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Note

Structure of the O-polysaccharide from the lipopolysaccharide of *Providencia stuartii* O43 containing an amide of D-galacturonic acid with L-serine

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Dedicated to Professor N. K. Kochetkov on the occasion of his 90th birthday

Abstract—The O-polysaccharide was obtained by mild acid degradation of the lipopolysaccharide of *Providencia stuartii* O43:H28 and studied by sugar and methylation analyses, Smith degradation and ^{1}H and ^{13}C NMR spectroscopy, including 2D ROESY, and H-detected ^{1}H, ^{13}C HSQC and HMBC experiments, as well as a NOESY experiment in a 9:1 H_2O/D_2O mixture to reveal correlations for NH protons. It was found that the polysaccharide is built up of linear tetrasaccharide repeating units containing an amide of D-galacturonic acid with L-serine [D-GalA6(L-Ser)] and has the following structure:

[3)-β-D-Gal*p*A6(L-Ser)-(1→3)-β-D-Glc*p*NAc-(1→2)-α-D-Rha*p*4NAc-(1→4)-β-D-Glc*p*A-(1→]$_n$

Keywords: *Providencia stuartii*; O-antigen; Lipopolysaccharide; Polysaccharide structure; D-Galacturonoyl-L-serine

Gram-negative bacteria of the genus *Providencia* are facultative pathogens, which are able to invade intestinal mucosa and other cell types and may cause intestinal infections. They may be responsible also for urinary tract infections. Currently, the genus *Providencia* is subdivided into five species: *P. alcalifaciens*, *P. rustigianii*, *P. heimbache*, *P. rettgerii* and *P. stuartii*. Particularly, *P. stuartii* is a well recognized pathogen that causes urinary tract infections in patients with chronic indwelling urinary catheters.[1] The serological O-specificity of *Providencia* is defined by the structure of the O-polysaccharide chain (O-antigen) of the lipopolysaccharide, which is considered also as a virulence factor of these bacteria. The serological classification scheme of *P. alcalifaciens*, *P. rustigianii* and *P. stuartii* includes 63 O-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, the O-polysaccharides structures of *P. stuartii* O4,[3] O18,[4] O33,[5] O47,[6] O49[7] and O57[8] have been established. Now we report on a novel structure for the O-polysaccharide of *P. stuartii* O43.

A high-molecular-mass polysaccharide was isolated by mild acid degradation of the lipopolysaccharide of *P. stuartii* O43 followed by GPC of the carbohydrate portion on Sephadex G-50. Sugar analysis by GLC of the acetylated alditols revealed 2-amino-2-deoxyglucose (GlcN) and 4-amino-4,6-dideoxymannose (Rha4N) in the ratio ~1:1. In addition, galacturonic acid (GalA) and glucuronic acid (GlcA) were identified by anion-exchange chromatography using a sugar analyzer, whereas

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amino acid analysis revealed serine. The D configuration of all monosaccharides and the L configuration of serine were determined by GLC of the acetylated (+)-2-octyl glycosides[9] (for sugars) or the acetylated (+)-2-octyl ester (for serine).

GLC–MS of the partially methylated alditol acetates derived from the methylated polysaccharide revealed 3-substituted GlcN and 2-substituted Rha4N. In addition to the amino sugars, a similar analysis after carboxyl-reduction of the methylated polysaccharide showed 2,3-di-*O*-methylglucose and a minor amount of 2,4-di-*O*-methylgalactose, which were evidently derived from 4-substituted GlcA and 3-substituted GalA, respectively. That only a small amount of 2,4-di-*O*-methylgalactose was detected could be accounted for by amidation of GalA to a large extent in the O-polysaccharide. The methylation data demonstrated the pyranose form GlcN, Rha4N and GalA, whereas that of the 4-substituted GlcA was shown by NMR spectroscopic data (see below).

The ^{13}C NMR spectrum of the polysaccharide (Fig. 1) contained signals for four sugar residues, including those for four anomeric carbons at δ 98.7–105.0, three nitrogen-bearing carbons (GlcNAc C-2, Rha4NAc C-4 and Ser C-2) at δ 56.9–58.3, one methyl group (C-6 of Rha) at δ 18.2, two *N*-acetyl groups at (CH_3 at δ 23.5 and 23.8, CO at δ 175.8 and 176.2) and three carboxyl groups (GalA C-6, GlcA C-6 and Ser C-1) at δ 171.0, 176.9 and 177.4. The ^{1}H NMR spectrum of the polysaccharide contained, inter alia, signals for four anomeric protons at δ 4.59–5.67, one methyl group (H-6 of Rha) at δ 1.11, and two *N*-acetyl groups at δ 2.01 and 2.02.

The 2D TOCSY spectrum of the polysaccharide revealed spin systems for four monosaccharide residues designated as **A**–**D** according to their sequence in the repeating unit established later (see below), and the COSY spectrum enabled differentiation between protons within each spin system (Table 1). The ^{13}C NMR spectrum was assigned using an ^{1}H, ^{13}C HSQC experiment (Table 2).

The configuration of the glycosidic linkages of β-GalA, β-GlcN and β-GlcA was established by $J_{1,2}$ coupling constant values of 7.0–7.6 Hz and confirmed by intraresidue H-1,H-3 and H1, H-5 correlation of the 1,3-diaxial protons in the 2D ROESY spectrum of the

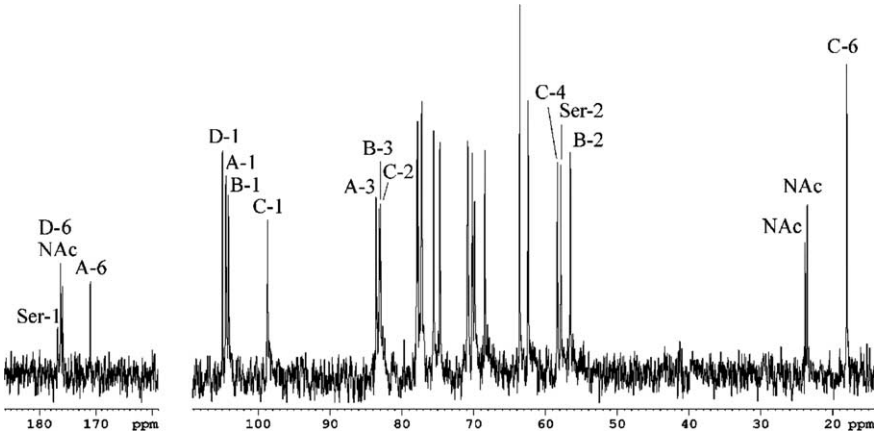

Figure 1. ^{13}C NMR spectrum of the O-polysaccharide of *P. stuartii* O43.

Table 1. ^{1}H NMR data (δ, ppm)

		H-1	H-2	H-3	H-4	H-5	H-6a	H-6b
Polysaccharide **1**								
→3)-β-D-Gal*p*A6(L-Ser)-(1→	**A**	4.59	3.69	3.85	4.47	4.25		
→3)-β-D-Glc*p*NAc-(1→	**B**	4.74	3.89	3.91	3.59	3.53	3.95	3.81
→2)-α-D-Rha*p*4NAc-(1→	**C**	5.67	3.66	3.69	3.56	3.76	1.11	
→4)-β-D-Glc*p*A-(1→	**D**	4.70	3.45	3.82	3.82	3.82		
L-Ser			4.35	3.88				
Oligosaccharide **2**								
β-D-Gal*p*A6(L-Ser)-(1→	**A**	4.55	3.57	3.73	4.26	4.26		
→3)-β-D-Glc*p*NAc-(1→	**B**	4.74	3.91	3.95	3.59	3.55	3.97	3.81
→2)-α-D-Rha*p*4NAc-(1→	**C**	5.33	3.70	3.77	3.61	3.95	1.16	
→3)-L-Erythronic acid	**D***		4.21	3.91	3.75, 3.82			
L-Ser			4.33	3.88				

Additional chemical shifts for *N*-acetyl groups are δ_H 2.01 and 2.02 in the polysaccharide **1**, δ_H 2.01 and 2.04 in the oligosaccharide **2**.

Table 2. ^{13}C NMR data (δ, ppm)

		C-1	C-2	C-3	C-4	C-5	C-6
Polysaccharide **1**							
→3)-β-D-Gal*p*A6(L-Ser)-(1→	**A**	104.5	70.9	83.6	69.9	75.6	171.0
→3)-β-D-Glc*p*NAc-(1→	**B**	104.2	56.9	83.2	70.2	77.2	62.4
→2)-α-D-Rha*p*4NAc-(1→	**C**	98.7	83.0	70.9	58.3	68.7	18.2
→4)-β-D-Glc*p*A-(1→	**D**	105.0	74.7	77.9[a]	77.8[a]	77.2[a]	176.9
L-Ser		177.4	57.9	63.6			
Oligosaccharide **2**							
β-D-Gal*p*A6(L-Ser)-(1→	**A**	104.7	71.7	73.6	70.4	75.9	171.2
→3)-β-D-Glc*p*NAc-(1→	**B**	104.0	56.5	82.8	70.1	77.0	62.2
→2)-α-D-Rha*p*4NAc-(1→	**C**	99.7	83.0	70.6	58.4	68.4	18.1
→3)-L-Erythronic acid	**D***	179.2	74.5	82.7	61.7		
L-Ser		175.9	58.2	63.6			

Additional chemical shifts for *N*-acetyl groups are δ_C 23.5, 23.8 (both CH_3), 175.8 and 176.2 (both CO) in the polysaccharide **1**, δ_C 23.5, 23.7 (both CH_3), 176.3 and 177.4 (both CO) in the oligosaccharide **2**.

[a] Assignment could be interchanged.

polysaccharide. The ROESY spectrum showed also an intraresidue α-Rha4NAc H-1,H-2 cross-peak at δ 5.67/3.66 with no H-1,H-3 and H-1,H-5 cross-peaks that are typical of α-linked monosaccharides having the *manno* configuration, including α-Rha4NAc.

Relatively low-field positions at δ 83.6, 83.2 and 83.0 of the signals for β-GalA C-3, β-GlcNAc C-3, α-Rha4NAc C-2, respectively, demonstrated the mode of substitution of the monosaccharides (compare the chemical shifts δ 73.79,[10] 74.81[10] and 71.1[11] for these carbons in the corresponding non-substituted monosaccharides). Although the ^{13}C NMR resonances of GlcA C-3, C-4 and C-5 at δ 77.9, 77.8 and 77.2 could not be assigned unambiguously owing to a coincidence of the H-3, H-4 and H-5 resonances, comparison with the C-3, C-4 and C-5 chemical shifts of free β-Glc*p*A (δ 76.53, 72.69

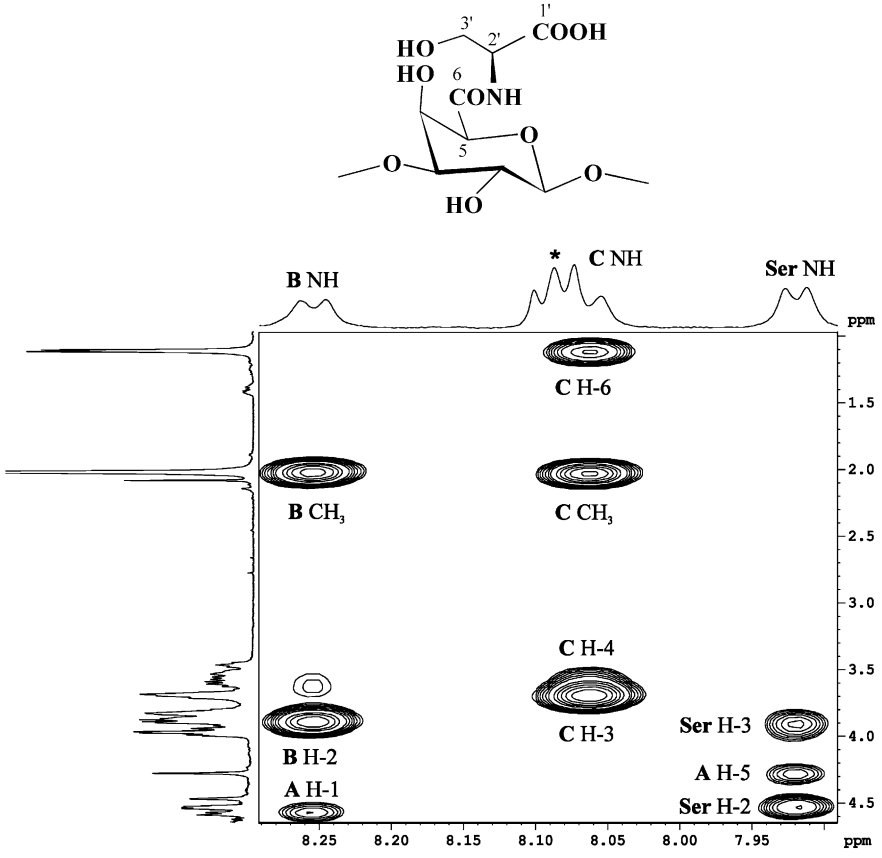

Figure 2. Part of a NOESY spectrum of the O-polysaccharide of *P. stuartii* O43 in a 9:1 H_2O/D_2O mixture. The corresponding parts of the ^{1}H NMR spectrum are shown along the axes. Arabic numerals refer to atoms in sugar residues denoted by letters as shown in Tables 1 and 2. Asterisk shows a signal of a pyridine impurity. The structure of the D-galacturonoyl-L-serine fragment is shown in the inset.

and 76.93,[10] respectively) showed that only the C-4 signal of GlcA shifted downfield and, hence, GlcA is 4-substituted. The position of serine was determined by a NOESY experiment on a polysaccharide solution in a H_2O/D_2O mixture (9:1), which showed a GalA H-5,Ser NH cross-peak at δ 4.25/7.92 (Fig. 2).

The ROESY spectrum of the polysaccharide showed a number of interresidue cross-peaks, from which the following correlations between the anomeric protons and protons at the linkage carbons could be recognized taking into account the glycosylation pattern determined earlier: β-GlcA H-1,β-GalA H-3 at 4.70/3.85; β-GalA H-1,β-GlcNAc H-3 at δ 4.59/3.91; β-GlcNAc H-1,α-Rha4NAc H-2 at 4.74/3.66 and α-Rha4NAc H-1, β-GlcA H-4 at 5.67/3.82. The sequence of the monosaccharides thus defined was confirmed by a ^1H, ^{13}C HMBC experiment (Fig. 3), which showed the following cross-peaks for the anomeric protons: β-GlcA H-1,β-GalA C-3 at δ 4.70/83.6; β-GalA H-1,β-GlcNAc C-3 at δ 4.59/83.2 (both strong); β-GlcNAc H-1,α-Rha4NAc C-2 at δ 4.74/83.0 and α-Rha4NAc H-1,β-GlcA C-4 at δ 5.67/77.8 (both strong).

Therefore, the O-polysaccharide of *P. stuartii* O43 has the structure **1** shown in Figure 4, which was confirmed independently by Smith degradation. The resultant oligosaccharide was isolated by GPC and its structure **2** shown in Figure 4 was established by NMR spectroscopy as described above for the O-polysaccharide (for the ^1H and ^{13}C NMR chemical shifts see Tables 1 and 2) and electrospray ionization MS (experimental and calculated monoisotopic molecular mass 789.265 Da).

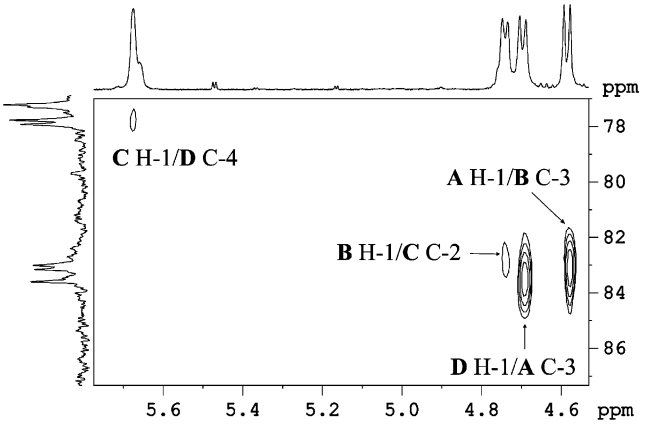

Figure 3. Part of an H-detected ^1H, ^{13}C HMBC spectrum of the O-polysaccharide of *P. stuartii* O43. The corresponding parts of the ^1H and ^{13}C NMR spectra are shown along the the horizontal and vertical axes, respectively. Arabic numerals refer to atoms in sugar residues denoted by letters as shown in Tables 1 and 2.

1. Experimental

1.1. Bacterial strain, isolation and degradation of the lipopolysaccharide

P. stuartii O43:H28 (strain 1746/51) 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 was isolated, in a yield of 5.7% of dry bacterial weight, by phenol-water extraction followed by dialysis of the extract without layer separation and purification by ultracentrifugation.

A portion of the lipopolysaccharide (200 mg) was heated with 2% AcOH (4 mL) for 2 h 20 min 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 24% of the lipopolysaccharide weight.

1.2. Smith degradation

The polysaccharide (30 mg) was oxidized with 0.1 M sodium metaperiodate in the dark for 72 h at 20 °C; after adding an excess of ethylene glycol, the product was reduced by an excess of $NaBH_4$ and desalted on a column of TSK HW-40 in 1% AcOH. The resultant modified polysaccharide was hydrolyzed with 2% AcOH for 2 h at 100 °C, and the products were fractionated on a TSK HW-40 gel column in 1% AcOH to give oligosaccharide **2** (18.4 mg).

1.3. Monosaccharide analysis

For sugar analysis, the polysaccharide (0.3 mg) was 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

[3)-β-D-Gal*p*A6(L-Ser)-(1→3)-β-D-Glc*p*NAc-(1→2)-α-D-Rha*p*4NAc-(1→4)-β-D-Glc*p*A-(1→]$_n$ **1**
A B C D

β-D-Gal*p*A6(L-Ser)-(1→3)-β-D-Glc*p*NAc-(1→2)-α-D-Rha*p*4NAc-(1→3)-L-EryA **2**
A B C D*

Figure 4. Structures of the O-polysaccharide of *P. stuartii* O43 (**1**) and the oligosaccharide derived by Smith degradation (**2**). EryA stands for erythronic acid.

GLC on a Hewlett–Packard HP 5880 chromatograph (Avondale, PA) equipped with a DB-5 fused-silica capillary (30 m × 0.25 mm) column and a temperature program of 160 °C (1 min)→260 °C at 3 °C min^{-1}. Uronic acids were analyzed using a Biotronik LC-2000 sugar analyzer as described.[12] Amino components were analyzed on a Biotronik LC-2000 amino acid analyzer using standard sodium citrate buffers.

For determination of the absolute configurations, the O-polysaccharide (0.5 mg) was (i) hydrolyzed with 2 M CF_3CO_2H (120 °C, 2 h) and N-acetylated (400 μL $NaHCO_3$, 60 μL Ac_2O, 0 °C, 1 h) (for amino sugars), (ii) subjected to methanolysis (1 mL MeOH, 0.1 mL AcCl, 16 h, 80 °C) (for uronic acids) or (iii) hydrolyzed with 10 M HCl (30 min, 80 °C) (for serine). The products were heated with (+)-2-octanol (100 μL) in the presence of CF_3CO_2H (15 μL) at 120 °C for 16 h, acetylated and analyzed by GLC as above.

1.4. Methylation analysis

Prior to methylation, the polysaccharide (1.2 mg) was treated with Amberlite IR-120 (H^+) to remove cations and lyophilized. Methylation of the polysaccharide 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, converted into the alditol acetates and analyzed by GLC–MS on a Hewlett–Packard HP 5989A instrument equipped with a 30-m HP-5MS column (Hewlett–Packard) using a temperature gradient of 150 °C (3 min)→320 °C at 5 °C min^{-1}.

1.5. Electrospray ionization MS

High-resolution negative ion electrospray ionization mass spectrum was obtained on a Fourier transform ion-cyclotron resonance mass spectrometer (ApexII, Bruker Daltonics, USA) equipped with a 7 T actively screened magnet and ion source. The sample was dissolved in 30:30:0.01 2-propanol–water–triethylamine mixture at a concentration of ~20 ng μL^{-1} and sprayed with a flow rate of 2 μL min^{-1}.

1.6. NMR spectroscopy

^1H and ^{13}C NMR spectra were recorded with a Bruker DRX-500 spectrometer at 55 and 70 °C in 99.96% ^2H_2O or a H_2O/D_2O (9:1) mixture. For experiments in ^2H_2O samples were freeze-dried twice from a ^2H_2O solution. 2D NMR experiments were performed using standard Bruker software. Internal TSP (δ_H 0) and external acetone (δ_C 31.45) were used as references. HDO and H_2O signals were suppressed by presaturation for 1 s. Mixing time of 100, 300 and 200 ms was used in ROESY, NOESY and TOCSY experiments, respectively.

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