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Structural studies of the O-antigen polysaccharide from the enteroinvasive *Escherichia coli* O173

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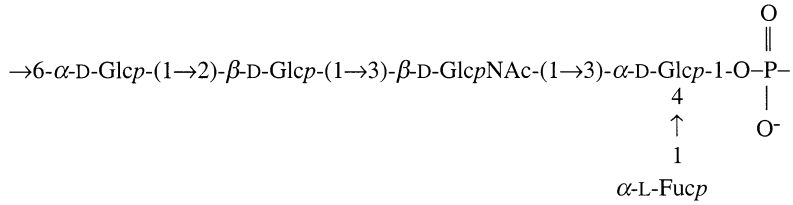
Abstract

The structure of the O-antigen polysaccharide (PS) from *Escherichia coli* O173 has been investigated. Sugar and methylation analyses, electrospray ionisation mass spectrometry together with ^{1}H, ^{31}P and ^{13}C NMR spectroscopy were the main methods used. The structure of the pentasaccharide repeating unit of the PS was found to be:



By treatment with 48% HF the phosphoric diester linkage was cleaved together with the glycosidic linkage of the fucosyl group, rendering a tetrasaccharide with the structure:

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

Keywords: *Escherichia coli*; Lipopolysaccharide; O-Antigen, structure; NMR

1. Introduction

Escherichia coli is a facultative anaerobic Gram-negative rod and is a predominant species in the colonic flora of animals and man. The species is subdivided into serotypes based on the immunogenicity of bacterial surface structures. Thus, the strains are usually designated as O:K:H serotypes, where O is the O-antigen, i.e., the polysaccharide (PS) portion of the lipopolysaccharide (LPS), K is the capsular PS and H the flagella antigen. As of today, more than 170 different O-antigens and over 80 capsular PSs have been identified within the species [1]. There are three general clinical syndromes that result from infections with pathogenic *E. coli*: (i) enteric/diarrhoeal, (ii) urinary tract infections, and (iii) septicaemia/meningitis. Only a limited number of O, K and H antigens and O:K:H serotypes are

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represented as pathogens in the different infections. The diarrhoeal strains can be further divided into enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC) and enteroaggregative *E. coli* (EAEC) [2]. The latest serotype, i.e., *E. coli* O173, was described by Ørskov and co-workers in 1991 [3]. It belongs to the group of enteroinvasive *E. coli* (EIEC) and was originally isolated from a child with diarrhoea. EIEC is a group of *E. coli* that may cause dysentery-like diseases in humans. Both EIEC and *Shigella* possess a large ($\sim$140 MDa) plasmid encoding for several outer membrane proteins involved in their invasion of epithelial cells [4]. In this study, we present the complete structure of the O-antigenic repeating unit of the LPS from the enteroinvasive *E. coli* O173.

2. Results and discussion

E. coli O173:K$^-$:H$^-$ was obtained from the Culture Collection in Gothenburg (Sweden) and grown in a glucose-containing TY medium. The LPS was isolated from the membrane by hot phenol/water extraction and delipidated under acidic conditions to yield a PS. A hydrolysate of the PS contained fucose, glucose and 2-amino-2-deoxyglucose in the ratio 23:63:14. Determination of the absolute configuration revealed that glucose and glucosamine have the D-configuration and fucose the L-configuration.

Four major components were identified from a methylation analysis, namely 2,3,4-tri-*O*-methyl-fucose derived from a terminal fucose, 3,4,6-tri-*O*-methyl-glucose from a 2-substituted glucose, 2,6-di-*O*-methyl-glucose from a 3,4-disubstituted glucose (pyranoid ringform identified by NMR spectroscopy, vide infra) and a 4,6-di-*O*-methyl-*N*-methyl-glucosamine derived from a 3-substituted glucosamine. A smaller but significant amount of peracetylated glucitol was also found in the analysis. Information from the component and methylation analyses indicate that the PS of *E. coli* O173 is built up by branched repeating units of at least four sugar residues.

In the ^{1}H NMR spectrum of the native PS the presence of phosphorus was indicated by an additional splitting of one anomeric signal (δ_H 5.44). The observation of one signal at δ_P -0.9 in the ^{31}P NMR spectrum corroborated the presence of a phosphoric diester group.

To obtain sequence information, the phosphoric diester linkage was cleaved by hydrolysis with 48% HF. The hydrolysate was fractionated by GPC and an oligosaccharide was isolated. The ^{13}C NMR spectrum of the oligosaccharide contained five signals in the anomeric region at δ_C 93.2, 97.1, 98.6, 102.3 and 102.4. The ^{1}H NMR spectrum (Fig. 1) showed, inter alia, five signals from anomeric protons at δ_H 5.29, 5.15, $\sim$4.84, 4.69 and 4.58 with the respective intensities 1:0.4:1:1:0.6. The combined evidence indicated that the material, derived from treatment of the PS with 48% HF, is an anomeric mixture of a tetrasaccharide. In the ^{1}H NMR spectrum, signals from minor amounts of material containing a fucosyl group were also detected. An ESIMS spectrum in the positive mode of the underivatised tetrasaccharide showed, inter alia, one peak at m/z 708 attributed to the pseudo molecular ion $[M+H]^+$. It corresponds to an oligosaccharide constituted by three hexoses and one N-acetylated hexosamine. The detected fragment ions in the MS/MS spectrum (Fig. 2) of the precursor ion m/z 708, together with the sequence of the sugars in the tetrasaccharide, are shown in Fig. 3. The fragments m/z 204 and m/z 366 can be attributed to a double cleavage process that is sometimes ob-

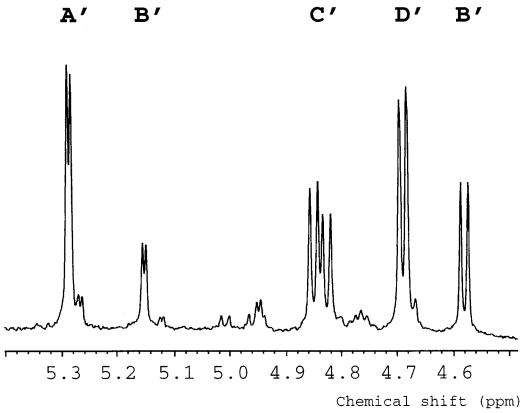


Fig. 1. The anomeric region of the ^{1}H NMR spectrum at 70 °C of tetrasaccharide **1**, obtained after treatment of the O-antigen PS from *E. coli* O173 with 48% HF (aq).

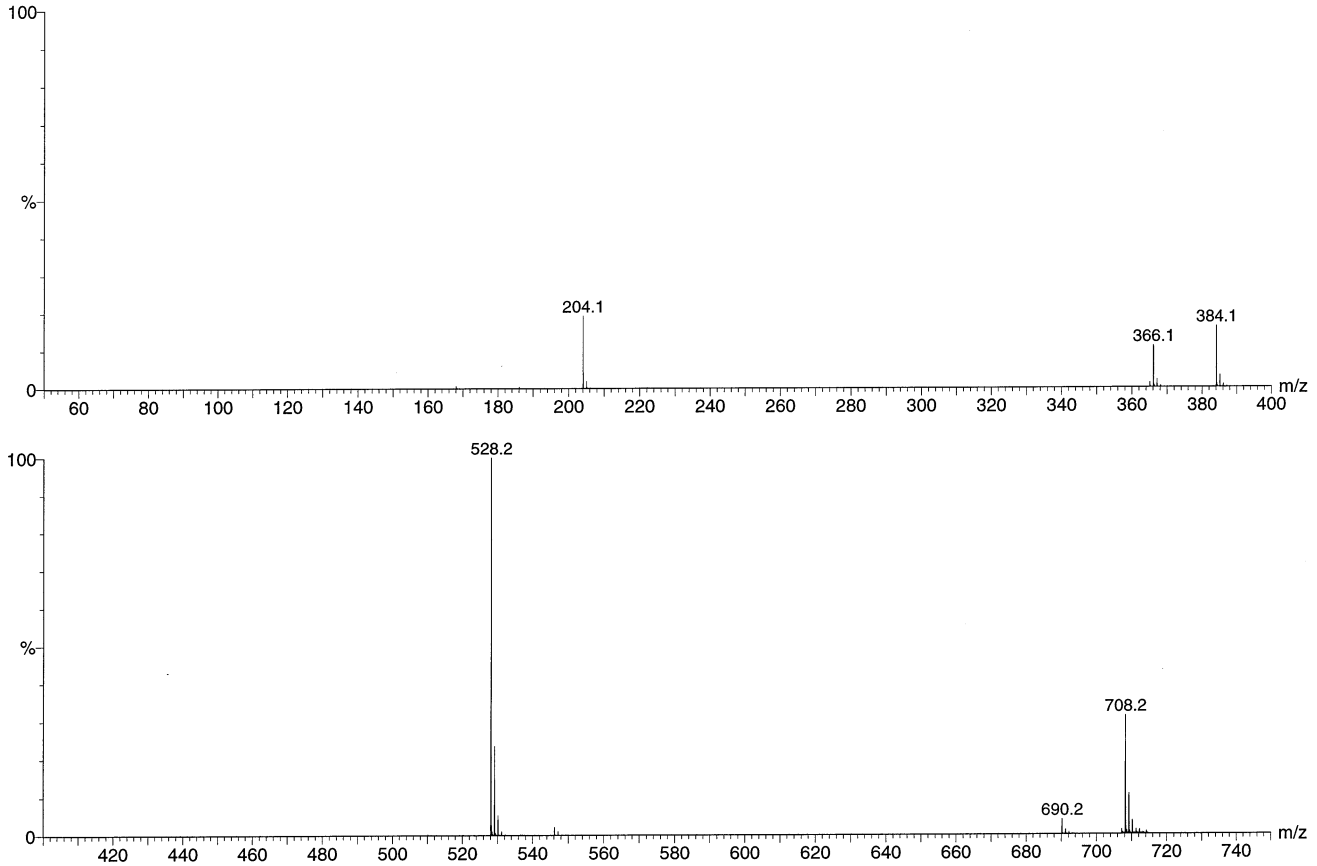


Fig. 2. The MS/MS spectrum of the precursor ion m/z 708, i.e., the pseudo molecular ion $[M+H]^+$ of tetrasaccharide **1**.

served for underivatised carbohydrates [5], i.e., A-type cleavages of the fragments m/z 384 and 546, respectively.

The ^1H and ^{13}C NMR signals of the tetrasaccharide were partially assigned using a combination of homonuclear and heteronuclear two-dimensional NMR experiments. The chemical shifts are reported in Table 1 together with the homonuclear coupling constants for the anomeric protons ($J_{H1,H2}$). From the chemical shifts of the ^1H and ^{13}C signals it was evident that all sugar residues are pyranosidic. The residues were denoted **A′** to **D′** according to the decreasing chemical shifts of their anomeric proton signals. The residue with an anomeric proton at δ_H 5.29 was denoted **A′**. Except for a downfield shift of the C-1 signal, the chemical shifts of residue **A′** showed good agreement with those of an unsubstituted α-D-glucose monosaccharide [6]. It was therefore assigned to the terminal glucosyl group. Residue **B′** (δ_H 5.15/4.58) was assigned to the reducing end, an α/β-mixture of a 3-substituted glucosyl residue. The substituted position was determined by a glycosylation shift, $\Delta\delta_{C\text{-}3} = 9.7$ for the α form and $\Delta\delta_{C\text{-}3} = 9.3$ for the β form. Residue **C′** was assigned to the 3-substituted *N*-acetylglucosamine residue, based on an upfield chemical shift of the C-2 signal due to linkage to nitrogen. A glycosylation shift of 5.8 ppm for the C-3 signal corroborated the substitution pattern of the sugar. The fourth residue, **D′**, was identified as a 2-substituted glucosyl residue due to a downfield chemical shift of C-2 ($\Delta\delta_{C\text{-}2} = 3.5$) as compared with unsubstituted β-D-glucose. By combining information from NMR and

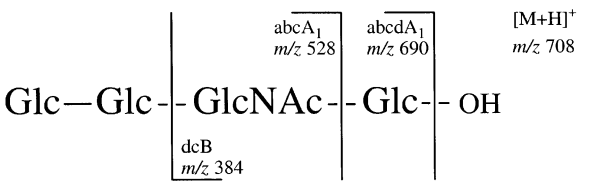


Fig. 3. Detected fragment ions in the MS/MS spectrum of the precursor ion m/z 708, and the sequence of the sugars in tetrasaccharide **1**.

Table 1
^{1}H and ^{13}C NMR chemical shifts (ppm) of tetrasaccharide **1** isolated after HF hydrolysis of the O-antigen PS from *E. coli* O173

Residue	^{1}H/^{13}C			
	1	2	3	4
α-D-Glc*p*-(1→	5.29 [3.7] [a]	3.50	3.72	3.42
A′	(0.06) [b]	(0.04)	(0.00)	(0.00)
	98.6	72.8	74.1	70.8
	(5.6) [b]	(0.3)	(0.3)	(0.1)
→3)-α-D-Glc*p*	5.15 [3.7]	3.55	3.78	3.44
B′	(−0.08)	(0.01)	(0.06)	(0.02)
	93.2	72.3	83.5	69.4
	(0.2)	(−0.2)	(9.7)	(−1.3)
→3)-β-D-Glc*p*NAc-(1→ [c]	4.85/4.83 [8.4]	3.77/3.78	4.01	3.57
C′	(0.13/0.11)	(0.12/0.13)	(0.45)	(0.11)
	102.4	56.6	80.6	69.7
	(6.5)	(−1.3)	(5.8)	(−1.4)
→2)-β-D-Glc*p*-(1→	4.69 [7.5]	3.46	3.55	3.42
D′	(0.05)	(0.21)	(0.05)	(0.00)
	102.3	78.7	75.9	70.8
	(5.5)	(3.5)	(−0.9)	(0.1)
→3)-β-D-Glc*p*	4.58 [8.2]	3.27	3.59	3.44
B′	(−0.06)	(0.02)	(0.09)	(0.02)
	97.1	75.0	86.1	69.4
	(0.3)	(−0.2)	(9.3)	(−1.3)

[a] $J_{H\text{-}1,H\text{-}2}$ values are given in Hz in square brackets.
[b] Chemical shift differences as compared to the corresponding monosaccharides [6].
[c] *N*-Acetyl signals at δ_H 2.03, δ_{CH_3} 23.5 and δ_{CO} 174.0.

MS, the sequence of the four sugars was determined. The anomeric configurations of the sugars were concluded from the chemical shifts together with the values of the anomeric coupling constants $J_{H\text{-}1,H\text{-}2}$. Thus, the complete structure of the tetrasaccharide **1** was revealed:

$$\alpha\text{-D-}\underset{\mathbf{A'}}{\text{Glc}}p\text{-}(1\rightarrow 2)\text{-}\beta\text{-D-}\underset{\mathbf{D'}}{\text{Glc}}p\text{-}(1\rightarrow 3)$$

$$\text{-}\beta\text{-D-}\underset{\mathbf{C'}}{\text{Glc}}p\text{NAc-}(1\rightarrow \underset{\mathbf{1}}{3})\text{-D-}\underset{\mathbf{B'}}{\text{Glc}}$$

The different chemical shifts observed for the signals from H-1 and H-2 of residue **C′** could thus be explained by the proximity to the reducing end. To further corroborate the sequence of the sugars, a NOESY experiment was performed. Inter-residue NOEs were observed defining the three glycosidic linkages, i.e., from H-1 in residue **A′** (δ_H 5.29) to H-2 in **D′** (δ_H 3.46), from H-1 in residue **D′** (δ_H 4.69) to H-3 in **C′** (δ_H 4.01) and from H-1 in **C′** (δ_H 4.85) to H-3 in **B′** (δ_H 3.59).

The ^{13}C NMR spectrum of the native PS contained, inter alia, one signal for a methyl group at δ_C 16.3, one signal for a methyl group of an *N*-acetyl function at δ_C 23.3, one signal for a carbon atom linked to nitrogen at δ_C 56.4 together with four signals for primary carbons at δ_C 60.4, 61.4, 62.6 and 65.1. Five signals were observed in the anomeric region, at δ_C 96.1, 98.8, 99.2, 101.8 and 102.0. One carbonyl signal was also visible in the ^{13}C NMR spectrum, at δ_C 175.5. The ^{1}H NMR spectrum (Fig. 4) showed, inter alia, six signals in the anomeric region of which five originated from anomeric protons (δ_H 5.44, 5.24, 4.97, 4.93 and 4.68). One signal was shown for a methyl group at δ_H 1.25 and one signal for an *N*-acetyl group at δ_H 2.06. All ^{13}C and ^{1}H NMR signals of the native PS were assigned and their chemical shifts are reported together with coupling constants for the anomeric atoms in Table 2. By analogy with the nomenclature applied for the tetrasaccharide, the five residues of the native PS were

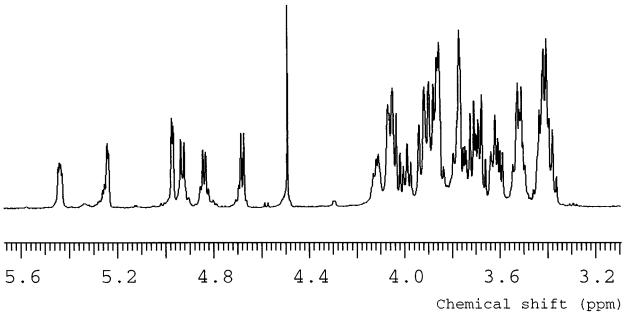


Fig. 4. Part of the ^{1}H NMR spectrum at 50 °C of the O-antigen PS from *E. coli* O173.

denoted **A** to **E**. The residue with an anomeric proton signal at δ_H 5.44, **B**, is the disubstituted glucosyl residue as shown by significant chemical shift differences ($\Delta\delta_{C\text{-}3} = 4.1$ and $\Delta\delta_{C\text{-}4} = 2.1$) compared with the monosaccharide. An extra splitting of the anomeric signal ($^3J_{H,P} = 6.7$ Hz) in **B′** is caused by a linkage to the phosphoric diester at this position. Residue **A**, with an anomeric proton at δ_H 5.24, is a 6-substituted glucosyl residue as deduced by a downfield chemical shift for C-6 ($\Delta\delta_{C\text{-}6} = 3.3$). The chemical shifts of the signals of residue **E** showed good agreement with those of free α-L-fucose and it was therefore assigned to the terminal fucosyl group. The *N*-acetylglucosamine was assigned to the residue with an anomeric proton signal at δ_H 4.93, which was thus denoted **C**. An upfield chemical shift of the C-2 signal in **C** is due to linkage to nitrogen. A glycosylation shift of 5.2 ppm for C-3 is in agreement with the previously deduced substitution pattern of this residue. The fifth residue, **D**, with an anomeric proton at δ_H 4.68, was assigned to the 2-substituted glucosyl residue based on a downfield chemical shift of its C-2 ($\Delta\delta_{C\text{-}2} = 3.6$).

The sequence of four of the five sugars, structural element **2**, was already known from the elucidation of the tetrasaccharide structure.

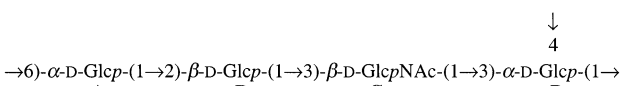
Table 2
^{1}H and ^{13}C NMR chemical shifts (ppm) of the O-antigen PS from *E. coli* O173

Residue	^{1}H/^{13}C					
	1	2	3	4	5	6
→3,4)-α-D-Glc*p*-(1P	5.44 [3.7] [a]	3.63	4.03	3.68	3.91	~3.86
B	(0.21) [c]	(0.09)	(0.31)	(0.26)	(0.07)	
	96.1 {176} [b]	73.4	77.9	72.8	73.2	60.4
	(3.1) [c]	(0.9)	(4.1)	(2.1)	(0.8)	(−1.4)
P6)-α-D-Glc*p*-(1→	5.24 [3.9]	3.52	3.73	4.06	3.51	4.06,4.12
A	(0.01)	(−0.02)	(0.01)	(0.64)	(−0.33)	
	98.8 {~175}	72.6	73.5	71.7	69.8	65.1
	(5.8)	(0.1)	(−0.3)	(1.0)	(−2.6)	(3.3)
α-L-Fuc*p*-(1→	4.97 [4.1]	3.76	3.86	3.77	4.84	1.25
E	(−0.23)	(−0.01)	(0.00)	(−0.04)	(0.64)	(0.04)
	99.2 {~173}	68.8	70.2	72.9	67.4	16.3
	(6.1)	(−0.3)	(−0.1)	(0.1)	(0.3)	(0.0)
→3)-β-D-Glc*p*NAc-(1→ [d]	4.93 [8.3]	3.78	3.99	3.39	3.42	3.61,3.93
C	(−0.21)	(0.13)	(0.34)	(−0.07)	(−0.04)	
	101.8 {~165}	56.4	80.0	70.1	76.1	62.6
	(6.0)	(−1.5)	(5.2)	(−1.0)	(−0.7)	(0.8)
→2)-β-D-Glc*p*-(1→	4.68 [7.5]	3.42	3.53	3.43	3.40	3.70,3.89
D	(0.04)	(0.17)	(0.03)	(0.01)	(−0.06)	
	102.0{~165}	78.8	75.7	70.6	76.7	61.4
	(5.2)	(3.6)	(−1.1)	(−0.1)	(−0.1)	(−0.4)

[a] $J_{H\text{-}1,H\text{-}2}$ values are given in Hz in square brackets.
[b] $J_{H\text{-}1,C\text{-}1}$ values are given in Hz in braces.
[c] Chemical shift differences as compared to the corresponding monosaccharides [6].
[d] *N*-Acetyl signals at δ_H 2.06, δ_{CH_3} 23.3 and δ_{CO} 175.5.

Table 3
Inter-residue NOEs and HMBC connectivities for the O-antigen PS from *E. coli* O173

Residue	Anomeric atom δ_H/δ_C	NOE to proton δ_H	HMBC to atom δ_H/δ_C
P6)-α-D-Glc*p*-(1→	5.24	D, H-2 3.42	
A			D, C-2 78.8
	98.8		D, H-2 3.42
α-L-Fuc*p*-(1→	4.97	B, H-4 3.68	
E			B, C-4 72.8
	99.2		B, H-4 3.68
→3)-β-D-Glc*p*NAc-(1→	4.93	B, H-3 4.03	
C			B, C-3 77.9
	101.8		B, H-3 4.03
→2)-β-D-Glc*p*-(1→	4.68	C, H-3 3.99	
D			C, C-3 80.0
	102.0		C, H-3 3.99



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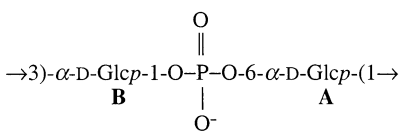
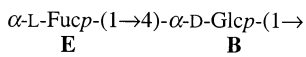
As mentioned above, the anomeric signal of residue **B** is split by the coupling to phosphorus. From an inverse detected $^{1}H,^{31}P$ correlated NMR experiment, cross-peaks between the phosphorus signal and four protons could be observed. These protons were the anomeric proton signal in residue **B**, δ_H 5.44, the two H-6 protons of residue **A**, δ_H 4.06 and 4.12, and the H-2 signal of residue **B**, δ_H 3.63. A structural element **3** in the backbone of the repeating unit of the PS was thus defined:



3

However, an *O*-methylated derivative originating from a 6-substituted glucosyl residue was not observed in the methylation analysis. The terminal fucosyl residue **E** is substituting position four of residue **B** as defined by two HMBC connectivities between H-1 in residue **E** (δ_H 4.97) and C-4 in residue **B** (δ_C 72.8) as well as C-1 in **E** (δ_C 99.2) and H-4 in **B** (δ_H 3.68). An NOE between H-1 in **E** (δ_H 4.97) and H-4 in **B** (δ_H 3.68) further corroborated disaccharide element **4**:



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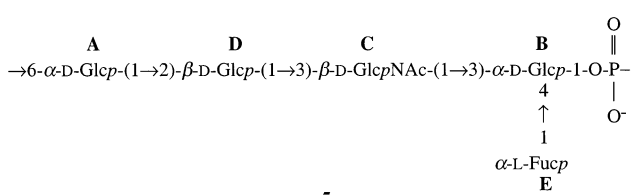
Inter-residue connectivities from the HMBC spectrum and NOEs from the NOESY spectrum (Table 3) corroborated the previously defined glycosidic linkages and thus the sequence of the sugars in the repeating unit was established. To confirm the anomeric configurations of the five sugars, a coupled ^{1}H, ^{13}C-gHSQC experiment was performed. The observed values of $J_{C\text{-}1,H\text{-}1}$ were approximately 175 Hz for the α-linked sugars, residues **A**, **B** and **E**, and 165 Hz for the β-linked sugars, residues **C** and **D** (Table 2). Thus, the structure of the pentasaccharide repeating unit from *E. coli* O173 was established as **5**:



5

The anionic character previously reported [1] for this O-antigen PS thus stems from the presence of a phosphoric diester linkage in the backbone of the polymer, a rare structural element in O-antigens from *E. coli*.

3. Experimental

Bacterial strain and conditions of growth.—The enteroinvasive *E. coli* O173:K^-:H^- strain CCUG 36541 was obtained from the Culture Collection University of Gothenburg, Sweden. Bacteria were grown in submerged culture to late exponential phase in 22 L of a tryptone/yeast extract medium [7] containing 1% glucose, using a 30-L fermentor (Belach AB) under constant aeration at 37 °C and pH 7.0. A preculture (3 L) in the same medium was used to inoculate the fermentor. All cultures were checked for purity at the end of the growth cycle. The bacteria were killed with 1% (mass/vol) formaldehyde. After incubation overnight at 4 °C, the cells were separated from the media by continuous-flow centrifugation using a CEPA model LE centrifuge at a cylinder speed of 35,000 rpm and a flow of 25 L/h (Carl Padberg Centrifugenbau). The bacterial mass was then removed from the cylinder, washed once with phosphate-buffered saline (0.01 M potassium phosphate, 0.14 M NaCl, pH 7.2), centrifuged (8000*g*, 4 °C, 20 min) and finally re-suspended in distilled water.

Preparation of lipopolysaccharide and lipid-free polysaccharide.—The LPS was extracted by the hot phenol/water method [8,9]. The aq phase was dialysed at 4 °C for 3–5 days against tap water, then overnight against distilled water, concd under diminished pressure and lyophilised. Contaminating nucleic acids were removed by treatment with nucleases, ribonuclease EC 3.1.27.5 (20 μg/mg LPS) and deoxyribonuclease EC 3.1.21.1 (4 μg/mg LPS) (Sigma), in 0.1 M sodium acetate, pH 5, and subsequent dialysis. The nucleic acid content was determined spectrophotometrically as described in Ref. [10] and the protein content was estimated according to Lowry and coworkers with BSA as standard [11]. The presences of nucleic acid and proteins were found to be < 5 and $< 0.5\%$, respectively.

Lipid-free PS was prepared by treatment of the LPS with 0.1 M sodium acetate, pH 4.2, at 100 °C for 5 h [12]. Lipid A was removed by centrifugation (10,000*g*, 4 °C, 20 min). The PS was purified by gel-permeation chromatography (GPC) on a Bio-Gel P-2 (Bio-Rad) column irrigated with pyridinium acetate buffer (0.07 M, pH 5.4). Column effluents were monitored using a differential refractometer (Waters, USA). The PS was further purified on a DEAE-Sepharose CL column (3×20 cm, Pharmacia) eluted with a gradient starting from 0.1 M NaCl and ending with 1 M NaCl. The PS was desalted on a Superdex 30 extra dense column (2×60 cm, Pharmacia) fitted to an FPLC system (Pharmacia) irrigated (1.60 mL/min) with pyridinium acetate buffer (0.07 M, pH 5.7). Column effluents were monitored using a differential refractometer (Waters 410).

Component analyses.—The PS was hydrolysed with 2 M trifluoroacetic acid (TFA) at 120 °C for 2 h. After reduction with sodium borohydride and acetylation, the samples were analysed by GLC [13]. The absolute configuration of the sugars present in the O-antigen of *E. coli* O173 was determined essentially as devised by Leontein and co-workers [14] and Gerwig and co-workers [15] detecting the acetylated (+)-2-butyl glycosides.

Methylation analysis.—The methylation analysis was performed as described earlier [16], using sodium methylsulfinylmethanide in Me_2SO (overnight) and methyl iodide [17]. The methylated compounds were purified by use of Sep-Pak C_{18} cartridges (Millipore) [18] and recovered using MeCN and EtOH. The purified methylated samples were then hydrolysed (2 M TFA, 120 °C, 2 h), the sugars converted into partially methylated alditol acetates and analysed by GLC–MS.

GLC and GLC–MS analyses.—Alditol acetates, partially methylated alditol acetates and acetylated 2-butyl glycosides were separated on an HP-5 fused silica column (0.20 mm × 25 m; Hewlett–Packard) using a temperature program of 170 °C (1 min) followed by 1 °C/min to 180 °C (1 min) and 4 °C/min to 250 °C. Hydrogen was used as carrier gas. The column was fitted to a Hewlett–Packard model 5890 series II gas chromatograph (Hewlett–Packard) equipped with a flame ionisation detector. GLC-MS analyses were performed on a Hewlett–Packard model 5970 mass spectrometer equipped with an HP-5MS fused silica column (0.20 mm × 25 m; Hewlett-Packard). A temperature program of

170 °C for 3 min followed by 3 °C/min to 250 °C was used with helium as carrier gas.

NMR spectroscopy.—NMR spectra of solutions in D_2O buffered with sodium phosphate (20 mM) at pD 8 were recorded at 50 °C for the PS and 70 °C for the tetrasaccharide, using Jeol GSX-270, Varian Inova 400 and Varian Inova 600 instruments. Data processing was performed using standard Jeol software or VNMR (Varian). Chemical shifts are reported in ppm relative to sodium 3-trimethylsilyl-(2,2,3,3-$^2H_4$)-propanoate (TSP, δ_H 0.00) or acetone (δ_C 31.00) as internal reference or phosphoric acid (2% H_3PO_4 in D_2O, δ_P 0.00) as external reference. ^1H,^1H-COSY [19], relayed ^1H,^1H-COSY [20] and ^1H,^1H-TOCSY [21] experiments (spin-lock times of 50 and 100 ms) were used to assign the proton signals and performed according to standard pulse sequences. For assignment of the ^{13}C-chemical shifts, a gradient version of ^1H,^{13}C-HSQC [22] and a ^1H,^{13}C-gHSQC-TOCSY [23,24] experiment with 30 and 60 ms spin-lock times were used. For sequence information, a NOESY experiment [25] with a mixing time of 100 ms and a gradient version of a ^1H,^{13}C-HMBC experiment [22,26] with a 90 ms delay for the evolution of long-range connectivities, were used. An inverse detected ^1H,^{31}P-correlated experiment was also performed [27] for the determination of the position of the phosphoric ester group.

Electrospray ionisation mass spectrometry.—ESIMS was performed on a Q-TOF instrument (Micromass) [28,29] in the positive mode. MS/MS was performed with a resolution of 4000, using collision-induced dissociation (CID) with argon gas.

HF hydrolysis.—The PS (30 mg) was dissolved in 48% HF (aq, 5 mL) and kept at 4 °C for 48 h [30]. Most of the solvent was evaporated with dry air at ambient temperature. The pH of the remaining solvent was adjusted to ~ 7 with a saturated $Ca(OH)_2$ solution. Solid material was filtered off and the solvent was evaporated under diminished pressure. The products were fractionated on a column of Superfine Bio-Gel P-2 (2×110 cm, Bio-Rad), fitted to an FPLC system (Pharmacia) irrigated (0.25 mL/min) with pyridinium acetate buffer (0.07 M, pH 5.7). Column effluents were monitored using a differential refractometer and an oligosaccharide was isolated.

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