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Note

Structure of the O-polysaccharide of *Providencia stuartii* O49

Ivan S. Bushmarinov,[a] Olga G. Ovchinnikova,[a,*] Nina A. Kocharova,[a] Aleksandra Blaszczyk,[b] Filipp 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, Russia*
[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-specific polysaccharide chain (O-antigen) of the lipopolysaccharide (LPS) of *Providencia stuartii* O49 was studied using sugar and methylation analyses along with ^{1}H and ^{13}C NMR spectroscopy, including two-dimensional COSY, TOCSY, ROESY, H-detected ^{1}H, ^{13}C HSQC and HMBC experiments. The polysaccharide was found to have the trisaccharide repeating unit with the following structure:

→6)-β-D-Gal*p*(1→3)-β-D-Gal*p*NAc(1→4)-α-D-Gal*p*(1→

Keywords: Lipopolysaccharide; O-Polysaccharide; Bacterial polysaccharide structure; *Providencia stuartii*

Bacteria of the genus *Providencia* belongs to the *Enterobacteriaceae* family; they are serologically related to *Escherichia coli*, *Proteus*, *Morganella*, *Salmonella* and *Shigella*. Currently, the genus consists of five species: *P. alcalifaciens*, *P. heimbachae*, *P. rettgerii*, *P. rustigianii* and *Providencia stuartii*. Being a component of the normal intestinal flora, in favourable conditions bacteria *P. stuartii* can cause urinary tract infections and *P. alcalifaciens* and *P. rustigianii* various infections, including traveller's diarrhoea. The serological O-specificity of *Providencia* is defined by the structure of the O-antigen (O-polysaccharide, PS), being a part of the lipopolysaccharide (LPS, endotoxin), one of the major components of the outer membrane of Gram-negative bacteria. Studies of the chemical structures and the serological specificity of the O-antigens aim at the elucidation of the molecular basis of the serological classification of *Providencia* species. The serological classification scheme of *P. alcalifaciens*, *P. rustigianii* and *P. stuartii* includes 62 O-serogroups.[1,2] At present, the O-polysaccharides structures of *P. stuartii* O4,[3] O18,[4] O33[5] have been established. Now we report on the structure of the O-polysaccharide of *P. stuartii* O49.

The lipopolysaccharide was isolated from bacterial cells by the phenol–water procedure[6] and degraded by mild acid hydrolysis to give the O-polysaccharide, which was isolated by GPC on Sephadex G-50. Monosaccharide analysis of the polysaccharide revealed Gal and GalN in the ratio ~2:1. An enzymatic assay with D-galactose oxidase showed that the O-polysaccharide contains D-Gal and D-GalN. The D-configuration of Gal was confirmed by GLC of the acetylated glycosides with (+)-2-octanol. Methylation analysis revealed 4-substituted Gal, 6-substituted Gal and 3-substituted GalN.

The ^{13}C NMR spectrum of the polysaccharide (Fig. 1) demonstrated a regular structure. It contained signals for three sugar residues, including those for three anomeric carbons at δ 99.8, 103.7 and 106.0, one nitrogen-bearing carbon at δ 52.7 (C-2 of GalN), two unsubstituted (δ 62.3

* Corresponding author. Tel.: +7-095-9383613; fax: +7-095-1355328; e-mail: olgao@hotmail.ru

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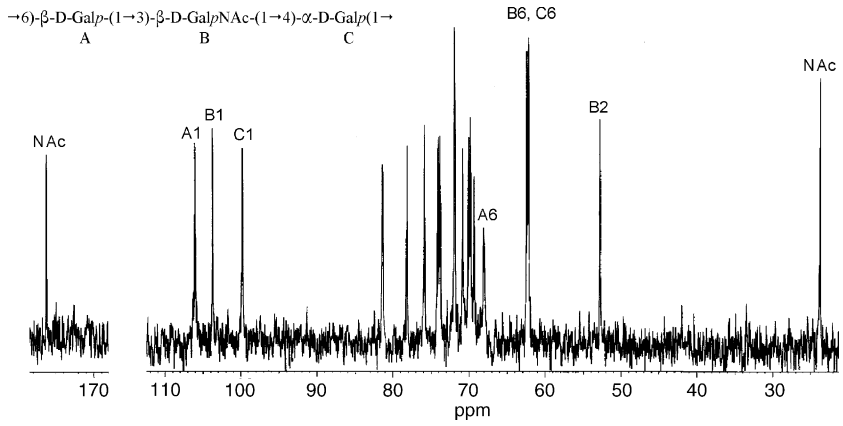

Figure 1. ^{13}C NMR spectrum of the O-polysaccharide of *P. stuartii* O49.

and δ 62.1) and one substituted $HOCH_2$ groups (δ 68.0) of hexoses and GalN residue (data of a DEPT-135 experiment, Fig. 2), 11 sugar-ring oxygen-bearing carbons in the region δ 70–82 and for one *N*-acetyl group at δ 23.7 (Me) and δ 176.2 (CO). Accordingly, the ^{1}H NMR spectrum contained signals for three anomeric protons at 4.96, 4.69 and 4.47 and a signal for one *N*-acetyl group at δ 2.05. As judged by the absence of signals within δ 82–88, all sugar residues are in pyranose form.[7]

The ^{1}H and ^{13}C NMR spectra of the O-polysaccharide were assigned using ^{1}H, ^{1}H COSY, TOCSY, ROESY, ^{1}H,^{13}C HSQC and ^{1}H,^{13}C HMBC experiments (Tables 1 and 2). The TOCSY spectrum demonstrated correlations of H-1 with all protons from H-2 to H-4 for all sugar residues. The COSY spectrum showed most of the correlations between the neighbouring protons within each spin system but as soon as there were no H-4,H-5 correlations in COSY, signals of H-5, H-6a and H-6b have been assigned using data of the ROESY experiment (Fig. 3). The ROESY spectrum showed the H-4,H-5 *intra*-residue cross-peaks for α-Gal*p*, β-Gal*p* and β-Gal*p*N at δ 4.14/3.89, 3.95/3.86 and 4.11/3.66, respectively. The signals of H-6a and H-6b were assigned basing on H-5,H-6a and H-5,H6b correlations in COSY (δ 3.89/3.77, 3.89/3.82 for α-Gal*p*, δ 3.86/3.88, 3.86/3.69 for β-Gal*p*, δ 3.66/3.77, 3.66/3.82 for β-Gal*p*NAc).

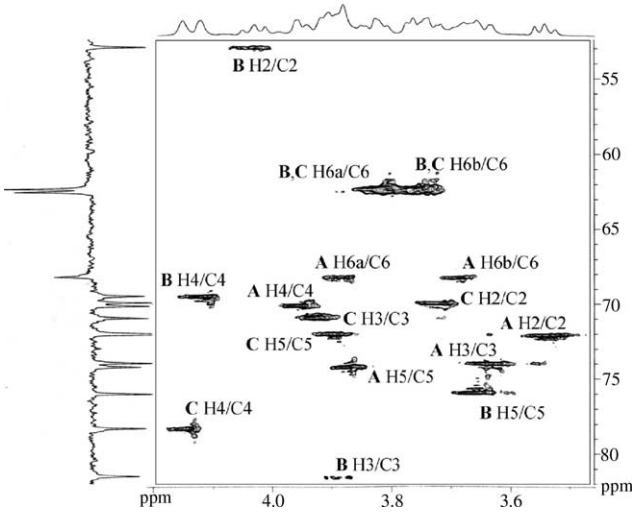

Figure 2. Part of an ^{1}H, ^{13}C HSQC spectrum of the O-polysaccharide of *P. stuartii* O49. The corresponding parts of the ^{1}H and DEPT spectra are shown along the axes. Arabic numerals refer to atoms in sugar residues denoted by capital letters as shown in Tables 1 and 2.

The $J_{4,5}$ and $J_{3,4}$ coupling constant values ~3 Hz showed the *galacto*-configuration for all sugar residues. The $J_{1,2}$ coupling constant values (~3 Hz for α-Gal*p*, ~8 Hz for β-Gal*p* and β-Gal*p*N) enabled determination of the anomeric configurations of monosaccharides, which are as follows: α-Gal*p*, β-Gal*p* and β-Gal*p*NAc. The spin system of β-Gal*p*N was distinguished by correlation of proton at the nitrogen-bearing carbon (H-2 at δ 4.02) to the corresponding carbon C-2 at δ 52.7 (data of the HSQC spectrum).

The ^{13}C NMR spectrum was assigned using a ^{1}H,^{13}C HSQC experiment (Fig. 2). Significant downfield displacement of the signals for C-4 α-Gal*p* (from δ 70.6 to δ 78.1), C-6 β-Gal*p* (from δ 62.2 to δ 68.0) and C-3 β-Gal*p*NAc (from δ 72.4 to δ 81.3), as compared to their position in the spectra of unsubstituted monomers,[8] revealed the substitution pattern. The ^{1}H, ^{13}C HMBC spectrum showed the following *inter*-residue cross-peaks: α-Gal*p* H-1,β-Gal*p* C-6 at δ 4.96/68.0, β-Gal*p* H-1,β-Gal*p*NAc C-3 at δ 4.47/81.3 and β-Gal*p*N H-1, α-Gal*p* C-4 at δ 4.69/78.1. These data confirmed the glycosylation pattern and revealed the monosaccharide sequence in the repeating unit.

Therefore, the O-polysaccharide of *P. stuartii* O49 has the following structure:

→6)-β-D-Gal*p*(1→3)-β-D-Gal*p*NAc(1→4)-
α-D-Gal*p*(1→

Table 1. ^{1}H NMR data (δ, ppm) of the O-polysaccharide of *P. stuartii* O49

Residue	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b
A → 6)-β-Gal*p*-(1 →	4.47	3.54	3.65	3.95	3.86	3.88	3.69
B → 3)-β-Gal*p*NAc-(1 →	4.69	4.02	~3.89	4.11	3.66	~3.77	~3.82
C → 4)-α-Gal*p*-(1 →	4.96	3.72	3.92	4.14	3.89	~3.77	~3.82

Chemical shift for NAc is δ 2.05.

Table 2. ^{13}C NMR data (δ, ppm) for the O-specific polysaccharide of *P. stuartii* O49

Residue	C-1	C-2	C-3	C-4	C-5	C-6
A → 6)-β-Gal*p*-(1 →	106.0	71.8	73.8	70.0	74.0	68.0
B → 3)-β-Gal*p*NAc-(1 →	103.7	52.7	81.3	69.3	75.8	62.1[a]
C → 4)-α-Gal*p*-(1 →	99.8	69.7	70.8	78.1	71.8	62.3[a]

Chemical shifts for NAc are δ 23.7 (CH_3) and 176.2 (CO).
[a]Assignment could be interchanged.

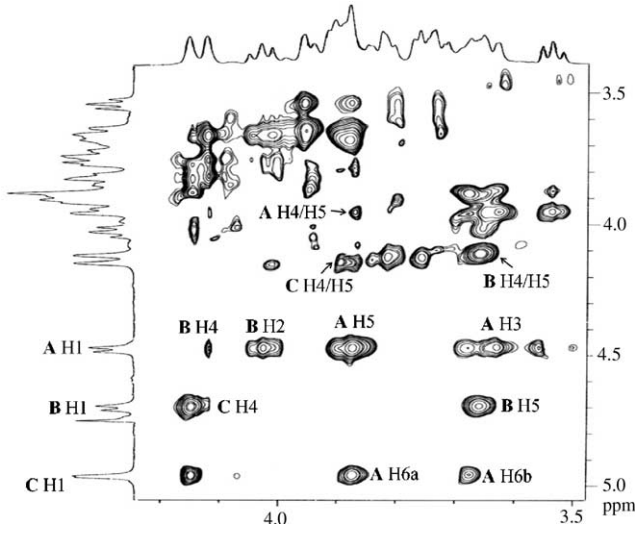

Figure 3. Part of a ROESY spectrum of the O-polysaccharide of *P. stuartii* O49. The corresponding part of the ^{1}H NMR spectrum is shown along the axes. Arabic numerals refer to atoms in sugar residues denoted by capital letters as shown in Tables 1 and 2.

1. Experimental

1.1. Bacterial strain and growth

Providencia stuartii O49:H4, strain 5875/52 was obtained from the Hungarian National Collection of Medical Bacteria (National Institute of Hygiene, Budapest) and 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 lyophilised.

1.2. Isolation of the lipopolysaccharide and O-polysaccharide

The lipopolysaccharide was isolated from bacterial cells by phenol/water extraction[6] and purified by treatment with cold aq 50% CCl_3COOH. After centrifugation, the supernatant was dialysed and freeze-dried.

The O-polysaccharide was obtained by mild acid hydrolysis of the lipopolysaccharide (200 mg) with 2% HOAc (3 mL) for 1 h at 100 °C followed by GPC of the water-soluble portion on a column (60 × 2.5 cm) of Sephadex G-50 in pyridinium acetate buffer (4 mL pyridine and 10 mL HOAc in 1 L water). The yield of the O-polysaccharide was 22.7% of the lipopolysaccharide weight.

1.3. Monosaccharide analysis

The polysaccharide (0.5 mg) was hydrolysed with 2 M CF_3CO_2H (0.5 mL, 120 °C, 2 h) and the alditol acetates derived were analysed by GLC using a temperature program from 180 to 290 °C at 10 °C min^{-1}. For determination of the absolute configuration, the O-polysaccharide (0.5 mg) was hydrolysed with 2 M CF_3CO_2H as above, N-acetylated (400 μL $NaHCO_3$, 60 μL Ac_2O, 0 °C, 1 h), subjected to 2-octanolysis[9] [100 μL (*S*)-2-octanol, 15 μL CF_3CO_2H, 120 °C, 16 h], acetylated and analysed by GLC as above. The O-polysaccharide (0.5 mg) was hydrolysed, treated with D-galactose oxidase in 0.1 M phosphate buffer pH 6.3 at 37 °C for 2 h,[10] reduced with $NaBH_4$, acetylated and analysed by GLC.

1.4. Methylation analysis

Methylation of the O-polysaccharide (0.5 mg) was performed according to the Hakomori procedure,[11] the product was recovered using a Sep-Pak cartridge, hydrolysed with 2 M CF_3CO_2H at 120 °C for 2 h, the partially methylated monosaccharides were reduced with $NaBH_4$, acetylated and analysed by GLC–MS.

1.5. NMR spectroscopy

NMR experiments were carried out at 50 °C for solutions in D_2O with internal TSP (δ_H 0) and external

acetone (δ_C 31.45) as references. The mixing time of 300 ms was used for ROESY. All spectra were recorded using Bruker DRX-500 NMR instrument and XwinNMR software on SGI Indy/Irix 5.3. The carbon chemical shifts data fit was performed using BIOPSEL software and database.[12]

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