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Note

The O-polysaccharide from the lipopolysaccharide of *Providencia stuartii* O44 contains L-quinovose, a 6-deoxy sugar rarely occurring in bacterial polysaccharides

Nina A. Kocharova,[a] Olga G. Ovchinnikova,[a,*] Filip V. Toukach,[a] Agnieszka Torzewska,[b] Alexander S. Shashkov,[a] Yuriy A. Knirel[a] and Antoni Rozalski[b]

[a]*N. D. Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, 119991 Moscow, Russian Federation*
[b]*Department of Immunobiology of Bacteria, Institute of Microbiology and Immunology, University of Lodz, PL 90-237 Lodz, Poland*

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Abstract—The O-polysaccharide (O-antigen) of *Providencia stuartii* O44:H4 (strain 3768/51) was obtained by mild acid degradation of the lipopolysaccharide and studied by sugar and methylation analyses along with ^{1}H and ^{13}C NMR spectroscopy, including 2D $^{1}H,^{1}H$ COSY, TOCSY, ROESY, and H-detected $^{1}H,^{13}C$ HSQC, and HMQC-TOCSY experiments. The O-polysaccharide was found to have a branched hexasaccharide repeating unit of the following structure:

β-D-Glc*p*A
1
↓
4
[3)-α-L-Fuc*p*-(1→3)-α-D-Glc*p*-(1→4)-α-L-Qui*p*-(1→3)-α-D-Glc*p*NAc-(1→4)-α-D-Gal*p*NAc-(1→]$_n$

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

Gram-negative bacteria of the genus *Providencia* are divided into five species, including *Providencia alcalifaciens*, *Providencia rustigianii*, *Providencia stuartii*, *Providencia heimbachae*, and *Providencia rettgerii*.[1] They are facultative pathogens that under favorable conditions cause enteric diseases, as well as wound and urinary-tract infections. Particularly, *Providencia stuartii* has been recognized as a pathogen with an increasing involvement in urinary tract infections primarily in nursing home patients with long-term urinary catheters in place. These infections are frequently persistent, difficult to treat, and may even result in fatal bacteremia. The serological classification scheme of three *Providencia* species, *P. alcalifaciens*, *P. rustigianii*, and *P. stuartii*, used in serotyping of clinical isolates, is based on the lipopolysaccharide (LPS, O-antigen, endotoxin) and flagella (H-antigens) and includes 63 serogroups.[2] Immunochemical studies of *Providencia* O-antigens aim at the creation of the molecular basis for the serological classification and cross-reactivity of *Providencia* strains and related bacteria, including *Proteus*. Recently, structures of the O-polysaccharides of the LPS of *P. stuartii* serogroups O4, O18, O33, O47, and O49 have been elucidated.[3–7] Now we report on the structure of the O-polysaccharide of *P. stuartii* O44.

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

* Corresponding author. Tel.: +7 095 9383613; fax: +7 095 1376148; e-mail: olgao@hotmail.ru

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lipopolysaccharide of *P. stuartii* O44. Sugar analysis obtained by GLC of the acetylated alditols revealed fucose (Fuc), quinovose (Qui), glucose (Glc), 2-aminodeoxyglucose (GlcN), and 2-aminodeoxygalactose (GalN) in the ratios ~0.4:0.6:1:0.7:0.6. In addition, glucuronic acid (GlcA) was identified by anion-exchange chromatography using a sugar analyzer. An enzymatic assay with D-glucose oxidase showed that Glc has the D configuration. The L configuration of the 6-deoxy sugars and the D configuration of the amino sugars and GlcA were determined by GLC of the acetylated (+)-2-octyl glycosides.

GLC–MS of the partially methylated alditol acetates derived from the methylated polysaccharide revealed a 4-substituted 6-deoxyhexose, a 3,4-disubstituted 6-deoxyhexose, a 3-substituted hexose, a 3-substituted hexosamine, and a 4-substituted hexosamine. In addition to these monosaccharides, similar analysis after carboxyl reduction of the methylated polysaccharide showed a 6-substituted hexose, which was evidently derived from a terminal nonreducing GlcA residue. The pyranose form of the 4-substituted monosaccharides was shown by NMR spectroscopic data (see below).

The ^{13}C NMR spectrum of the polysaccharide (Fig. 1) demonstrated a regular structure. It contained anomeric signals for six sugar residues, three of which were separated and resonated at δ 99.6, 100.0, and 105.1 and three others overlapped at δ 100.5. There were also signals for two nitrogen-bearing carbons at δ 50.9 and 54.7 (C-2 of *N*-acetylhexosamines), three unsubstituted $HOCH_2$ groups of hexoses and hexosamines at δ 61.1 (2 C) and 62.0 (data of a DEPT-135 experiment), two methyl groups (C-6 of 6-deoxy sugars) at δ 16.2 and 17.8, one carboxyl group of GlcA at δ 175.3, 22 sugar-ring oxygen-bearing carbons in the region δ 68-84, some of the signals being overlapped, and two *N*-acetyl groups at δ 23.4, 23.6 (both Me), 175.7 and 175.9 (both CO). Accordingly, the ^{1}H NMR spectrum (data not shown) contained signals for six anomeric protons at δ 4.55, 4.86, 5.01, 5.03, 5.24, and 5.27, and two *N*-acetyl groups at δ 2.04 and 2.07. As judged by the absence of signals within δ 84–88 from the ^{13}C NMR spectrum, all sugar residues are in the pyranose form.[8]

The ^{1}H and ^{13}C NMR spectra of the polysaccharide were assigned using ^{1}H,^{1}H COSY, TOCSY, ROESY (Fig. 2), ^{1}H,^{13}C HSQC (Fig. 3), and ^{1}H,^{13}C HMQC-TOCSY experiments. The TOCSY spectrum showed correlation from H-6 to all protons of Qui, from H-1 to H-5 of Fuc, and from H-1 to H-4 for the other sugar residues. The COSY spectrum showed most of correlations between the neighboring protons within each spin system. The spin system of GlcA was identified by the absence of any H-6 signal, and the spin systems of GlcN and GalN were distinguished using the characteristic chemical shifts of C-2 as a nitrogen-bearing carbon, which were determined based on the ^{1}H,^{13}C HSQC data (Fig. 3). Using these data, protons and carbons of all six sugar residues were fully assigned (Table 1).

The small $J_{1,2}$ coupling constant values (<3 Hz, signals not resolved) showed that all residues except GlcA are α-linked, whereas GlcA showing $J_{1,2}$ 7.2 Hz is β-linked. Significant downfield displacements in the ^{13}C NMR spectrum of the signals for α-Fuc*p* C-3 (from δ 70.4 to 75.2), α-Fuc*p* C-4 (from δ 73.0 to 81.5), α-Glc*p*

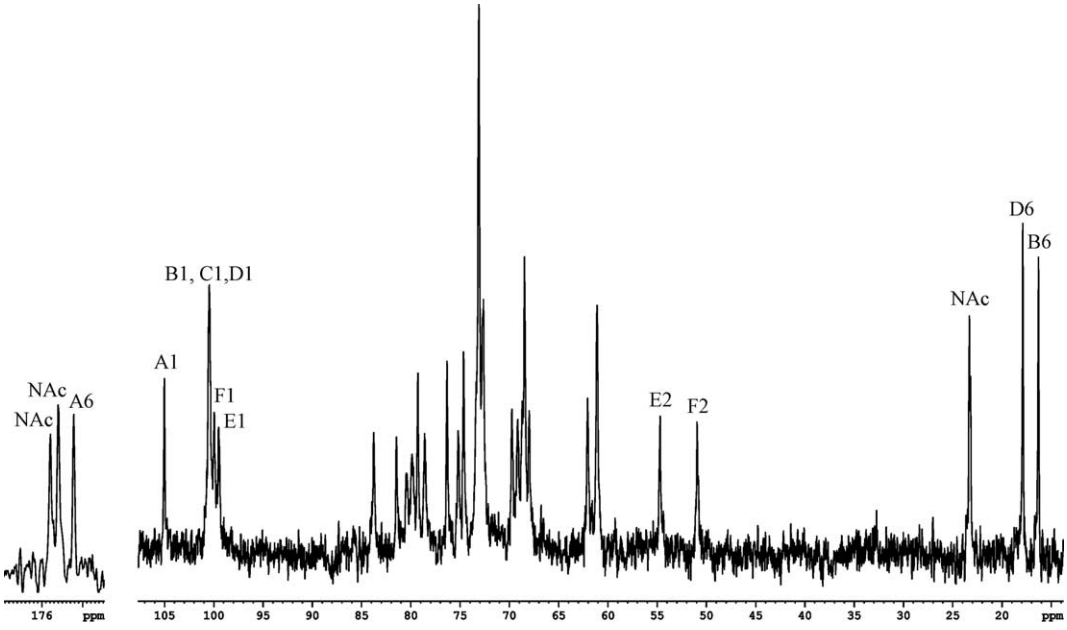

Figure 1. ^{13}C NMR spectrum of the O-polysaccharide from *P. stuartii* O44. Arabic numerals refer to atoms of sugar residues denoted by letters as shown in Table 1.

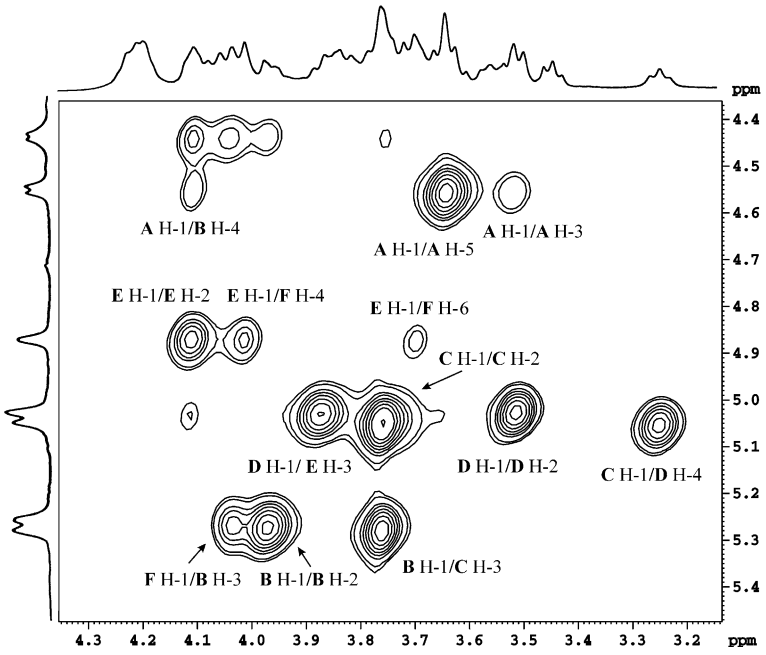

Figure 2. Part of a 2D ROESY spectrum of the O-polysaccharide from *P. stuartii* O44. The corresponding parts of the ^{1}H NMR spectrum are shown along the axes. Arabic numerals refer to atoms in sugar residues denoted by letters as shown in Table 1.

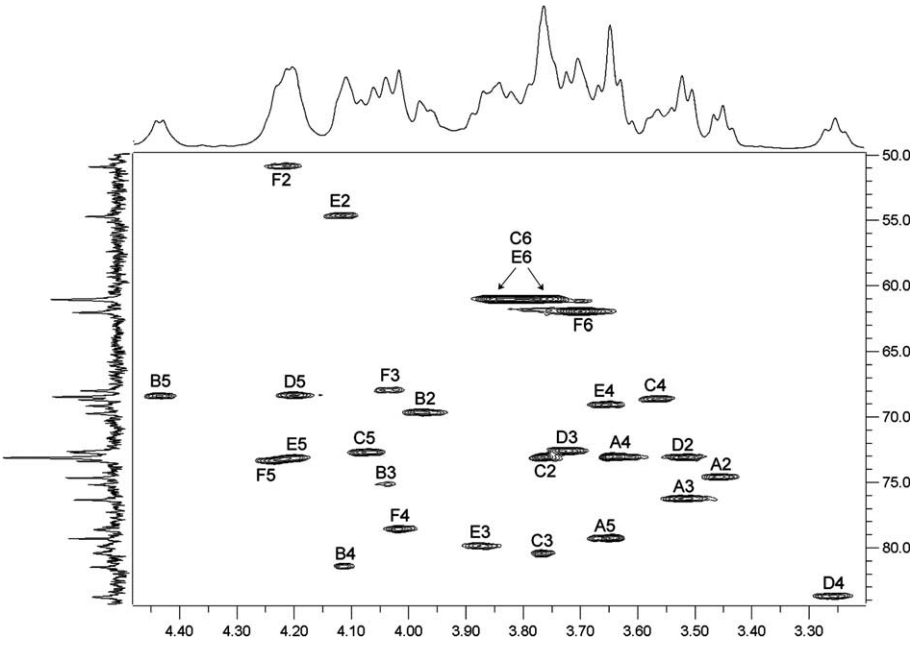

Figure 3. Part of an ^{1}H, ^{13}C HSQC spectrum of the O-polysaccharide from *P. stuartii* O44. The corresponding parts of the ^{1}H and ^{13}C NMR spectra are shown along the axes. Arabic numerals refer to atoms of sugar residues denoted by letters as shown in Table 1.

C-3 (from δ 74.0 to 80.5), α-Qui*p* C-4 (from δ 76.4 to 83.8), α-Glc*p*NAc C-3 (from δ 72.0 to δ 79.9), and α-Gal*p*NAc C-4 (from δ 69.9 to 78.6), as compared with their positions in the spectra of the corresponding unsubstituted monomers,[9] revealed the substitution pattern in the repeating unit. In addition to intra-residue cross-peaks (α-Fuc H-1,H-2 at δ 5.27/3.97; α-Glc H-1, H-2 at δ 5.03/3.78; α-Qui H-1,H-2 at δ 5.01/3.52; α-GlcNAc H-1,H-2 at δ 4.86/4.12; β-GlcA H-1,H-3 and H-1,H-5 at δ 4.55/3.53 and 4.55/3.66, respectively), the ROESY spectrum showed the following inter-residue cross-peaks: α-Fuc H-1, α-Glc H-3 at δ 5.27/3.78; α-Glc H-1, α-Qui H-4 at δ 5.03/3.26; α-Qui H-1, α-GlcNAc H-3 at δ 5.01/3.88; α-GlcNAc H-1, α-GalNAc H-4

Table 1. ^{1}H NMR and ^{13}C NMR data of the O-polysaccharide of *P. stuartii* O44 (δ, ppm)

Sugar residue		H-1	H-2	H-3	H-4	H-5	H-6a	H-6b
β-D-Glc*p*A-(1→	**A**	4.55	3.46	3.53	3.63	3.66		
→3,4)-α-L-Fuc*p*-(1→	**B**	5.27	3.97	4.05	4.11	4.43	1.30	
→3)-α-D-Glc*p*-(1→	**C**	5.03	3.78	3.78	3.57	4.08	3.84	3.78
→4)-α-L-Qui*p*-(1→	**D**	5.01	3.52	3.72	3.26	4.20	1.31	
→3)-α-D-Glc*p*NAc-(1→	**E**	4.86	4.12	3.88	3.67	4.20	3.84	3.78
→4)-α-D-Gal*p*NAc-(1→	**F**	5.24	4.22	4.03	4.02	4.24	3.70	
		C-1	C-2	C-3	C-4	C-5	C-6	
β-D-Glc*p*A-(1→	**A**	105.1	74.7	76.4	73.1	79.3	175.3[a]	
→3,4)-α-L-Fuc*p*-(1→	**B**	100.5	69.8	75.2	81.5	68.5	16.2	
→3)-α-D-Glc*p*-(1→	**C**	100.5	73.1	80.5	68.6	72.7	61.1	
→4)-α-L-Qui*p*-(1→	**D**	100.5	73.1	72.6	83.8	68.5	17.8	
→3)-α-D-Glc*p*NAc-(1→	**E**	99.6	54.7	79.9	69.2	73.1	61.1	
→4)-α-D-Gal*p*NAc-(1→	**F**	100.0	50.9	68.0	78.6	73.2	62.0	

Additional chemical shifts for the *N*-acetyl groups are δ_H 2.04, 2.07, δ_C 23.4, 23.6 (both CH_3) and 175.7[a], 175.9[a] (both CO).
[a] Assignment could be interchanged.

at δ 4.86/4.02; α-GalNAc H-1, α-Fuc H-3 at δ 5.24/4.05; β-GlcA H-1, α-Fuc H-4 at δ 4.55/4.11. These data confirmed the glycosylation pattern and revealed the monosaccharide sequence in the repeating unit.

With the known sequence, the L configuration of the 6-deoxy sugars was confirmed by glycosylation effects on the ^{13}C NMR chemical shifts.[10] The different absolute configurations of α-Fuc and α-Glc residues were confirmed by the negative β-glycosylation effect on C-4 of α-Glc (δ −2.3, while it should be near zero if the absolute configurations were the same). In the same way, the different absolute configurations of α-Glc and α-Qui residues were confirmed by the negative β-glycosylation effect on C-3 of α-Qui (δ −1.0, while it should be zero or slightly positive if the absolute configurations were the same).

The data obtained established that the O-polysaccharide of *P. stuartii* O44 has the structure shown below. Remarkably, the polysaccharide contains L-quinovose, a 6-deoxy sugar that to the best of our knowledge, has not been hitherto found in bacterial polysaccharides.

β-D-Glc*p*A
1
↓
4
[3)-α-L-Fuc*p*-(1→3)-α-D-Glc*p*-(1→4)-α-L-Qui*p*-(1→3)-α-D-Glc*p*NAc-(1→4)-α-D-Gal*p*NAc-(1→]$_n$

1. Experimental

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

Providencia stuartii O44:H4 (strain 3768/51) 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 (yield of 5.2% of dry bacterial weight) was isolated by phenol–water extraction and purified by ultracentrifugation.

A portion of the lipopolysaccharide (150 mg) was treated with 2% AcOH (3 mL) for 1 h at 100 °C and the carbohydrate-containing supernatant was fractionated on a column of Sephadex G-50 (60 × 2.5 cm) in pyridinium acetate buffer (4 mL pyridine and 10 mL AcOH in 1 L water). The yield of the polysaccharide was 11% of the lipopolysaccharide weight.

1.2. Sugar analyses

The polysaccharide was hydrolyzed with 10 M HCl for 30 min at 80 °C. The 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). They were analyzed by GLC on a Hewlett–Packard HP 5880 chromatograph equipped with a Ultra-2 column (Hewlett–Packard) using a temperature gradient of 10 °C min^{-1} from 180 to 290 °C. GlcA was analyzed using a Biotronik LC-2000 sugar analyzer as described.[11]

For the determination of the GlcA absolute configuration, the polysaccharide was subjected to methanolysis (1 mL MeOH, 0.1 mL AcCl, 16 h, 80 °C) followed by

(+)-2-octanolysis, acetylation and analysis by GLC. For determination of the absolute configuration of glucose, the polysaccharide was hydrolyzed, treated with D-glucose oxidase, reduced with $NaBH_4$, acetylated and analyzed by GLC. For determination of the absolute configurations of the other monosaccharides, the polysaccharide was hydrolyzed, N-acetylated (400 μL $NaHCO_3$, 60 μL Ac_2O, 0 °C, 1 h), subjected to (+)-2-octanolysis[12] [100 μL (+)-2-octanol, 15 μL CF_3CO_2H, 120 °C, 16 h], acetylated and analyzed by GLC as above.

1.3. Methylation analyses

Prior to methylation, the polysaccharide was treated with an Amberlite IR-120 (H^+) cation-exchange resin for a better solubility in dimethyl sulfoxide and lyophilized. Methylation was performed according to the Hakomori procedure,[13] the products were recovered using a Sep-Pak cartridge 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 of the permethylated polysaccharide with 10 M HCl for 30 min at 80 °C, followed by conversion into the alditol acetates. They were analyzed by GLC–MS using Hewlett–Packard 5971A system equipped with an HP-1 glass capillary column (0.2 mm × 12 m) and applying the temperature program from 150 to 270 °C at 8 °C min^{-1}.

1.4. NMR spectroscopy

Samples were freeze-dried twice from a ^2H_2O soln and dissolved in 99.96% ^2H_2O. NMR experiments were carried out using a Bruker DRX-500 instrument at 30 °C with internal TSP (δ_H 0) and external acetone (δ_C 31.45) as references. XwinNMR software on SGI Indy/Irix 5.3 were used to acquired and process the NMR data. A mixing time of 300 ms was used in the ROESY experiment.

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