] Linkage carbons are indicated in bold.

with an equal volume of aq 2% phenol. The suspension was stirred (24 h) at 4 °C, dialysed (6000–8000 MW cut-off) against running water for 48 h to remove phenol, and the slurry was freeze-dried. The dried bacterial cells (20 g) were suspended in 200 mL aq 50 mM Na EDTA containing 0.05% w/v NaN_3 and stirred in a Waring blender at top speed for 1 min. Hen egg-white lysozyme (0.15 g, Sigma, 50,000 units/mg) was added and the suspension stirred for 16 h at 4 °C, after which the suspension was heated for 10 min at 37 °C and blended again for 3 min. The volume was adjusted to 400 mL with aq 20 mM $MgCl_2$ and bovine pancreas ribonuclease (Sigma) was added to a final concentration of 1 μg/mL. After incubating the suspension for 10 min at 37 °C and then for 10 min at 60 °C, the suspension was freeze-dried and the dried cells were extracted using a modified version of the phenol extraction method [7]. The solid material was resuspended in 400 mL H_2O, the suspension was heated to 70 °C and added to an equal volume of a 90% w/v solution of phenol, also at 70 °C. The resulting suspension was stirred at 70 °C for 30 min, cooled to room temperature (rt) and dialysed against running H_2O (6000–8000 MW cut-off) for 4 days. The solution was filtered to remove solid matter and freeze-dried. The crude polysaccharide was resuspended in 100 mL H_2O and centrifuged at 105,000*g* for 16 h in a Beckman model L8-80M ultracentrifuge. The pellet was collected, resuspended in 50 mL H_2O and centrifuged a second time. The final pellet (16 g) was suspended in 1% AcOH (100 mL) and heated at 90 °C for 90 min, after which the solid material was removed by centrifugation (25,000 rpm) and the supernatant was dialysed against running water (3500 MW cut-off) for 24 h and freeze-dried. The crude mixture of O-antigen, KDO and core oligosaccharide (992 mg) was purified by GPC on Sephacryl S-200 SF to yield 768 mg of the O-polysaccharide, PS.

Partial hydrolysis.—O116 PS (40 mg) was hydrolysed with aq. 0.5 M CF_3CO_2H (20 mL) at 100 °C for 1 h and the hydrolysate in water (5 mL) containing MeOH (0.5 mL) was N-reacetylated by treatment with Ac_2O (2 mL) for 2 h at rt [22]. After concentration of the solution, the residue in water (2 mL) was treated with NH_3 (0.5 mL) before being subjected to GPC on Toyopearl TSK-40. Two fractions were obtained, one containing mainly a tetrasaccharide (3 mg) and the second comprising pure Qui4NAc (9.5 mg). The tetrasaccharide was rechromatographed under the same conditions to obtain a purer sample.

The identity of the Qui4NAc was confirmed by NMR data and had $[\alpha]_D + 43°$ (*c*. 0.95, H_2O).

NMR spectroscopy.—Samples were deuterium-exchanged by freeze-drying several times from D_2O and then examined as solutions in 99.99% D_2O containing a trace of acetone as internal standard (δ 2.230 for ^1H and 31.07 ppm for ^{13}C). Spectra were recorded at 35 °C on a Bruker AMX-400 spectrometer equipped with an X32 computer. The parameters used for 2D experiments performed on the PS were as follows: COSY45 [256 × 2048 data matrix, zero-filled to 1024 data points in t_1; 96 scans per t_1 value; spectral width, 1811.6 Hz; recycle delay, 1.0 s; unshifted sine-bell filtering in t_1 and t_2]; HOHAHA [256 × 1024 data matrix, zero-filled to 1024 data points in t_1; 96 scans per t_1 value; spectral width, 1811.6 Hz; recycle delay, 1.0 s; mixing time 90 ms; shifted sine-squared filtering in t_1 and t_2]; NOESY [512 × 1024 data matrix, zero-filled to 1024 data points in t_1; 80 scans per t_1 value; spectral width, 1811.6 Hz; recycle delay, 1.0 s; mixing time 100 ms; shifted sine-squared filtering in t_1 and t_2]; HMQC and HMBC [256 × 4096 data matrix, zero-filled to 1024 data points in t_1; 88 or 80 scans per t_1 value; recycle delay 1.0 s; fixed delay 3.45 ms; spectral width in t_1 11,068.2 Hz (HMQC) and 17,106.0 Hz (HMBC) and in t_2 1811.6 Hz; shifted sine-squared filtering in t_1 and t_2]; HMQC-TOCSY [512 × 4096 data matrix, zero-filled to 1024 data points in t_1; 76 or 80 scans per t_1 value; recycle delay 1.0 s; fixed delay 3.45 ms; mixing delay 25 or 90 ms; spectral width 1811.6 Hz in t_2 and 11,068.2 Hz in t_1; shifted sine-squared filtering in t_1 and t_2].

The parameters used for 2D experiments performed on Qui4NAc were as follows: COSY [256 × 2048 data matrix, zero-filled to 1024 points in t_1; 140 scans per t_1 value; spectral width 1818.2 Hz; recycle delay 1.0 s; unshifted sine-bell filtering in t_1 and t_2]; HMQC [256 × 4096 data matrix, zero-filled to 1024 data points in t_1; 96 scans per t_1 value; spectral width 1742.2 Hz in t_2 and 11,068.2 Hz in t_1; recycle delay 1.0 s; fixed delay 3.45 ms; shifted sine-squared filtering in t_1 and t_2].

The parameters used for 2D experiments performed on the tetrasaccharide were as follows: COSY [256 × 2048 data matrix, zero-filled to 1024 data points in t_1; 160 scans per t_1 value; spectral width 1818.2 Hz; recycle delay 1.0 s; unshifted sine-bell filtering in t_1 and t_2]; HOHAHA [256 × 4096 data matrix, zero-filled to 1024 data points in t_1; 128 scans per t_1 value; spectral width 1818.2 Hz; recycle delay 1.0 s; mixing times 39 and 90 ms; shifted sine-squared filtering in t_1 and t_2]; HMQC [256 × 2048 data matrix, zero-filled to 1024 data points in t_1; 180 scans per t_1 value; spectral width 1818.2 Hz in t_2 and 11,068.2 Hz in t_1; recycle delay 1.0 s; fixed delay 3.45 ms; shifted sine-squared filtering in t_1 and t_2]; NOESY [512 × 2048 data matrix, zero-filled to 1024 data points in t_1; 128 scans per t_1 value; spectral width 1818.2 Hz; recycle delay 1.0 s; mixing time 800 ms; shifted sine-squared filtering in t_1 and t_2].

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