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Structure of the *N*-acetyl-L-rhamnosamine-containing O-polysaccharide of *Proteus vulgaris* TG 155 from a new *Proteus* serogroup, O55

Anna N. Kondakova,[a] Katarzyna Kolodziejska,[b] Krystyna Zych,[b] Sof'ya N. Senchenkova,[a] Alexander S. Shashkov,[a] Yuriy A. Knirel,[a,*] Zygmunt Sidorczyk[b]

[a] *N. D. Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, Leninsky Prospekt 47, Moscow 119991, Russian Federation*
[b] *Department of General Microbiology, Institute of Microbiology and Immunology, University of Lódz, Lódz, Poland*

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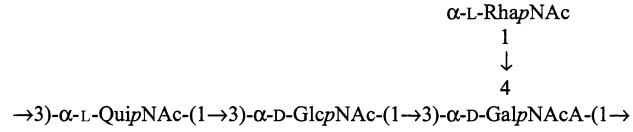
Abstract

The O-polysaccharide of the lipopolysaccharide (LPS) of *Proteus vulgaris* TG 155 was found to contain 2-acetamido-2,6-dideoxy-L-mannose (*N*-acetyl-L-rhamnosamine, L-RhaNAc), a monosaccharide that occurs rarely in Nature. The following structure of the O-polysaccharide was established by NMR spectroscopy, including 2D COSY, TOCSY, ROESY and ^{1}H,^{13}C HSQC experiments, along with chemical methods:



Rabbit polyclonal O-antiserum against *P. vulgaris* TG 155 reacted with both core and O-polysaccharide moieties of the homologous LPS but showed no cross-reactivity with other LPS from the complete set of serologically different *Proteus* strains. Based on the unique O-polysaccharide structure and the serological data, we propose classifying *P. vulgaris* TG 155 into a new, separate *Proteus* O-serogroup, O55.

Keywords: *Proteus vulgaris*; Lipopolysaccharide; O-polysaccharide structure; Serological classification; Rhamnosamine

1. Introduction

Gram-negative bacteria of the genus *Proteus* are human opportunistic pathogens, which under favorable conditions cause mainly wounds and urinary tract infections. The latter sometimes lead to severe complications, such as acute or chronic pyelonephritis and formation of bladder and kidney stones.[1] It has been also reported that *Proteus* surface antigens are involved in rheumathoid arthritis.[2]

Proteus rods are subdivided into five species, *P. mirabilis*, *P. vulgaris*, *P. penneri*, *P. hauseri* and *P. myxofaciens*, and three unnamed *Proteus* genomospecies 4–6.[3,4] Based on the serological specificity of the lipopolysaccharide (LPS, O-antigen), strains of *P. mirabilis* and *P. vulgaris* are divided into 49 O-serogroups,[5] and 11 additional O-serogroups have been proposed.[6] Recently, the classification scheme of *Proteus* has been extended by creation of new serogroups, including those for strains of *P. penneri*.[7–9]

* Corresponding author. Tel.: +7-095-9383613; fax: +7-095-1355328.
E-mail address: knirel@ioc.ac.ru (Y.A. Knirel).

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In this paper, we report on the structure of the O-polysaccharide of the LPS from *P. vulgaris* TG 155, which is one of the strains that earlier have been considered as candidates for new *Proteus* O-serogroups.[6] The data obtained support the expediency of classifying this strain into a new *Proteus* serogroup.

2. Results and discussion

The LPS was isolated from dried bacterial cells of *P. vulgaris* TG 155 by the phenol–water extraction procedure. Mild acid degradation of the LPS with dilute acetic acid, followed by GPC on Sephadex G-50 resulted in a high-molecular mass O-polysaccharide.

The ^{13}C NMR spectrum of the polysaccharide (Fig. 1, Table 1) contained signals for four anomeric carbons at δ 95.1–98.6, four nitrogen-bearing carbons at δ 48.7–54.5 (C-2 of amino sugars), 12 oxygen-bearing carbons in the region δ 69.1–77.0, one unsubstituted HO*C*H$_2$–C group at δ 61.8 (C-6 of a hexose or a hexosamine), two *C*H$_3$–C groups at δ 17.8 and 17.9 (C-6 of 6-deoxyhexoses), four *N*-acetyl groups (CH$_3$ at δ 23.3 and 23.8, 3C's and 1C, respectively) and five CO groups in the region δ 173.7–175.8 (C-6 of a hexuronic acid and four *N*-acetyl groups). The ^{1}H NMR spectrum of the polysaccharide (Table 2) contained, inter alia, signals for four anomeric protons in the region δ 4.91–5.13, two CH$_3$–C groups at δ 1.18 (H-6 of 6-deoxyhexoses) and four *N*-acetyl groups at δ 1.87–2.05. These data suggest that the polysaccharide has a tetrasaccharide repeating unit, which, most likely, consists of two residues of 6-deoxy-*N*-acetylamino sugars and one residue each of an *N*-acetylhexosamine and an *N*-acetylhexosaminuronic acid.

Monosaccharide analysis using amino acid analyser showed two peaks with the intensity ratio 1:2, coinciding with the authentic samples of GlcN and 2-amino-2,6-dideoxyhexose (QuiN), respectively. GLC of the acetylated (*S*)-2-octyl glycosides revealed the D configuration of GlcN and the L configuration of QuiNAc. The ^{1}H and ^{13}C NMR spectra of RhaNAc, which was isolated by partial acid hydrolysis of the O-polysaccharide, were assigned using 2D ^{1}H,^{1}H COSY, TOCSY and H-detected ^{1}H,^{13}C HSQC experiments (Tables 1 and 2). The $^3J_{H,H}$ values determined from the ^{1}H NMR spectrum were indicative of the *manno* configuration of the monosaccharide.[10] Hydrolysis with 2 M HCl converted RhaNAc to RhaN·HCl, which coincided with QuiN in analysis on an amino acid analyser. The specific optical rotation value of RhaN·HCl, $[\alpha]_D$ +21.6°, showed the L configuration of the monosaccharide (compare published data[11] $[\alpha]_D$ −23.0° (water) for D-RhaN·HCl). The D configuration of GalNAcA was established by glycosylation effects in the ^{13}C NMR spectrum of the O-polysaccharide (see below).

The ^{1}H and ^{13}C NMR spectra of the polysaccharide were assigned as described above for RhaNAc (Tables 1 and 2), and four sugar spin systems were identified. All sugars had small $J_{1,2}$ coupling constant values of 3–4 Hz, which showed that GlcNAc, GalNAc and QuiNAc are α-linked. This conclusion was confirmed, and the anomeric configuration of RhaNAc established, by the 2D ROESY spectrum, which showed a H-1,H-2 correlation for each monosaccharide but no H-1,H-3 or H-1,H-5 correlations, which are typical of β-linked pyranoses.

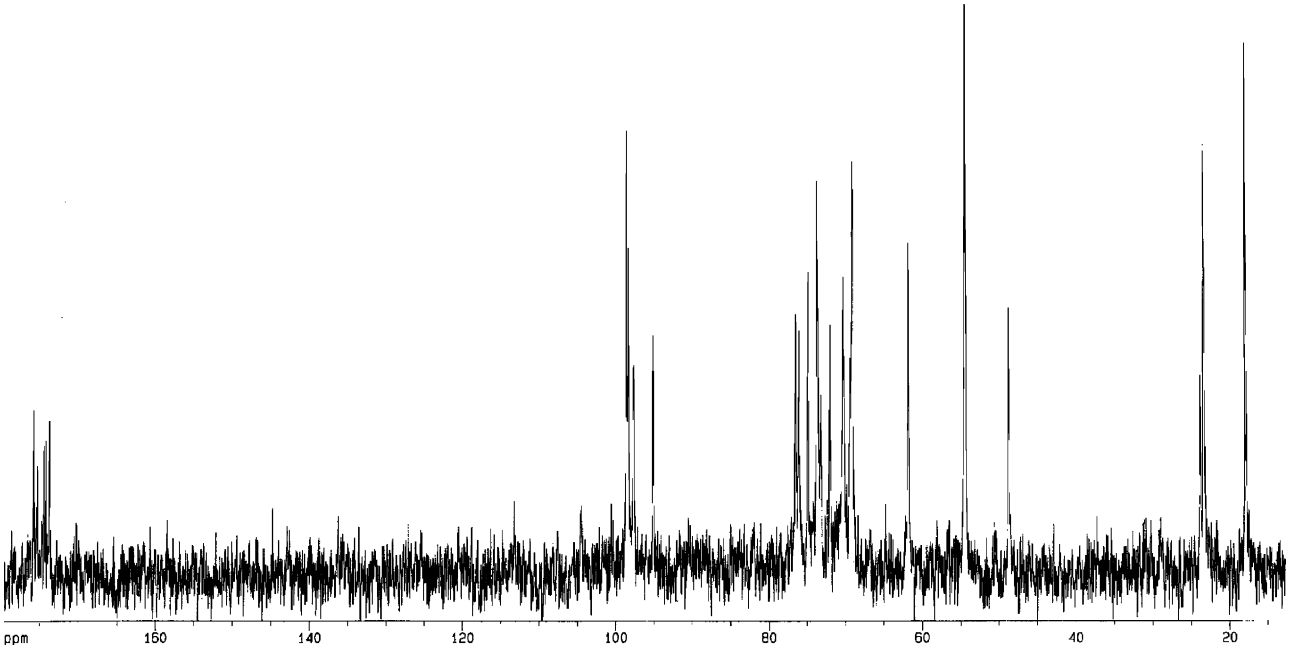


Fig. 1. 125-MHz ^{13}C NMR spectrum of the O-polysaccharide of *P. mirabilis* TG 155.

Table 1
^{13}C NMR data[a]

Sugar residue	C-1	C-2	C-3	C-4	C-5	C-6
Polysaccharide						
→3,4)-α-D-Gal*p*NAcA-(1→	98.4	48.7	73.7	70.4	72.0	175.3[b]
→3)-α-L-Qui*p*NAc-(1→	97.7	54.5	77.0	74.9	69.0	17.8[c]
→3)-α-D-Glc*p*NAc-(1→	95.1	54.4	76.6	69.1	73.8	61.8
α-L-Rha*p*NAc-(1→	98.6	54.4	69.4	73.2	70.3	17.9[c]
Monosaccharide						
α-L-Rha*p*NAc	94.1	54.7	69.6	73.6	69.3	17.9
β-L-Rha*p*NAc	94.0	55.4	72.9	73.3	73.7	17.9

[a]The chemical shifts (δ, ppm) for NAc are δ 23.3, 23.8 (3C's and 1C, respectively; all CH_3), 173.7,[b] 174.2,[b] 174.4[b] and 175.8[b] (all CO); [b,c]Assignments could be interchanged.

In the TOCSY spectrum, GlcNAc, QuiNAc and RhaNAc showed correlations between H-1 and H-2,3,4,5,6 and GalNAcA between H-1 and H-2,3,4 and between H-4 and H-5. The assignment within each spin system was performed using the COSY spectrum. All monosaccharides were identified by characteristic $J_{2,3}$, $J_{3,4}$ and $J_{4,5}$ coupling constant values that were partially determined from the 1D ^{1}H NMR spectrum and partially estimated from the 2D NMR spectra. Particularly, the *galacto* configuration of GalNAcA followed from low $J_{3,4}$ and $J_{4,5}$ values and the *manno* configuration of RhaNAc from a low $J_{2,3}$ value (each ≤ 3 Hz). GalNAcA was distinguished by low-field positions of the signals for H-4 and H-5 at δ 4.58 and 4.86, respectively, which are typical of galacturonic acid and its derivatives. The position of the amino group in all amino sugars was confirmed by correlations of the protons at the nitrogen-bearing carbons (H-2) to the corresponding carbons (C-2) at δ 48.7–54.5 in the HSQC spectrum.

The ROESY spectrum showed the following correlations between the anomeric protons and protons at the linkage carbons: GalNAcA H-1,QuiNAc H-3 at δ 5.08/3.80; QuiNAc H-1,GlcNAc H-3 at δ 4.91/3.58; GlcNAc H-1,GalNAcA H-3 at δ 5.07/4.04; and RhaNAc H-1,GalNAcA H-4 at δ 5.14/4.58. These data showed that the polysaccharide is branched and defined the linkage positions and the monosaccharide sequence in the repeating unit.

α-Effects of glycosylation in the ^{13}C NMR spectrum of the polysaccharide confirmed the substitution pattern. The following signals were shifted downfield as compared with their positions in the spectra of the corresponding nonsubstituted monosaccharides[12,13]: C-3 of α-GalNAcA to δ 73.7 (+4.6 ppm), C-3 of α-QuiNAc to δ 77.0 (+5.0 ppm), C-3 of α-GlcNAc to δ 6.6 (+4.9 ppm, respectively). In accordance with the terminal position of α-RhaNAc, the ^{13}C NMR chemical shifts for this sugar were close to those for the unsubstituted monosaccharide (Table 1). The chemical shift for C-4 of the 3,4-disubstituted α-GalNAcA residue was also close to that of the nonsubstituted monosaccharide (δ 70.4 and 71.1,[13] respectively). This can be accounted for by a β-effect of glycosylation caused by substitution at position 3 and by a steric hindrance in the branched trisaccharide fragment consisting of three *N*-acetylamino sugars.[14]

In the ^{13}C NMR spectrum of the polysaccharide, the β-effect on C-4 of α-L-Qui*p*NAc caused by glycosylation at position 3 was negative and relatively large by

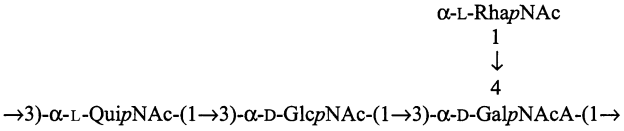
Table 2
^{1}H NMR data[a]

Sugar residue	H-1	H-2	H-3	H-4	H-5	H-6
Polysaccharide						
→3,4)-α-D-Gal*p*NAcA-(1→	5.08	4.58	4.04	4.58	4.86	
→3)-α-L-Qui*p*NAc-(1→	4.91	4.07	3.80	3.19	4.09	1.18
→3)-α-D-Glc*p*NAc-(1→	5.07	4.09	3.58	3.56	3.53	3.74, 3.84
α-L-Rha*p*NAc-(1→	5.14	4.36	4.02	3.32	3.62	1.18
Monosaccharide						
α-L-Rha*p*NAc	5.07	4.31	4.02	3.39	3.93	1.31
β-L-Rha*p*NAc	5.00	4.45	3.78	3.29	3.45	1.30

[a]The chemical shifts (δ, ppm) for NAc are δ 1.87, 1.91, 2.01 and 2.05.

modulus (−2.2 ppm), thus indicating different absolute configurations of the monosaccharides in the α-L-Qui*p*NAc-(1→3)-Gal*p*NAcA disaccharide, i.e., the D configuration of GalNAcA (in case of the same absolute configuration, the β-effect of glycosylation on C-4 would be about +1 ppm).[13,14]

Based on these data, it was concluded that the tetrasaccharide repeating unit of the O-polysaccharide of *P. vulgaris* TG 155 has the following structure:



The O-polysaccharide studied contains a rarely occurring monosaccharide, 2-acetamido-2,6-dideoxy-L-mannose (*N*-acetyl-L-rhamnosamine), which has not been hitherto found in *Proteus* LPS but in a few other bacterial polysaccharides, including the LPS of *Escherichia coli* O3[15] and the capsular polysaccharide of *Vibrio vulnificus*.[16] As most other *Proteus* O-antigens,[8] the O-polysaccharide of *P. vulgaris* TG 155 is acidic, but the acidic component, 2-acetamido-2-deoxy-D-galacturonic acid, is found for the first time in *Proteus* polysaccharides.

The LPS's of 130 *Proteus* strains, including 28 strains of *P. vulgaris*, 37 strains of *P. mirabilis* and 65 strains of *P. penneri,* were tested with rabbit polyclonal O-antiserum against *P. vulgaris* TG 155. From them, only the homologous LPS reacted in enzyme immunosorbent assay and passive immunohemolysis (reciprocal titres 512,000 and 51,200, respectively). Both LPS and alkali-treated LPS inhibited the reaction in both assays (minimal inhibiting dose 0.5–1.0 ng). In a Western blot (Fig. 2), *P. vulgaris* TG 155 O-antiserum reacted strongly with slow-migrating bands of the homologous LPS and weaker with fast-migrating bands, thus showing the occurrence of antibodies against both O-polysaccharide chain and the LPS core.

Therefore, the serological data are consistent with the structure of the O-polysaccharide, which is unique among known structures of bacterial polysaccharides. Based on these data, we propose classifying *P. vulgaris* TG 155 into a new, separate *Proteus* serogroup, O55, in which at present this strain is the single representative.

Fig. 2. Western blot of the LPS of *P. vulgaris* TG 155 with the homologous O-antiserum.

3. Experimental

3.1. Bacterial strain, growth, and isolation of the lipopolysaccharide

P. vulgaris TG 155 and 11 more *P. mirabilis* and *P. vulgaris* strains[6] were kindly provided by Professor J.L. Penner (Department of Medical Genetics, University of Toronto, Canada). 65 *P. penneri* strains were from the collection of the Department of General Microbiology (University of Lódz, Poland) and 37 *P. mirabilis* and 28 *P. vulgaris* strains were from the Czech National Collection of Type Cultures (CNCTC, Prague). Dry bacterial cells were obtained from aerated liquid cultures as described.[17] The bacterial mass was harvested at the end of the logarithmic growth phase, centrifuged, washed with water, and lyophilized. The LPS was isolated from dried cells by the phenol–water method[18] and purified using the CCl_3CO_2H precipitation procedure as described.[19]

3.2. Mild acid degradation of the lipopolysaccharide

The LPS (100 mg) was hydrolysed with 2% aq HOAc at 100 °C for 2 h, and a lipid precipitate was removed by

centrifugation at 13,000*g* for 20 min. The carbohydrate portion was fractionated by GPC on a column (56 × 2.6 cm) of Sephadex G-50 in 0.05 M pyridinium acetate buffer pH 4.5 with monitoring by a Knauer differential refractometer to give a high-molecular mass O-polysaccharide (28 mg).

3.3. Monosaccharide analysis

The O-polysaccharide was hydrolysed with 2 M CF_3CO_2H (120 °C, 2 h), and the sugars were identified using a Biotronik LC-2000 amino acid analyser on a column (0.4 × 22 cm) of Ostion LG AN B cation-exchange resin at 80 °C in 0.35 M sodium citrate buffer, pH 5.28. The absolute configurations of the monosaccharides were determined by GLC of the acetylated (*S*)-2-octyl glycosides[20,21] using a Hewlett–Packard 5989A instrument equipped with an HP-5 capillary column using a temperature gradient 150 (3 min) → 320 °C at 5 °C min^{-1}.

3.4. Isolation and identification of L-RhaNAc

The O-polysaccharide (16.5 mg) was hydrolysed with 0.01 M HCl (3 mL, 1 h, 100 °C), and the products were fractionated by GPC on a column (40 × 2.6 cm) of Sephadex G-50 (S) in 0.1 M NH_4HCO_3 buffer (7.91 g NH_4HCO_3 and 10 mg NaN_3 in 1 L water) at 30 mL h^{-1} monitored with a Knauer differential refractometer to give a polymer and a monosaccharide. The polymer was further degraded with 0.05 M HCl under the same conditions, and the products were fractionated by GPC as described above to give an additional amount of the monosaccharide, which showed essentially the same ^1H NMR spectrum as the monosaccharide from the first hydrolysis. The total yield of the monosaccharide fraction (RhaNAc) was 2.2 mg. RhaNAc was hydrolysed with 2 M HCl (100 °C, 2 h), and the solution was evaporated in vacuum to give RhaN·HCl, $[\alpha]_D^{20}$ +21.6° (*c* 0.25, water).

3.5. NMR spectroscopy

Samples were deuterium-exchanged by freeze-drying 3 times from D_2O and then examined as solutions in 99.96% D_2O at 60 °C. Spectra were recorded on Bruker DRX-500 and DRX-600 spectrometers. 2D NMR spectra were obtained using standard Bruker software, and the XWINNMR 2.6 program (Bruker) was used to acquire and process the NMR data. The parameters used for 2D experiments were essentially the same as described previously.[22] A mixing time of 200 ms was used in TOCSY and ROESY experiments.

3.6. Rabbit antiserum and serological assays

Polyclonal O-antiserum was obtained by immunization of rabbits with heat-inactivated bacteria of *P. vulgaris* TG 155 according to the published procedure.[23] SDS-PAGE, electrotransfer of the LPS from gels to nitrocellulose sheets, immunostaining and absorption experiments were carried out as described.[24] LPS was used as the antigen in an enzyme immunosorbent assay. Passive immunohemolysis was performed with increasing amounts (from 2 to 200 μg) of alkali-treated LPS.[23]

Acknowledgements

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