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Note

The structure of the O-polysaccharide from the lipopolysaccharide of *Providencia alcalifaciens* O30

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Abstract—The O-polysaccharide was obtained by mild acid degradation of the lipopolysaccharide of *Providencia alcalifaciens* O30. Studies by sugar and methylation analyses along with ^{1}H and ^{13}C NMR spectroscopy, including two-dimensional ^{1}H,^{1}H COSY, TOCSY, ROESY, and H-detected ^{1}H,^{13}C HSQC, HMBC, and HMQC-TOCSY experiments, showed that the polysaccharide has a linear pentasaccharide repeating unit of the following structure:

$$[\rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{A-}(1\rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{A-}(1\rightarrow 3)\text{-}\alpha\text{-D-Fuc}p\text{NAc4N-}(1\rightarrow 2)\text{-}\beta\text{-D-Qui}p\text{4NFo-}(1\rightarrow 2)\text{-}\beta\text{-D-Rib}f\text{-}(1\rightarrow]_n$$

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

Lipopolysaccharide (LPS) is one of the main components of the outer surface of Gram-negative bacteria, including *Providencia*. The O-polysaccharide (O-antigen), which together with the core region is the polysaccharide fragment of LPS, determines part of the immunospecificity of the cell. Bacteria of the genus *Providencia* are facultative pathogens, which are able to invade intestinal mucosa and other cell types and may cause intestinal and urinary tract infections.[1] Structural and immunochemical studies of *Providencia* O-antigens aim at the creation of the molecular basis for the serological classification of *Providencia* strains and their cross-reactivity with related bacteria, including *Proteus*. Currently, the genus *Providencia* is subdivided into five species: *Providencia alcalifaciens*, *Providencia rustigianii*, *Providencia heimbache*, *Providencia rettgerii*, and *Providencia stuartii*. The existing serological classification scheme of *P. alcalifaciens*, *P. rustigianii*, and *P. stuartii* includes 63 O-serogroups.[2] At present, 23 O-polysaccharide structures have been established. Now, we report on a novel structure of the O-polysaccharide of *P. alcalifaciens* O30.

A high-molecular-mass polysaccharide, eluted immediately after the void volume on GPC on Sephadex G-50, was isolated by mild acid degradation of the lipopolysaccharide of *P. alcalifaciens* O30. Sugar analysis using GLC of the acetylated alditols showed the presence of ribose (Rib) and traces of glucose (Glc) and 2-amino-2-deoxyglucose (GlcN) (both from core). Sugar analysis of N-acetylated polysaccharide showed an additional peak of 4-amino-4,6-dideoxyglucose (Qui4N), the Qui4N and Rib ratios being ~0.5:1.0, respectively. Analysis of the polysaccharide hydrolysate

Abbreviations: GlcA, glucuronic acid; FucNAc4N, 2-acetamido-4-amino-2,4,6-trideoxygalactose; Qui4NFo, 4-formamido-4,6-dideoxyglucose.

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by anion-exchange chromatography using a sugar analyzer resulted in identification of a trace amount of glucuronic acid (GlcA). Later NMR spectroscopic studies demonstrated the presence of an additional monosaccharide, namely, 2,4-diamino-2,4,6-trideoxygalactose (FucN4N), which was fully destroyed during acid hydrolysis of the polysaccharide. The D configuration of Rib was determined by GLC of the acetylated glycosides with (+)-2-octanol, whereas the D configuration of the other monosaccharides was confirmed by ^{13}C NMR spectroscopy (see below).

GLC–MS of the partially methylated alditol acetates derived from the methylated N-acetylated polysaccharide revealed the presence of 2-substituted Qui*p*4N, 2-substituted Rib*f* and 3-substituted Fuc*p*N4N. In addition to these monosaccharides, similar analysis after carboxyl-reduction of the methylated polysaccharide showed the presence of 4,6-disubstituted Glc*p*, which was evidently derived from 4-substituted Glc*p*A. The pyranose form of GlcA was inferred from NMR spectroscopic data (see below).

The ^{13}C NMR spectrum of the polysaccharide (Fig. 1, pH 2) demonstrated a regular structure. It contained signals for five sugar residues, including those for five anomeric carbons at δ 98.0–106.6, three nitrogen-bearing carbons (FucN4N C-2, C-4, and Qui4N C-4) at δ 48.7, 56.3, and 56.8, two methyl groups of 6-deoxy sugars (FucN4N and Qui4N C-6) at δ 16.4 and 18.2, one *N*-acetyl group (CH_3 at δ 23.4, CO at δ 175.7), and one *N*-formyl group at δ 165.9 as well as 19 sugar-ring oxygen-bearing carbons in the region δ 60.9–85.1, some of which overlapped. The total number of signals in the spectrum and the presence of a signal at δ 83.5, which is typical of C-4 of a furanose residue,[3] confirmed the occurrence of ribose in the furanose form.

Accordingly, the ^{1}H NMR spectrum (Fig. 2, pH 7) contained, inter alia, five signals at δ 4.46–5.36, which belong to five anomeric protons; an additional signal in this region, that for H-5 of FucNAc4N, found by 2D NMR spectroscopic studies (see below), overlapped with the HDO signal at δ 4.71. There were also signals for two methyl groups of 6-deoxy sugars (FucNAc4N and Qui4N H-6) at δ 1.23 and 1.24, *N*-acetyl group at δ 2.03 and *N*-formyl group at δ 8.22 and 8.02 (major and minor peaks for the *Z* and *E* isomers, respectively).[4]

The 2D TOCSY spectrum of the O-polysaccharide revealed spin systems for five monosaccharide residues designated as **A**–**E** according to their sequence in the repeating unit established later, and the COSY spectrum enabled differentiation between protons within each spin system (Table 1). The spin systems were ascribed to particular monosaccharides by characteristic coupling constant values estimated from the 2D NMR spectra, and those of amino sugars were identified by correlation of proton at the nitrogen-bearing carbons to the corresponding carbons in the 2D H-detected ^{1}H,^{13}C HSQC spectrum. The same spectrum was used also for the assignment of the ^{13}C NMR chemical shifts of the polysaccharide shown in Table 1.

The β configuration of the glycosidic linkages of both GlcA and Qui4NFo (units **A**, **B**, and **D**, respectively) was established by $J_{1,2}$ coupling constant values of 7.5–8.0 Hz. This conclusion was confirmed by correlations between the H-1,H-3 and H-1,H-5 pairs of 1,3-diaxial protons in the 2D ROESY spectrum of the polysaccharide (Fig. 3). Relatively small $J_{1,2}$ coupling constant value (<3 Hz, signal was not resolved) showed that FucNAc4N (unit **C**) is α-linked. The β configuration of Rib*f* (unit **E**) followed from the C-1 chemical shift of δ 106.6.[3]

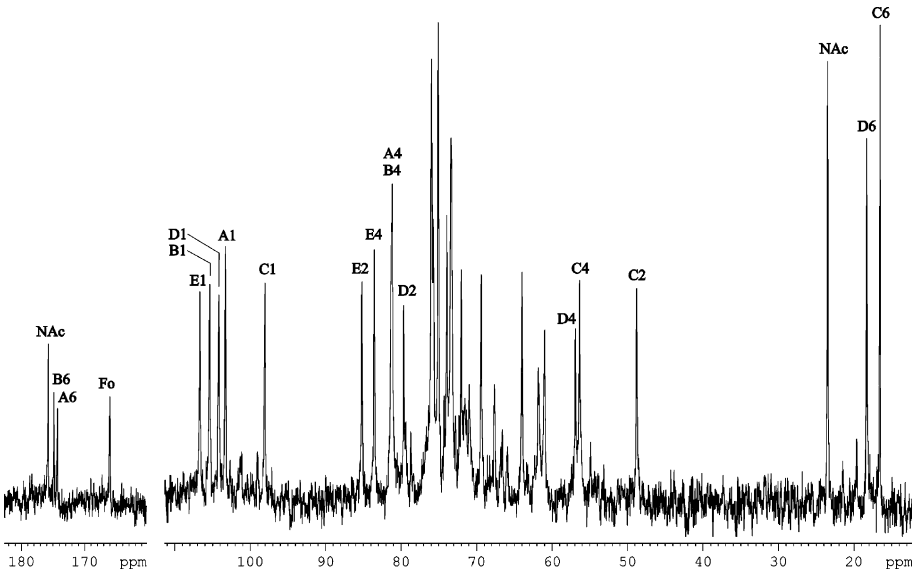

Figure 1. ^{13}C NMR spectrum of the O-polysaccharide of *P. alcalifaciens* O30.

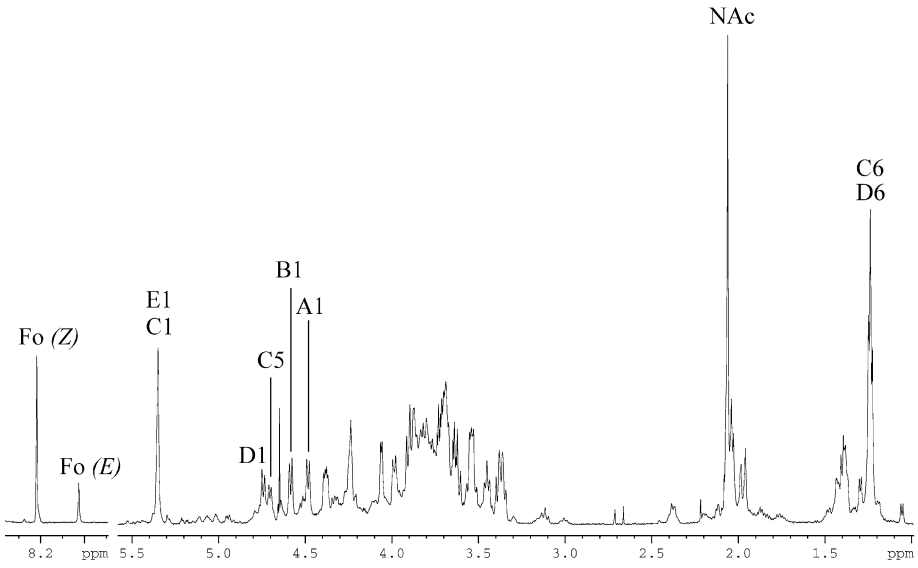

Figure 2. ^{1}H NMR spectrum of the O-polysaccharide of *P. alcalifaciens* O30.

Table 1. ^{1}H NMR and ^{13}C NMR data of the O-polysaccharide of *P. alcalifaciens* O30 (δ_H at pH 7, δ_C at pH 2; ppm)

Sugar residue		H-1	H-2	H-3	H-4	H-5 (H-5a)	H-6 (H-5b)
→4)-β-D-Glc*p*A-(1→	**A**	4.46	3.35	3.52	3.52	3.73	
→4)-β-D-Glc*p*A-(1→	**B**	4.57	3.37	3.61	3.68	3.85	
→3)-α-D-Fuc*p*NAc4N-(1→	**C**	5.35	4.23	4.23	3.88	4.71	1.23
→2)-β-D-Qui*p*4NFo-(1→	**D**	4.75	3.44	3.69	3.68	3.80	1.24
→2)-β-D-Rib*f*-(1→	**E**	5.36	4.07	4.38	3.98	3.69	3.84
		C-1	C-2	C-3	C-4	C-5	C-6
→4)-β-D-Glc*p*A-(1→	**A**	103.2	73.8	75.0 (−2)	81.1	75.9	174.2[a]
→4)-β-D-Glc*p*A-(1→	**B**	105.3 (+8)	73.3	75.0 (−2)	81.1	75.9	174.7[a]
→3)-α-D-Fuc*p*NAc4N-(1→	**C**	98.0	48.7	75.6	56.3	63.9	16.4
→2)-β-D-Qui*p*4NFo-(1→	**D**	104.1	79.6	73.3 (+0.5)	56.8	71.9	18.2
→2)-β-D-Rib*f*-(1→	**E**	106.6	85.1	69.3	83.5	60.9	

Additional chemical shifts for the *N*-acetyl group are δ_H 2.03, δ_C 23.4 (CH_3) and 175.7 (CO) and for the *N*-formyl group δ_H 8.22 and 8.02 (major and minor signals for the *Z* and *E* isomers, respectively), δ_C 165.9. The key ^{13}C NMR glycosylation effects used for determination of the absolute configuration of the monosaccharides[5] are given in parentheses.

[a] Assignment could be interchanged.

Relatively low-field positions of the signals for C-4 of units **A** and **B** at δ 81.1 and 81.1, C-3 of unit **C** at δ 75.6 and C-2 of units **D** and **E** at δ 79.6 and 85.1, respectively, gave an indication of the glycosylation pattern in the repeating unit.

The ROESY spectrum of the polysaccharide (Fig. 3) showed *inter*-residue cross-peaks between the anomeric protons and protons at the linkage carbons, which, taking into account the positions of glycosylation of the monosaccharides (see above), could be interpreted as follows: β-GlcA H-1 (**A**),β-GlcA H-4 (**B**) at δ 4.46/3.68; β-GlcA H-1 (**B**),α-FucN4N H-3 at δ 4.57/4.23; α-FucN4N H-1,β-Qui4N H-2 at δ 5.35/3.44; β-Qui4N H-1,β-Rib H-2 at δ 4.75/4.07, and β-Rib H-1,β-GlcA H-4 (**A**) at δ 5.36/3.52. These data confirmed the glycosylation pattern and established the monosaccharide sequence in the repeating unit.

With the known monosaccharide sequence and absolute configuration of Rib, the D configuration of the other constituent sugars was determined by analysis of glycosylation effects on the ^{13}C NMR chemical shifts using known rules[5] (Table 1).

Additional NMR spectra of the polysaccharide were recorded at pH 9 and 2 and compared with each other. The pH decrease resulted in downfield displacement of the H-5 signals of units **A** and **B**, which are mostly sensitive to pH in uronic acids, from δ 3.73 and 3.81 to δ 3.81 and 3.90, respectively, and in downfield displacement of the H-4 signal of unit **B** from δ 3.65 to 3.71, whereas the other signals (except for protons of

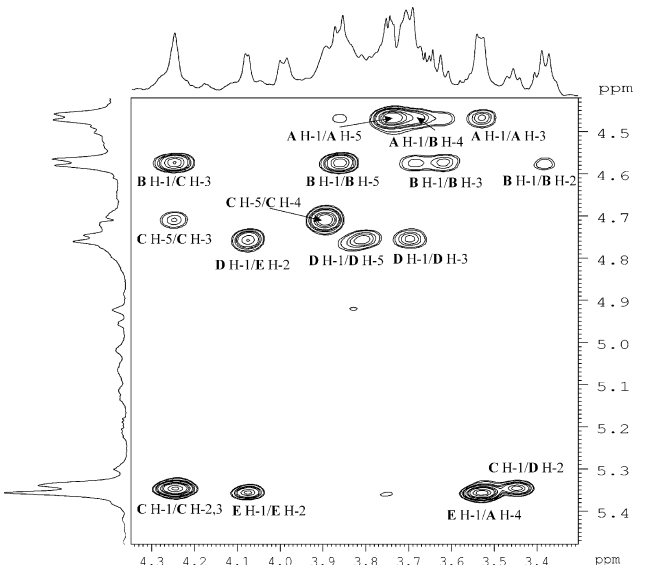

Figure 3. Part of a 2D ROESY spectrum of the O-polysaccharide of *P. alcalifaciens* O30. The corresponding parts of the ^{1}H NMR spectrum are shown along the axes. Sugar residues are denoted by letters as shown in Table 1.

FucNAc4N, see below) in the spectrum shifted by <0.03 ppm. This finding showed that both GlcA residues have the free carboxyl group. A poor release of GlcA on acid hydrolysis of the polysaccharide could be accounted for by a high stability of the glycosidic linkages of two GlcA residues linked to each other.

Relatively large low-field shifts of the signals for FucNAc4N C-3 and C-5 (from δ 75.6 and 63.9 at pH 2 to δ 79.6 and 67.7 at pH 9, respectively) and a small high-field shift of FucNAc4N C-4 (from δ 56.3 to 55.2) demonstrated that the amino group at C-4 of FucNAc4N is not acylated. An ^{1}H,^{13}C HMBC experiment showed a Qui4N C-4,Fo H-1 cross-peak at δ 56.8/8.22, indicating that Qui4N is N-formylated and, hence, FucN4N is 2-N-acetylated.

These data established the following structure of the O-polysaccharide of *P. alcalifaciens* O30:

A **B** **C** **D** **E**

[→4)-β-D-Glc*p*A-(1→4)-β-D-Glc*p*A-(1→3)-α-D-Fuc*p*NAc4N-(1→2)-β-D-Qui*p*4NFo-(1→2)-β-D-Rib*f*-(1→]$_n$

1. Experimental

1.1. Bacterial strain, isolation, and degradation of the lipopolysaccharide

P. alcalifaciens O30:H19, strain 19372, 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 in a yield of 4.4% of dry bacterial weight was isolated by phenol–water extraction and purified by ultracentrifugation.

A portion of the lipopolysaccharide (182 mg) was heated with 2% acetic acid (4 mL) for 3 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 6% of the lipopolysaccharide weight.

1.2. Monosaccharide analysis

Prior to sugar analysis, the polysaccharide (10 mg) was N-acetylated (400 μL $NaHCO_3$, 60 μL Ac_2O, 0 °C, 1 h), treated with Amberlite IR-120 (H^+) and lyophilized.

The intact and N-acetylated polysaccharides were hydrolyzed with 10 M HCl for 30 min at 80 °C. Alditol acetates were prepared by reduction with an excess of $NaBH_4$ (20 °C, 2 h) followed by acetylation (0.2 mL Ac_2O, 0.2 mL pyridine, 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 10 °C min^{-1} from 180 to 290 °C.

For determination of the absolute configuration, the intact O-polysaccharide (0.8 mg) was hydrolyzed with 10 M HCl as above, subjected to 2-octanolysis[6] [100 μL (*S*)-2-octanol, 15 μL CF_3CO_2H, 120 °C, 16 h], acetylated and analyzed by GLC as above.

Uronic acids were analyzed using a Biotronik LC-2000 sugar analyzer as described.[7]

1.3. Methylation analysis

Prior to methylation, the polysaccharide (1.5 mg) was N-acetylated, treated with an Amberlite IR-120 (H^+) resin and lyophilized. Methylation of the polysaccharide was performed according to the Hakomori procedure,[8] the products were recovered by Sep-Pak and divided into two parts, one of which was reduced with $LiBH_4$ in aq 70% 2-propanol (20 °C, 2 h). Partially methylated monosaccharides were obtained by hydrolysis with 10 M HCl as above, 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}.

1.4. NMR spectroscopy

Samples were freeze-dried twice from a ^2H_2O soln and dissolved in 99.96 % ^2H_2O with internal TSP (δ_H 0) and external acetone (δ_C 31.45) as references. ^1H and ^{13}C NMR spectra were recorded with a Bruker DRX-500 spectrometer at 30 °C. 2D NMR experiments were performed using standard Bruker software. Mixing time of 300 ms and spin-lock time of 30 ms were used in ROESY and TOCSY, HMQS-TOCSY experiment, respectively. Other NMR experimental parameters were essentially as previously described.[9]

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