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D- and L-Aspartic Acids: New Non-sugar Components of Bacterial Polysaccharides

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Abstract—For the first time in bacterial polysaccharides, residues of D- and L-aspartic acids were identified as N-acyl substituents of 4-amino-4,6-dideoxy-D-glucose in the O-antigens of enterobacteria of the genera *Providencia* and *Proteus*.

Key words: *Providencia*, *Proteus*, aspartic acid, O-antigen, bacterial polysaccharide structure

Amino acids are known as components of a number of specific bacterial polysaccharides, including capsular polysaccharides and lipopolysaccharides of Gram-negative bacteria [1]. They contribute to the polysaccharide charge and may play a role in manifesting of the immunospecificity of bacterial polysaccharide antigens [2]. Glycine, D- and L-alanine, L-serine, and various N-acyl derivatives thereof, as well as various hydroxy and C-methyl derivatives of 5-oxoproline, have been found as N-acyl substituents of amino sugars. In addition, L-alanine, L-serine, L-threonine, D-allothreonine, L-glutamic acid, L-lysine, and N^{ε}-[(*R*)- and (*S*)-1-carboxyethyl]-L-lysine occur as amino components that amidate, by the α-amino group, the carboxyl group of uronic acids. Ester-linked D-alanine is common in teichoic acids. Here we report, for the first time in bacterial polysaccharides, on identification of residues of D- and L-aspartic acids as N-acyl substituents of an amino sugar in the O-polysaccharides (O-antigens) of the lipopolysaccharides of *Providencia* and *Proteus*, which define the serospecificity of these bacteria.

MATERIALS AND METHODS

Bacterial strain, growth, and isolation of the lipopolysaccharides. *Proteus mirabilis* O38, strain PrK 64/57 was from the Czech National Collection of Type Cultures (Institute of Epidemiology and Microbiology, Prague). *Providencia alcalifaciens* O4:H4, strain 884 came from the Hungarian National Collection of Medical Bacteria (National Institute of Hygiene, Budapest). Dry bacterial cells were obtained from aerated liquid cultures as described [3], the bacterial mass was harvested at the end of the logarithmic growth phase, centrifuged, washed with water, and lyophilized. The lipopolysaccharide was isolated from dried cells by the phenol/water method [4] and purified using the CCl_3CO_2H precipitation procedure as described [5].

Mild acid degradation of lipopolysaccharides. The lipopolysaccharide each of *P. mirabilis* O38 (180 mg) and *P. alcalifaciens* O4 (150 mg) was hydrolyzed with aqueous 2% HOAc at 100°C for 3 and 6 h, respectively, and a lipid precipitate was removed by centrifugation at 13,000*g* for 20 min. The carbohydrate portion was fractionated by gel chromatography 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 differential refractometer (Knauer, Germany) to yield a high-molecular-mass polysaccharide (48 and 41 mg from *P. mirabilis* O38 and *P. alcalifaciens* O4, respectively).

Compositional analysis. The polysaccharides were hydrolyzed with 2 M CF_3CO_2H (120°C, 2 h). Amino components were identified using a Biotronik LC-2000 amino acid analyzer on a column (0.4 × 22 cm) of Ostion LG AN B cation-exchange resin at 80°C in 0.2 M sodium citrate buffer, pH 3.25, for amino acids and 0.35 M sodium citrate buffer, pH 5.28, for amino sugars and ethanolamine.

Chemical modifications. The polysaccharide of *P. mirabilis* O38 (10 mg) was dephosphorylated with aqueous 48% HF (0.2 ml, 4°C, 24 h), and the products were

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isolated by gel chromatography on a column (90 × 2.5 cm) of TSK HW-40 in aqueous 1% HOAc with monitoring by the differential refractometer to give a dephosphorylated polysaccharide (4.5 mg).

The polysaccharide of *P. mirabilis* O38 (7.5 mg) in a 10 : 1 : 1 (v/v) mixture of methanol, chloroform, and concentrated HCl was stirred for 7 days at 20°C. The solution was evaporated, MeOH (1.5 ml) was added and evaporated to remove HCl, and the procedure was repeated three times. Then, 2 ml imidazole buffer [6] was added, the mixture was cooled to 0°C, supplemented with $NaBH_4$ (400 mg), stirred for 1.5 h at 0°C, and neutralized with concentrated HOAc. After evaporation, a carboxyl-reduced polysaccharide (6.5 mg) was isolated by gel chromatography on Sephadex G-50.

The polysaccharide of *P. alcalifaciens* O4 (20 mg) was oxidized with 0.1 M $NaIO_4$ (1.5 ml, 20°C, 64 h). After treatment with ethylene glycol (0.05 ml) for 30 min, the product was reduced with $NaBH_4$ (50 mg, 16 h), neutralized with aqueous 50% HOAc, evaporated, boric acid was removed by evaporation with MeOH (3 × 1.5 ml), and an oligosaccharide 3 (6 mg) and amino alditol 2 (1 mg) were isolated by gel chromatography on TSK-HW40.

The polysaccharide of *P. mirabilis* O38 (27 mg) was treated with anhydrous HF for 1 h at 20°C. HF was evaporated in vacuum and absorbed using a cartridge with solid NaOH, and the products were dissolved in water and fractioned by gel chromatography on TSK HW-40 in aqueous 1% HOAc to give four compounds in yields 4.0, 5.0 (disaccharide 5), 1.8, and 0.9 mg in order of elution.

The polysaccharide of *P. mirabilis* O38 (33 mg) was treated with anhydrous triflic acid (0.2 ml) for 2 h at 20°C. After neutralization with aqueous 5% ammonia and evaporation, the reaction products were fractioned by gel chromatography on TSK HW-40 in water to give monosaccharide 6, which was reduced with $NaBH_4$ in water and, after acidification with concentrated HOAc, phosphorylated amino alditol 7 (2.5 mg) was isolated by gel chromatography on TSK HW-40 in aqueous 1% HOAc.

NMR-spectroscopy. Samples were deuterium-exchanged by freeze-drying from D_2O and then examined as solutions in 99.96% D_2O at 50°C using a DRX-500 spectrometer (Bruker, Germany). Experiments in an 85 : 15 H_2O–D_2O mixture were performed at 58°C. Internal acetone (δ_H 2.225, δ_C 31.45) and external aqueous 85% H_3PO_4 (δ_P 0) were used as references. The parameters used for 2D experiments were essentially the same as described [7]. A mixing time of 100 and 200 msec was used in TOCSY and ROESY experiments, respectively, and a delay of 60 msec in an HMBC experiment.

Mass-spectrometry. Negative ion electrospray ionization (ESI) mass spectra were obtained using an APEX II Instrument (Bruker Daltonics, USA) equipped with a 7 Tesla actively shielded magnet and an Apollo ion source. Samples were dissolved at a concentration of ~10 ng/μl in a 50 : 50 : 0.001 (v/v) mixture of 2-propanol, water, and triethylamine and sprayed at a flow rate of 2 μl/min. Capillary entrance voltage was set to 3.8 kV, and dry gas temperature to 150°C.

RESULTS

The O-polysaccharides were obtained by mild acid degradation of the lipopolysaccharides isolated from dry cells of *Providencia alcalifaciens* O4 and *Proteus mirabilis* O38 by the phenol–water procedure [4]. Full acid hydrolysis of the polysaccharides followed by analysis using an amino acid analyzer revealed the presence of aspartic acid. GLC of the acetylated (+)-2-octyl esters indicated that aspartic acid from *P. alcalifaciens* O4 has the L configuration and that from *P. mirabilis* O38 has the D configuration. The presence of aspartic acid was confirmed by ^1H- and ^{13}C-NMR spectra of the polysaccharides, which showed characteristic signals at δ_H 2.64, 2.85 (H3), 4.50 (H2); δ_C 40.0 (C3), 53.5 (C2), 174.5 (C4), 178.5 (C1) (*P. alcalifaciens* O4); and δ_H 2.73 (2H, H3), 4.74 (H2); δ_C 38.4 (C3), 50.7 (C2), 172.7 (C4), 175.6 (C1) (*P. mirabilis* O38).

Structural studies, including monosaccharide and methylation analyses along with 2D ^1H- and ^{13}C-NMR spectroscopic studies showed that the *P. alcalifaciens* O4 polysaccharide has a branched pentasaccharide repeating unit with two residues each of D-galactose and D-glucosamine in the main chain and a residue of 4-amino-4,6-dideoxy-D-glucose (D-Qui4N) in the side chain (Fig. 1). The *P. mirabilis* O38 polysaccharide has a linear tetrasaccharide repeating unit containing one residue each of D-glucose, D-galacturonic acid, D-glucosamine, D-Qui4N and ethanolamine phosphate (Fig. 2). Each polysaccharide contains also one aspartyl and three acetyl groups.

A NOESY experiment with samples of each polysaccharide dissolved in a 85 : 15 H_2O–D_2O mixture revealed correlations with protons of NH-groups, which are absent from the spectra measured in D_2O. They showed correlation between, and, hence, a spatial proximity of, NH4 of Qui4N and CH_2 (H3) of Asp. An HMBC experiment showed a correlation between CO of an N-acetyl group and CHN (H2) of Asp. Therefore, aspartic acid is N-acetylated and attached by COOH4 to N4 of Qui4N. The mode of the attachment of the amino acid was confirmed by carboxyl-reduction [6] of the *P. mirabilis* O38 polysaccharide. It resulted in conversion of the aspartyl group into a 3-amino-4-hydroxybutyryl group. This followed from the appearance of a spin system for the latter at δ 2.38 (2H, H2), 4.28 (H3), and 3.57 (2H, H4) in the ^1H-NMR spectrum and a signal for an additional $HOCH_2$-C group near δ 61.0 in the ^{13}C-NMR spectrum.

Further evidence was obtained by selective cleavages of the polysaccharides. Smith degradation of the *P. alcalifaciens* O4 polysaccharide 1 afforded amino alditol 2 and

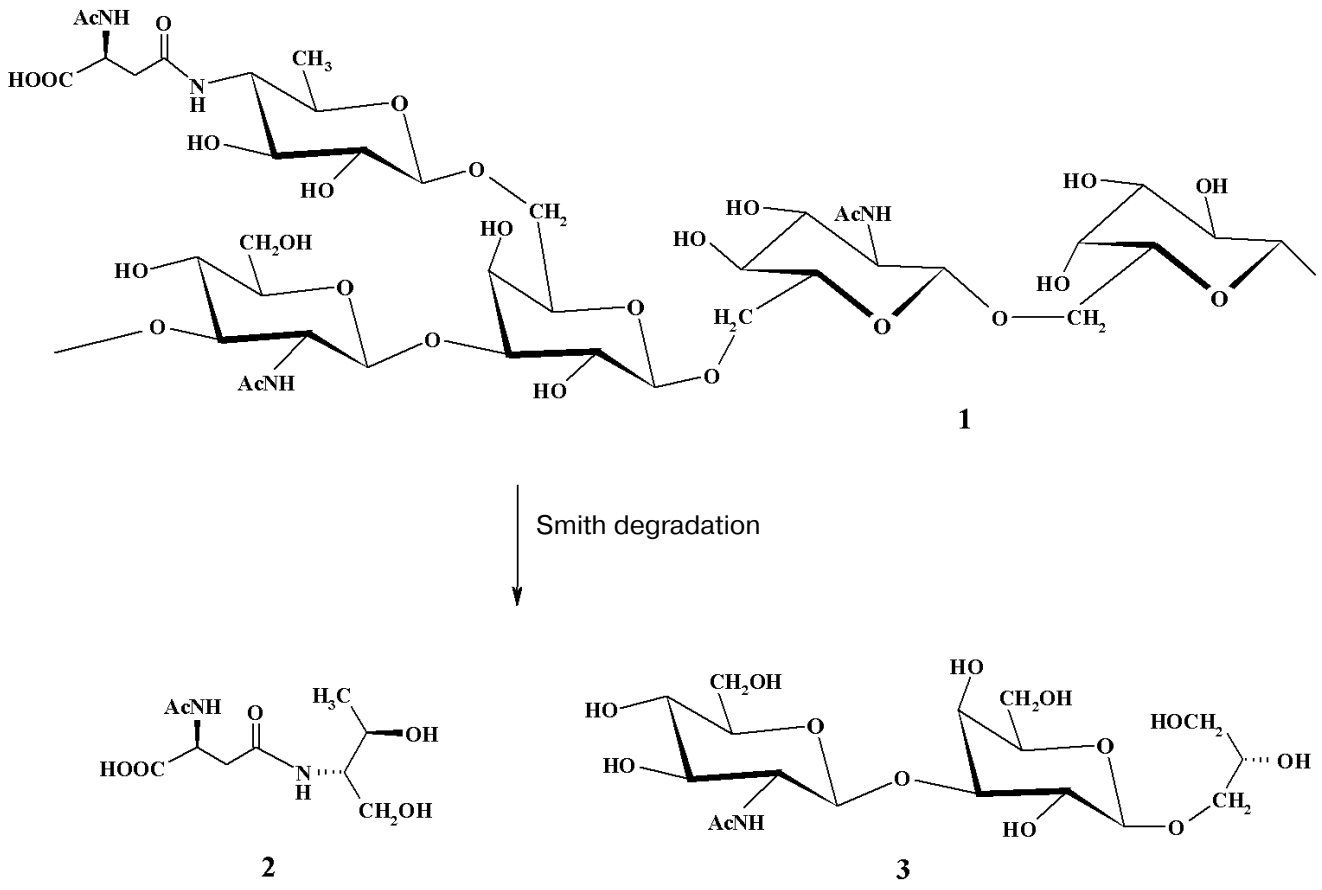

Fig. 1. Smith degradation of the O-polysaccharide of *Providencia alcalifaciens* O4 (1).

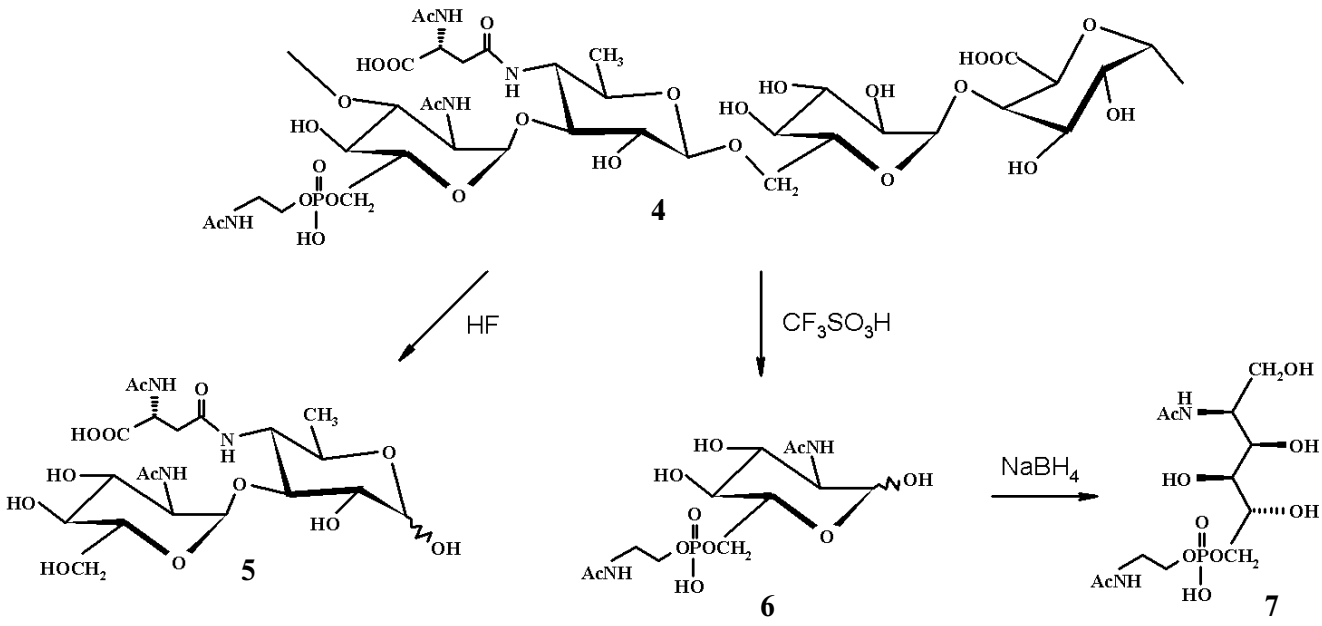

Fig. 2. Selective solvolysis of the O-polysaccharide of *Proteus mirabilis* O38 (4).

oligosaccharide 3 (Fig. 1). Compound 2 contained a remainder of the destroyed Qui4N residue with an N-linked N-acetylaspartyl group. Structure of 2 was determined by assignment of its ^{1}H- and ^{13}C-NMR spectra (table), using COSY and H-detected ^{1}H,^{13}C HMQC experiments, and molecular mass determination (262.11 daltons) by ESI MS, which was in agreement with the calculated mass.

The *P. mirabilis* O38 polysaccharide 4 was cleaved with either anhydrous HF [8] or CF_3SO_3H (triflic acid) [9] to give a GlcN→Qui4N disaccharide 5 and a phosphorylated GlcN derivative 6, respectively. Compound 6 was reduced with borohydride into a phosphorylated amino alditol 7. The structures of the degradation products were established using 2D ^{1}H- and ^{13}C-NMR spectroscopy (table) and confirmed by ESI MS, which demonstrated the expected molecular masses of 523.20 (for 5) and 386.11 daltons (for 6), respectively. Elucidation of the structures of these products revealed the N-acylation pattern of all amino components of the polysaccharide and, particularly, confirmed the attachment of aspartic acid to Qui4N. Noteworthy also is the presence of an N-acetylated 2-aminoethyl phosphate group, which is another uncommon component of bacte-

^{1}H- and ^{13}C-NMR data (δ, ppm)

Residue	H-1	H-2	H-3	H-4	H-5	H-6	CH_3CO	
Amino alditol 2								
C(4)-fragment of Qui4N	3.63	3.87	3.87	1.16				
	3.74							
AcAsp		4.50	2.63				2.00	
			2.83					
Disaccharide 5								
α-GlcNAc-(1→	5.08	3.85	3.71	3.53	4.11	3.80	2.01	
						3.80		
→3)-α-Qui*p*4N	5.18	3.64	3.90	3.75	3.84	1.09		
→3)-β-Qui*p*4N	4.61	3.34	3.66	3.75	3.52	1.14		
AcAsp		4.61	2.62				2.06	
			2.71					
Amino alditol 7								
α-GlcNAc-ol	3.61	4.08	3.97	3.66	3.85	3.98	2.01	
	3.71					4.06		
AcEtn*P*	3.93	3.41					2.04	
	C-1	C-2	C-3	C-4	C-5	C-6	*C*H_3CO	CH_3*C*O
Amino alditol 2								
C(4)-fragment of Qui4N	61.8	57.7	68.0	19.5				
AcAsp		53.3	39.7				23.8	
Disaccharide 5								
α-GlcpNAc-(1→	98.4	55.0	72.3	70.9	73.0	61.4	23.3[a]	
→3)-α-Qui4N	93.5	72.2	76.0	57.9	67.7	17.9		
→3)-β-Qui4N	97.0	74.8	79.0	57.9	72.4	17.9		
AcAsp		52.3	39.2				23.6[a]	
Amino alditol 7								
α-GlcNAc-ol	61.7	55.2	69.6	71.4	71.3	68.1	23.5[a]	175.3
AcEtnP	65.5	41.4					23.3[a]	175.3

[a] Assignment could be interchanged.

rial polysaccharides. Formation of 6 showed that, as opposite to anhydrous HF, triflic acid is a useful reagent for isolation of phosphorylated sugar derivatives.

According to our preliminary data, an N-acetyl-D-aspart-4-yl derivative of Qui4N is present also in the O-polysaccharide of *P. alcalifaciens* O33.

This is the first report of the presence of N-linked dicarboxylic amino acids in bacterial polysaccharides, which endow them with the negative charge. Aspartic acid is the second amino acid, after alanine, which is found to occur in polysaccharides in both enantiomeric forms. Elucidation of the full structures of the aspartic acid-containing O-polysaccharides will be reported in detail elsewhere.

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