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Note

# Structural determination of the O-antigenic polysaccharide from *Escherichia coli* O166

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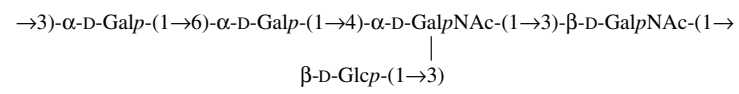
**Abstract**—The O-antigen of the lipopolysaccharide from *Escherichia coli* O166 has been determined by component analysis together with 1D and 2D NMR spectroscopy techniques. The polysaccharide has pentasaccharide repeating units consisting of D-glucose (1), D-galactose (2) and *N*-acetyl-D-galactosamine (2) with the following structure:




In the $^1$H NMR, spectrum resonances of low intensity were observed. Further analysis of these showed that they originate from the terminal part of the polysaccharide, thereby revealing that the repeating unit has a 3-substituted *N*-acetyl-D-galactosamine residue at its reducing end.


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

*Escherichia coli* strains causing diarrhoeal diseases are divided into six well-described categories: enteropathogenic *E. coli* (EPEC); enterotoxigenic *E. coli* (ETEC); enteroinvasive *E. coli* (EIEC); enterohemorrhagic *E. coli* (EHEC); enteroaggregative *E. coli* (EAEC) and diffusely adherent *E. coli* (DAEC).[1] The different virulence attributes help the bacteria to cause diseases by various mechanisms.

*E. coli* O166 is a rather common serotype isolated from patients with diarrhoea. This serotype has been described as EAEC possessing the heat-stable enterotoxin 1 (EAST 1) gene.[2] In addition, the *E. coli* O166 was found as a cause of an outbreak of diarrhoea in Japan in 1996.[3,4] The O166 serotype has also been classified as EHEC when isolated from cattle,[5] sheep,[6,7] and from the environment.[8,9] Furthermore, O166 has been identified as ETEC in New Caledonia.[10] We herein present the structure of the O-polysaccharide from an *E. coli* O166 reference strain, that is, the original O166 strain described by Ørskov et al. in 1984.[11]

*E. coli* O166 was obtained from The International *Escherichia* and *Klebsiella* Centre and was grown in an 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 glucose, galactose and 2-amino-2-deoxygalactose in the ratio 4:4:5. Determination of the absolute configuration showed that these sugars have the D-configuration.

The anomeric region in the $^1$H NMR spectrum of the *E. coli* O166 PS showed seven resonances, five of which

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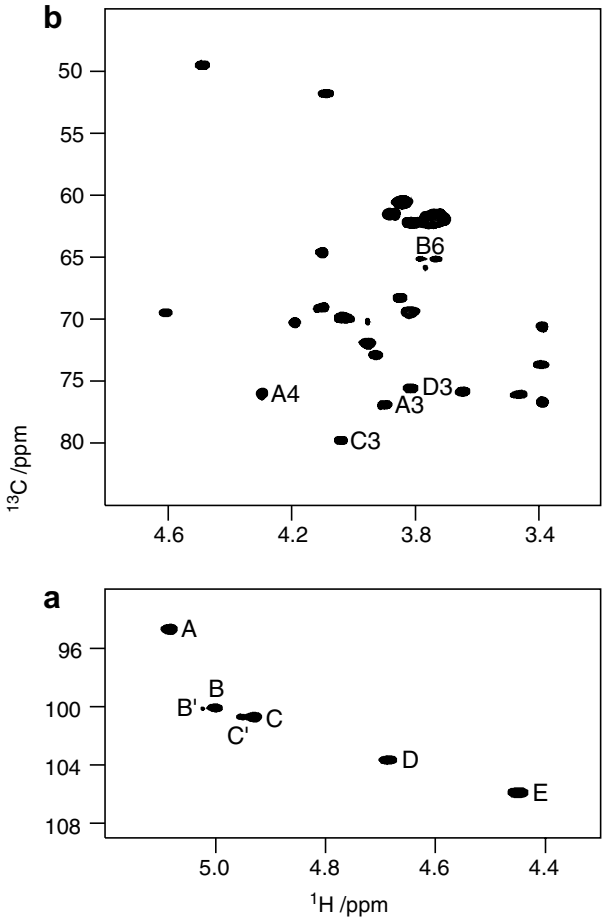
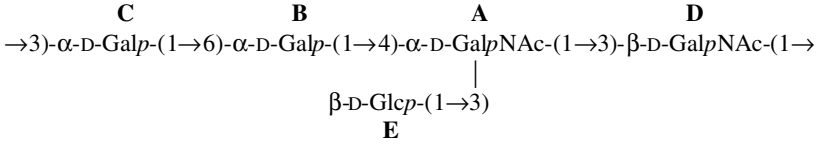



**Figure 1.** Sections of the $^{1}$H,$^{13}$C HSQC NMR spectrum of the O-antigen PS from *E. coli* O166 showing (a) the anomeric region and (b) the region for ring atoms and hydroxymethyl groups. Resonances from substitution positions are annotated.

subsequently could be assigned to anomeric ones denoted **A**–**E**, in decreasing order of their $^{1}$H chemical shift (5.08, 5.00, 4.93, 4.68 and 4.45 ppm). Additional resonances were found, inter alia, at $\delta_H$ 2.05 (3H) and 2.01 (3H) indicating that the aminosugars are N-acetylated. The $^{1}$H,$^{13}$C HSQC spectrum of the PS showed in the region for anomeric resonances five major peaks corresponding to hexopyranosyl residues (Fig. 1a). Resonances at $\delta_C$ 49.5 and 51.7 confirmed the presence of two aminosugars in the repeating unit of the O-antigen (Fig. 1b). The $^{1}$H and $^{13}$C NMR resonances were assigned using 2D NMR techniques and the chemical shifts are compiled in Table 1. The assignments of the chemical shifts of the *N*-acetyl groups to the aminosugars are based on chemical shift similarities to the corresponding monosaccharides. Residues **A**–**C** are α-linked since $J_{H\text{-}1,H\text{-}2}$ and $J_{H\text{-}1,C\text{-}1}$ coupling constants are ~4 and ~175 Hz, respectively, and residues **D** and **E** are β-linked since the corresponding coupling constants are ~8 and ~162 Hz, respectively. From the analysis of $^{1}$H,$^{1}$H TOCSY spectra and $^{1}$H and $^{13}$C chemical shifts, residues **A**–**D** have the *galacto*-configuration whereas residue **E** has the *gluco*-configuration. Furthermore, the chemical shifts of resonances from C-2 atoms reveal that residues **A** and **D** are the aminosugars.

Carbon-13 glycosylation shifts are indicative of substitution positions[12] and the linkage pattern for the sugar residues can consequently be identified. Residue **A** has large glycosylation shifts of $\Delta\delta_C$ 8.5 and 6.4, for C-3 and C-4, respectively, which shows it to be →3,4)-α-D-Gal*p*NAc-(1→. Residue **B** was assigned to →6)-α-D-Gal*p*-(1→ due to the glycosylation shift of C-6, $\Delta\delta_C$ 3.1. Residues **C** and **D** both have significant glycosylation shifts for C-3, $\Delta\delta_C$ 9.7 and 3.6, respectively, revealing them as →3)-α-D-Gal*p*-(1→ and →3)-β-D-Gal*p*NAc-(1→. Finally, residue **E** was shown to be a terminal β-D-Glc*p* residue.

The sequence of the sugar residues in the O-antigen repeating unit was determined from $^{1}$H,$^{1}$H NOESY (Fig. 2) and $^{1}$H,$^{13}$C HMBC experiments (Table 1). Three-bond heteronuclear correlations were observed that define the tetrasaccharide element **B**–**A**–**D**–**C** in which residue **A** is substituted at position O-4. Likewise residue **E** is linked to O-3 in residue **A** as deduced from the $^{1}$H,$^{13}$C HMBC spectrum. $^{1}$H,$^{1}$H NOE correlations consistent with the above were also present. The last structural element **C**–**B** was confirmed by NOE correlations from the anomeric proton in residue **C** to the H-6 protons as well as to H-4 in residue **B**. Thus, the structure of the repeating unit of the O-antigen polysaccharide from *E. coli* O166 is




Notable chemical shift displacements were observed for C-4 in **D**, $\Delta\delta_C$ −4.3, and the low glycosylation shift for C-1 in **A**, $\Delta\delta_C$ 2.8, both of which are due to the proximity between the anomeric proton in residue **A** and H-4 in residue **D** (an NOE was observed, cf. Table 1 and Fig. 2). This is known as the γ-gauche effect.[13] Furthermore, the chemical shift displacement of H-5 in **B**, $\Delta\delta_H$ 0.58, should be due to the stereo-chemical arrangement and conformational preferences of the residues at the

**Table 1.** $^{1}H$ and $^{13}C$ NMR chemical shifts (ppm) of the resonances from the O-antigen polysaccharide of *E. coli* O166 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 |
| →3,4)-α-D-Gal*p*NAc-(1→[a] | **A** | 5.08 [3.9] | 4.49 | 3.90 | 4.29 | 3.93 | ~3.84 | H3, **D** | C3, **D** |
| | | (−0.20) | (0.30) | (−0.05) | (0.24) | (−0.20) | | H4, **D** | |
| | | 94.7 {176} | 49.5 | 76.9 | 76.0 | 72.9 | 60.5 | | H3, **D** |
| | | (2.8) | (−1.7) | (8.5) | (6.4) | (1.5) | (−1.6) | | |
| →6)-α-D-Gal*p*-(1→ | **B** | 5.00 [4.0] | 3.82 | 4.03 | 4.09 | 4.61 | 3.73, 3.79 | H4, **A** | C4, **A** |
| | | (−0.22) | (0.04) | (0.22) | (0.14) | (0.58) | | H6, **A** | |
| | | 100.1 {174} | 69.5 | 69.9 | 69.0 | 69.5 | 65.1 | | H4, **A** |
| | | (6.9) | (0.2) | (−0.2) | (−1.3) | (−1.8) | (3.1) | | |
| →3)-α-D-Gal*p*-(1→ | **C** | 4.93 [4.0] | 3.85 | 4.04 | 4.19 | 3.95 | ~3.73 | H4, **B** | |
| | | (−0.29) | (0.07) | (0.23) | (0.24) | (−0.08) | | H6, **B** | |
| | | 100.7 {174} | 68.3 | 79.8 | 70.3 | 72.0 | 62.0 | | |
| | | (7.5) | (−1.1) | (9.7) | (0.0) | (0.7) | (0.0) | | |
| →3)-β-D-Gal*p*NAc-(1→[b] | **D** | 4.68 [8.5] | 4.08 | 3.82 | 4.10 | 3.64 | 3.75, 3.81 | H3, **C** | C3, **C** |
| | | (0.00) | (0.18) | (0.05) | (0.12) | (−0.08) | | | |
| | | 103.6 {163} | 51.7 | 75.6 | 64.6 | 75.9 | 62.2 | | H3, **C** |
| | | (7.3) | (−3.1) | (3.6) | (−4.3) | (−0.1) | (0.3) | | |
| β-D-Glc*p*-(1→ | **E** | 4.45 [7.7] | 3.39 | 3.46 | 3.39 | 3.39 | 3.73, 3.88 | H3, **A** | C3, **A** |
| | | (−0.19) | (0.14) | (−0.04) | (−0.03) | (−0.07) | | | |
| | | 105.9 {162} | 73.7 | 76.1 | 70.6 | 76.7 | 61.5 | | H3, **A** |
| | | (9.1) | (−1.5) | (−0.7) | (−0.1) | (−0.1) | (−0.3) | | |

$J_{H\text{-}1,H\text{-}2}$ values are given in hertz in square brackets and $J_{H\text{-}1,C\text{-}1}$ values in braces. Chemical shift differences as compared to the corresponding monosaccharides are given in parenthesis.

[a] Chemical shifts for NAc are $\delta_H$ 2.01; $\delta_C$ 22.9 and 175.4.

[b] Chemical shifts for NAc are $\delta_H$ 2.05; $\delta_C$ 23.2 and 175.8.

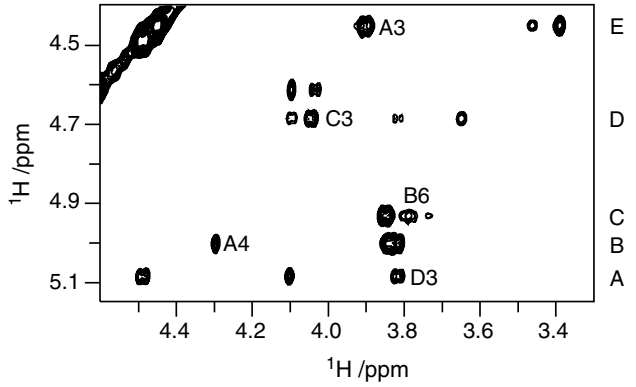



**Figure 2.** Part of the $^{1}H,^{1}H$ NOESY NMR spectrum of the O-antigen PS from *E. coli* O166.

branching region of the repeating unit, which are similar to those in the O-antigen PS from *E. coli* O142.[14,15]

Analysis of the LPS preparation by SDS/PAGE showed a ladder pattern due to LPS with different numbers of repeating units (data not shown). The $^{1}H,^{13}C$ HSQC NMR spectrum contains peaks of low intensities (Fig. 1a), which have chemical shifts similar of those from residues **A**–**C** and will be referred to as **A′**–**C′** (Table 2). Integration of the resonances in the $^{1}H$ NMR spectrum revealed that the PS preparation consisted of ~6 repeating units on average. The proton and carbon chemical shifts of residue **C′** indicated it to be an unsubstituted α-D-Gal*p* residue, which locates it on the terminus of the non-reducing end of the PS. Consequently, the terminal end of the PS has the sequence **C′**–**B′**–**A′**,

**Table 2.** Selected $^{1}H$ and $^{13}C$ NMR chemical shifts (ppm) of the resonances from the terminal repeating unit of the O-antigen polysaccharide from *E. coli* O166

| Sugar residue | | $^{1}H/^{13}C$ | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 |
| →3,4)-α-D-Gal*p*NAc–(1→ | **A′** | 5.09 | 4.50 | 3.90 | 4.29 | | |
| | | 94.7 | 49.5 | 76.9 | 76.0 | | |
| →6)-α-D-Gal*p*-(1→ | **B′** | 5.03 [4.0] | 3.82 | 4.02 | 4.12 | 4.56 | 3.77 |
| | | 100.2 | 70.1 | 70.2 | 69.1 | 69.4 | 65.8 |
| α-D-Gal*p*-(1→ | **C′** | 4.96 [3.9] | 3.78 | 3.88 | 3.95 | | |
| | | 100.7 | 69.3 | 70.2 | 70.2 | | |

$J_{H\text{-}1,H\text{-}2}$ values are given in hertz in square brackets.

confirmed by $^{1}H,^{1}H$ NOE correlations between H-1 in **C′** and H-6 in **B′** as well as between H-1 in **B′** and H-4 in **A′**. From this it is evident that the repeating unit of the O-antigen has an *N*-acetyl-D-galactosamine residue at its reducing end.[16] Whether the glucosyl group **E** in the side chain of the repeating unit is biosynthetically added on the periplasmic side of the membrane[17,18] remains an interesting question to be addressed in the future.

## 1. Experimental

### 1.1. Bacterial strains and conditions of growth

The enterotoxigenic *E. coli* O166 strain 3866-54 lot 101 was obtained from The International *Escherichia* and *Klebsiella* Centre (WHO), Statens Serum Institute, Copenhagen, Denmark. The bacterium was grown in a Luria Bertani (LB) medium.

### 1.2. Preparation of lipopolysaccharide and lipid-free polysaccharide

The LPS were extracted and delipidated as previously described.[19,20]

### 1.3. Component analyses

The PS was hydrolyzed with 2 M trifluoroacetic acid at 120 °C for 2 h. After reduction with $NaB^{2}H_4$ 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.[21]

### 1.4. GLC analyses

Alditol acetates and acetylated 2-butyl glycosides were separated on an HP-5 column using a temperature program of 180 °C for 1 min, 3 °C $min^{-1}$ to 210 °C, 10 min at 210 °C except for the galactosyl derivatives, which were separated on a DB-225 column with a temperature program of 180 °C for 1 min, 3 °C $min^{-1}$ to 210 °C, 1 min at 210 °C, 4 °C $min^{-1}$ to 230 °C, 10 min at 230 °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 in $D_2O$ solution were recorded at 25 °C using Varian Inova 600 and 800 spectrometers equipped with 5 mm PFG triple-resonance probes and on a Bruker DRX 500 MHz spectrometer equipped with a 5 mm PFG triple-resonance CryoProbe. Data processing was performed using vendor-supplied software. Chemical shifts are reported in ppm using internal sodium 3-trimethylsilyl-(2,2,3,3-$^{2}H_4$)-propanoate (TSP, $\delta_H$ 0.00) or external 1,4-dioxane in $D_2O$ ($\delta_C$ 67.40) as references. The PS (30 mg) was dissolved in 0.7 mL $D_2O$. Total correlation spectroscopy (TOCSY)[22] with mixing times of 30, 60 and 90 ms, nuclear Overhauser effect spectroscopy (NOESY)[23] with mixing times of 50 and 100 ms, gradient selected heteronuclear single quantum coherence (gHSQC),[24] gradient selected heteronuclear multiple-bond correlation (gHMBC)[25] with a mixing time of 50 ms, and HSQC–TOCSY[26] 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.[27]

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