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# Structure of the O-specific polysaccharide of *Proteus vulgaris* O45 containing 3-acetamido-3,6-dideoxy-D-galactose

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**Abstract**

An O-specific polysaccharide was isolated by mild acid degradation of the lipopolysaccharide of *Proteus vulgaris* O45 and studied by sugar and methylation analyses along with $^{1}H$ and $^{13}C$ NMR spectroscopy, including 2D COSY, TOCSY, ROESY, H-detected $^{1}H,^{13}C$ HSQC and HMBC experiments. The following structure of the pentasaccharide repeating unit of the polysaccharide was established:

→6)-α-D-Glc*p*NAc-(1→4)-α-D-Gal*p*NAc-(1→4)-α-D-Gal*p*A-(1→3)-β-D-Glc*p*NAc-(1→2)-β-D-Fuc*p*3NAc4Ac-(1→

where Fuc3NAc4Ac is 3-acetamido-4-*O*-acetyl-3,6-dideoxygalactose. A cross-reactivity of anti-*P. vulgaris* O45 serum was observed with several other *Proteus* lipopolysaccharides, which contains Fuc3N derivatives. © 2002 Published by Elsevier Science Ltd.

*Keywords:* *Proteus vulgaris*; Lipopolysaccharide; O-antigen, Bacterial polysaccharide structure; 3-Acetamido-3,6-dideoxy-D-galactose

## 1. Introduction

Bacteria of the genus *Proteus* cause urinary tract infections, which can lead to severe complications, such as pyelonephritis and formation of bladder and kidney stones. Recently, structures of the O-specific polysaccharide chains (O-antigens) of the lipopolysaccharides (LPS) of a number of *Proteus* strains have been established.[1–6] The structure of the O-antigen of *P. vulgaris* O45 has been reported,[7] but later it was found that the strain studied was erroneously classified in serogroup O45 and belonged actually to serogroup O47 (authors' unpublished data). Now we report on the structure of the O-specific polysaccharide of strain CCUG 4680 from *Proteus* serogroup O45.

## 2. Results and discussion

The O-specific polysaccharide was obtained by mild acid degradation of the LPS isolated from dried cells by the phenol–water procedure.[8] Sugar analysis after full acid hydrolysis of the polysaccharide revealed GalA, GlcN, GalN and 3-amino-3,6-dideoxygalactose (Fuc3N). GLC of the acetylated glycosides with a chiral alcohol showed that all sugars have the D configuration.

Methylation analysis of the polysaccharide resulted in identification of 3,6-deoxy-4-*O*-methyl-3-(*N*-methyl)acetamidogalactose, 2-deoxy-3,6-di-*O*-methyl-2-(*N*-methyl)acetamidogalactose, 2-deoxy-4,6-di-*O*-methyl-2-(*N*-methyl)acetamidoglucose and 2-deoxy-3,4-di-*O*-methyl-2-(*N*-methyl)acetamidoglucose. Therefore, the O-specific polysaccharide is linear and contains 3-substituted Fuc3N, 4-substituted GalN, 3-substituted GlcN and 6-substituted GlcN.

The $^{13}C$ NMR spectrum of the polysaccharide was typical of a regular polymer (Fig. 1). It contained

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signals for five anomeric carbons at $\delta$ 99.6–104.2, four nitrogen-bearing carbons (C-2 of GlcN and GalN and C-3 of Fuc3N) at $\delta$ 51.1–56.1, two non-substituted HO*C*$H_2$–C groups at $\delta$ 61.1 and 62.5, and one substituted group at $\delta$ 69.6 (C-6 of GalN and GlcN; data of a DEPT experiment), one *C*$H_3$–C group (C-6 of Fuc3N) at $\delta$ 16.4, one COOH group (C-6 of GalA) at $\delta$ 173.6, three *N*-acetyl groups at $\delta$ 23.1–23.5 ($CH_3$), one *O*-acetyl group at $\delta$ 21.3 ($CH_3$) and 16 other carbons in the region $\delta$ 68.3–82.9. Accordingly, the $^1$H NMR spectrum of the polysaccharide contained six signals at $\delta$ 4.58–5.36 for five anomeric protons and H-4 of Fuc3N (see below), one $CH_3$–C group (H-6 of Fuc3N) at $\delta$ 1.40, one *O*-acetyl and three *N*-acetyl groups at $\delta$ 1.91–2.10 and other signals in the region of $\delta$ 3.45–4.39.

Therefore, the polysaccharide has a pentasaccharide repeating unit containing one residue each of D-Fuc3NAc, D-GalNAc, D-GalA, two residues of D-GlcNAc and an *O*-acetyl group.

The $^1$H and $^{13}$C NMR spectra of the polysaccharide were assigned using 2D COSY, TOCSY, ROESY, H-detected $^1$H,$^{13}$C HSQC and HMBC experiments (Tables 1 and 2). The spin systems for GalA and GlcNAc residues were identified by correlations of H-1 to H-2,3,4,5 in the TOCSY spectrum; β-Glc*p*NAc showed also an H-1,H-6a,6b correlation, and there were present correlations of H-1 to H-2,3,4 for GalNAc residue. The spin system for Fuc3NAc was recognised by the following correlations: (i) H-1,H-2,3,4 and H-5,H-6 correlations in the TOCSY spectrum; (ii) H-4,C-4 in the HSQC spectrum; and (iii) H-6,C-4 and H-6,C-5 in the HMBC spectrum. Correlations of the H-2 signals at $\delta$ 3.77, 3.98, and 4.27 to the signals of nitrogen-bearing carbons (C-2) at $\delta$ 56.1, 55.2, and 51.1 in the $^1$H,$^{13}$C HSQC spectrum demonstrated GlcNAc and GalNAc residues, and an H-3,C-3 correlation at $\delta$ 4.19/54.6 confirmed the Fuc3NAc residue.

The $J_{1,2}$ coupling constant values of $\sim$3 Hz indicated that GalNAc, GalA and one of the GlcNAc

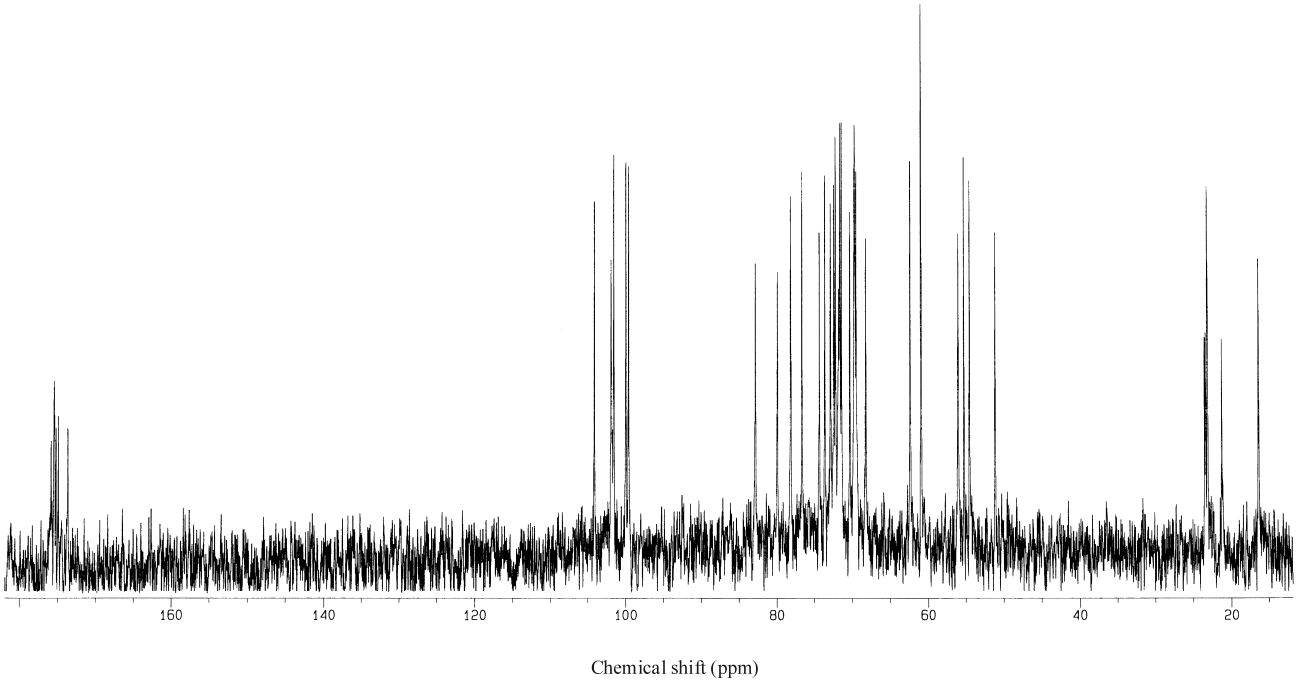


Fig. 1. 125-MHz $^{13}$C NMR spectrum of the O-specific polysaccharide of *P. vulgaris* O45.

Table 1
$^1$H NMR data of the O-specific polysaccharide ($\delta$, ppm)

| Sugar residue | H-1 | H-2 | H-3 | H-4 | H-5 | H-6a, 6b |
|---|---|---|---|---|---|---|
| →6)-α-D-Glc*p*NAc-(1→ | 4.97 | 3.98 | 3.88 | 3.97 | 4.20 | 3.92, 4.03 |
| →4)-α-D-Gal*p*NAc-(1→ | 4.99 | 4.27 | 4.01 | 4.08 | 4.36 | 3.66, 3.71 |
| →4)-α-D-Gal*p*A-(1→ | 5.36 | 3.87 | 3.95 | 4.39 | 4.37 | |
| →3)-β-D-Glc*p*NAc-(1→ | 4.69 | 3.77 | 3.79 | 3.66 | 3.45 | 3.79, 3.96 |
| →2)-β-D-Fuc*p*3NAc4Ac-(1→ | 4.58 | 3.82 | 4.19 | 5.05 | 3.99 | 1.40 |

The chemical shifts for OAc and NAc groups are in the region $\delta$ 1.91–2.10 (Me).

Table 2
$^{13}$C NMR data of the polysaccharide ($\delta$, ppm)

| Sugar residue | C-1 | C-2 | C-3 | C-4 | C-5 | C-6 |
|---|---|---|---|---|---|---|
| →6)-α-D-Glc*p*NAc-(1→ | 99.6 | 55.2 | 71.8 | 69.9 | 72.5 | 69.6 |
| →4)-α-D-Gal*p*NAc-(1→ | 99.9 | 51.1 | 68.3 | 78.2 | 71.9 | 61.1 |
| →4)-α-D-Gal*p*A-(1→ | 101.7 | 69.5 | 70.5 | 80.1 | 72.9 | 173.6 |
| →3)-β-D-Glc*p*NAc-(1→ | 102.0 | 56.1 | 82.9 | 72.3 | 76.8 | 62.5 |
| →2)-β-D-Fuc*p*3NAc4Ac-(1→ | 104.2 | 74.5 | 54.6 | 73.7 | 71.6 | 16.4 |

The chemical shifts for OAc and NAc are $\delta$ 21.3 and $\delta$ 23.1–23.5 (Me), respectively, 174.9–175.8 (CO).

→4)-α-D-Gal*p*NAc-(1→4)-α-D-Gal*p*A-(1→3)-β-D-Glc*p*NAc-(1→2)-β-D-Fuc*p*3NAc4Ac-(1→6)-α-D-Glc*p*NAc-(1→

O-Antigen of *Proteus vulgaris* O45

α-D-Glc*p*
1
↓
2
→4)-β-D-Glc*p*A3Ac-(1→3)-α-D-Glc*p*NAc-(1→2)-β-D-Fuc*p*3N(*R*-3HOBu)-(1→6)-α-D-Glc*p*4Ac-(1→

O-Antigen of *Proteus penneri* 16 (O17)[13,14]

Etn*P*
|
6
→4)-β-D-Glc*p*A-(1→3)-α-D-Glc*p*NAc-(1→2)-β-D-Fuc*p*3N(*R*-3HOBu)-(1→6)-α-D-Glc*p*-(1→

O-Antigen of *Proteus mirabilis* O17[4]

α-Fuc*p*3N(*R*-3HOBu)-(1→4)-α-Gal*p*-(1→6)-β-Glc*p*-(1→3)-α-Gal*p*6*P*Etn-(1→

Outer core region of the LPS of *Proteus penneri* 16[15]

Fig. 2. Structures of the O-antigens of cross-reactive *Proteus* strains and the outer core region of *P. penneri* 16. Fuc3NAc, 3-acetamido-3,6-dideoxygalactose; Fuc3N(*R*-3HOBu), 3,6-dideoxy-3-[(*R*)-3-hydroxybutyramido]galactose; *P*Etn, 2-aminoethyl phosphate.

residues are α-linked, whereas the $J_{1,2}$ values of 7–8 Hz showed that Fuc3NAc and the second GlcNAc residue are β-linked. The pyranose form of all monosaccharide residues was inferred by the $^{3}J_{H,H}$ coupling constant values and the absence from the $^{13}$C NMR spectrum of any signals for non-anomeric sugar ring carbons at a lower field than $\delta$ 83.[9]

A relatively low-field position of the signals for C-2 of Fuc3NAc, C-6 of α-GlcNAc, C-4 of GalNAc and GalA and C-3 of β-GlcNAc at $\delta$ 74.5, 69.6, 78.2, 80.1 and 82.9, respectively, as compared with their positions in the corresponding non-substituted monosaccharides,[10,11] demonstrated the modes of glycosylation of the monosaccharides.

A ROESY experiment revealed strong interresidue cross-peaks between the anomeric protons and protons at the linkage carbons at $\delta$ 4.58/3.92, 4.03; 4.97/4.08; 4.99/4.39; 5.36/3.79 and 4.69/3.82, which were assigned to Fuc3NAc H-1/α-GlcNAc H-6a,6b; α-GlcNAc H-1/GalNAc H-4; GalNAc H-1/GalA H-4; GalA H-1/β-GlcNAc H-3 and β-GlcNAc H-1/Fuc3NAc H-2 correlations, respectively. These data were in agreement with the glycosylation pattern and defined the sequence of the monosaccharide residues in the repeating unit.

The location of the *O*-acetyl group was determined by a significant downfield displacement of the H-4,C-4 cross-peak of Fuc3NAc from $\delta$ 3.72/70.9 in the non-O-acetylated monosaccharide[11] to $\delta$ 5.05/73.7 in the polysaccharide studied. This displacement was due to a deshielding effect of the *O*-acetyl groups and indicated stoichiometric O-acetylation of Fuc3NAc at O-4. Upfield shifts of the signals for C-3 and C5 of Fuc3NAc by 1.3 and 0.8 ppm, respectively, confirmed the O-acetylation pattern.[12]

On the basis of the data obtained, it was concluded that the O-specific polysaccharide (O-antigen) of *P. vulgaris* O45 has the structure shown in Fig. 2. The polysaccharide is distinguished by the presence of a rarely occurring component of bacterial polysaccharides, Fuc3NAc. Previously, this amino sugar N-acylated with a (*R*)-3-hydroxybutyryl group has been

found in the O-specific polysaccharides of *Proteus* strains from serogroup O17.[4,13,14]

Polyclonal rabbit anti-*P. vulgaris* O45 serum reacted with the homologous LPS in the passive hemolysis test (PHT) and enzyme immunosorbent assay (EIA) at high titres of 1:102,400 and 1:1,024,000, respectively. From a number of other *Proteus* LPS with known O-antigen structures, the LPS of *P. penneri* 16 (serogroup O17)[14] cross-reacted with anti-*P. vulgaris* O45 serum at titres 1:25,600 and 1:32,000 in PHT and EIA, respectively. In Western blot, anti-*P. vulgaris* O45 serum recognized both slow- and fast-migrating bands of the homologous LPS, which correspond to high- and low-molecular mass LPS species with and without big-chain O-antigen, respectively. Western blot confirmed the serological relatedness of *P. vulgaris* O45 and *P. penneri* 16 and, in addition, showed also a cross-reactivity of anti-*P. vulgaris* O45 serum with the LPS of three more strains, *P. penneri* 18, *P. mirabilis* PrK 32/57 and *P. vulgaris* PrK 33/67, all belonging to serogroup O17. Anti-*P. vulgaris* O45 serum recognized both high- and low-molecular-mass LPS species of *P. penneri* 16, thus showing sharing of both O-antigen and core epitopes. Absorption of anti-*P. vulgaris* O45 serum with the LPS of *P. penneri* 28, which has the same core structure[15] but a different O-antigen structure[13,14] compared to *P. penneri* 16, reduced significantly the reactivity with the LPS of *P. vulgaris* O45 and *P. penneri* 16 in PHT (titres 1:25,600 and 1:800, respectively).

Comparison of the O-antigen structures of *P. vulgaris* O45 on one hand and *P. penneri* 16 and *P. mirabilis* O17 on the other hand (Fig. 2) revealed the presence of similar disaccharide fragments →2)-β-D-Fuc*p*3NAc-(1→6)-α-D-Glc*p*NAc-(1→ and →2)-β-D-Fuc*p*3N(*R*-3HOBu)-(1→6)-α-D-Glc*p*-(1→, respectively, which may account for the serological relatedness of these strains. A terminal Fuc3NAc residue in the outer core region of the LPS of *P. penneri* 16 (Fig. 2) may be responsible for the cross-reactivity of anti-*P. vulgaris* O45 serum with low-molecular LPS species of *P. penneri* 16 observed in Western blot. Importantly, the cross-reactivity of anti-*P. vulgaris* O45 serum in PHT and EIA is markedly weaker compared to the reactivity with the homologous LPS. This finding and the unique polysaccharide structure established in this work confirmed the expedience of classification of *P. vulgaris* strain CCUG 4680 in a separate *Proteus* serogroup, O45.

## 3. Experimental

### 3.1. Bacterial strain, cultivation and isolation of the lipopolysaccharide

*P. vulgaris* O45, strain CCUG 4680, came from the Culture Collection of the University of Göteborg (Göteborg, Sweden). The bacteria were cultivated under aerobic conditions in nutrient broth (BTL, Lodz, Poland). The bacterial mass was harvested at the end of the logarithmic growth phase, centrifuged, washed with water and lyophilised.

The LPS was isolated from dried bacterial cells by hot phenol–water extraction[8] and purified by treatment with DNAse and RNAse and ultracentrifugation as described.[8,16]

### 3.2. Isolation of the O-specific polysaccharide

Delipidation of the LPS (75 mg) was performed with aq 2% HOAc at 100 °C until lipid precipitation. The precipitate was removed by centrifugation (13,000*g*, 20 min), and the supernatant was fractionated on a column (56 × 2.6 cm) of Sephadex G-50 (S) (Pharmacia, Sweden) in 0.05 M pyridinium acetate buffer pH 4.5 monitored using a Waters differential refractometer (USA). A high-molecular-mass polysaccharide was obtained in a yield of 33% of the LPS weight.

### 3.3. Chemical analyses

The polysaccharide was hydrolysed with 2 M $CF_3CO_2H$ (120 °C, 2 h). Amino sugars were identified by GLC of the alditol acetates on a Hewlett–Packard 5890 chromatograph equipped with an Ultra-2 column using a temperature gradient of 150–290 °C at 3 °C/min. Uronic acid was analysed by GLC of the acetylated methyl glycosides prepared by methanolysis of the polysaccharide with 1 M HCl in MeOH (80 °C, 16 h), followed by acetylation with a 1:1 pyridine–$Ac_2O$ mixture (80 °C, 2 h). The absolute configurations of the monosaccharides were determined by GLC of the acetylated (−)-2-octyl glycosides according to the published method.[17,18]

### 3.4. Methylation analysis

Methylation of the polysaccharide was performed with $CH_3I$ in $Me_2SO$ in the presence of sodium methylsulfinylmethanide.[19] Partially methylated monosaccharides were derived by hydrolysis under the same conditions as in sugar analysis, reduced with $NaBH_4$, acetylated and analysed by GLC–MS on a Hewlett–Packard 5890 chromatograph equipped with a NERMAG R10-10L mass spectrometer (France) under the same chromatographic conditions as in the sugar analysis.

### 3.5. NMR spectroscopy

NMR spectra were recorded with a Bruker DRX-500 spectrometer for a solution in $D_2O$ at 50 °C using internal acetone ($\delta_H$ 2.225, $\delta_C$ 31.45) as reference. Standard Bruker software (XWINNMR 1.2) was used to

acquire and process the NMR data. A mixing time of 200 and 100 ms was used in 2D TOCSY and ROESY experiments, respectively.

### 3.6. Serological techniques

Polyclonal rabbit anti-*P. vulgaris* O45 serum was obtained by immunization of New Zealand white rabbits with heat-killed bacteria as described.[20] The passive hemolysis test with alkali-treated LPS and the enzyme immunosorbent assay with LPS as antigen, sodium deoxycholate polyacrylamide gel electrophoresis and Western blot were performed as described previously.[21]

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