# Structures of new acidic O-specific polysaccharides of the bacterium *Proteus mirabilis* serogroups O26 and O30

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**Abstract The polysaccharide chains of the lipopolysaccharides of the *Proteus mirabilis* serogroups O26 and O30 were studied using sugar and methylation analysis and $^{1}H$ and $^{13}C$ NMR spectroscopy, including two-dimensional correlation spectroscopy and rotating-frame NOE spectroscopy. The polysaccharides were found to be acidic due to the presence of D-galacturonic acid and its amide with L-lysine in serogroup O26 or D-glucuronic acid in serogroup O30, and the structures of their tetrasaccharide repeating units were established. The O26-specific polysaccharide is structurally and serologically related to the O-specific polysaccharide of *P. mirabilis* O28, which includes amides of D-GalA with L-lysine and L-serine [Radziejewska-Lebrecht, J. et al. (1995) Eur. J. Biochem. 230, 705–712].**

*Key words:* Lipopolysaccharide; O-Antigen structure; Bacterial acidic polysaccharide; Hexuronic acid; L-Lysine; *Proteus mirabilis*

## 1. Introduction

Bacteria of the genus *Proteus* are a common cause of wound and urinary tract infections; the latter may result in pyelonephritis, bacteremia, and formation of kidney stones. According to the serological classification based on the specificity of the outer membrane lipopolysaccharide (LPS, O-antigen), strains of *Proteus mirabilis* and *Proteus vulgaris* are divided into 49 O-serogroups [1,2].

In addition to common monosaccharides (glucose, 2-amino-2-deoxyglucose, L-*glycero*-D-*manno*-heptose, 3-deoxy-D-*manno*-octulosonic acid), LPS contains galacturonic acid (GalA) in all *Proteus* O-serogroups and glucuronic acid (GlcA) in many of them [3]. In a study of the O-specific polysaccharide chain of *P. mirabilis* strain 1959 (OXK group) LPS, GalA was shown to occur as an amide with lysine [4]. Later, the same amide, $N^{\varepsilon}$-(D-galacturonoyl)-L-lysine, was found in the O-specific polysaccharide of *P. mirabilis* serogroup O28 [5], while in some other *Proteus* O-specific polysaccharides including amides of D-GalA with L-alanine, L-serine or L-threonine, or an amide of D-GlcA with L-lysine were shown to present [6]. The structures of a number of hexuronic acid-containing polysaccharides, including the amino acid-containing polysaccharides, have been established [6].

Now we report the structures of two new acidic O-specific polysaccharides of *P. mirabilis* serogroups O26 and O30, which are frequently isolated from clinical material [2]. Of them, the polysaccharide of serogroup O30 contains D-GlcA and that of serogroup O26 includes D-GalA and an amide of D-GalA with L-lysine.

## 2. Materials and methods

### 2.1. Bacteria, growth, isolation of lipopolysaccharides and O-specific polysaccharides

Bacterial strains PrK 49/57 and PrK 53/57 of *P. mirabilis* serogroups O26 and O30, respectively, were derived from the Czech National Collection of Type Cultures (Institute of Microbiology and Epidemiology, Prague) and grown as described [7]. LPS was isolated by phenol-water extraction [8] and purified by ultracentrifugation followed by digestion with nucleases [9]. After degradation of LPS with 1% HOAc at 100°C [10], O-specific polysaccharide was isolated by gel chromatography on a column (40 cm × 2.5 cm) of Sephadex G-50 in 0.05 M pyridinium acetate buffer (pH 4.5) monitored with a Knauer differential refractometer.

### 2.2. Chemical methods

Hydrolysis of the polysaccharides was performed with 2 M $CF_3CO_2H$ at 121°C for 2 h. Amino components in the hydrolysates were identified by cation-exchange chromatography using a Biotronik LC-2000 amino acid analyzer and the standard 0.35 M sodium citrate buffer (pH 5.28). Neutral and acidic monosaccharides were identified by anion-exchange chromatography using a Biotronik LC-2000 sugar analyzer as described [11]. Absolute configurations of monosaccharides were determined after conversion into acetylated (*S*)-2-octyl or (*S*)-2-butyl glycosides [12,13] using GLC on a Hewlett-Packard 5890 chromatograph equipped with a glass capillary column (25 m × 0.2 mm) of Ultra 2 stationary phase. Similarly, L-lysine was identified by GLC after conversion into (*S*)-2-octyl ester by treatment of (*S*)-2-octanol in the presence of a drop of concentrated $CF_3CO_2H$ at 130°C for 6 h followed by trifluoroacetylation.

Methylation of the O30-specific polysaccharides was performed as described [14], partially methylated monosaccharides were derived by hydrolysis with 2 M $CF_3CO_2H$ at 100°C for 4 h, converted conventionally into alditol acetates, and analyzed by GLC/MS using a Hewlett-Packard 5791 instrument equipped with a capillary column (12 m × 0.2 mm) of HP-1 and published data [15,16] for identification of mass spectra.

*O*-Deacetylation was performed with 12% aqueous ammonia at 50°C for 3 h, ammonia was removed by concentration of the solution in vacuum. Carboxyl reduction of the O30-specific polysaccharide was performed as described [17].

### 2.3. NMR spectroscopy

NMR spectra were run with Bruker WM-250 ($^{1}H$) and Bruker AM-300 ($^{13}C$) spectrometers for solutions in $D_2O$ at 45 and 70°C, respectively, with acetone ($\delta_H$ 2.225, $\delta_C$ 31.45) as internal standard. Standard Bruker software was applied to obtain two-dimensional NMR spectra. A rotating-frame NOE (ROESY) experiment was carried out on a modified Bruker WM-250 spectrometer using the proposed pulse sequence [18] and a mixing time of 0.23 s; HDO signal was suppressed by irradiation during 1 s. An H-detected $^{1}H,^{13}C$ heteronuclear multi-quantum coherence (HMQC) experiment was performed on a Bruker AM-300 spectrometer equipped with a BSV-3 generator as described [19].

$^{1}H$ NMR spectra were assigned using sequential, selective spin decoupling, two-dimensional COSY, and COSY with one-step relayed

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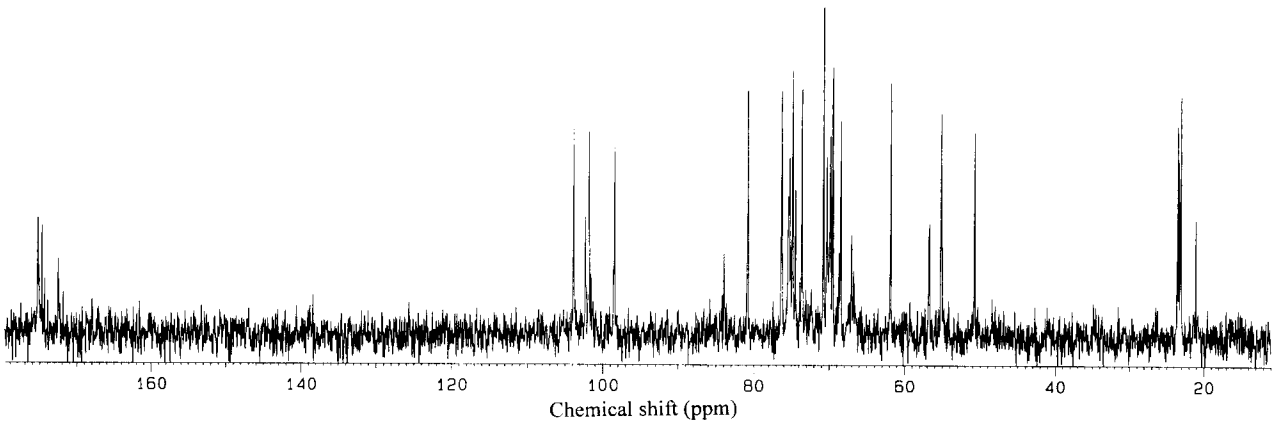



Fig. 1. $^{13}C$ NMR spectrum of the O30-specific polysaccharide.

coherence transfer. $^{13}C$ NMR spectra were assigned using HMQC for the O26-specific polysaccharide and $^{1}H,^{13}C$ heteronuclear COSY and attached-proton test [20] for the O30-specific polysaccharides. Spin systems of sugars with the *gluco* and *galacto* configurations were differentiated by the $^{3}J$ coupling constant values. The typical position in the region δ 50–57 of the $^{13}C$ NMR signals for carbons bearing nitrogen demonstrated the amino sugars. The assignments are given in Tables 1 and 2.

## 3. Results

### 3.1. Elucidation of the structure of the O30-specific polysaccharide

Sugar analysis of the polysaccharide revealed 2-amino-2-deoxy-D-glucose and 2-amino-2-deoxy-D-galactose in the 2:1 ratio, as well as D-glucuronic acid.

The $^{13}C$ NMR spectrum of the polysaccharide (Fig. 1) showed the absence of strict regularity due to nonstoichiometric *O*-acetylation (a peak for $CH_3$ of an *O*-acetyl group was present at δ 21.3). *O*-Deacetylation with aqueous ammonia resulted in a modified polysaccharide (PS) with a $^{13}C$ NMR spectrum typical of a regular polymer (Table 1). The spectrum contained, inter alia, signals for four anomeric carbons at δ 98.6–103.8, three carbons bearing nitrogen (C2 of amino sugars) at δ 50.9–56.9, and three *N*-acetyl groups at δ 23.3–23.8 ($CH_3$) and 175.0–175.5 (CO). Accordingly, the $^{1}H$ NMR spectrum of PS (Table 2) displayed signals for four anomeric protons at δ 4.49–4.92 and three *N*-acetyl groups at δ 2.02–2.07. Therefore, PS has a tetrasaccharide repeating unit, and the three amino sugars present are *N*-acetylated.

Methylation analysis of PS revealed 2-amino-2-deoxy-3,4-di-*O*-methylglucose, 2-amino-2-deoxy-4,6-di-*O*-methylglucose, and 2-amino-2-deoxy-3,4-di-*O*-methylgalactose. When PS was carboxyl-reduced prior to methylation, in addition, 2,3-di-*O*-methylglucose was identified, which was derived from GlcA. These data indicated that PS is linear, one of the GlcNAc residues is 3-substituted, the other GlcNAc and GalNAc are 6-substituted, and GlcA is 4-substituted.

The pyranose form of the four sugars was identified by the coupling constants of the ring protons [21] in the $^{1}H$ NMR spectrum of PS. The coupling constant values $J_{1,2}$ 7–8 Hz showed that both ClcNAc residues and GlcA are β-linked, while a smaller coupling constant $J_{1,2}$ 3.5 Hz proved GalNAc to be α-linked.

A ROESY experiment with PS showed correlation peaks between GlcA H1 and GalNAc H6 at δ 4.49/4.01 (weak) and 4.49/3.86 (strong). Similarly, GalNAc H1 gave correlation peaks with GlcNAc H6 at δ 4.92/4.00 (weak) and 4.92/3.70 (strong). These data revealed the sequence GlcA-(1→6)-GalNAc-(1→6)-GlcNAc. Chemical shifts for H1 of two GlcNAc residues were close (δ 4.57 and 4.58), and their correlation

Table 1
Data of $^{13}C$ NMR spectra of the *O*-deacetylated polysaccharides (PS)

| Residue | Chemical shift (δ, ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | C1 | C2 | C3 | C4 | C5 | C6 | $CH_3CON$ | $CH_3CON$ |
| *P. mirabilis* O30 | | | | | | | | |
| →4)-β-Glc*p*A-(1→ | 103.8 | 74.0 | 75.1 | 81.1 | 77.9 | [a] | | |
| →6)-α-Gal*p*NAc-(1→ | 98.6 | 50.9 | 68.7 | 69.7 | 70.9 | 70.1 | 23.8 | 175.5 |
| →6)-β-Glc*p*NAc-(1→ | 102.5 | 56.9 | 74.8 | 70.9 | 75.4 | 67.0 | 23.5 | 175.2 |
| →3)-β-Glc*p*NAc-(1→ | 101.7 | 55.2 | 84.2 | 70.1 | 76.6 | 62.1 | 23.3 | 175.0 |
| *P. mirabilis* O26 | | | | | | | | |
| →4)-α-Gal*p*A-(1→ | 100.9 | 69.9 | 70.7 | 77.5 | 72.5 | 170.8 | | |
| →4)-α-Gal*p*-(1→ | 96.7 | 69.3 | 70.4 | 78.5 | 72.6 | 63.0 | | |
| →3)-β-Gal*p*A-(1→ | 104.2 | 69.9 | 79.0 | 67.5 | 76.3 | 174.8 | | |
| →3)-β-Glc*p*NAc-(1→ | 102.1 | 55.9 | 85.4 | 71.4 | 76.1 | 61.4 | 23.6 | 175.5 |
| Lys | 179.0 | 55.8 | 32.6 | 23.2 | 27.9 | 40.6 | | |

Assignment of the signals for the *N*-acetyl groups in the spectrum of the *P. mirabilis* O30 PS could be interchanged.
[a]Not found.

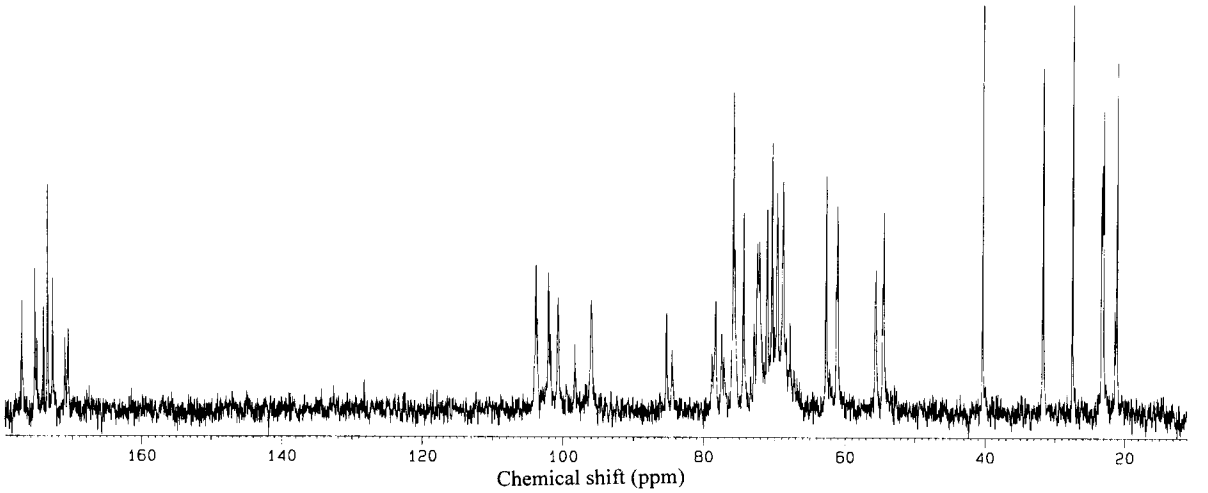


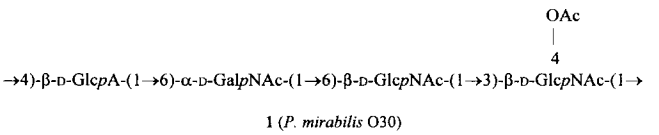

Fig. 2. $^{13}C$ NMR spectrum of the O26-specific polysaccharide.

peaks with GlcA H4 at δ 3.76 and GlcNAc H3 at δ 3.71 appeared on the same line. However, taking into account the linear character of PS, these data were sufficient to establish the complete sequence of the sugar residues shown below.

Downfield displacements by 5–10 ppm of the signals for C4 of GlcA, C3 of the one and C6 of the other GlcNAc residue, and C6 of GalNAc in the $^{13}C$ NMR spectrum of PS (Table 1), as compared with their positions in the spectra of the corresponding nonsubstituted monosaccharides [22], confirmed the substitution pattern of PS. Computer-assisted analysis [23] showed that the structure established is the only linear structure compatible with the experimental $^{13}C$ NMR spectrum of PS.

A marked decrease of the signals at δ 84.2 and 76.6, which belong to C3 and C5 of 3-substituted GlcNAc, and an increase of the signals at δ 81.1 and 75.4 were observed in the $^{13}C$ NMR spectrum of the initial polysaccharide as compared with the spectrum of the *O*-deacetylated polysaccharide. These changes were caused by *O*-acetylation of part of the GlcNAc residues at position 4 [24]. As judged by the relative intensities of the signals for the *O*-acetyl and *N*-acetyl groups in the $^{13}C$ NMR spectrum of the initial polysaccharide, the degree of *O*-acetylation was about 70%.

Therefore, the data obtained suggested that the tetrasaccharide unit of the O30-specific polysaccharide has the structure **1**.




**1** (*P. mirabilis* O30)

### 3.2. Elucidation of the structure of the O26-specific polysaccharide

Sugar analysis of the polysaccharide led to identification of D-galactose, D-galacturonic acid, 2-amino-2-deoxy-D-glucose, and L-lysine.

The $^{13}C$ NMR spectrum of the polysaccharide (Fig. 2) looked complex owing to nonstoichiometric *O*-acetylation (a peak for $CH_3$ of an *O*-acetyl group was present at δ 21.4) but could be simplified by *O*-deacetylation with aqueous ammonia. As judged by the $^{13}C$ NMR spectrum (Table 1), the *O*-deacetylated polysaccharide (PS) is a regular polymer built up of tetrasaccharide repeating units (there were signals for four anomeric carbons in the region δ 96.7–104.2). The spectrum pointed to the presence of GlcNAc (C2 at δ 55.9, NAc at δ 23.6 and 175.5), carboxyl-free GalA (C6 at δ 174.8), and

Table 2
Data of $^1H$ NMR spectra of the *O*-deacetylated polysaccharides (PS)

| Residue | Chemical shift (δ, ppm) | | | | | | |
|---|---|---|---|---|---|---|---|
| | H1 | H2 | H3 | H4 | H5 | H6 | $CH_3CON$ |
| *P. mirabilis* O30 | | | | | | | |
| →4)-β-Glc*p*A-(1→ | 4.49 | 3.35 | 3.58 | 3.76 | 3.68 | | |
| →6)-α-Gal*p*NAc-(1→ | 4.92 | 4.19 | 3.96 | 4.03 | 4.11 | 4.01, 3.86 | 2.07 |
| →6)-β-Glc*p*NAc-(1→ | 4.58 | 3.70 | 3.60 | 3.58 | 3.63 | 4.00, 3.70 | 2.06 |
| →3)-β-Glc*p*NAc-(1→ | 4.57 | 3.77 | 3.72 | 3.52 | 3.50 | 3.93, 3.75 | 2.02 |
| *P. mirabilis* O26 | | | | | | | |
| →4)-α-Gal*p*A-(1→ | 5.19 | 3.72 | 4.06 | 4.38 | 4.92 | | |
| →4)-α-Gal*p*-(1→ | 5.04 | 3.87 | 4.01 | 4.12 | 4.20 | 3.80 | |
| →3)-β-Gal*p*A-(1→ | 4.44 | 3.60 | 3.76 | 4.42 | 4.02 | | |
| →3)-β-Glc*p*NAc-(1→ | 4.75 | 3.78 | 3.68 | 3.41 | 3.39 | 3.90, 3.64 | 2.02 |
| Lys | | 4.20 | 1.82, 1.68 | 1.44 | 1.70 | 3.03 | |

Assignment of the signals for the *N*-acetyl groups in the spectrum of the *P. mirabilis* O30 PS could be interchanged.

GalA amidated by lysine (C6 of GalA at δ 170.8, C-1,2,3,4,5,6 of lysine at δ 179.0, 55.8, 32.6, 23.2, 27.9, 40.6, respectively; cf. published data for $N^{\varepsilon}$-(D-galacturonoyl)-L-lysine [5]).

Based on the coupling constant values determined from the $^1$H NMR spectrum of PS, the four sugar constituents are in the pyranose form, two of them are α-linked (Gal and one of the GalA residues, $J_{1,2}$ 7–8 Hz), and two others β-linked (GlcNAc and the second GalA residue, $J_{1,2}$ 3.5–4 Hz). On changing the pD of a PS solution from 5 to 2, the H5 signal of β-GalA shifted from δ 4.02 to 4.16, while that of α-GalA at δ 4.92 did not. Hence, β-GalA has the free carboxyl group and α-GalA is amidated by lysine. The attachment of lysine via its α-amino group followed from a low field position of the signal for H2 of lysine at δ 4.20 in the spectrum of PS (Table 2), as compared with its position at δ 3.70 in the spectrum of the nonsubstituted amino acid [25].

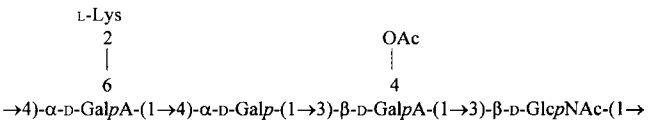
The two-dimensional ROESY spectrum of PS showed correlation peaks β-GalA H1/β-GlcNAc H3 at δ 4.44/3.68 and β-GlcNAc H1/α-GalA H4 at δ 4.75/4.38 which pointed to the presence of a fragment β-GalA-(1→3)-β-GlcNAc-(1→4)-α-GalA. Two correlation peaks, with β-GalA H3 and H4 at δ 3.76 and 4.42, respectively, were present for α-Gal H1 at δ 5.04. This suggested the fragment α-Gal-(1→3)-β-GalA since no NOE contact could be observed between H1′ and H3 in 1,4-linked disaccharides but between H1′ and H4 in 1,3-linked galactose disaccharides [26].

Proton α-GalA H1 at δ 5.19 also afforded two correlation peaks with α-Gal H4 and H6 at δ 4.12 and 3.80, respectively. Since PS contained no 6-substituted sugar residues (data of the attached-proton test [20]) and NOE on H6 is typical of a number of 1,4-linked disaccharides [26], this demonstrated the fragment α-GalA-(1→4)-α-Gal. An additional correlation peak between α-GalA H5 and α-Gal H2 at δ 4.92/3.87 pointed to a conformation where these protons are in close proximity.

The substitution pattern of PS thus established was confirmed by significant downfield displacements of the signals for C3 of β-GalA and β-GlcNAc and C4 α-GalA and α-Gal to δ 79.0, 85.4, 77.5, and 78.5, respectively, in the $^{13}$C NMR spectrum of PS, as compared with their position in the spectra of the corresponding nonsubstituted monosaccharides [22].

Comparison of the $^1$H NMR spectra of the initial and *O*-deacetylated polysaccharides revealed a shift of the signal for β-GalA H4 from δ 4.42 in the latter to δ 5.08 in the former, which was caused by a deshielding effect of the *O*-acetyl group. *O*-Acetylation of β-GalA at position 4 followed also from a downfield displacement of the signal for β-GalA C4 from δ 67.5 in the $^{13}$C NMR spectrum of PS to the region δ 68.5–71 in the spectrum of the initial polysaccharide and from an upfield displacement of the signal for β-GalA C3 from δ 79.0 to the region 74.5–76.5 [24]. In addition to this major *O*-acetyl group, which is present in almost, if not fully, stoichiometric amount, the presence of minor *O*-acetyl groups is likely, which may account for the structural heterogeneity of the polysaccharide revealed by the $^{13}$C NMR spectrum (see above).

On the basis of the data obtained, it was concluded that the O26-specific polysaccharide consists mainly of the repeating units having the structure **2**

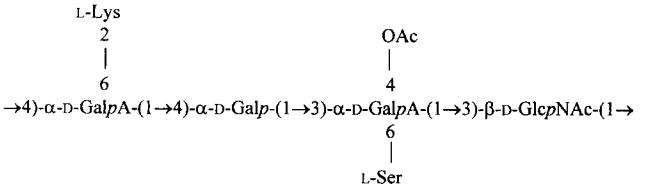



**2** (*P. mirabilis* O26)

## 4. Discussion

Both O-specific polysaccharides studied are acidic, which is characteristic for LPS of many enterobacteria [27] and especially for those of *Proteus*, almost all of which contain negatively charged groups [6,27,28]. Most often these are carboxyl groups of GalA or GlcA. As for amides of hexuronic acids with amino acids, such as $N^{\varepsilon}$-(D-galacturonoyl)-L-lysine present in the O26-specific polysaccharide, these are unique components of *Proteus* LPS [6]. Of them, amides with L-lysine endow the polysaccharide chain with both positively and negatively charged groups that may improve adaptation of the bacteria to changes of environmental pH or contribute in another way to the bacterial virulence, such as has been shown for bacterial capsular polysaccharides with the oppositely charged groups [29].

The structure **2** of the O26-specific polysaccharide much resembles the structure **3** of the O-specific polysaccharide of *P. mirabilis* O28 which has been established earlier [5] and differs from the structure **2** in that the *O*-acetylated GalA residue carries L-serine and has a different configuration of the glycosidic linkage.




**3** (*P. mirabilis* O28)

Immunochemical analysis of the O28-specific polysaccharide using synthetic antigens, amides of D-GalA with various L-amino acids, revealed the immunodominant role of a lysine-containing epitope but not a serine-containing epitope [5]. Anti-O26 antibodies were found to cross-react with *P. mirabilis* O28 and strain 1959 (OXK group) cells, most likely, due to the presence of a common lysine-associated epitope as well (Kaca, W., Swierzko, A. and Cedzynski, M., unpublished data). Therefore, $N^{\varepsilon}$-(D-galacturonoyl)-L-lysine plays an important role in serological specificity of a number of *Proteus* O-antigens.

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