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Note

Structure of the O-polysaccharide from the lipopolysaccharide of *Providencia alcalifaciens* O29

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Abstract—The O-polysaccharide was obtained by a mild acid degradation of the lipopolysaccharide of *Providencia alcalifaciens* O29. Structural studies were performed using sugar and methylation analyses along with ^{1}H and ^{13}C NMR spectroscopy, including two-dimensional ^{1}H, ^{1}H COSY, TOCSY, ROESY, H-detected ^{1}H, ^{13}C HSQC and HMBC experiments. On the basis of the data obtained, the following structure of the branched tetrasaccharide repeating unit of the O-polysaccharide was established:

$$[\rightarrow 3)\text{-}\alpha\text{-L-Fuc}p\text{NAc-}(1\rightarrow 3)\text{-}\alpha\text{-D-Glc}p\text{NAc-}(1\rightarrow 6)\text{-}\alpha\text{-D-Glc}p\text{NAc-}(1\rightarrow]_n$$
$$\begin{array}{c} 4 \\ \uparrow \\ 1 \\ \beta\text{-D-Glc}p \end{array}$$

Keywords: *Providencia alcalifaciens*; O-antigen; Lipopolysaccharide; Polysaccharide structure

Providencia is the genus of bacteria in the family of Enterobacteriaceae, which, together with two closely related genera *Proteus* and *Morganella*, is included in the tribe *Proteae*. Strains of three *Providencia* species, *P. alcalifaciens*, *P. rustigianii* and *P. stuartii*, are classified into 63 O-serogroups.[1] *Providencia* are facultative pathogens, which under favorable conditions may cause various infections, mainly urinary tract infections, wound infections and enteric diseases.[1,2] The serological O-specificity of *Providencia* is defined by the structure of the O-polysaccharide chain (O-antigen) of the lipopolysaccharide. Immunochemical studies of *Providencia* O-antigens aim at creation of the molecular basis for the serological classification and cross-reactivity of *Providencia* strains and related bacteria, including *Proteus*. At present, O-polysaccharide structures of 24 *Providencia* O-serogroups have been established. Now we report on a novel structure of the O-polysaccharide of *P. alcalifaciens* O29.

A high-molecular-mass polysaccharide, eluted immediately after the void volume on GPC on Sephadex G-50, was isolated by a mild acid degradation of the lipopolysaccharide of *P. alcalifaciens* O29. Sugar analysis using GLC of the acetylated alditols demonstrated the presence of 2-amino-2,6-dideoxygalactose (FucN), GlcN and Glc in the ratio ~0.9:2.0:2.4. Later studies

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showed that part of the Glc was derived from the lipopolysaccharide core. The L configuration for FucN and the D configuration for Glc and GlcN were determined by GLC of the acetylated (*S*)-2-octyl glycosides. Methylation analysis, including GLC–MS of the partially methylated alditol acetates, revealed the presence of 3-substituted FucN, 3,4-disubstituted GlcN, 6-substituted GlcN, and terminal Glc. These data confirmed also the pyranose form of all monosaccharides except for the disubstituted GlcN residue, whose pyranose form was inferred from NMR spectroscopy (see below).

The ^{13}C NMR spectrum of the polysaccharide (Fig. 1) demonstrated a regular structure. It contained signals for four sugar residues, including those for four anomeric carbons at δ 98.2 (doubly intense; resolved in the ^{1}H, ^{13}C HSQC spectrum), 100.7 and 102.7, three nitrogen-bearing carbons (C-2 of FucN and two GlcN) at δ 49.8–55.3, one methyl group (FucN C-6) at δ 16.8, other sugar carbons in the region δ 60.8–77.8, and three *N*-acetyl groups at δ 174.7, 175.0, 175.5 (all CO), 23.3 and 23.7 (doubly intense) (all CH_3). The absence of signals in the region δ 82–88 confirmed the pyranose form of all sugar residues.[3]

Accordingly, the ^{1}H NMR spectrum contained signals for four anomeric protons at δ 4.52–5.08, one methyl group (FucN H-6) at δ 1.19, other sugar protons in the region δ 3.01–4.79, and three *N*-acetyl groups at δ 1.96, 1.98, and 2.06.

The ^{1}H and ^{13}C NMR spectra of the polysaccharide were assigned using COSY, TOCSY, ROESY, ^{1}H, ^{13}C HSQC (Fig. 2) and ^{1}H, ^{13}C HMBC experiments (Table 1). The signals for C-2 and C-6 of FucN (unit **A**) were revealed by their characteristic[4] chemical shifts (δ 49.8 and 16.8, respectively). The spin system of Fuc*p*N was distinguished using the TOCSY and COSY spectra, which showed correlations between H-1, H-2, H-3 and H-4 signals; that for H-2 being assigned by correlation with the attached carbon (C-2) at δ 4.26/49.8 shown by the HSQC spectrum. The H-6, C-5 and H-6, C-4 correlations in the HMBC spectrum enabled the assignment of the remaining Fuc*p*N signals. A small $J_{1,2}$ coupling constant of <3 Hz demonstrated the α configuration of Fuc*p*N. A low-field displacement of the C-3 signal to δ 75.9 and an upfield displacement of the C-2 signal to δ 49.8 for the Fuc*p*NAc residue in the polysaccharide, as compared with the corresponding chemical shifts in the non-substituted Fuc*p*NAc[4] (δ 68.7 and 51.2, respectively), showed the substitution of unit **A** at position 3.

Two GlcN residues (units **B** and **C**) were identified by the H-2, C-2 cross-peaks at δ 4.21/55.3 and δ 3.95/55.1 in the HSQC spectrum. The TOSCY and COSY spectra showed all expected correlations within the Glc*p*N **B** spin system and both proton and carbon signals of this residue were easily assigned. A small $J_{1,2}$ value of <3 Hz demonstrated that Glc*p*N **B** is α-linked. The ROESY spectrum (see below) showed the substitution of Glc*p*N **B** at position 3, and the substitution at position 4 was inferred by a downfield displacement of the C-4 signal to δ 74.4, as compared with the value δ 71.4 in the non-substituted α-Glc*p*N.[4] The glycosylation modes of Fuc*p*N **A** and Glc*p*N **B** determined by the NMR data were in agreement with the methylation analysis data (see above).

Assignment for the Glc*p*N **C** residue was complicated by the complete overlap of the H-3 and H-4 signals and was aided by the methylation data, which indicated that the second GlcN residue (i.e., unit **C**) is 6-substituted; hence, the C-6 signal at δ 65.3 belongs[4] to unit **C**. This finding enabled the assignment of the H-6 signals by

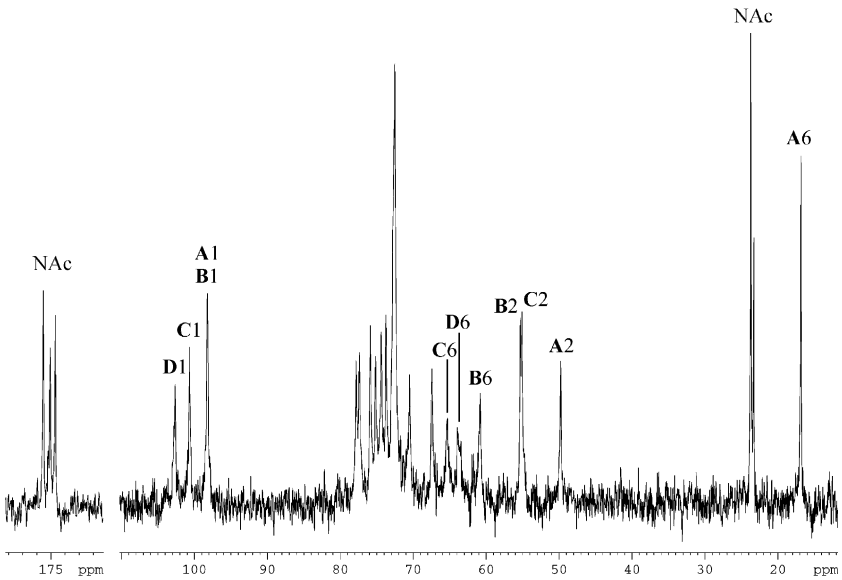

Figure 1. The ^{13}C NMR spectrum of the O-polysaccharide of *P. alcalifaciens* O29.

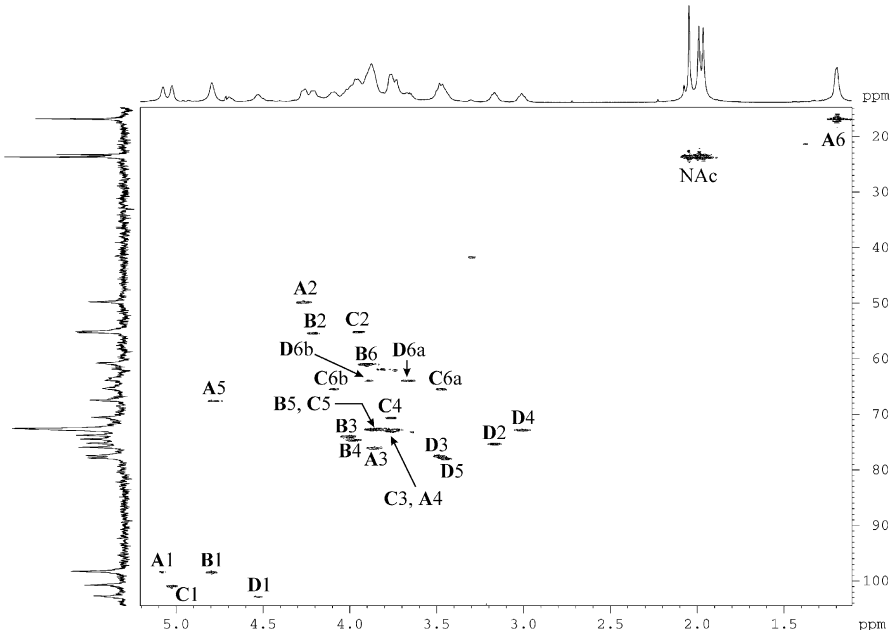

Figure 2. Part of a ^{1}H, ^{13}C HSQC spectrum of the O-polysaccharide of *P. alcalifaciens* O29. The corresponding parts of the ^{1}H and ^{13}C NMR spectra are shown along the axes. Arabic numerals refer to atoms in sugar residues denoted by capital letters as shown in Table 1.

Table 1. ^{1}H and ^{13}C NMR data of the O-polysaccharide of *P. alcalifaciens* O29 (δ, ppm). Additional chemical shifts for the *N*-acetyl groups are δ_H 1.96, 1.98, 2.06; δ_C 23.3, 23.7 (all CH_3), 174.7, 175.0, 175.5 (all CO)

Sugar residue		H-1	H-2	H-3	H-4	H-5	H-6a	H-6b
→3)-α-L-Fuc*p*NAc-(1→	**A**	5.08	4.26	3.86	3.76	4.79	1.19	
→3,4)-α-D-Glc*p*NAc-(1→	**B**	4.79	4.21	4.00	3.98	3.85	3.90	3.90
→6)-α-D-Glc*p*NAc-(1→	**C**	5.01	3.95	3.75	3.75	3.86	3.46	4.09
β-D-Glc*p*-(1→	**D**	4.52	3.17	3.48	3.01	3.45	3.66	3.88
		C-1	C-2	C-3	C-4	C-5	C-6	
→3)-α-L-Fuc*p*NAc-(1→	**A**	98.2	49.8	75.9	72.5	67.4	16.8	
→3,4)-α-D-Glc*p*NAc-(1→	**B**	98.2	55.3	73.7	74.4	72.5	60.8	
→6)-α-D-Glc*p*NAc-(1→	**C**	100.7	55.1	72.5	70.5	72.5	65.3	
β-D-Glc*p*-(1→	**D**	102.7	75.2	77.4	72.5	77.8	63.9	

H-6a, C-6 and H-6b, C-6 correlations at δ 3.46/65.3 and δ 4.09/65.3 in the HSQC spectrum following the assignment of the other Glc*p*N **C** protons by correlations between H-1, H-2 and H-3 as well as between H-6, H-5 and H-4 in the TOCSY and COSY spectra. A $J_{1,2}$ coupling constant of <3 Hz showed that unit **C** is α-linked as well.

The TOCSY spectrum displayed correlations of H-1 of Glc*p* (unit **D**) with the protons from H-2 to H-5, and the COSY spectrum showed correlations between all neighboring protons of this residue. The chemical shift of Glc*p* H-1 (δ 4.52) demonstrated the β configuration of unit **D**. (The H-1 signals were wide but non-resolved, and the $J_{1,2}$ coupling constant could not be determined.) The ^{13}C NMR chemical shifts for unit **D**, which were assigned using the HSQC spectrum, are characteristic for non-substituted β-Glc*p*[4] and, therefore, this residue occupies the terminal position in the side chain.

The ROESY spectrum (Fig. 3) displayed the following interresidue cross-peaks: Fuc*p*N **A** H-1, Glc*p*N **B** H-3 at δ 5.08/4.00; Glc*p*N **B** H-1, Glc*p*N **C** H-6a at δ 4.79/3.46, and Glc*p*N **C** H-1, Fuc*p*N **A** H-3 at δ 5.01/3.86. The expected Glc*p* **D** H-1, Glc*p*N **B** H-4 cross-peak was not observed, most likely, owing to sterical hindrance in 3,4-disubstituted GlcNAc, which remotes proton H-4 of unit **B** from proton H-1 of unit **D**. Instead, there was a Glc*p* H-1, Glc*p*N **B** H-6 cross-peak at δ 4.52/3.90, which is typical of some (1→4)-linked disaccharides.[5] These data established the monosaccharide sequence in the repeating unit.

Based on the data obtained, the structure of the O-polysaccharide of *P. alcalifaciens* O29 is shown in Chart 1.

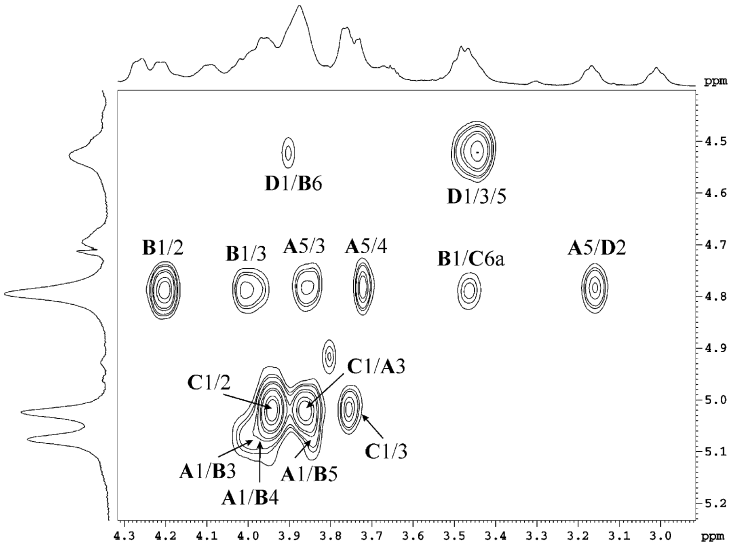

Figure 3. Part of a 2D ROESY spectrum of the O-polysaccharide of *P. alcalifaciens* O29. The corresponding parts of the ^{1}H NMR spectrum are shown along the axes. Arabic numerals refer to protons in sugar residues denoted by capital letters as shown in Table 1.

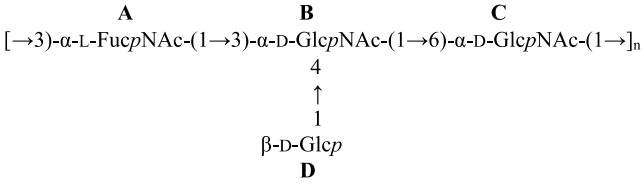

Chart 1. Structure of the O-polysaccharide of *P. alcalifaciens* O29.

1. Experimental

1.1. Bacterial strain, isolation and degradation of the lipopolysaccharide

Providencia alcalifaciens O29:H2, strain 575/48, obtained from the Hungarian National Collection of Medical Bacteria (National Institute of Hygiene, Budapest), was cultivated under aerobic conditions in tryptic soy broth supplemented with 0.6% yeast extract. The bacterial mass was harvested at the end of the logarithmic growth phase, centrifuged, washed with distilled water and lyophilized. The lipopolysaccharide was isolated in a yield of 3.5% of dry bacterial weight by phenol–water extraction, followed by dialysis of the extract without layer separation and purified by treatment with cold aq 50% CCl_3CO_2H; the aq layer was dialyzed and freeze-dried.

A portion of the lipopolysaccharide (115 mg) was heated with 2% AcOH for 2.5 h at 100 °C and the carbohydrate-containing supernatant was fractionated on a column of Sephadex G-50 (60 × 2.5 cm) in 0.05 M pyridinium acetate buffer. The yield of the polysaccharide was 8% of the lipopolysaccharide weight.

1.2. Chemical methods

For sugar analysis, the polysaccharide was hydrolyzed with 10 M HCl (80 °C, 30 min), the products were reduced with an excess of $NaBH_4$ (2 h, 20 °C), acetylated by Ac_2O/Py (1:1, 100 °C, 1 h) and analyzed by GLC on a Hewlett-Packard HP 5890 chromatograph equipped with a Ultra-2 column (Hewlett-Packard) using a temperature gradient of 5 °C min^{-1} from 160 to 290 °C.

For determination of the absolute configurations of sugars by GLC, the O-polysaccharide was hydrolyzed with 10 M HCl as above, N-acetylated (400 μL $NaHCO_3$, 60 μL Ac_2O, 0 °C, 1 h), subjected to (+)-2-octanolysis[6] [100 μL (+)-2-octanol, 15 μL CF_3CO_2H, 120 °C, 16 h] and acetylated.

Methylation of the polysaccharide was performed according to the Hakomori procedure;[7] the products were recovered by Sep-Pak. Partially methylated monosaccharides were derived by hydrolysis with 10 M HCl, converted into the alditol acetates and analyzed by GLC–MS on a TermoQuest Finnigan model Trace series GC 2000 instrument equipped with an EC-1 column (0.32 mm × 30 m) using a temperature gradient from 150 °C (2 min) to 250 °C at 10 °C min^{-1}.

1.3. NMR spectroscopy

Samples were freeze-dried twice from a $^{2}H_2O$ soln and dissolved in 99.96% $^{2}H_2O$, with internal TSP (δ_H 0) and external acetone (δ_C 31.45) as references. ^{1}H and ^{13}C NMR spectra were recorded at 30 °C using Bruker DRX-500 NMR instrument and XwinNMR software on SGI Indy/Irix 5.3. Mixing time of 300 ms and spin-lock time of 30 ms were used in ROESY and TOCSY experiment, respectively. Other NMR experimental parameters were essentially as previously described.[8]

Acknowledgements

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