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Note

Structure of an O-acetylated acidic O-specific polysaccharide of *Proteus vulgaris* O46

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Abstract

An acidic O-specific polysaccharide was obtained by mild acid degradation of the lipopolysaccharide of *Proteus vulgaris* O46 and studied by chemical methods (O-deacetylation, sugar and methylation analyses, partial solvolysis) and ^{1}H and ^{13}C NMR spectroscopy. Solvolysis of the O-deacetylated polysaccharide with trifluoromethanesulfonic acid resulted in a α-D-Glc*p*NAc-(1→3)-D-GlcA disaccharide that demonstrated the usefulness of this reagent for selective cleavage of heteropolysaccharides. The following structure for the polysaccharide was established:

→4)-α-D-Glc*p*6Ac(1→3)-β-D-Glc*p*A4Ac-(1→3)-α-D-Glc*p*NAc-(1→3)-β-D-Glc*p*A4Ac-(1→

where the degree of O-acetylation is ~65% at position 6 of Glc and 80–95% at position 4 of GlcA residues.

Keywords: *Proteus vulgaris*; Lipopolysaccharide; O-Specific polysaccharide, structure; O-Acetylation; Triflic acid solvolysis; Selective cleavage

Bacteria of the genus *Proteus* are a common cause of urinary tract infections, which can lead to severe complications, such as acute or chronic pyelonephritis and formation of bladder and kidney stones. Two medically important species, *Proteus mirabilis* and *P. vulgaris*, are classified into 60 O-serogroups [1,2]. The serological O-specificity of *Proteus* is defined by the structure of the polysaccharide chain of the outer-membrane lipopolysaccharide (O-antigen). Chemical and immunochemical studies of the O-antigens are important for the creation of the molecular basis for classification of *Proteus* strains. In most *P. mirabilis* O-serogroups studied so far, the O-specific polysaccharides contain acidic or both acidic and basic components, such as uronic acids, their amides with lysine and some other amino acids, phosphate groups and phosphate-linked amino components [3]. Now we report the structure of a new acidic O-specific polysaccharide of *P. vulgaris* O46, which has a tetrasaccharide repeating unit with three O-acetylation sites

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The O-specific polysaccharide was obtained by mild acid degradation of the lipopolysaccharide isolated from *P. vulgaris* O46 by the phenol/water procedure [4]. Sugar analysis after full acid hydrolysis of the polysaccharide revealed the presence of Glc, GlcN and GlcA. GLC analysis of acetylated glycosides with (*S*)-2-butanol and (*R*)-2-octanol showed that all three monosaccharides have the D configuration.

Methylation analysis of the polysaccharide resulted in identification of 2,3,6-tri-*O*-methyl-

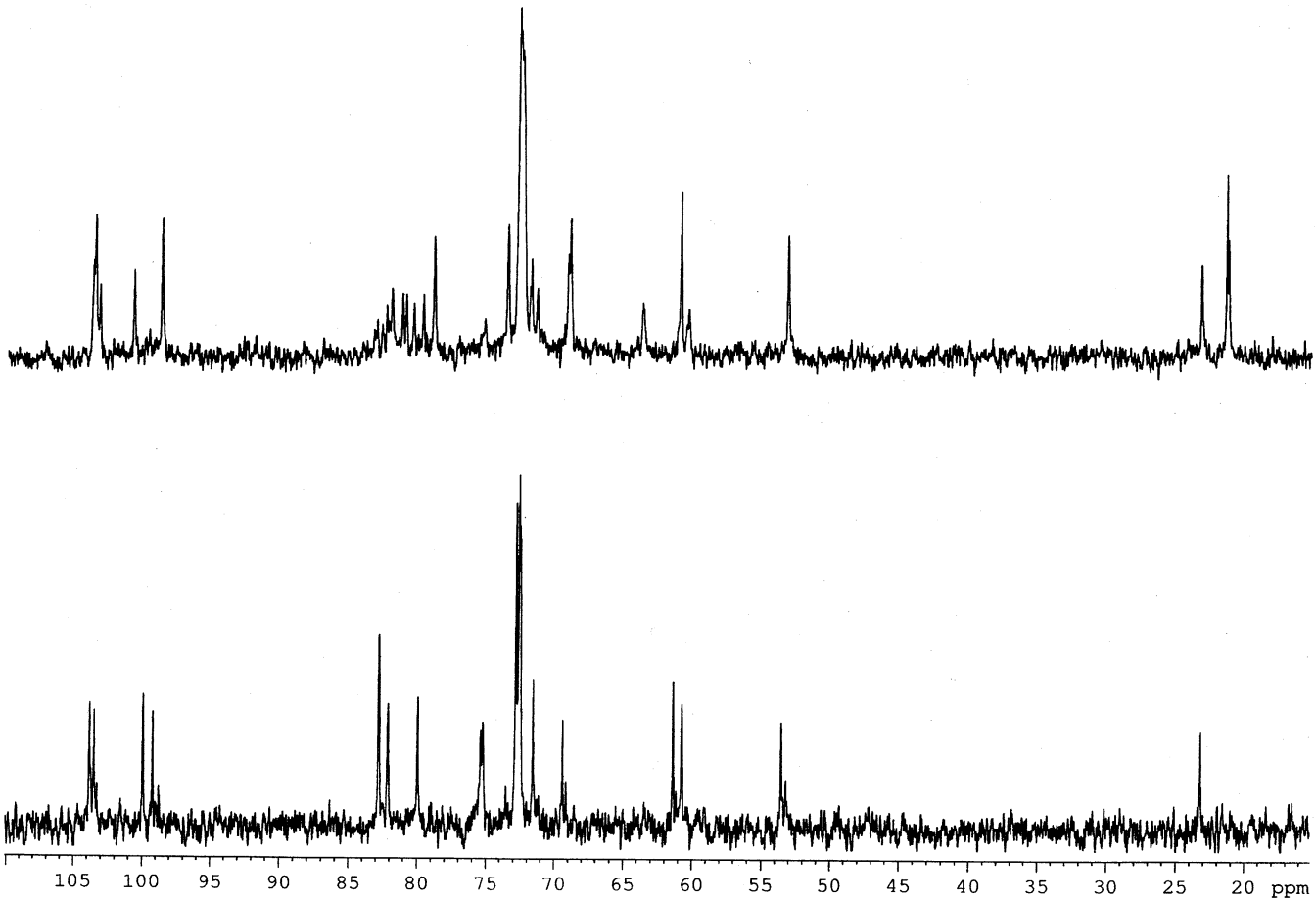

Fig. 1. 125-MHz ^{13}C NMR spectra of the O-specific polysaccharide (top) and O-deacetylated polysaccharide (bottom).

Table 1
^{1}H NMR data (δ, ppm) [a]

Sugar residue	Proton					
	H-1	H-2	H-3	H-4	H-5	H-6a,b
O-Deacetylated polysaccharide						
→4)-α-D-Glc*p*-(1→	5.34	3.60	3.87	3.66	4.13	3.82, 3.88
→3)-β-D-Glc*p*A^{II}-(1→	4.60	3.48	3.70	3.85	4.01	
→3)-α-D-Glc*p*NAc-(1→	5.25	4.12	3.93	3.64	4.04	3.83
→3)-β-D-Glc*p*A^{I}-(1→	4.60	3.48	3.69	3.83	4.01	
Disaccharide (reduced)						
α-D-Glc*p*NAc-(1→	5.11	3.95	3.78	3.52	3.80	3.82, 3.86
→4)-L-gulonic acid		4.10	4.00	3.99	3.99	3.72, 3.80

[a] The chemical shifts for NAc are δ 2.02 and 2.07 in the polysaccharide and the disaccharide, respectively.

Table 2
^{13}C NMR data (δ, ppm) [a]

Sugar residue	Carbon					
	C-1	C-2	C-3	C-4	C-5	C-6
O-Deacetylated polysaccharide						
→4)-α-D-Glc*p*-(1→	99.9	72.6	72.8	80.0	71.5	60.7
→3)-β-D-Glc*p*A^{II}-(1→	103.5	72.5	82.8	72.8	75.4	173.6
→3)-α-D-Glc*p*NAc-(1→	99.2	53.5	82.1	69.4	72.8	61.4
→3)-β-D-Glc*p*A^{I}-(1→	103.8	72.5	82.8	72.6	75.2	173.6
Disaccharide (reduced)						
α-D-Glc*p*NAc-(1→	100.3	55.1	72.1	71.2	73.9	61.6
→4)-L-gulonic acid	173.6	73.1	72.9	80.2	72.9	63.8

[a] The chemical shifts for NAc are δ 23.4 (Me), 175.1 and 175.3 (CO) in the polysaccharide and the disaccharide, respectively. Assignment of the signals with the chemical shifts difference $\leq$0.3 ppm could be interchanged.

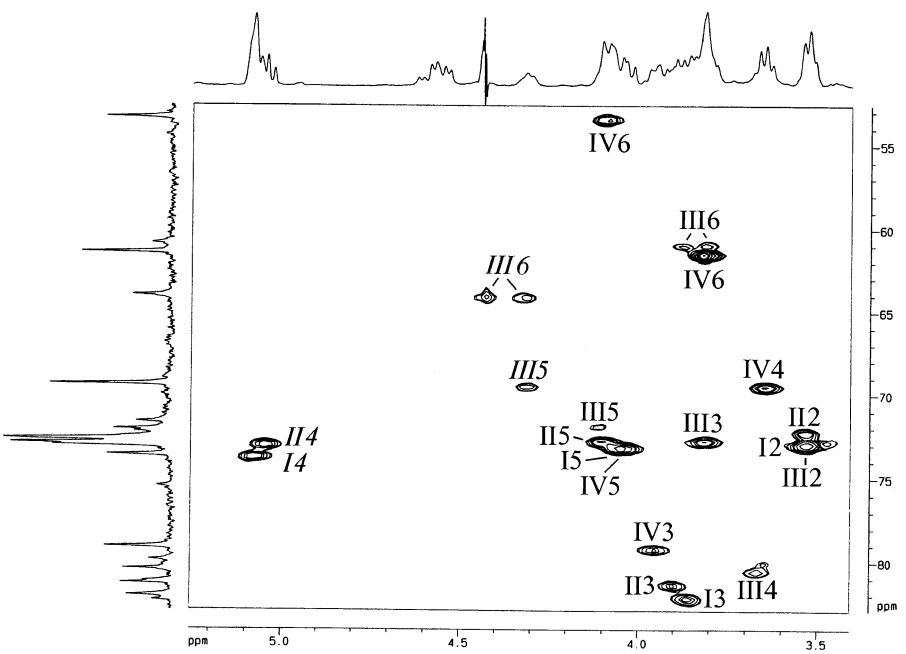

Fig. 2. Part of a 2D ^{1}H,^{13}C HMQC spectrum of the O-specific polysaccharide. The corresponding parts of the ^{1}H and ^{13}C NMR spectra are displayed along the horizontal and vertical axes, respectively. Arabic numerals refer to the atoms in sugar residues denoted by Roman numerals as follows: I, GlcA^{I}; II, GlcA^{II}; III, Glc, IV, GlcNAc. Designations for H-4,C-4 of 4-O-acetylated GlcA^{I} and GlcA^{II}, H-5,C-5 and H-6,C-6 of 6-*O*-acetylated Glc are italicised.

glucose and 2-deoxy-4,6-di-*O*-methyl-2-(*N*-methyl)acetamidoglucose. When the methylated polysaccharide was carboxyl-reduced prior to hydrolysis, 2,4-di-*O*-methylglucose was identified in addition to the sugars mentioned above, which was evidently derived from GlcA. Therefore, the polysaccharide is linear and contains three-substituted residues of GlcN and GlcA and four-substituted residues of Glc.

The ^{13}C NMR spectrum of the polysaccharide (Fig. 1, top) contained signals having different intensities, most likely, owing to non-stoichiometric O-acetylation (there were signals for CH_3 of *O*-acetyl groups at δ 21.4 and 21.5). The ^{1}H NMR spectrum of the polysaccharide showed signals for *O*-acetyl and *N*-acetyl groups at δ 2.13, and 2.02 in the ratio 2.1:1, respectively.

The ^{13}C NMR spectrum of the O-deacetylated polysaccharide (Fig. 1, bottom) contained major signals for four anomeric

carbons at δ 99.2–103.8, one carbon bearing nitrogen (C-2 of GlcN) at δ 53.5, two HO*C*H_2–C groups (C-6 of Glc and GlcN) at δ 60.7 and 61.4, two COOH groups (C-6 of GlcA) at δ 173.6, 15 other sugar ring carbons at δ 69.4–82.8, and one *N*-acetyl group (CH_3 at δ 23.4, CO at δ 175.1). The absence from the ^{13}C NMR spectrum of signals in the region δ 83–88 characteristic for furanosides [5], showed that all monosaccharides are in the pyranose form. The ^{1}H NMR spectrum of the polysaccharide contained major signals for four anomeric protons at δ 4.60–5.34, one *N*-acetyl group at δ 2.03, and other signals at δ 3.48–4.13. Both ^{13}C and ^{1}H NMR spectra of the O-deacetylated polysaccharide contained minor series of signals, which were absent from the spectra of the initial polysaccharide and, therefore, reflected a structural heterogeneity introduced by mild alkaline O-deacetylation.

Therefore, the polysaccharide has a tetrasaccharide repeating unit containing one residue each of D-glucose and 2-acetamido-2-deoxy-D-glucose and two residues of D-glucuronic acid.

The ^{1}H NMR spectrum of the O-deacetylated polysaccharide was assigned using 2D COSY, TOCSY, NOESY, and H-detected ^{1}H,^{13}C heteronuclear multiple-quantum coherence (HMQC) experiments (Table 1). The TOCSY spectrum showed cross-peaks of H-1 with H-2,3,4,5,6 of Glc and GlcNAc and with H-2,3,4,5 of both GlcA residues (GlcA^{I} and GlcA^{II}). The assignments within each spin system were made using the COSY spectrum. The spin system of GlcNAc was distinguished from that of Glc by a correlation of the proton at carbon bearing nitrogen at δ 4.12 to the corresponding carbon at δ 53.5. The $J_{1,2}$ coupling constant values of $\sim$3 Hz indicated that Glc and GlcNAc are α-linked, whereas the $J_{1,2}$ values of $\sim$8 Hz showed that both GlcA^{I} and GlcA^{II} are β-linked.

The ^{1}H,^{13}C HMQC experiment allowed full assignment of the ^{13}C NMR spectrum of the O-deacetylated polysaccharide (Table 2). Relatively low-field positions at δ 80–83 of the signals for C-3 of GlcNAc, GlcA^{I} and GlcA^{II} and C-4 of Glc, as compared with their positions in the spectra of the corresponding non-substituted monosaccharides [6], confirmed the glycosylation pattern.

In the NOESY spectrum of the O-deacetylated polysaccharide, the direct assignment of cross-peaks between the transglycosidic protons was complicated by coincidences of H-1 and H-3 signals of GlcA^{I} and GlcA^{II} at δ 4.60 and 3.69–3.70, respectively. This problem could be overcome taking into account the modes of the sugar substitution, which were determined independently by methylation analysis and ^{13}C NMR chemical shift data (see above). Thus, the cross-peaks at δ 4.60/3.66, 5.34/3.70, 4.60/3.93 and 5.25/3.69 could be assigned to the GlcA^{I} H-1,Glc H-4, Glc H-1,GlcA^{II} H-3, GlcA^{II} H-1,GlcNAc H-3 and GlcNAc H-1,GlcA^{I} H-3 correlations, respectively. These data established the full sequence of the monosaccharide residues in the polysaccharide.

In order to obtain an oligosaccharide fragment(s), the O-deacetylated polysaccharide was solvolysed with trifluoromethanesulfonic (triflic) acid. Previously, this new reagent for selective cleavage of glycosidic linkages has been successfully applied to structural analysis of a polysaccharide from *Pseudoalteromonas* sp. KMM 634 [7]. Solvolysis resulted in a disaccharide containing GlcNAc and GlcA. The latter sugar, which occupied the reducing end of the disaccharide, was reduced with $NaBH_4$ to gulonic acid.

COSY and TOCSY experiments allowed assignment of the spin systems for both monosaccharide components of the reduced disaccharide, starting from H-1 of α-GlcNAc at δ 5.11 (d, $J_{1,2}$ 3.5 Hz) and H-2 of gulonic acid at δ 4.10 (d, $J_{2,3}$ 7.1 Hz) (Table 1). Then, the ^{13}C NMR spectrum of this disaccharide was assigned using a ^{1}H,^{13}C HMQC experiment (Table 2). The spectrum showed, inter alia, signals for one anomeric carbon (C-1 of GlcNAc) at δ 100.3, two HO*C*H_2–C groups (C-6) at δ 61.6 and 63.8, one carbon bearing nitrogen at δ 55.1 (C-2 of GlcNAc), one COOH group (C-1 of gulonic acid) at δ 173.6, and one *N*-acetyl group (CH_3 at δ 23.4, CO at δ 175.3). A low field position of the signal for C-4 indicated substitution of gulonic acid at position 4 and, hence, GlcA in the initial disaccharide was substituted at position 3.

Therefore, solvolysis of the polysaccharide with triflic acid resulted in the α-D-Glc*p*NAc-(1→3)-D-GlcA disaccharide, thus confirming the polysaccharide structure. This finding again demonstrated the usefulness of triflic acid for selective cleavage of heteropolysaccharides, especially N-acylated hexosaminoglycans since it does not split off the *N*-acyl groups. The glycosidic linkage of α-GlcNAc turned out to be more stable as compared with the linkages of α-Glc and β-GlcA, the observation resembling the behaviour of the sugar glycosides towards solvolysis with anhydrous HF [8]. In contrast, on aqueous acid hydrolysis, one could expect formation of oligo(di)saccharides with GlcA at the non-reducing end [9].

Positions of the *O*-acetyl groups were determined by a $^{1}H,^{13}C$ HMQC experiment on the initial polysaccharide (Fig. 2). As compared with the $^{1}H,^{13}C$ HMQC spectrum of the O-deacetylated polysaccharide, this showed a displacement of about two thirds of the Glc H-6a,C-6 and H-6b,C-6 cross-peaks from δ 3.82/60.7 and 3.88/60.7 to 4.32/64.0 and 4.43/64.0, respectively. This displacement was due to a deshielding effect of the *O*-acetyl group and indicated partial (~65%) O-acetylation of Glc at position 6. Similar low-field displacements were observed for the H-4,C-4 cross-peaks of GlcA^{I} and GlcA^{II} from δ 3.83/72.6 and 3.85/72.8 to δ 5.08/73.7 and 5.05/73.0, respectively. The O-acetylation pattern was confirmed by upfield shifts by 0.8–2.6 ppm of the signals for C-5 of Glc and C-3,5 of GlcA^{I} and GlcA^{II}, respectively, which were caused by β-effects of O-acetylation [10]. As judged by the intensities of the residual C-5 signals from the non-acetylated GlcA residues at δ 75.2 and 75.4 in the ^{13}C NMR spectrum of the initial polysaccharide, the degree of O-acetylation of GlcA^{I} and GlcA^{II} was estimated as 80 and 95%, respectively.

On the basis of the data obtained, it was concluded that the O-specific polysaccharide of *P. vulgaris* O46 has the following structure:

→4)-α-D-Glc*p*6Ac(1→3)-β-D-Glc*p*A4Ac-(1→3)-α-D-Glc*p*NAc-(1→3)-β-D-Glc*p*A4Ac-(1→

Interestingly, the same carbohydrate backbone, but different O-acetylation pattern, was found in the O-specific polysaccharide of *P. vulgaris* O37. These data, as well as the serological relatedness of these two *Proteus* O-serogroups, will be reported elsewhere.

1. Experimental

Bacterial strain, isolation and degradation of lipopolysaccharide.—*P. vulgaris* O46, strain PrK 72/57, came from the Czech National Collection of Type Cultures (Institute of Epidemiology and Microbiology, Prague). The bacterium was cultivated under aerobic conditions in nutrient broth (BTL, Lodz, Poland). The bacterial mass was harvested at the end of the logarithmic growth phase, centrifuged, washed with water, and lyophilised.

Lipopolysaccharide was isolated from dried bacterial cells by hot phenol/water extraction [4] and purified by treatment with DNase and RNase and ultracentrifugation as described [11].

Delipidation of the lipopolysaccharide (100 mg) was performed with aq 2% HOAc at 100 °C until lipids were precipitated. The precipitate was removed by centrifugation (13,000*g*, 20 min), and the supernatant was fractionated by GPC on a column (56 × 2.6 cm) of Sephadex G-50 (S) (Pharmacia, Sweden) in 0.05 M pyridinium acetate buffer (pH 4.5), with monitoring using a Knauer differential refractometer (Germany). The yield of a high-molecular-mass O-specific polysaccharide was 23% of the lipopolysaccharide weight.

Sugar analysis.—The polysaccharide was hydrolysed with 2 M CF_3COOH (120 °C, 2 h). Amino sugars were identified using a Biotronik LC-2000 amino acid analyser (Germany) equipped with a column (0.4 × 22 cm) of Ostion LG AN B cation-exchange resin using 0.35 M sodium citrate buffer (pH 5.28) at 80 °C. Neutral sugars and uronic acids were identified using a Biotronik LC-2000 sugar analyser (Germany) on a column (0.4 × 15 cm) of Dionex A × 8 anion-exchange resin at 70 °C using 0.5 M sodium borate buffer (pH 8.0) or 0.04 M sodium phosphate buffer (pH 2.4) [12], respectively. The absolute configura-

tions of the monosaccharides were determined by GLC of acetylated (*S*)-2-butyl glycosides (for GlcN and GlcA) or (*R*)-2-octyl glycosides (for Glc) according to the published method [13,14] modified as described [15]. GLC was performed with a Hewlett–Packard Model 5890 chromatograph (USA) equipped with an Ultra 2 capillary column (Hewlett–Packard) using a temperature gradient of 160 °C (1 min) to 290 °C at 10 °C/min.

Methylation analysis.—Methylation of the polysaccharide was performed with CH_3I in Me_2SO in the presence of sodium methylsulfinylmethanide [16]. A portion of the methylated polysaccharide was reduced with $LiBH_4$ in aq 70% 2-propanol (20 °C, 2 h). Partially methylated monosaccharides were derived by hydrolysis under the same conditions as in sugar analysis, converted into alditol acetates, and analysed by GLC-MS [17].

O-Deacetylation.—The initial polysaccharide (23 mg) was treated with aq 12.5% ammonia at 37 °C for 16 h, the solution was desalted on a column (90 × 2.5 cm) of TSK HW-40 (S) (E. Merck, Germany) in water and freeze-dried to give an O-deacetylated polysaccharide (17 mg).

Solvolysis with triflic acid.—O-Deacetylated polysaccharide (17 mg) was treated with anhyd CF_3SO_3H at −4 °C for 40 min. After neutralisation with aq 25% ammonia at 0 °C, the reaction products were reduced with $NaBH_4$ and fractionated by GPC on TSK HW-40 (S) to give a disaccharide (6.5 mg).

NMR spectroscopy.—NMR spectra were recorded with a Bruker DRX-500 spectrometer (Germany) for solutions in D_2O at 30 and 50 °C for the disaccharide and the polysaccharides, respectively, using internal acetone (δ_H 2.225, δ_C 31.45) as reference. Standard Bruker software (XWINNMR 1.2) was used to acquire and processed the NMR data. A mixing time of 200 ms was used in 2D TOCSY and NOESY experiments.

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