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Structural studies on the *Shigella*-like *Escherichia coli* O121 O-specific polysaccharide

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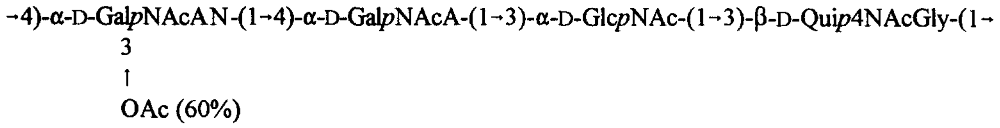
Abstract

The O-specific polysaccharide isolated from the lipopolysaccharide (LPS) of *Escherichia coli* O121 by mild acid hydrolysis has been studied using mainly NMR spectroscopy. The polysaccharide was treated with mild base to yield a *O*-deacetylated polysaccharide which contained D-GlcNAc, D-GalNAcA, D-GalNAcAN (2-acetamido-2-deoxy-D-galacturonamide) and D-Qui4NAcGly (where D-Qui4N is 4-amino-4,6-dideoxy-D-glucose) in equimolar proportions. The presence of the amide was confirmed by recording the ^{1}H NMR spectrum of the *O*-deacetylated polysaccharide at different pH values. The *O*-acetyl group was located on O-3 of the GalNAcAN and the structure of the polysaccharide can be written as



$$\rightarrow 4)\text{-}\begin{array}[t]{l}\alpha\text{-D-Gal}p\text{NAcAN}\\ \quad 3\\ \quad\uparrow\\ \quad\text{OAc (60\%)}\end{array}\text{-}(1\rightarrow 4)\text{-}\alpha\text{-D-Gal}p\text{NAcA-}(1\rightarrow 3)\text{-}\alpha\text{-D-Glc}p\text{NAc-}(1\rightarrow 3)\text{-}\beta\text{-D-Qui}p\text{4NAcGly-}(1\rightarrow$$

This structure is almost identical to that previously reported for the O-specific polysaccharide of *Shigella dysenteriae* type 7 LPS, the only difference being that *O*-acetylation is stoichiometric in the latter. © 1997 Elsevier Science Ltd.

Keywords: *E. coli*; *Shigella*; O-Specific polysaccharide; Structure; Lipopolysaccharide

1. Introduction

O-Antigens (lipopolysaccharides, LPS) of *Escherichia coli* show serological cross-reactions with O-antigens of bacteria belonging to several different genera, e.g. *Shigella*, *Salmonella*, and *Klebsiella* [1]. Although most *E. coli* O-specific polysaccharides are composed only of neutral sugars, some contain acidic sugars and in this respect they resemble the O-polysaccharides of certain strains of *Shigella*. A number of these enteroinvasive *E. coli* strains cause dysentery-like diseases and have O-antigens which are closely related to those of *Shigella boydii* and *Shigella dysenteriae* [1]. This cross-reactivity can be explained in part by the structural similarity of their

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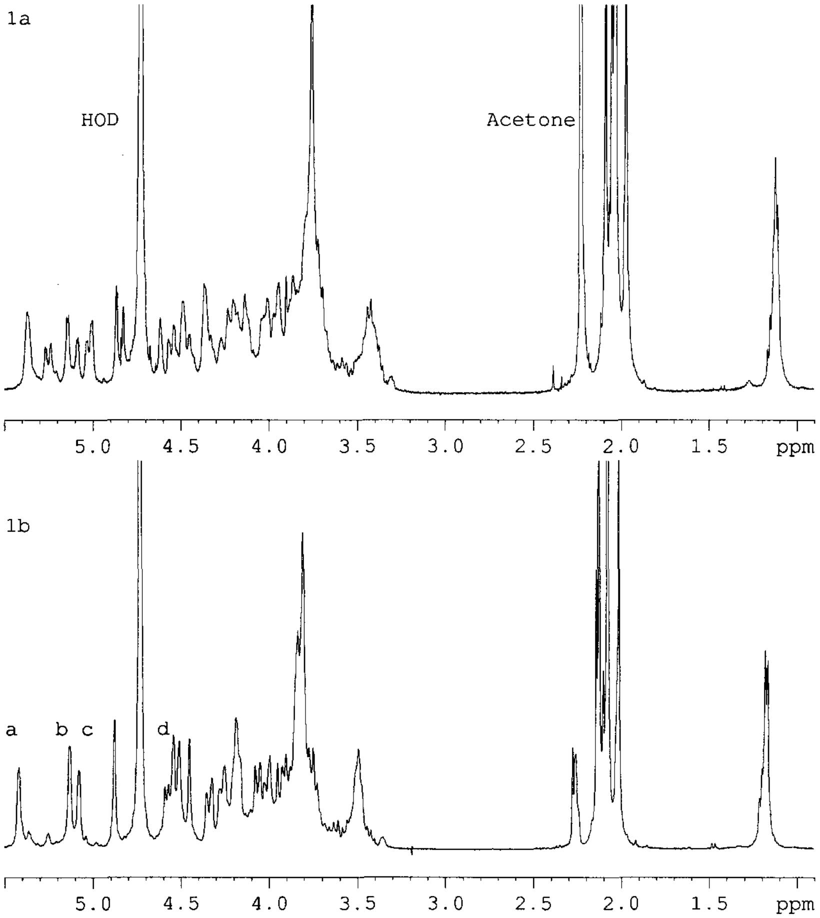

Fig. 1. ^{1}H NMR spectra of *E. coli* O121 **PS** (1a, 30 °C) and **DPS** (1b, 30 °C).

surface carbohydrate antigens, e.g. *E. coli* O124 [2] and O58 [3] O-specific polysaccharides are identical to those of *S. dysenteriae* type 3 and type 5, respectively. We now report the elucidation of the structure of the *Shigella*-like O-polysaccharide of *E. coli* O121, a strain isolated from calf septicaemia, not associated with any K-antigen, and which was reported earlier to cross-react serologically with *E. coli* O101, O115, and O116 [1]. This cross-reactivity, however, has recently been reported to have been lost [1].

2. Results and discussion

Isolation and composition of the polysaccharide. —*E. coli* O121 bacteria were grown in Luria-Bertani broth at 37 °C for 25 h, after which the cells were killed with phenol and were isolated by centrifugation and then washed. The LPS was extracted from the lysozyme and ribonuclease-treated cells using the hot aqueous phenol method [4]. 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 polysaccharide (Fig. 1a) recorded at 30 °C was complex and showed at least ten signals in the anomeric region between δ 4.50 and 5.40, two doublets for methyl protons of 6-deoxy sugars at δ 1.12 and 1.13, together with methyl proton signals for acetyl groups at δ 2.09, 2.05, 2.03, and 1.97. Many of the signals were not present in stoichiometric amounts, e.g. the acetyl resonances were present in the ratios 1.0:0.6:2.0:1.0. Treatment of the polysaccharide with 0.1 M NaOH at 40 °C for 4 h afforded a *O*-deacetylated polysaccharide (**DPS**) which gave a much simpler ^{1}H NMR spectrum (Fig. 1b) and identified the signal at δ 2.05 above as arising from *O*-acetyl groups in the native

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[1] F. Scheutz, WHO International Escherichia and Klebsiella Centre, Copenhagen, personal communication.

polysaccharide. The ^{1}H NMR spectrum of the **DPS**, recorded at 45 °C, had three H-1 signals typical for α-linked hexopyranoses at δ 5.37 ($J_{1,2}$ 3.2 Hz), 5.09 ($J_{1,2}$ 3.4 Hz), and 5.04 ($J_{1,2}$ 3.0 Hz), a signal for an unresolved doublet at δ 4.83, and a signal for H-1 of a β-linked hexopyranose at δ 4.54 ($J_{1,2}$ 8.0 Hz). In addition, signals were observed for the methyl protons of a 6-deoxy sugar at δ 1.13 ($J_{5,6}$ 5.9 Hz), and for *N*-acetyl groups at δ 2.08, 2.03 (6 H), and 1.97.

The ^{13}C NMR spectrum of the **DPS** (Fig. 2), recorded at 45 °C, contained four signals for anomeric carbons, three for α-linked hexopyranoses at 97.30, 99.47, and 99.54 ppm, and one for a β-linked hexopyranose at 104.56 ppm. These results show that one of the signals in the anomeric region of the ^{1}H NMR spectrum arose from a non-anomeric proton and that the polysaccharide therefore is based on a tetrasaccharide repeating unit. The ^{13}C NMR spectrum of the **DPS** also contained a signal for the carbon of a methyl group of a 6-deoxy sugar at 17.25 ppm, signals for the methyl carbons of *N*-acetyl groups at 22.99, 22.95, 22.89, and 22.49 ppm, signals for four carbon atoms carrying nitrogen at 57.33, 52.84, 50.75, and 50.21 ppm, a signal at 43.73 ppm (probably a methylene carbon), a signal for a hydroxymethyl carbon at 60.64 ppm, and signals for seven carbonyl groups at 175.61, 175.54 (2 C), 174.73, 173.93, 172.39, and 172.29 ppm. These data suggest that the tetrasaccharide repeating unit consists of two hexuronic acids, a 6-deoxyhexose, and a hexose, some or all of which contain acetamido groups.

Solvolysis of the polysaccharide with anhydrous HF for 3 h at 24 °C and GLC examination of the derived alditol acetates showed the presence of GlcN, while methanolysis of the polysaccharide, followed by reduction of the methoxycarbonyl groups, prior to HF solvolysis and alditol acetate preparation, showed the presence of GlcN and GalN. These results demonstrate that GalNA, and possibly GlcNA, is present in the repeating unit. The GlcN and GalN residues were shown to have the D configuration by GLC examination of the acetylated (–)-2-octyl glycosides prepared from a hydrolysate of the polysaccharide after reduction of the carboxyl groups.

2D NMR spectroscopy of the ***DPS***.—The identities of the 6-deoxyhexose and uronic acids, and the linkage positions and sequence of the residues in the repeating unit were established by 2D NMR experiments recorded on a solution of the **DPS** at 45 °C. The residues were labelled **a**–**d** in order of the decreasing chemical shift of their anomeric protons. The ^{1}H resonances of each residue were assigned by COSY [5] and HOHAHA [6] experiments. The COSY contour plot allowed the assignment of H-1,2,3,4,5 of residues **a** and **b**, and H-1,2,3,4,5,6 of **c**. For residue **d**, H-1,2,3 could be traced from H-1, while starting at the deoxy function allowed H-6,5,4 to be traced, revealing that H-3 and H-5 overlapped as did H-2 and H-4. In the HOHAHA contour plot the H-1 tracks for residues **a**, **b**, and **c** showed the cross-peaks for H-2,3,4,5. The H-1 track for **d** showed signals only at δ 3.78, 3.45, and 1.10, and the H-6 track showed signals at δ 4.54, 3.78, and 3.45, thus con-

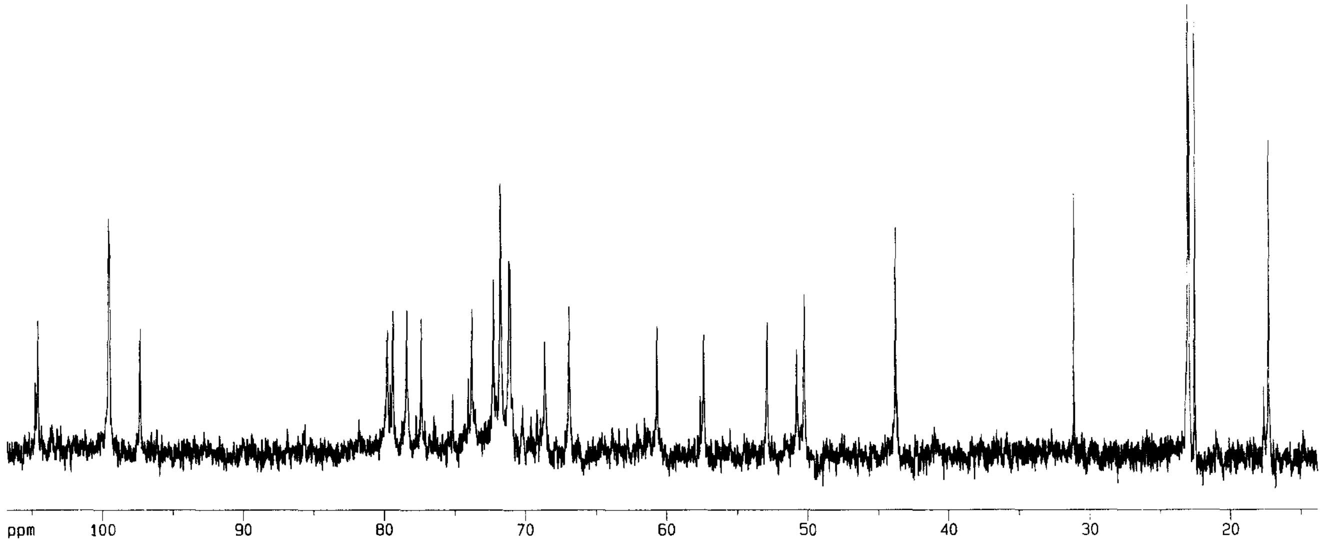


Fig. 2. Partial ^{13}C NMR spectrum of *E. coli* O121 **DPS**.

Table 1
^{1}H and ^{13}C chemical shift data [a] for *E. coli* O121 *O*-deacetylated polysaccharide **DPS**

Residue		H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5/C-5	H-6a/C-6	H-6b	CH_2 Gly
→ 4)-α-D-GalNAcA	**a**	5.37	4.22	4.01	4.46	4.41			
					4.39 [b]	4.11 [b]			
		99.47	50.21	66.87	**78.37** [c]	71.03	172.39		
					79.63 [b]	72.46 [b]			
→ 4)-α-D-GalNAcAN	**b**	5.09	4.29	4.13	4.50	4.83			
					4.50 [b]	4.82 [b]			
		99.54	50.75	68.56	**79.35**	71.73	173.93		
					79.55 [b]	71.77 [b]			
→ 3)-α-D-GlcNAc	**c**	5.04	3.97	3.86	3.70	4.15	3.82	3.80	
		97.30	52.84	**79.74**	71.79	72.22	60.64		
→ 3)-β-D-Qui4NAcGly	**d**	4.54	3.45	3.78	3.78	3.45	1.10		3.76
		104.56	73.76	**77.71**	57.33	71.73	17.25		43.73

[a] Chemical shifts in ppm relative to acetone at δ 2.23 for ^{1}H and at δ 31.07 for ^{13}C, at 45 °C and pH 1.5.
[b] Data recorded at pH 8.0.
[c] Linkage carbons are indicated in bold.

firming the overlap of two sets of signals for this residue. The ^{13}C resonances were assigned from a ^{1}H–^{13}C HMQC [7] and HMBC [8] experiment. A DEPT-HMQC [9] experiment confirmed that the cross-peaks at 43.73/3.76 and 60.64/3.82, 3.80 ppm emanated from CH_2 groups, and an HMQC-TOCSY [10] experiment then confirmed all the assignments. The data are collected in Table 1. The sequence of the residues in the **DPS** was determined from NOESY [11] and HMBC experiments. The carbonyl and methyl resonances of the acetyl substituents of residues **a** and **c** were assigned from the long-range two- and three-bond ^{1}H–^{13}C connectivities observed in the HMBC experiment (Fig. 3). This experiment also identified the acyl substituent of the 4-amino-4-deoxy sugar **d** as *N*-acetylglycyl.

By comparison of the NMR data for the residues in **DPS** with those for methyl glycosides [12–14] it was possible to identify **a** and **b** as → 4)-α-D-Gal*p*NAcA, **c** as → 3)-α-D-Glc*p*NAc and **d** as → 3)-β-Qui*p*4NAcGly.

The NOESY experiment showed interresidue NOEs, from which the sequence of the **DPS** was established, as well as intraresidue NOEs which confirmed the anomeric configurations of the residues and which substantiated their identities, e.g. H-1,H-3 and H-1,H-5 NOEs for the β-linked unit, H-1,H-2 NOEs for the α-linked units, and H-3,H-4 NOEs for the residues with the *galacto* configuration. The data

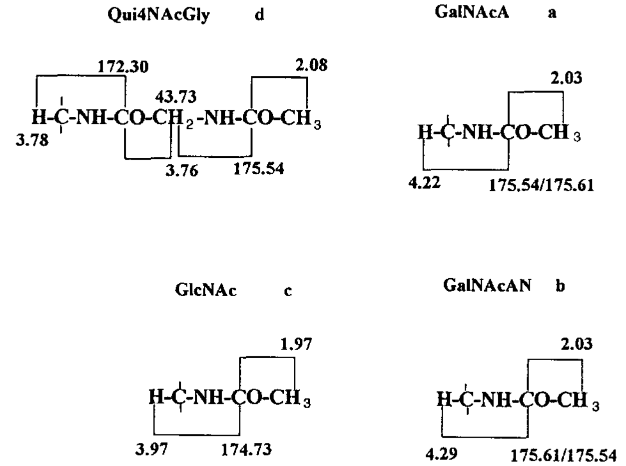
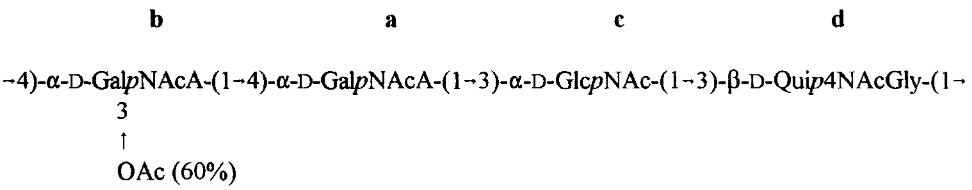


Fig. 3. Two- and three-bond ^{1}H–^{13}C correlations for residues **a–d** in the **DPS**. Values in ppm.

Table 2
NOE data for *E. coli* O121 **DPS**

Proton at δ	NOE to
4.54 **d1** [a]	3.78 **d3**, 3.45 **d5**
5.37 **a1**	4.22 **a2**, 3.86 **c3**
5.09 **b1**	4.29 **b2**, 4.46 **a4**, 4.41 **a5**
5.04 **c1**	3.97 **c2**, 3.77 **d3**
4.83 **b5**	4.50 b4, 4.13 **b3**, 4.22 **a2**
4.50 **b4**	4.13 **b3**
4.46 **a4**	4.41 **a5**, 4.01 **a3**
4.41 **a5**	4.01 **a3**
3.45 **d5**	3.78 **d3**
4.15 **c5**	3.82 **c6a**, 3.86 **c3**
1.10 **d6**	3.78 **d4**, 3.45 **d5**

[a] **d1** connotes H-1 of residue **d**, etc.



$$\underset{\substack{3\\ \uparrow \\ \text{OAc (60\%)}}}{\overset{\mathbf{b}}{\rightarrow 4)\text{-}\alpha\text{-D-Gal}p\text{NAcA}}}\text{-}\overset{\mathbf{a}}{(1\rightarrow 4)\text{-}\alpha\text{-D-Gal}p\text{NAcA}}\text{-}\overset{\mathbf{c}}{(1\rightarrow 3)\text{-}\alpha\text{-D-Glc}p\text{NAc}}\text{-}\overset{\mathbf{d}}{(1\rightarrow 3)\text{-}\beta\text{-D-Qui}p\text{4NAcGly}}\text{-}(1\rightarrow$$

are collected in Table 2 and allow the following sequence to be written for the sugars in the repeating unit of **DPS**: → 4)-**b**-(1 → 4)-**a**-(1 → 3)-**c**-(1 → 3)-**d**-(1 →.

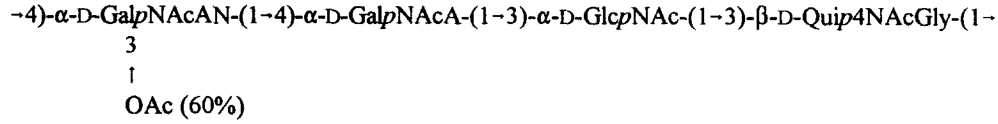
The Qui4NAc unit was considered to have the D configuration by virtue of the small β-effect of glycosylation (-0.3 ppm) on the chemical shift of C-4 (^{13}C chemical shifts for β-Qui4NAc from [15] and compared with *E. coli* O121 Qui4NAc at pH 8). This small shift difference indicated that the absolute configurations of **c** and **d** were the same, i.e. D-D. A larger shift difference would be expected for a D-L situation for the two monosaccharides [16].

The location of the *O*-acetyl group in the repeating unit of the **PS** was determined after analysis of the ^{1}H chemical shifts obtained from its COSY spectrum. The chemical shift differences for unit **b** in the two polymers indicated that the *O*-acetyl group was located on position three of this unit as evidenced by the strong downfield shift of H-3 and the smaller downfield shifts of H-2 and H-4 (H-2, $\Delta\delta$ 0.25; H-3, $\Delta\delta$ 1.12; H-4, $\Delta\delta$ 0.12) in the spectrum of the **PS**, compared to those in the **DPS**. The above data suggest that the repeating unit of the **PS** is as shown above.

This repeating unit is almost identical to that reported for *Shigella dysenteriae* type 7 O-polysaccharide [15] and, indeed, when the ^{13}C chemical shifts of the two are compared, there is extremely close correlation. In the *S. dysenteriae* O-polysaccharide repeating unit, however, the GalNAcA residue carrying the *O*-acetyl group is present as a simple amide of ammonia. This results in the unusual chemical shift for H-5 of this unit at δ 4.83. When the pH of the NMR sample of *S. dysenteriae* polysaccharide is changed from 1 to 5, the signal for H-5 of the galacturonamide remains unaltered in contrast to that for H-5 of GalNAcA, which shifts from δ 4.63 to 4.40. Analogous shifts are observed when the pH of the *E. coli* O121 **DPS** sample is varied in a similar manner; in this case, when the pH is changed from 1.5 to 8.0, the chemical shift for H-5 of residue **a** changes from δ 4.41 to 4.11. Under these conditions the chemical shift of the signal for H-5 of **b** remains virtually unchanged (see Table 1). The effects of a change in pH on the ^{13}C chemical shifts of C-5 and C-4 of the amidated and free acid forms of unit **b** (Table 1) are also consistent with those observed for free and amidated GalNAcAN and GalNFmAN in *Pseudomonas aeruginosa* O4 polysaccharide [17]. Due to the fact that the resonance for H-5 of unit **b** in the **DPS** behaves similarly to that for the GalNAcAN in the *S. dysenteriae* type 7 polymer, unit **b** was identified as being present in the amide form also. The repeating unit of the *E. coli* O-specific polysaccharide can therefore be written as shown below.

Further evidence that the O-polysaccharides of *E. coli* O121 and *S. dysenteriae* are almost also identical is provided by their optical rotations, + 146° and + 144°, respectively.

The above data suggest that the repeating units of the O-polysaccharides of *E. coli* O121 and *S. dysenteriae* are essentially the same, the only difference being that *O*-acetylation is stoichiometric in the latter. It is possible that some loss of *O*-acetyl groups from the O121 polymer could have occurred during isolation and purification. This report thus represents further evidence of the similarity of the genera *Escherichia* and *Shigella* with respect to the composition of their surface antigens. Several serological cross-reactions are known to occur between the two genera. Some examples are *E. coli* O124 with *S.*



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dysenteriae type 3, *E. coli* O112ac with *S. dysenteriae* type 2 and *S. boydii* 1 and 15, *E. coli* O136 with *S. dysenteriae* type 3 and *S. boydii* 1, *E. coli* O164 with *S. dysenteriae* type 3, and *E. coli* O143 with *S. boydii* 8 [1,18]. It will be necessary to determine the structures of these antigens to determine if these interactions indeed can be ascribed to common sugar sequences in the polymers.

3. Experimental

General methods.—Analytical GLC and GLC–MS were performed as previously described [19]. GPC was performed on a column (2.6 × 90 cm) of Sephacryl™ S-200 SF using aq 0.1 M NaOAc, pH 5.0, as eluent. Polysaccharide samples were solvolysed with anhyd HF at room temperature for 3 h and the resulting fluorides were hydrolysed with aq 50% HOAc. Alditol acetates were prepared by reduction of the products of hydrolysis with $NaBH_4$ followed by acetylation with 2:1 Ac_2O–pyridine at 100 °C for 1 h. Samples were methanolysed by refluxing with methanolic 3% HCl for 16 h and the resulting methoxycarbonyl groups were reduced with $NaBH_4$ in dry MeOH. Acetylated (−)-2-octyl glycosides were prepared from a hydrolysate of the methanolysed, carboxyl-reduced polysaccharide according to the method of Leontein et al. [20].

Preparation of E.coli *O121 O-polysaccharide.*—A culture of *E. coli* O121:H10 bacteria (culture no. 39w) was obtained from the WHO International Escherichia and Klebsiella Centre, Copenhagen, and was propagated in liquid culture as described previously [21]. Dried bacterial cells (15 g) were suspended in 150 mL aq 50 mM Na EDTA containing 0.05% NaN_3 and stirred in a Waring blender at top speed for 1 min. Hen egg white lysozyme (0.1 g, Sigma Chemical Corp., 50,000 units/mg) was added and the suspension stirred for 16 h at 4 °C, after which the suspension was heated for 20 min at 37 °C and blended again for 3 min. The volume was then adjusted to 300 mL with aq 20 mM $MgCl_2$ and bovine pancreas ribonuclease (Sigma Chemical Corp.) 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 cells were extracted using the hot aqueous phenol method [4]. The phenol layer was extracted twice and the aqueous layers were combined and dialysed against running water for 4 days. The retentate was centrifuged at 105,000 × *g* for 14 h in a Beckman model L8-80M ultracentrifuge, the pellet was collected, resuspended and recentrifuged. The final pellet was suspended in 1% HOAc and heated for 90 min at 90 °C, following which the solid material was removed by centrifugation (5000 rpm) and the supernatant was dialysed against running water for 24 h and lyophilised. The O-polysaccharide **PS** (120 mg) was then purified on Sephacryl™ S-200 SF. The **PS** had $[\alpha]_D$ +146° (*c* 0.52, water), measured on a Perkin–Elmer model 141 polarimeter. A solution of the **PS** (25 mg) in 0.1 M NaOH was heated for 4 h at 40 °C, dialysed, and lyophilised to give a *O*-deacetylated polymer **DPS**.

NMR spectroscopy.—Samples of polysaccharide were deuterium exchanged several times by lyophilising from D_2O and then examined as solutions in 99.99% D_2O containing a trace of acetone as internal standard (δ 2.23 for ^1H and δ 31.07 for ^{13}C). Spectra were recorded at 30 °C (Fig. 1) and at 45 °C (all 2D experiments) on a Bruker AMX-400 spectrometer using UXNMR software. The 2D pulse programmes were as follows: *a*, COSY-45; *b*, HOHAHA using a MLEV-17 pulse sequence for mixing (89 ms); *c*, phase-sensitive NOESY using TPPI, mixing time 200 ms; *d*, HMQC using TPPI and GARP decoupling during acquisition; *e*, DEPT-HMQC using TPPI and a read angle of 180° to give CH and CH_3 negative and CH_2 positive, with GARP decoupling during acquisition; *f*, HMQC-TOCSY using TPPI and with a MLEV-17 pulse sequence for mixing (25 and 89 ms), with GARP decoupling; *g*, HMBC with GARP decoupling, mixing delay 60 ms. A 1.0 s relaxation delay was applied in all cases. The spectral width in f_1 and f_2 for *a–c* was 2283 Hz, for *d–f* 2283 and 14,000 Hz, and for *g* 2283 and 20,826 Hz, respectively. The data matrix for *a* was 256 × 2048 points, zero filled to 1024 × 2048, and a sine window function applied prior to Fourier transform (FT); the matrices for *b* and *c* were 512 × 2048 points, zero filled to 1024 × 2048, and a shifted sine squared function applied prior to FT; matrices for *d–g* were 256 × 4096 points, zero filled to 1024 × 2048, and a shifted sine squared function applied prior to FT.

Acknowledgements

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