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Note

Structural determination of the O-antigenic polysaccharide from the verocytotoxin-producing *Escherichia coli* O176

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Abstract—The structure of the O-antigen polysaccharide (PS) from *Escherichia coli* O176 has been determined. Component analysis together with ^{1}H and ^{13}C NMR spectroscopy was 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:

→4)-α-D-Man*p*-(1→2)-α-D-Man*p*-(1→2)-β-D-Man*p*-(1→3)-α-D-Gal*p*NAc-(1→

Cross-peaks of low intensity from α-linked mannopyranosyl residues were present in the ^{1}H, ^{1}H TOCSY NMR spectra and further analysis of these showed that they originate from the terminal part of the polysaccharide. Consequently, the biological repeating unit has a 3-substituted *N*-acetyl-D-galactosamine residue at its reducing end. The repeating unit of the *E. coli* O176 O-antigen is similar to those from *E. coli* O17 and O77, thereby explaining the reported cross-reactivities between the strains, and identical to that of *Salmonella cerro* (O:6, 14, 18).

Keywords: *Escherichia coli*; *Salmonella cerro*; Lipopolysaccharide; NMR; Biological repeating unit

Escherichia coli is the predominating gram-negative, facultative anaerobic rod in the gut of humans and warm-blooded animals. Most of the strains are harmless members of the normal gut flora. However, there are a number of strains that cause disease in man. Verocytotoxin-producing *E. coli* (VTEC) is one of the categories of diarrhoea causing strains. VTEC strains occur naturally in different animals, particularly cattle, and may act as a reservoir for human infections.[1] The transmission of VTEC from animals to man often occurs by contaminated food or water. In addition, due to a very low infectious dose (100–200 organisms), person to person transmission is a high risk factor.[2] Usually, the VTEC strains belong to a restricted number of serotypes and produce one or two different verocytotoxins (VT1 and/or VT2). The most characterized VTEC serotype is *E. coli* O157:H7 and in the 1990s it was ranked as the cause of the fourth most costly food borne disease in USA.[3] However, during the past 15 years the epidemiology of VTEC has shifted and other non-O157 serotypes are frequently isolated. The *E. coli* O176 was isolated from a calf without symptoms and classified as VTEC.[4] The strain was designated as *E. coli* O176:H- and was positive for the genes encoding the VT1 as well as for the enterohaemolysin. We herein elucidate the structure of the *E. coli* O176 O-antigen polysaccharide.

E. coli O176:H- 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 mannose,

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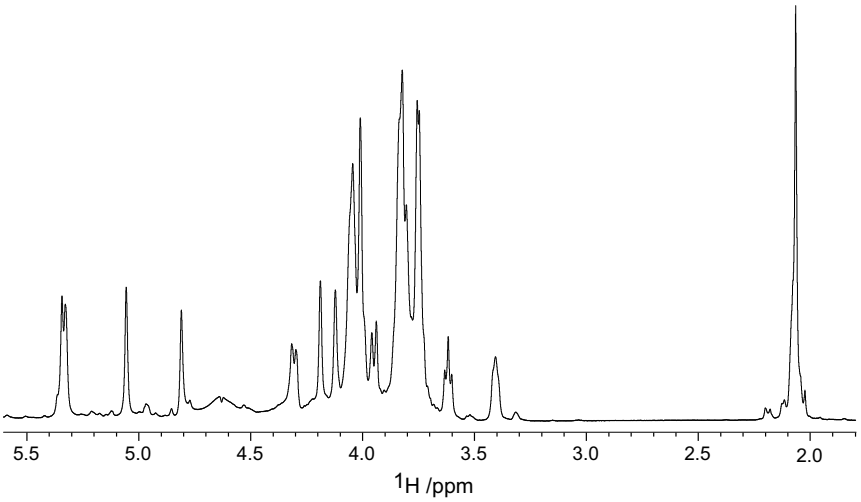


Figure 1. The ^{1}H NMR spectrum of the O-antigen PS from *E. coli* O176.

glucose, galactose, 2-amino-2-deoxyglucose, 2-amino-2-deoxygalactose and heptose in the ratio 73:6:2:2:15:2. Determination of the absolute configuration of the two main components showed D-Man and D-GalN, the remaining sugars being attributed to the core.

The anomeric region in the ^{1}H NMR spectrum of the *E. coli* O176 PS had four resonances that could be assigned to anomeric protons with H-1 chemical shifts of 5.35, 5.33, 5.06 and 4.81 ppm (Fig. 1). The corresponding sugar residues were then denoted **A**–**D**. A resonance was found, inter alia, at δ_{H} 2.05 (3H) indicating that the aminosugar is N-acetylated. The ^{1}H, ^{13}C HSQC spectrum of the PS showed in the region for anomeric resonances four cross-peaks corresponding to hexopyranosyl residues (Fig. 2). A cross-peak at δ_{C} 4.31/δ_{C} 49.97 confirmed the presence of the aminosugar in the repeating unit of the O-antigen. The ^{1}H and ^{13}C NMR resonances were assigned using 2D NMR techniques. In the resonance-assignments, two-[5] and three-bond heteronuclear intra-residue correlations present in the ^{1}H, ^{13}C HMBC spectrum were of particular importance. 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. Residues **A**–**C** are α-linked since $J_{H\text{-}1,C\text{-}1}$ coupling constants are ~175 Hz and residue **D** is β-linked as the corresponding coupling constant is 160 Hz.[6] From the analysis of ^{1}H, ^{1}H TOCSY spectra and ^{1}H and ^{13}C chemical shifts, residues **A**, **C** and **D** have the *manno*-configuration whereas residue **B** has the *galacto*-configuration. The chemical shifts of the resonances from the C-2 atoms reveal that residue **B** is the aminosugar.

The substitution positions and the linkage pattern for the sugar residues can be identified from ^{13}C NMR glycosylation shifts.[7] Residue **A** has a significant glycosylation shift $\Delta\delta_{C}$ 7.27 for C-2, which reveals it to be →2)-α-D-Man*p*-(1→. Residue **B** was assigned to →3)-α-D-Gal*p*NAc-(1→ due to the large glycosylation shift of C-3, $\Delta\delta_{C}$ 9.00. Residue **C** has a glycosylation shift for C-4 of $\Delta\delta_{C}$ 6.69, thus assigning it to be →4)-α-D-Man*p*-(1→. Finally, residue **D** was shown to be →2)-β-D-Man*p*-(1→, with a glycosylation shift for C-2 of $\Delta\delta_{C}$ 4.60.

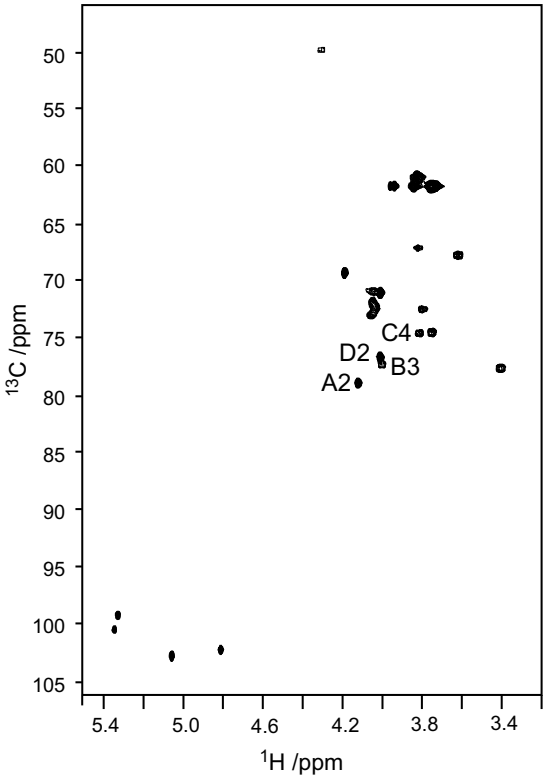


Figure 2. The ^{1}H, ^{13}C HSQC NMR spectrum of the O-antigen PS from *E. coli* O176. Resonances from substitution positions are annotated.

The sequence of the sugar residues in the O-antigen repeating unit was determined from ^{1}H, ^{1}H NOESY

Table 1. ^{1}H and ^{13}C NMR chemical shifts (ppm) of the resonances from the O-antigen polysaccharide of *E. coli* O176 and inter-residue correlations from ^{1}H, ^{1}H NOESY and ^{1}H, ^{13}C HMBC spectra

Sugar residue		$^{1}H/^{13}C$						Correlation to atom (from anomeric atom)	
		1	2	3	4	5	6	NOE	HMBC
→2)-α-D-Man*p*-(1→	**A**	5.35 [n.r.]	4.12	4.04	3.82	4.05	3.75, 3.84	H-2, **D**	C-2, **D**
		(0.17)	(0.18)	(0.18)	(0.14)	(0.23)		H-5, **C**	
		100.46 {175}	78.96	70.98	67.21	73.07	61.88		H-2, **D**
		(5.52)	(7.27)	(−0.27)	(−0.73)	(−0.27)	(−0.11)		
→3)-α-D-Gal*p*NAc-(1→[a]	**B**	5.33 [~3.5]	4.31	4.00	4.19	4.03	~3.75	H-4, **C**	C-4, **C**
		(0.05)	(0.12)	(0.05)	(0.14)	(−0.10)			
		99.18 {177}	49.97	77.40	69.38	72.45	61.88		H-4, **C**
		(7.23)	(−1.19)	(9.00)	(−0.18)	(1.09)	(−0.23)		
→4)-α-D-Man*p*-(1→	**C**	5.06 [n.r.]	4.01	4.045	3.82	3.80	~3.82	H-2, **A**	C-2, **A**
		(−0.12)	(0.07)	(0.185)	(0.14)	(−0.02)			
		102.69 {173}	71.15	71.96	74.63	72.53	61.05		H-2, **A**
		(7.75)	(−0.54)	(0.71)	(6.69)	(−0.81)	(−0.94)		
→2)-β-D-Man*p*-(1→	**D**	4.81 [n.r.]	4.01	3.75	3.62	3.41	3.74, 3.95	H-3, **B**	C-3, **B**
		(−0.08)	(0.06)	(0.09)	(0.02)	(−0.03)			
		102.20 {160}	76.73	74.57	67.84	77.69	61.88		H-3, **B**
		(7.65)	(4.60)	(0.54)	(0.15)	(0.69)	(−0.11)		

$J_{H\text{-}1,\ H\text{-}2}$ and $J_{H\text{-}1,C\text{-}1}$ values are given in Hz in square brackets and in braces, respectively. n.r. = not resolved.
Chemical shift differences as compared to the corresponding monosaccharides are given in parenthesis.
[a] Chemical shifts for NAc are δ_H 2.06; δ_C 22.90 and 174.84.

(Fig. 3) and ^{1}H, ^{13}C HMBC experiments (Table 1). Three-bond heteronuclear correlations were observed that define the tetrasaccharide elements **C**–**A**–**D**–**B** (1→2)-α-D-Man*p*-(1→.[8] The structure of the repeating unit of the O-antigen polysaccharide from *E. coli* O176 is

C **A** **D** **B**
→4)-α-D-Man*p*-(1→2)-α-D-Man*p*-(1→2)-β-D-Man*p*-(1→3)-α-D-Gal*p*NAc-(1→

in which residue **C** is substituted at position O-4. ^{1}H, ^{1}H NOE correlations across glycosidic linkages, consistent with the above, were also present. In addition, a strong NOE was observed between H-1 in residue **A** and H-5 in residue **C**. This NOE is anticipated for the disaccharide structural element α-D-Man*p*-

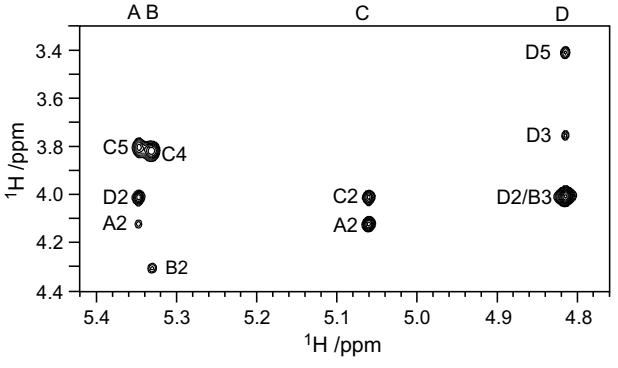
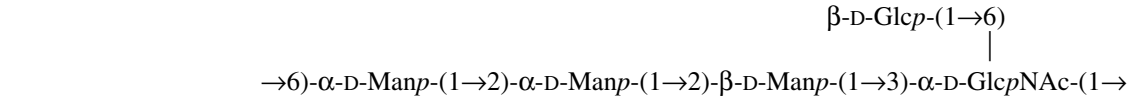


Figure 3. Part of the ^{1}H, ^{1}H NOESY NMR spectrum of the O-antigen PS from *E. coli* O176.

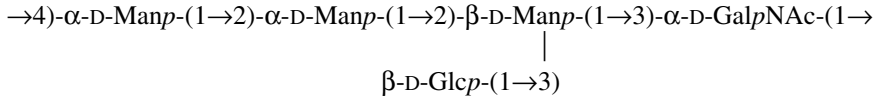
The ^{1}H, ^{1}H TOCSY NMR experiment with a mixing time of 30 ms resulted in a spectrum in which cross-peaks of low intensities were also observed. Chemical shifts similar to those of anomeric protons from residues **A** and **C** were present, viz, at 5.37 ppm and shifted by +0.002 ppm compared to that of the anomeric proton from residue **C**, respectively. Corresponding cross-peaks to H-2 protons were observed at 4.11 and 4.08 ppm. The latter is in excellent agreement with the chemical shift of H-2 in the terminal residue of α-D-Man*p*-(1→2)-α-D-Man*p*-OMe,[9] indicating that these resonances derive from the terminal residues in the O-antigen polysaccharide. Consequently, the biological repeating unit in O-antigen is defined, having a →3)-α-D-Gal*p*NAc-(1→ residue at its reducing end.[10] Integration of the resonances at 5.37 and 5.35 ppm in the ^{1}H NMR spectrum revealed that the PS preparation consisted of ~10 repeating units on average.

Cross-reactivity[4] has been reported between *E. coli* O176 and *E. coli* O17 as well as *E. coli* O77.[11,12] The structures of the O-antigens of the two latter have the same back-bone consisting of four sugar residues, but

that of *E. coli* O17 has a glucosyl group as a side-chain as shown below:



The differences to the *E. coli* O176 O-antigen linear back-bone are (i) in the O17 and O77 structures there is instead an *N*-acetyl-D-glucosamine residue and (ii) the O-antigens are polymerized at O-6 of the terminal α-D-Man*p*-(1→2)-residue in the undecaprenyl-PP-oligosaccharide. Notably, the *E. coli* O176 O-antigen has the same linear back-bone as that of the *E. coli* strain 73-1 O-antigen, which also has a glucosyl group as a side-chain.[13] The pentasaccharide repeating unit of the O-antigen from *E. coli* strain 73-1 is given below:



Likewise, the *E. coli* strain 73-1 O-antigen is reminiscent of those from *Salmonella carrau* (O:6, 14, 24)[14] and *E. coli* O73.[15] Thus, the relationships of the O-antigen structures of the *E. coli* O176 and strain 73-1 O-antigens are similar to those of the 'O77 group', including *E. coli* O73. Finally, the O-antigen structure of *E. coli* O176 is identical to that of *Salmonella cerro* (O:6, 14, 18) recently reported by Vinogradov et al.[16]

1. Experimental

1.1. Bacterial strains and conditions of growth

The *E. coli* O176:H-, isolate E29518-83, was obtained from the International *Escherichia* and *Klebsiella* Centre (WHO), Statens Serum Institut, Copenhagen S, Denmark. 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 re-suspended in distilled water.

1.2. Preparation of lipopolysaccharide and lipid-free polysaccharide

The lipopolysaccharide (LPS) was extracted by the hot phenol/water method.[17] 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.[18] Lipid A was removed by centrifugation (10,000*g*, 20 min, 4 °C). The PS was further purified by gel-permeation chromatography.

1.3. Component analyses

The PS was hydrolyzed with 2 M trifluoroacetic acid at 120 °C for 2 h. After reduction with NaB^2H_4 and acetylation, the sample was analyzed by GLC. The absolute configurations of the sugars present in the O-antigen were determined by derivation of the sugars as their acetylated (+)-2-butyl glycosides essentially as described.[19]

1.4. GLC analyses

Alditol acetates and acetylated 2-butyl glycosides were separated on an HP-5 column using a temperature programme of 180 °C for 1 min, 3 °C min^{-1} to 210 °C, 10 min at 210 °C. Hydrogen was used as carrier gas. The columns were fitted to a Hewlett–Packard model 5890 series II gas chromatograph equipped with a flame ionization detector.

1.5. NMR spectroscopy

NMR spectra of the PS (33 mg) in D_2O solution (0.6 mL) were recorded at 40 °C using a Varian Inova 600 spectrometer equipped with 5 mm PFG triple-resonance probe. Data processing was performed using

vendor-supplied software. Chemical shifts are reported in ppm using internal sodium 3-trimethylsilyl-(2,2,3,3-$^2H_4$)-propanoate (TSP, δ_H 0.00) or external 1,4-dioxane in D_2O (δ_C 67.40) as references. Total correlation spectroscopy (TOCSY)[20] with mixing times of 10, 30, 60 and 100 ms, nuclear Overhauser effect spectroscopy (NOESY)[21] with mixing times of 50 and 100 ms, gradient selected heteronuclear single quantum coherence (gHSQC),[22] gradient selected heteronuclear multiple-bond correlation (gHMBC)[23] with a mixing time of 50 ms and HSQC–TOCSY[24] experiments with mixing times of 20 and 50 ms were used in the assignment of spectra. The chemical shifts were compared to those of the corresponding monosaccharides.[25]

Acknowledgements

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