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Note

Structural determination of the O-antigenic polysaccharide from *Escherichia coli* O74

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Dedicated to Professor Dr. Hans Kamerling on the occasion of his 65th birthday

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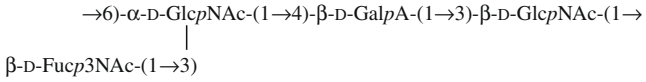
ABSTRACT

The structure of the O-antigen polysaccharide (PS) from *Escherichia coli* O74 has been determined. Component analysis, together with ^{1}H and ^{13}C NMR spectroscopy as well as ^{1}H,^{15}N-HSQC experiments were employed to elucidate the structure. Inter-residue correlations were determined by ^{1}H,^{1}H-NOESY and ^{1}H,^{13}C-heteronuclear multiple-bond correlation experiments. The PS is composed of tetrasaccharide repeating units with the following structure:



Cross-peaks of low intensity from an α-linked *N*-acetylglucosamine residue were present in the NMR spectra, and spectral analysis indicates that they originate from the penultimate residue in the polysaccharide. Consequently, the biological repeating unit has a 3-substituted *N*-acetyl-D-glucosamine residue at its reducing end. The ^{1}H, ^{13}C and ^{15}N NMR chemical shifts of the α- and β-anomeric forms of D-Fucp3NAc are also reported. The repeating unit of the *E. coli* O74 O-antigen is identical to that of the capsular polysaccharide from *E. coli* K45.

Escherichia coli is a Gram-negative rod and a predominant facultative anaerobic species in the colonic flora of animals and in 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, that is, the polysaccharide portion of the lipopolysaccharide (LPS); K is the capsular polysaccharide and H is the flagella antigen. As of today, more than 180 different O-antigens and over 100 capsular polysaccharides have been identified within the species.[1,2] 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 represented as pathogens in the different infections. The diarrhoeal strains can be further divided into different virotypes based on the type of virulence factors they express and hence on the diarrhoeal disease they cause. The *E. coli* O74 is an O-serotype that has shown cross-reactivity with *E. coli* O2 and the LPS from both strains contain D-Fuc3NAc.[3,4] In this report, we describe the structure of the *E. coli* O74 O-polysaccharide.

E. coli O74:K-:H39 was grown in LB medium. The LPS was isolated from the bacterial membrane by hot phenol/water extraction and delipidated under mild acidic conditions to yield a polysaccharide (PS). Sugar analysis of the polysaccharide revealed 3-amino-3-deoxyfucose and 2-amino-2-deoxyglucose as the major components as well as glucose and galactose as minor components attributed to the core. Galacturonic acid was identified by methanolysis. Determination of the absolute configuration of the three main components utilized authentic standards and showed D-Fuc3N, D-GlcN and D-GalA.

In the ^{1}H NMR spectrum of the *E. coli* O74 PS (Fig. 1) there are four resonances that could be assigned to anomeric protons with ^{1}H chemical shifts of 4.91, 4.59, 4.49 and 4.46 ppm; the corresponding sugar residues were then denoted **A**–**D**, respectively. Resonances were also found, inter alia, at δ_H 2.04 (3H), 2.05 (3H) and 2.07 (3H) indicating that the amino sugars are N-acetylated. The resonance at δ_H 1.25 (3H) should consequently be assigned to H-6 of D-Fuc3NAc. The chemical shifts of the sugar residues in the PS were assigned using a combination of 1D and 2D NMR techniques. The ^{1}H,^{13}C-HSQC spectrum (Fig. 2) of the PS showed in the region for anomeric resonances four cross-peaks corresponding to hexopyranosyl residues. The ^{1}H and ^{13}C NMR chemical shifts of the four sugars in the repeating unit of the O-antigen are compiled in Table 1. Residue **A** is α-linked since the $J_{H\text{-}1,C\text{-}1}$ coupling constant

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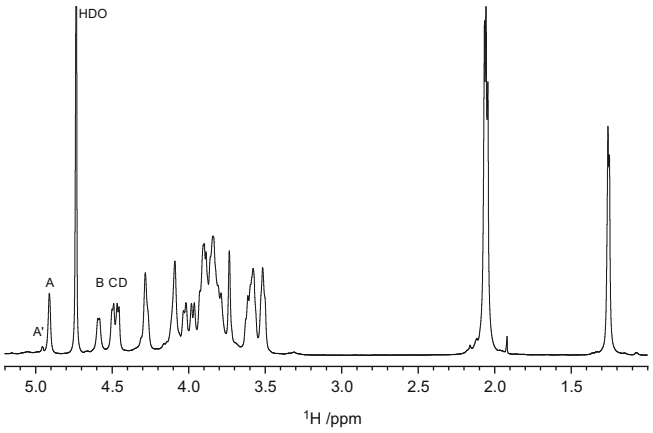


Figure 1. The ^{1}H NMR spectrum of the O-antigen PS from *E. coli* O74.

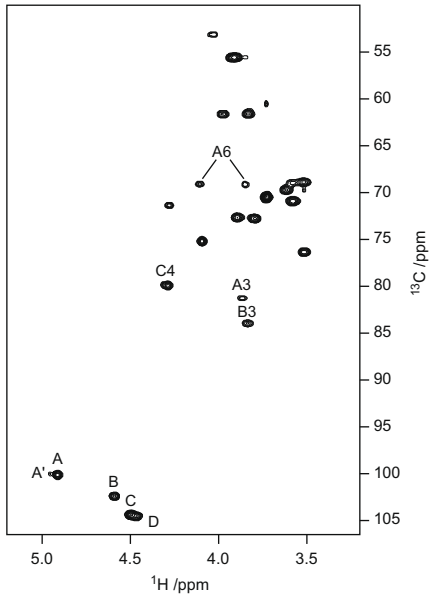


Figure 2. Part of the ^{1}H,^{13}C-HSQC NMR spectrum of the O-antigen PS from *E. coli* O74. Resonances from anomeric and substitution positions are annotated.

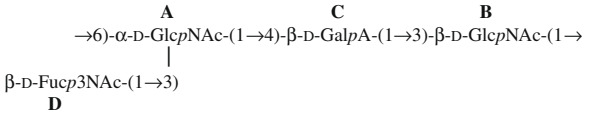
is 173 Hz and residues **B**–**D** are β-linked as the corresponding coupling constant is ~160 Hz.[5] From the analysis of ^{1}H,^{1}H-TOCSY spectra and ^{1}H and ^{13}C chemical shifts, residues **A** and **B** have the *gluco*-configuration whereas residues **C** and **D** have the *galacto*-configuration. Both residues **A** and **B** have C-2 chemical shifts that show that they correspond to D-GlcNAc residues. Differentiation of the two remaining residues **C** and **D** to D-GalA and D-Fuc3N, respectively, were readily performed by ^{1}H,^{1}H-NOESY and ^{1}H,^{13}C-HMBC spectra. The ^{1}H and ^{13}C chemical shifts of α-D-Fuc*p*3NAc and β-D-Fuc*p*3NAc were also recorded (Table 2) since glycosylation shifts are highly indicative of substitution patterns in sugars.[6]

The ^{15}N NMR chemical shifts, together with their corresponding amide protons, were determined from 1D ^{1}H NMR and 2D ^{1}H,^{15}N-HSQC NMR spectra. Due to fast proton exchange at 30 °C, the spectra were recorded at 10 °C. The ^{1}H,^{15}N-HSQC spectrum (Fig. 3) further supports the existence of three amino sugars in the repeating unit. The resonances were assigned (Table 1) to their corresponding sugar residues using ^{1}H,^{1}H-TOCSY and ^{1}H,^{1}H-NOESY experiments, thereby confirming two D-GlcNAc residues and one residue with the amino function at C-3, that is, D-Fuc3NAc.

A band-selective, constant-time ^{1}H,^{13}C-HMBC experiment was performed with a selective pulse centred at the carbonyl resonance frequency.[7] The resulting spectrum lends further evidence of the two D-GlcNAc residues with correlations from a carbonyl resonance at δ_C 175.5 to δ_H 4.03 (H-2 in **A**) and to δ_H 3.90 (H-2 in **B**). The resonance at δ_C 175.1 ppm has a correlation to δ_H 3.92 confirming the *N*-acetyl group at C-3 in **D**. Finally, the existence of the uronic acid was confirmed by a correlation from δ_C 174.0 to δ_H 4.09 (H-5 in **C**).

The substitution positions for the sugar residues were identified from ^{13}C NMR glycosylation shifts.[6] Residue **A** has significant glycosylation shifts $\Delta\delta_C$ 9.6 and 7.3 for C-3 and C-6, respectively. This result shows that residue **A** is disubstituted and corresponds to →3,6)-α-D-Glc*p*NAc-(1→. Residue **B** was assigned to →3)-β-D-Glc*p*NAc-(1→ due to the large C-3 glycosylation shift, $\Delta\delta_C$ 9.2. Residue **C** has a glycosylation shift for C-4 of $\Delta\delta_C$ 8.7, thereby assigning it to be →4)-β-D-Gal*p*A-(1→. Finally, residue **D** was shown to be β-D-Fuc*p*3NAc-(1→ since only C-1 has a large glycosylation shift.

The sequence of the sugar residues in the O-antigen repeating unit was determined from ^{1}H,^{1}H-NOESY and ^{1}H,^{13}C-HMBC experiments (Table 1). Three-bond heteronuclear correlations were observed that define the trisaccharide element **A**–**C**–**B** which corresponds to the backbone of the PS. Residue **D** is linked to O-3 in **A**. ^{1}H,^{1}H-NOE correlations across the glycosidic linkages, consistent with the above, were also present. The structure of the repeating unit of the O-antigen polysaccharide from *E. coli* O74 is thus:



In the ^{1}H NMR spectrum a peak of low intensity, denoted **A′**, was observed at 4.95 ppm (Fig. 1). Subsequent analysis of the spin system originating from the anomeric resonance of this residue using, for example, ^{1}H,^{1}H-TOCSY and ^{1}H,^{13}C-HMBC experiments, revealed that it corresponds to an *N*-acetylglucosamine residue, similar to **A**, but with slightly altered chemical shifts (H-1′ to H-5′: 4.95, 4.06, 3.90, 3.60 and 4.15 ppm; C-1′ to C-5′: 100.0, 53.1, 81.2, 68.9 and 72.7 ppm). In particular, the resonance from H-5′ has an altered (lower) chemical shift from that which is observed in the PS. The C-5′ resonance is present at a higher chemical shift, that is, the ^{13}C β-effect on glycosylation, ca 1 ppm upfield (cf. Table 1), is absent for the **A′** residue. These results indicate that **A′** corresponds to →3)-α-D-Glc*p*NAc-(1→, the penultimate residue in the PS. Consequently, the biological repeating unit in O-antigen is defined, having a →3)-β-D-Glc*p*NAc-(1→ residue at its reducing end.[8] Integration of the resonances at 4.95 and 4.91 ppm in the ^{1}H NMR spectrum revealed that the PS preparation consisted of ~13 repeating units on average.

Cross-reactivity has been reported between *E. coli* O74 and *E. coli* O2 as well as to *E. coli* K45.[9] In the LPS of *E. coli* O2, which *inter alia* contains three L-Rha*p* residues in the repeating unit of its O-antigen, D-Fuc*p*3NAc is α-linked, whereas in the CPS of *E. coli* K45 it is β-linked. Notably, the repeating unit in the LPS of *E. coli* O74 determined herein is identical to that of the CPS from *E. coli* K45.[10] The genetic relationships, biosynthesis and assembly machinery for the LPS in *E. coli* O74 and the CPS in *E. coli* K45, the capsule of which should be classified as belonging to group 4,[11] may now be investigated in detail knowing the structure of the repeating unit. D-Fuc*p*3NAc is an unusual sugar, but it has been found in, for example, the O-antigenic polysaccharides from *Proteus vulgaris* O45 and *Xanthomonas campestris* strain 8004.[12–14]

Table 1
^{1}H, ^{13}C and ^{15}N NMR chemical shifts (ppm) of the resonances from the O-antigen polysaccharide of *E. coli* O74 and interresidue correlations from ^{1}H,^{1}H-NOESY and ^{1}H,^{13}C-HMBC spectra

Sugar residue		$^{1}H/^{13}C$								$^{1}H/^{15}N$	Correlation to atom (from anomeric atom)	
		1	2	3	4	5	6	Me	CO	NH[b]	NOE	HMBC
→3,6)-α-D-Glc*p*NAc-(1→	A	4.91 [3.8][a]	4.03	3.86	3.58	4.28	3.84, 4.10	2.04		8.03	H-4, C	C-4, C
		(−0.30)	(0.15)	(0.11)	(0.09)	(0.42)		(−0.02)				
		100.1 {173}	53.1	81.3	68.9	71.4	69.1	23.2	175.5	122.0		H-4, C
		(8.3)	(−1.9)	(9.6)	(−2.4)	(−1.1)	(7.3)	(0.3)	(0.4)			
→3)-β-D-Glc*p*NAc-(1→	B	4.59 [8.3][a]	3.90	3.83	3.61	3.52	3.83, 3.97	2.07		8.49	H-6a, A	C-6, A
		(−0.13)	(0.25)	(0.27)	(0.15)	(0.06)		(0.01)			H-6b, A	
		102.4 {161}	55.6	84.0	69.7	76.4	61.6	23.2	175.5	122.2		
		(6.6)	(−2.3)	(9.2)	(−1.4)	(−0.4)	(−0.3)	(0.1)	(0.0)			
→4)-β-D-Gal*p*A-(1→	C	4.49 [7.1][a]	3.59	3.79	4.29	4.09					H-3, B	C-3, B
		(−0.07)	(0.08)	(0.10)	(0.06)	(0.06)						
		104.3 {∼160}	70.9	72.8	79.9	75.2	174.0					H-3, B
		(7.4)	(−1.7)	(−1.0)	(8.7)	(−1.2)	(−1.6)					
β-D-Fuc*p*3NAc-(1→	D	4.46 [7.7][a]	3.52	3.92	3.73	3.89	1.25	2.05		8.35	H-3, A	C-3, A
		(−0.18)	(0.05)	(−0.01)	(0.03)	(0.02)	(0.02)	(−0.01)		(0.02)		
		104.6 {∼160}	68.8	55.6	70.5	72.6	16.3	22.8	175.1	125.1		H-3, A
		(7.0)	(−1.7)	(−0.1)	(−0.4)	(−0.1)	(−0.1)	(0.0)	(0.0)	(0.1)		

Spectra were recorded at 30 °C unless stated otherwise.
[a] $J_{H-1,H-2}$ values are given in hertz in square brackets and $J_{H-1,C-1}$ values in braces. Chemical shift differences as compared to the corresponding monosaccharides are given in parentheses.
[b] NMR spectra were acquired at 10 °C.

Table 2
^{1}H, ^{13}C and ^{15}N NMR chemical shifts (ppm) of the α- and β-anomeric forms of D-Fuc*p*3NAc at 65 °C

Sugar	$^{1}H/^{13}C$								$^{1}H/^{15}N$
	1	2	3	4	5	6	Me	CO	NH[b]
α-D-Fuc*p*3NAc	5.21 [3.7][a]	3.82	4.16	3.75	4.25	1.18	2.06		8.26
	92.67	66.99	51.81	71.29	67.12	16.25	22.90	175.22	125.4
β-D-Fuc*p*3NAc	4.64 [7.8][a]	3.47	3.93	3.70	3.87	1.23	2.06		8.33
	97.65	70.46	55.74	70.93	72.70	16.35	22.84	175.09	125.0

[a] $J_{H-1,H-2}$ values are given in hertz in square brackets.
[b] NMR spectra were acquired at 10 °C.

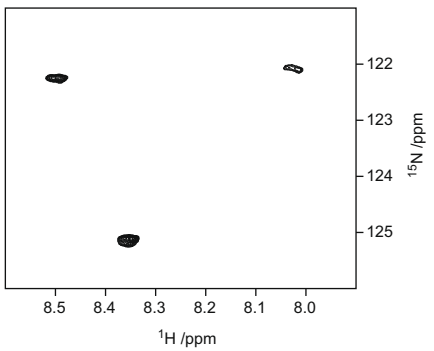


Figure 3. The ^{1}H,^{15}N-HSQC NMR spectrum of the O-antigen PS from *E. coli* O74.

1. Experimental

1.1. Bacterial strain and conditions of growth

The *E. coli* O74:K-:H39 strain CCUG 11376 was obtained from the Culture Collection University of Gothenburg, Sweden. The bacteria were grown in a submerged culture to late exponential phase in 15 L of a Luria–Bertani (LB) broth, using a 30-L fermentor (Belach AB) under constant aeration at 37 °C and pH 7.0. A preculture (1.5 L) in the same medium was used to inoculate the fermentor. The culture was checked for purity at the end of the growth cycle. The bacteria were killed with 1% (v/v) formaldehyde. After incubation for 16 h 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 29,000*g* and a flow of 25 L h^{-1} (Carl Padberg Centrifugenbau, Lahr, Germany). The bacterial mass was then removed from the cylinder, washed once with NaCl/P_i (0.01 M potassium phosphate, 0.14 M NaCl, pH 7.2), centrifuged (8000*g*, 4 °C, 20 min) and finally resuspended in distilled water.

1.2. Preparation of the lipopolysaccharide and lipid-free polysaccharide

The lipopolysaccharide (LPS) was extracted by the hot phenol–water method.[15] The aqueous phase was dialyzed at 4 °C for 3–5 days against tap water, overnight against distilled water, concentrated under diminished pressure and lyophilized. Contaminating nucleic acids were removed by ultracentrifugation (100,000*g*, 4 h, 4 °C). Lipid-free polysaccharide (PS) was prepared by treatment of the LPS with 0.1 M sodium acetate, pH 4.2, at 100 °C for 5 h.[16] Lipid A was removed by centrifugation (10,000*g*, 20 min, 4 °C). The PS was further purified by gel-permeation chromatography.

1.3. Preparation of D-Fuc3NAc

3-Acetamido-3-deoxy-D-fucose was prepared from ethyl 3-amino-2,4-di-*O*-benzyl-3-deoxy-1-thio-β-D-fucopyranoside[17] (17 mg) by acetylation of the amino group with Ac_2O. The *S*-ethyl group at the anomeric position was then removed using NIS as a promoter and H_2O as the nucleophile. The benzyl groups were

removed by catalytic hydrogenolysis using $Pd(OH)_2/C$ and H_2 (g). All steps were performed consecutively without purification in between due to the limited amount of material. The final purifications were carried out on a size-exclusion gel plug of Bio-Gel P-2 and then on a 20 mm C-18 Sep-Pak® column eluted with H_2O to give the target compound (5 mg). The monosaccharide was freeze-dried from D_2O three times prior to preparation of the NMR sample.

1.4. Component analyses

The PS was hydrolyzed with 2 M TFA at 120 °C for 30 min. The sample was then reduced with $NaBH_4$ and acetylated, after which it was analyzed by GLC. The uronic acid was identified through methanolysis under acidic conditions (MeOH, AcCl, 85 °C, overnight) and was analyzed with GLC as an acetylated methyl glycoside methyl ester derivative. The absolute configuration of the sugar components was determined by GLC analysis of their acetylated (+)-2-butyl glycoside derivatives ((+)-2-butanol, AcCl, 85 °C, overnight) essentially as described.[18]

1.5. GLC analyses

Alditol acetates and the acetylated alcohol glycosides were separated on a HP-5 column using a temperature programme of 180 °C for 1 min, 3 °C min^{-1} up to 230 °C and then 5 min at 230 °C. Hydrogen gas was used as carrier gas. The column was fitted to a Hewlett–Packard model 5890 series II gas chromatograph equipped with a flame-ionization detector.

1.6. NMR spectroscopy

NMR spectra of the PS (2 mg) in D_2O solution (0.55 mL) were recorded at 30 °C on a Bruker AVANCE 500-MHz spectrometer equipped with a 5-mm Cryoprobe. Data processing was performed using vendor-supplied software. To detect amide protons the PS was dissolved in a 9:1 mixture of H_2O–D_2O, and the spectra were recorded at 10 °C. Chemical shifts are reported in ppm using external sodium 3-methylsilyl-(2,2,3,3-$^2H_4$)-propanoate (TSP, δ_H 0.00) or 1,4-dioxane in D_2O (δ_C 67.40) as references.

The assignments of the ^1H and ^{13}C resonances of the PS were obtained by analysis of both 1D ^1H and ^{13}C NMR spectra, together with 2D NMR spectra from ^1H,^{13}C-HSQC experiments, including a multiplicity-edited version,[19] ^1H,^1H-TOCSY experiments[20] with mixing times 10, 30, 60, 90 and 120 ms, ^1H,^1H-NOESY experiments[21] with mixing times of 50 and 100 ms and a band-selective constant-time ^1H,^{13}C-HMBC experiment.[7] The latter was performed with a 50-ms *J*-evolution delay, and the carbonyl resonances were selectively excited using a 2.5 ms 180° Gaussian Cascade pulse. The spectrum was collected with 1344 scans per increment over 6 ppm (^1H) × 9 ppm (^{13}C region centred at 175 ppm) with $2k \times 80$ data points, respectively. The spectrum was processed using a 90°-shifted squared sine-bell window function in F_2 and a 90°-shifted sine-bell function in F_1. The direct dimension was zero-filled once and the resolution in F_1 was enhanced by forward linear prediction. The interresidue correlations were assigned using ^1H,^1H-NOESY experiments with the above-mentioned mixing times and a ^1H,^{13}C-HMBC experiment[22,23] with a 50-ms delay for evolution of long-range couplings. The chemical shifts were compared to those of the corresponding monosaccharides.[24]

The amide ^1H and ^{15}N NMR chemical shifts were determined from a 1D ^1H NMR experiment with water suppression using excitation sculpting[25] (selective square pulse with a length of 2 ms) and a ^1H,^{15}N-HSQC experiment[26] with water suppression using WATERGATE[27] with a selective sinc pulse of 1.2 ms and gradients of 10 G cm^{-1}. The latter spectrum was recorded over 10 ppm (^1H) × 20 ppm (^{15}N region centred at 120 ppm) with $2k \times 256$ data points, respectively, and was processed using 90°-shifted squared sine-bell window functions in both dimensions. The direct dimension was zero-filled twice and the resolution in F_1 was enhanced by forward linear prediction. The assignments of the chemical shifts to the corresponding sugar residue were accomplished by analysis of spectra from ^1H,^1H-TOCSY (mixing times 10, 30 and 60 ms) and ^1H,^1H-NOESY (mixing time 100 ms) experiments with water suppression by excitation sculpting (selective square pulse with a length of 2 ms). The ^{15}N NMR chemical shifts were indirectly referenced as described by Wishart et al.[28] using $^{15}N/^1H$ = 0.10132900.[29]

The assignments of the ^1H and ^{13}C NMR chemical shifts of D-Fuc*p*3NAc were performed at 65 °C using the same experiments as mentioned above on a sample containing the monosaccharide (2 mg) in D_2O solution (0.55 mL). The amide ^1H and ^{15}N NMR chemical shifts were determined at 10 °C in 9:1 H_2O–D_2O.

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