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Structure of the acidic O-specific polysaccharide from *Proteus vulgaris* O39 containing 5,7-diacetamido-3,5,7,9-tetradeoxy-L-*glycero*-L-*manno*-non-2-ulosonic acid

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Dedication to Professor Ernst T. Rietshchel on the occasion of his 60th anniversary

Abstract

The O-specific polysaccharide of *Proteus vulgaris* O39 was found to contain a new acidic component of *Proteus* lipopolysaccharides, 5,7-diacetamido-3,5,7,9-tetradeoxy-L-*glycero*-L-*manno*-non-2-ulosonic acid (di-*N*-acetylpseudaminic acid, Pse5Ac7Ac). The following structure of the polysaccharide was determined by NMR spectroscopy, including 2D $^{1}H,^{1}H$ COSY, TOCSY, ROESY, and $^{1}H,^{13}C$ HMQC experiments, along with selective cleavage of the polysaccharide by solvolysis with anhydrous trifluoromethanesulfonic (triflic) acid:

→8)-β-Pse*p*5Ac7Ac-(2→3)-α-L-Fuc*p*NAc-(1→3)-α-D-Glc*p*NAc-(1→

The structure established is unique among the O-specific polysaccharides, which is in accordance with classification of the strain studied into a separate *Proteus* serogroup. © 2001 Published by Elsevier Science Ltd.

Keywords: *Proteus vulgaris*; O-specific polysaccharide; 5,7-Diacetamido-3,5,7,9-tetradeoxy-L-*glycero*-L-*manno*-non-2-ulosonic acid; Pseudaminic acid; Selective cleavage; Triflic acid solvolysis

1. Introduction

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 *Proteus vulgaris*, are classified into 60 O-serogroups.[1,2] The serological O-specificity of *Proteus* is defined by the structure of the polysaccharide chain (O-antigen) of the outer-membrane lipopolysaccharide (LPS). In most *Proteus* O-serogroups studied so far, the O-specific polysaccharides contain acidic or both acidic

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and basic components, such as uronic acids, their amides with lysine and some other amino acids, ether-linked lactic acid, acetal-linked pyruvic acid, phosphate groups and phosphate-linked amino components.[3] Now we report on the structure of the acidic O-specific polysaccharide of *P. vulgaris* O39, which contains a new component of *Proteus* polysaccharides, di-*N*-acetyl derivative of 5,7-diamino-3,5,7,9-tetradeoxy-L-*glycero*-L-*manno*-non-2-ulosonic acid called pseudaminic acid.

2. Results and discussion

The LPS was extracted from bacterial cells of *P. vulgaris* O39, strain PrK 65/57, by the phenol/water procedure.[4] Mild acid degradation of the LPS with dilute acetic acid at 100 °C resulted in polysaccharide and oligosaccharide fractions, which were separated by GPC on Sephadex G-50. However, the yield of the polysaccharide (PS1) was poor (about 12%) compared to that from LPSs of other *Proteus* strains studied earlier (usually 20–30%). In an attempt to improve the yield, an alternative approach was applied for the LPS degradation, namely deamination with nitrous acid, which can be performed at ambient temperature. This method made use of the presence in the outer part of the core of many *Proteus* LPSs of a residue of glucosamine or galactosamine with the free amino group,[5–7] which is cleaved upon deamination.[8] As a result, a high-molecular-mass polysaccharide (PS2) was obtained in a slightly higher yield (14%). Further studies showed that both polysaccharides have the identical repeating unit, and, hence, no acid-labile component was lost during mild-acid degradation of the LPS, neither any component of the repeating unit with the free amino group was cleaved upon deamination. PS1 gave better-resolved NMR spectra, and all NMR experiments were performed with this preparation, whereas both PS1 and PS2 were used in chemical studies.

Sugar analysis of PS1 using an amino acid analyser and GLC–MS of the acetylated glycosides with (+)-2-octanol revealed approximately equal amounts of 2-amino-2-deoxy-D-glucose and 2-amino-2,6-dideoxy-L-fucose (L-FucN). GLC–MS of the monosaccharide derivatives obtained by hydrolysis of the borohydride-reduced PS2 followed by acetylation resulted in identification of 1,3,4,6-tetra-*O*-acetyl-2,5-anhydromannitol. This was evidently derived from 2,5-anhydromannose at the reducing end of PS2 that had been obtained by deamination of glucosamine;[8] hence, a GlcN residue with the free amino group is present in the LPS core of *P. vulgaris* O39.

The ^{13}C NMR spectrum of PS1 (Table 1) contained signals for three anomeric carbons at δ 98.5–103.9. A DEPT-135 experiment (Fig. 1) showed that the signal at δ 103.9 belonged to a carbon having no proton at-

Table 1
^{13}C NMR data (δ in ppm)

Sugar residue	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9
Polysaccharide									
→3)-α-L-Fuc*p*NAc-(1→	98.6	49.0	74.4	72.7	67.4	16.6			
→3)-α-D-Glc*p*NAc-(1→	98.5	54.7	77.5	69.5	73.4	61.8			
→8)-β-Pse*p*5Ac7Ac-(2→	172.8	103.9	37.3	67.4	49.4	73.2	54.1	76.7	14.6
Trisaccharide **7**									
α-D-Glc*p*NAc-(1→	97.1	55.0	72.1	71.2	73.2	61.6			
→8)-β-Pse*p*5Ac7Ac-(2→			36.2	67.9	49.3	73.4	53.8	75.0	14.0
→3)-L-FucNAc-ol	62.4	51.8	74.1	76.6	67.7	20.1			
Disaccharide **9**									
β-Pse*p*5Ac7Ac-(2→			36.8 (37.0)	67.9 (67.6)	49.6 (49.1)	74.7 (74.9)	54.9 (54.6)	69.5 (69.3)	17.4 (17.6)
→3)-L-FucNAc-ol	62.7	52.1	74.4	77.0	67.7	20.0			

Chemical shifts for NAc are δ 23.2–23.6 (Me), 174.1–175.7 (CO). Data of 5-acetamido-3,5,7,9-tetradeoxy-7-[(*R*)-3-hydroxybutyramido]-β-L-*glycero*-L-*manno*-non-2-ulopyranosidonic acid[11] are given in parentheses.

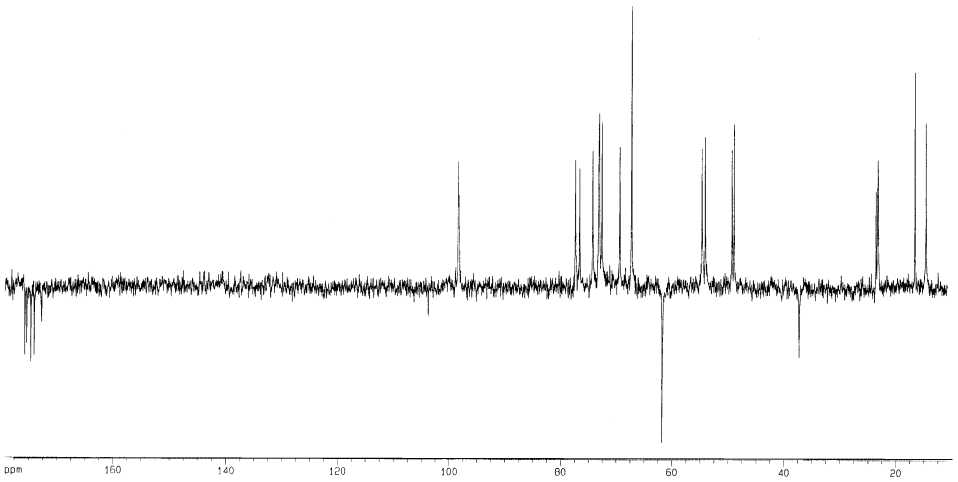


Fig. 1. 125-MHz DEPT-135 ^{13}C NMR spectrum of the O-specific polysaccharide of *P. vulgaris* O39.

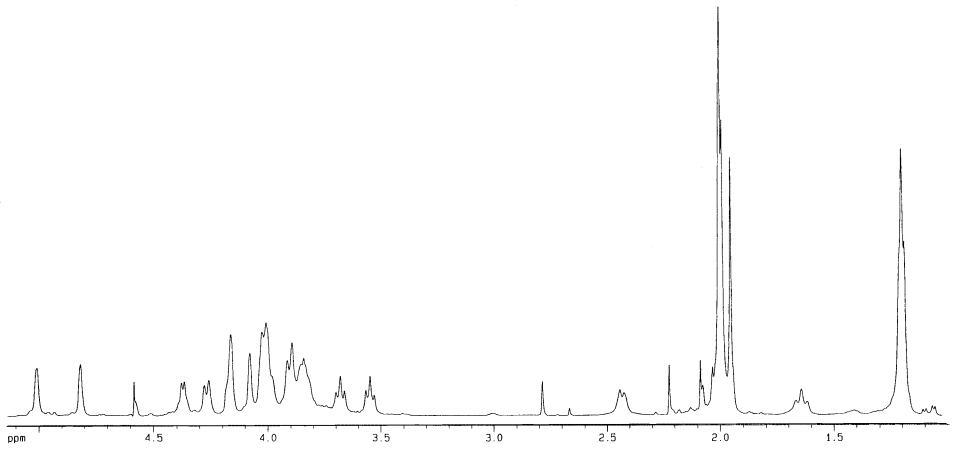


Fig. 2. 500-MHz ^{1}H NMR spectrum of the O-specific polysaccharide of *P. vulgaris* O39.

tached, and hence, a keto sugar is present. In the spectrum, there were also signals for one carboxyl group at δ 172.8, one unsubstituted HO*C*H_2–C-group at δ 61.8, four carbons bearing nitrogens at δ 49.0–54.7, and nine carbons bearing oxygen at δ 67.4–77.5. One signal at δ 37.3 belonged to a C–*C*H_2–C group and two signals at δ 14.6 and 16.6 to *C*H_3–C groups of deoxy sugars. The signals at δ 23.2–23.6 (Me) and 174.1–175.7 (CO) indicated the presence of four *N*-acetyl groups. The total number of signals in the spectrum suggested the presence in the repeating unit of a higher, nine-carbon sugar along with two hexose derivatives. The absence from the ^{13}C NMR spectrum of any signals for non-anomeric sugar carbons at a lower field than δ 81 demonstrated the pyranoid form of all sugar residues.[9]

A low-field region of the ^{1}H NMR spectrum of PS1 (Fig. 2, Table 2) contained signals for two anomeric protons at δ 4.81 and 5.01. A high-field region of the spectrum contained signals for one C–CH_2–C group in a pyranose ring (δ 1.64 and 2.44) and two CH_3–C groups (δ 1.19 and 1.20) of deoxy sugars, as well as signals for four *N*-acetyl groups at δ 1.96–2.01. In the HMBC spectrum, the methylene protons gave cross-peaks with the carboxyl carbon at δ 172.8 and the anomeric carbon of the keto sugar at δ 103.9, thus demonstrating H-3_{ax} and H-3_{eq} of a 3-deoxyald-2-ulosonic acid.

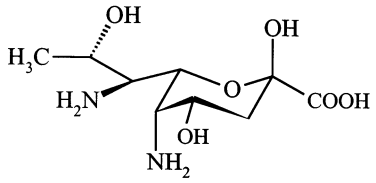
The ^{1}H and ^{13}C NMR spectra were assigned using 2D ^{1}H,^{1}H COSY, TOCSY, and H-detected ^{1}H,^{13}C HMQC experiments (Tables 1 and 2). From three spin-systems present, two were assigned to Glc*p*NAc and Fuc*p*NAc on

the basis of characteristic $J_{2,3}$, $J_{3,4}$ and $J_{4,5}$ coupling constant values. Relatively low $J_{1,2}$ coupling constant values of ~ 3 Hz showed that both amino sugars are α-linked. The third sugar spin system was assigned to a 3,9-dideoxynon-2-ulosonic acid. An $^{1}H,^{13}C$ HMQC experiment revealed correlation of protons at carbons bearing nitrogen (H-5 and H-7) to the corresponding carbons (C-5 and C-7) at δ 49.4 and 54.1; hence, a 5,7-diamino-3,5,7,9-tetradeoxynon-2-ulosonic acid is present. This might be either a new isomer or one of the known sugars of this class: pseudaminic acid (L-*glycero*-L-*manno* isomer **1**), legionaminic acid (D-*glycero*-D-*galacto* isomer **2**), or either of epimers of legionaminic acid (L-*glycero*-D-*galacto* isomer **3** or D-*glycero*-D-*talo* isomer **4**) (Fig. 3).[10–14]

Table 2
^{1}H NMR data (δ in ppm)

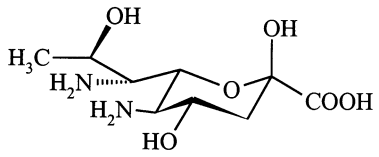
Sugar residue	H-1(1a) H-1b	H-2	H-3(3_{ax}) H-3_{eq}	H-4	H-5	H-6	H-7	H-8	H-9
Polysaccharide									
→3)-α-D-Glc*p*NAc-(1→	4.82	4.03	3.67	3.53	3.97	3.84			
→8)-β-Pse*p*5Ac7Ac-(2→			1.64 2.44	3.84	4.18	3.89	4.23	4.00	1.20
→3)-α-L-Fuc*p*NAc-(1→	5.01	4.19	4.03	4.06	4.36	1.19			
Trisaccharide ***7***									
α-D-Glc*p*NAc-(1→	5.00	3.85	3.65	3.50	3.92	3.86			
→8)-β-Pse*p*5Ac7Ac-(2→			1.79 2.45	3.96	4.17	3.65	4.32	4.17	1.19
→3)-L-FucNAc-ol	3.59 3.67	4.24	4.17	3.58	3.97	1.18			
Disaccharide ***9***									
β-Pse*p*5Ac7Ac-(2→			1.74 2.50	3.89	4.19	3.79	4.05	4.14	1.19
→3)-L-FucNAc-ol	3.57 3.66	4.23	4.09	3.65	4.03	1.20			

Chemical shifts for NAc are δ 1.95–2.04.



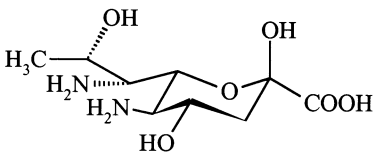
L-*glycero*-L-*manno*

1



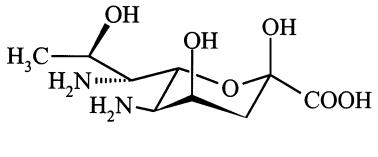
D-*glycero*-D-*galacto*

2



L-*glycero*-D-*galacto*

3



D-*glycero*-D-*talo*

4

Fig. 3. Naturally occurring isomers of 5,7-diamino-3,5,7,9-tetradeoxynon-2-ulosonic acid.

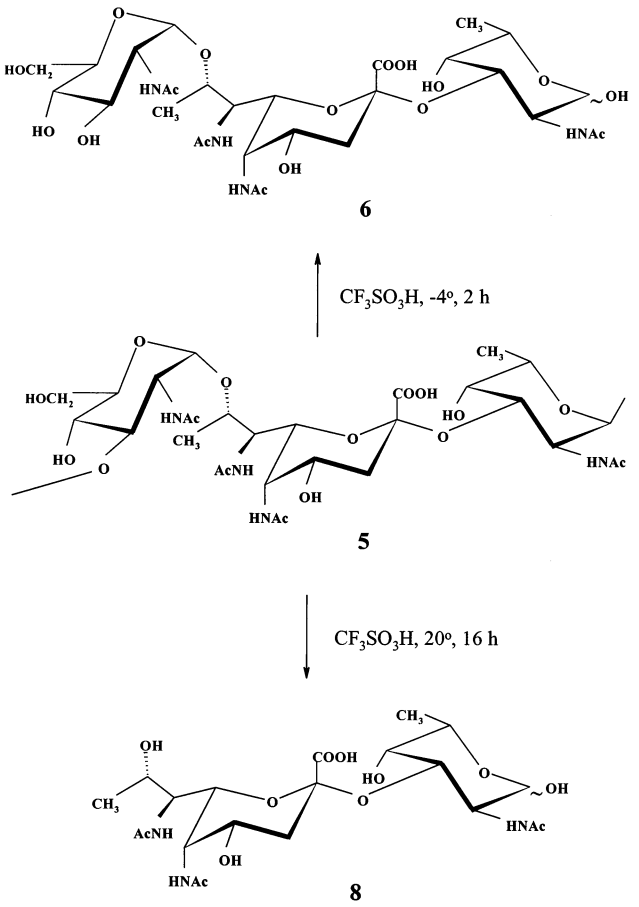


Fig. 4. Selective cleavage of the O-specific polysaccharide of *P. vulgaris* O39 by solvolysis with triflic acid.

The coupling constant values for the higher sugar were similar to those published for pseudaminic acid (Pse)[10,11] and different from the data of the other known stereoisomers.[12–14] Particularly, $J_{3ax,4} \sim 11$, $J_{4,5} < 2$ and $J_{5,6} < 2$ Hz indicated that H-4 occupies the axial position and H-5 the equatorial position, and, hence, the C-4,5,6 fragment has the lyxo configuration. A large $J_{6,7}$ value of ~ 10 Hz showed the erythro configuration of the C-6,7 fragment (higher sugars with the threo configuration are characterised by a small $J_{6,7}$ value of < 2 Hz).[10–12] The ^{13}C NMR chemical shift δ 14.6 suggested the erythro configuration of the C-7,8 fragment,[10–12] which was confirmed by the NMR data of oligosaccharides derived from the polysaccharide (see below). A relatively large difference (0.77 ppm) between the chemical shifts of H-3_{ax} and H-3_{eq} is typical of the axial orientation of the carboxyl group in glycosides of 3-deoxynon-2-ulosonic acids.[10–12] Therefore, the non-2-ulosonic acid present has the same general configuration as pseudaminic acid and is β-linked. The trisaccharide repeating-unit of the polysaccharide contains thus one residue each of α-D-Glc*p*NAc, α-L-Fuc*p*NAc, and β-Pse*p*5Ac7Ac.

A low-field position of the signals for C-3 of GlcNAc and FucNAc at δ 77.5 and 74.4 indicated that both sugars are substituted at position 3 (compare published data for the corresponding nonsubstituted monosaccharides[15,16]). The position of the C-8 signal of Pse at δ 76.7 suggested that pseudaminic acid is substituted at position 8 (in nonsubstituted Pse C-8 resonates at δ 69–70[11]). The glycosylation pattern was confirmed and the monosaccharide sequence determined by a 2D ROESY experiment, which showed cross-peaks for FucNAc H-1,GlcNAc H-3 at δ 5.01/3.67 and GlcNAc H-1, Pse5Ac7Ac H-8 at δ 4.82/4.00. These data showed that the trisaccharide repeating unit of the O-specific polysaccharide of *P. vulgaris* O39 has structure **5** (Fig. 4).

To confirm the configuration of pseudaminic acid and the structure of the repeating unit, PS1 was cleaved with anhydrous trifluoromethanesulfonic (triflic) acid[17] (Fig. 4). Solvolysis at -4 °C for 2 h resulted in oligosaccharide **6**, which was isolated by GPC and reduced with borohydride (Fig. 4). The negative-ion mode electrospray ionisation mass spectrum of the reduced product **7** showed an intense peak of the $[M-H]^-$ pseudomolecular ion at m/z 725.36, and, hence, **6** is a trisaccharide containing all components of the initial polysaccharide, including the *N*-acetyl groups (calculated molecular mass 726.32 Da). Solvolysis of PS1 under more drastic conditions (20 °C, 16 h) gave a disaccharide **8** containing Pse5Ac7Ac and FucNAc, which was also reduced with borohydride to glycosyl-alditol **9**.

The structures of **7** and **9** were confirmed by ^{13}C and ^{1}H NMR spectra (Tables 1 and 2), which were assigned using 2D COSY, TOCSY, and $^{1}H,^{13}C$ HMQC experiments as described above for the polysaccharide. The signal for C-6 of FucNAc was characteristically shifted from δ 16.6 in the polysaccharide to δ 20.0–20.1 in the oligosaccharides, thus

indicating that this sugar was converted into the corresponding alditol (FucNAc-ol). Therefore, oligosaccharidesis **6** and **8** have the structures shown in Fig. 4, which confirmed the structure **5** of the polysaccharide.

It is worth noting that solvolysis of the polysaccharide with triflic acid could be performed directionally: under milder conditions, the glycosidic linkage of FucNAc was cleaved highly selectively, whereas the linkages of GlcNAc was stable but could be cleaved under more drastic conditions. The ketosidic linkage of Pse5Ac7Ac was the most stable. Importantly, triflic acid did not cleave amidic linkages, and the *N*-acetyl groups of the amino sugars were not affected. The same products could be expected to result from solvolysis of the polysaccharide with anhydrous hydrogen fluoride,[18] but an advantage of triflic acid is that no special equipment is necessary to perform solvolysis. Methanesulfonic acid was also tested as a reagent for selective cleavage of the polysaccharide and was found to be less active compared to triflic acid: it cleaved the linkage of FucNAc only partially at -4 °C and gave a $\sim$1:2 mixture of trisaccharide **6** and disaccharide **8** in the conditions in which triflic acid gave only the disaccharide.

The ^{13}C NMR chemical shifts for the terminal residue of the non-2-ulosonic acid in disaccharide **9** (Table 1) were close to those for pseudaminic acid.[11] Since the chemical shifts are known to be sensitive to the general configuration,[10–12,14] they confirmed the identity of these sugars. The C-6 chemical shift (δ 74.2) confirmed the β configuration of the ketosidic linkage (in α-linked pseudaminic acid C-6 would resonate at δ 71.2–72.2).[10] The absolute L-*glycero*-L-*manno* configuration of the non-2-ulosonic acid followed from a relatively high by the absolute value β-effect on the C-9 chemical shift caused by glycosylation at position 8 with α-D-Glc*p*NAc [-3.5 ppm, as determined by comparison of the data for oligosaccharides **7** and **9** (Table 1)]. This conclusion was based on published data of the β-effect of glycosylation on the C-4 chemical shift in model compounds *O*-(α-D-galactopyranosyl)-L-allothreonine and -D-allothreonine (-3.95 and -1.2 ppm, respectively).[19]

These data finally confirmed that the polysaccharide of *P. vulgaris* O39 contains di-*N*-acetylpseudaminic acid. Previously, derivatives of pseudaminic acid has been reported as components of the LPSs of *Pseudomonas aeruginosa* and some other bacterial polysaccharides,[20–22] but found for the first time in the O-specific polysaccharides of *Proteus* in this work.

Proteus strains from different O-serogroups are known to cross-react with anti-*Proteus* O-sera due to the presence of common epitopes in their LPSs.[3] In order to reveal serological relationships of the strain studied, rabbit polyclonal anti-*P. vulgaris* O39 serum was tested with LPSs of various *Proteus* strains. In passive hemolysis test and enzyme immunosorbent assay, O-antiserum reacted with the homologous LPS at a high titre of 1:25,600 and 1:1,024,000, respectively. A marked cross-reaction was observed in passive hemolysis test with LPS of *P. mirabilis* O6 and *P. vulgaris* O8 (titres 1:6,400 and 1:3,200, respectively), and a relatively weaker cross-reaction in enzyme immunosorbent assay (titre 1:16,000 for both strains). In Western blot after deoxycholate polyacrylamide gel electrophoresis, anti-*P. vulgaris* O39 serum reacted with slowly and fast migrating bands of the homologous LPS, which corresponded to high- and low-molecular-mass species containing and lacking the O-specific polysaccharide chain, respectively (data not shown). Western blot revealed also cross-reactivity with high-molecular-mass LPS species of *P. mirabilis* O6 (strong) and *P. vulgaris* O8 (weak).

These data indicated that the cross-reactive LPSs share an epitope with *P. vulgaris* O39 LPS, which is located in the polysaccharide chain. Comparison of the structures of the three O-specific polysaccharides (Fig. 5) suggested that, most likely, the common epitope is associated with the α-L-Fuc*p*NAc-(1→3)-D-Glc*p*NAc disaccharide fragment. A marked serological cross-reactivity between LPS of *P. mirabilis* O6 and *P. vulgaris* O8 and the corresponding O-antisera has been reported and attributed to the same common disaccharide fragment in the O-specific polysaccharides.[23] Remarkably, in all cross-reactive LPS, FucNAc is substituted with an acidic monosac-

charide (pseudaminic acid in *P. vulgaris* O39 or D-glucuronic acid in two other strains), and it is not excluded that the negative charge of the acidic sugar is important for the cross-reactivity. Charged constituents, including D-glucuronic acid in *P. mirabilis* O6 LPS,[24] have been demonstrated to be involved in the manifesting of the serological specificity of many *Proteus* strains.[3]

The present serological data showed that *P. vulgaris* O39 LPS shares are only minor epitopes, whereas the major epitope(s), as well as the structure of the O-antigen on the whole are unique among bacteria, which is in agreement with classification of *P. vulgaris* strain PrK 65/57 in a separate *Proteus* serogroup.

3. Experimental

Bacterial strain, growth, and isolation of the lipopolysaccharide.—*P. vulgaris* O39 strain PrK 65/57 came from the Czech National Collection of Type Cultures (Institute of Epidemiology and Microbiology, Prague). The bacteria were cultivated under aerobic conditions in nutrient broth (BTL, Łodz, Poland). The bacterial mass was harvested at the end of the logarithmic growth phase, centrifuged, washed with water, and lyophilised.

LPS was isolated from dried cells of *P. vulgaris* O39 using the phenol–water method[4] and purified by treatment with cold aq 50% CCl_3CO_2H;[25] the aqueous layer was dialysed and freeze-dried.

Degradation of the lipopolysaccharide

Mild acid hydrolysis. The LPS (187 mg) was hydrolysed with aq 1% HOAc at 100 °C for 4 h and a lipid precipitate was removed by centrifugation at 13,000*g* for 20 min. The supernatant was fractionated by GPC on Sephadex G-50 in 0.05 M pyridinium acetate buffer (pH 4.5) with monitoring by a Knauer differential refractometer to give a polysaccharide (PS1, 22 mg).

Deamination. The LPS (100 mg) was treated with aq 1% $NaNO_2$ (5 ml) and aq 10% HOAc (5 ml) at 25 °C for 3 h, a lipid precipitate was removed by centrifugation, and the supernatant was fractionated by GPC on Sephadex G-50 as described above to give a polysaccharide (PS2, 14 mg).

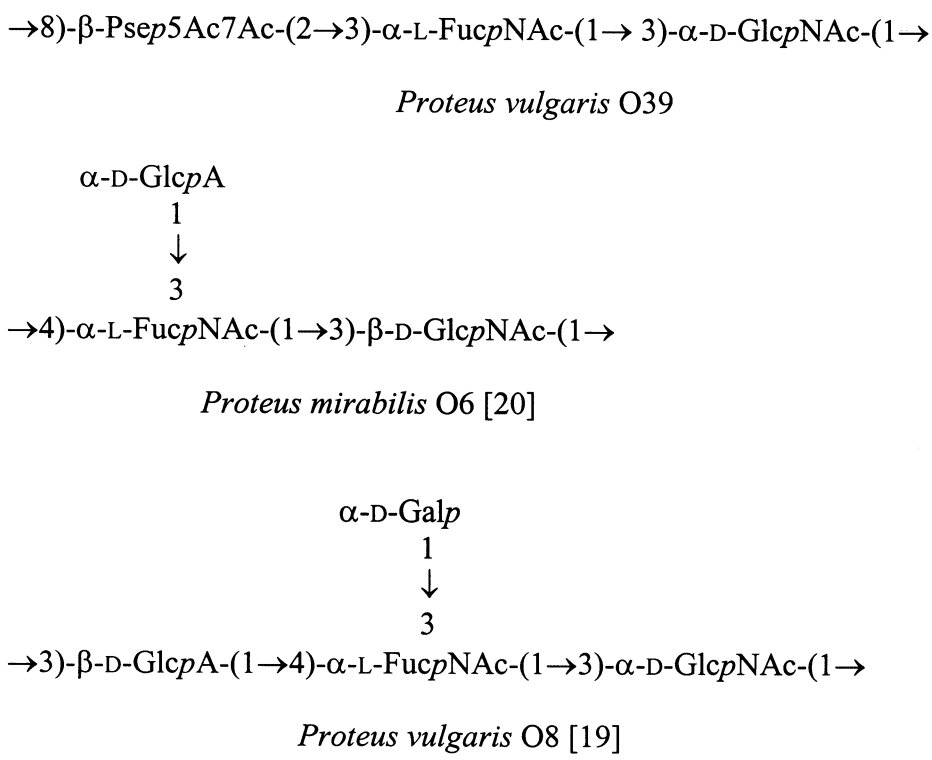


Fig. 5. Structures of the cross-reactive O-antigens of *Proteus*.

Sugar analysis.—PS1 was hydrolysed with 2 M CF_3COOH (120 °C, 2 h), and amino sugars were identified on a Biotronik LC-2000 amino acid analyser (Germany) using a column (0.4 × 22 cm) of an Ostion LG AN B cation-exchange resin and 0.35 M sodium citrate buffer (pH 5.28) at 80 °C. PS2 was reduced with $NaBH_4$ in water, acidified with conc HOAc to pH 5, desalted by GPC on a column (52 × 1.6 cm) of TSK HW-40 (S) in aq 1% AcOH, hydrolysed as above, the monosaccharides were acetylated and analysed by GLC–MS on a DB-5 fused-silica capillary column using Hewlett–Packard 5890 chromatograph (USA) equipped with a NERMAG R10-10L mass spectrometer (France) at a temperature gradient of 160 °C (1 min) to 290 °C at 3 °C/min. For determination of the absolute configurations, PS1 was methanolysed with 1 M HCl–MeOH (80 °C, 16 h). The products were acetylated with acetanhydride in pyridine (100 °C, 1 h), subjected to (+)-2-octanolysis in the presence of CF_3COOH (120 °C, 16 h), acetylated, and analysed by GLC–MS as described above.

Selective cleavage of the polysaccharide

Solvolysis with triflic acid. PS1 (19 mg) was treated with triflic acid (0.5 mL) at −4 °C for 2 h in anhydrous conditions. After neutralisation with aq 5% ammonia at 0 °C and evaporation, and the reaction products were desalted by GPC, reduced with $NaBH_4$, and fractionated by GPC on TSK HW-40 (S) in aq 1% AcOH monitored using a Knauer differential refractometer (Germany) to give trisaccharide **7** (3.6 mg). Solvolysis of PS1 (7 mg) at 20 °C for 16 h followed by a similar work-up and borohydride reduction afforded disaccharide **9** (1.2 mg).

Solvolysis with methanesulfonic acid. PS2 (8 mg) was treated with methanesulfonic acid at −4 °C for 2 h, and a polymeric material (3.9 mg) and lower oligosaccharides (3.3 mg) were isolated as above. The former was further treated with methanesulfonic acid at 20 °C for 16 h and, after borohydride reduction and GPC, disaccharide **7** (1.9 mg) and trisaccharide **9** (0.9 mg) were obtained.

Mass spectrometry.—Electrospray-ionisation mass spectrometry was performed in the negative-ion mode using a VG Quattro triple quadrupole mass spectrometer (Micromass, Altrincham, Cheshire, UK) with MeCN as the mobile phase at a flow rate of 10 μL/min. Samples were dissolved in aq 50% MeCN at a concentration about 50 pmol/μL, and 10 μL was injected via a syringe pump into the electrospray source.

NMR spectroscopy.—Samples were deuterium-exchanged by freeze-drying three times from D_2O and then examined as solutions in 99.96% D_2O at 40 °C for polysaccharides and 30 °C for oligosaccharides. Spectra were recorded on a Bruker DRX-500 MHz spectrometer (Germany) equipped with an SGI INDY computer workstation. Two-dimensional spectra were obtained using standard Bruker software, and XWINNMR 2.1 program (Bruker) was used to acquire and process NMR data. The parameters used for 2D experiments were essentially the same as described previously.[26] A mixing time of 300 ms was used in TOCSY and ROESY experiments.

Serological techniques.—Rabbit polyclonal anti-*P. vulgaris* O4 serum was obtained as described.[27] Serological tests, polyacrylamide gel-electrophoresis, and Western blot were performed as described previously.[27] LPS and alkali-treated LPS were used as antigen in enzyme immunosorbent assay and passive hemolysis test, respectively.

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