Eur. J. Biochem. 230, 705–712 (1995)
© FEBS 1995

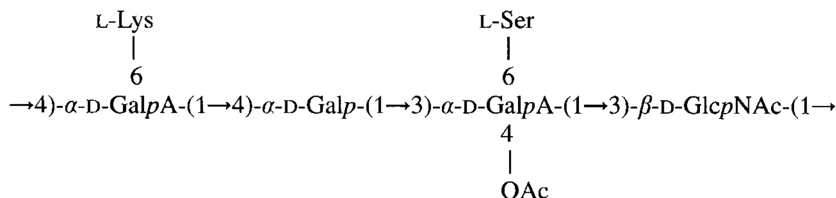
Structure and epitope characterisation of the O-specific polysaccharide of *Proteus mirabilis* O28 containing amides of D-galacturonic acid with L-serine and L-lysine

Joanna RADZIEJEWSKA-LEBRECHT[1], Alexander S. SHASHKOV[2], Eugeny V. VINOGRADOV[2], Horst GROSSKURTH[3], Beata BARTODZIEJSKA[1], Antoni ROZALSKI[1], Wieslaw KACA[1], Leonid O. KONONOV[2], Anatoly Y. CHERNYAK[2], Hubert MAYER[4], Yuriy A. KNIREL[2] and Nikolay K. KOCHETKOV[2]

[1] Institute of Microbiology and Immunology, University of Lodz, Poland
[2] N. D. Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, Moscow, Russia
[3] Max-Planck-Institut für Medizinische Forschung, Heidelberg, Germany
[4] Max-Planck-Institut für Immunobiologie, Freiburg, Germany

(Received 2 December 1994/23 February 1995) – EJB 94 1863/5

The O-specific polysaccharide of *Proteus mirabilis* O28 was found to contain D-galactose, D-galacturonic acid (GalA), 2-acetamido-2-deoxy-D-glucose, L-serine, L-lysine, and *O*-acetyl groups in molar ratios 1:2:1:1:1:1, the amino acids being linked via their α-amino group to the carboxyl group of GalA. The polysaccharide was studied using ^{1}H- and ^{13}C-NMR spectroscopy, including selective spin-decoupling, one-dimensional total correlation spectroscopy, two-dimensional homonuclear correlation spectroscopy (COSY), heteronuclear ^{13}C,^{1}H COSY, one-dimensional NOE, and two-dimensional rotating-frame NOE spectroscopy and partial acid hydrolysis followed by borohydride reduction, methylation, and GLC/MS analysis of the derived glycosyl alditols. The following structure of the repeating unit was established:



Epitope specificity of the *P. mirabilis* O28 polysaccharide was analysed using a homologous rabbit polyclonal antiserum in quantitative precipitation, passive immunohemolysis, and inhibition of passive immunohemolysis. Study with related synthetic glycopolymers (2-acrylamidoethyl glycosides of amides of α-D-GalA with amino acids copolymerised with acrylamide) showed the importance of D-GalA(L-Lys) for manifesting serological specificity of the O-antigen. Serological cross-reactions between *P. mirabilis* O28, S1959, and R14/S1959 (a transient-like form) are discussed.

Keywords. Proteus mirabilis; lipopolysaccharide; O antigen; structure; epitope.

Gram-negative bacteria of the genus *Proteus* are important pathogens which cause mainly wound and urinary tract infections. The latter sometimes lead to acute or chronic pyelonephritis as well as bacteremia and formation of kidney stones. Several constituents of these bacteria, e.g. fimbriae, urease, IgA protease, hemolysins, and lipopolysaccharide (LPS, endotoxin, O-antigen) were identified as pathogenic factors [1–4]. Basing on O-antigens, *Proteus* strains are classified into 49 O-serogroups [5]. Some of them, including *P. mirabilis* O28, are recognised to be most important from the clinical point of view.

Correspondence to Y. A. Knirel, N. D. Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, Leninsky Pr. 47, B-334 Moscow 117913, Russia
Fax: +7095 1355328.

Abbreviations. COSY, correlation spectroscopy; GalA, galacturonic acid; GalA(Lys), *N*-(galacturonoyl)lysine; GalA(Ser), *N*-(galacturonoyl)serine; LPS, lipopolysaccharide; LPS-OH, alkali-treated lipopolysaccharide; ROESY, rotating-frame NOE spectroscopy; TOCSY, total correlation spectroscopy.

A number of *Proteus* O-antigens have been found to contain amino acids, such as L-lysine [6–8], L-alanine [8, 9], and L-threonine [10], which are amidically linked to the carboxyl group of D-glucuronic acid [8] or D-galacturonic acid [6–10]. Some of these amides play an important role in immunospecificity of LPSs [6, 7, 10–13]. We now report the structure and epitope specificity of the O-specific polysaccharide of *P. mirabilis* O28, which includes two amides of D-galacturonic acid: with L-serine and L-lysine in the repeating unit.

EXPERIMENTAL PROCEDURES

General methods. These are described in the accompanying paper [10].

Bacterial strains. *P. mirabilis* O28 (strain 51/57) was derived from the Czechoslovak National Collection of Type Cultures. *P. mirabilis* S1959 and the mutant strains R4/O28 (Rc), R5/O28, R14/S1959 (a transient-like form), R13/S1959 (Ra),

R110/S1959 (Ra), and R45/S1959 (Re) were from the collection of the Institute of Microbiology and Immunology (Lodz).

Isolation and degradation of LPS. LPSs from *P. mirabilis* O28, S1959, and R14/S1959 were obtained according to the Westphal procedure [14]. LPSs from the R-mutant strains were isolated using the petroleum ether/chloroform/phenol procedure [15]. Alkali-treated LPS (LPS-OH) was prepared by saponification of LPS with 0.25 M sodium hydroxide (56°C, 2 h). LPS was degraded by hydrolysis with 1% acetic acid (100°C); the lipid precipitate was removed by centrifugation, and the supernatant was fractionated on a column of Sephadex G-50 in pyridine acetate pH 4.5 to give a high-molecular-mass fraction (O-specific polysaccharide), a fraction of the core oligosaccharide, and a low-molecular-mass fraction which was not further investigated.

Synthetic antigens. Synthetic 2-acrylamidoethyl α-glycosides of amides of D-GalA with various amino acids, representing partial structures of *P. mirabilis* O-antigens and their analogues, were copolymerised with acrylamide to give high-molecular-mass polyacrylamide-based glycopolymers. The details of this procedure have been reported [16].

Serological techniques. Antisera against *P. mirabilis* O28, S1959, and R14 were obtained by immunisation of New Zealand white rabbits with heat-killed bacteria (100°C, 2.5 h) as described earlier [17].

Quantitative precipitation was performed using a micro method as reported earlier [18]. Passive hemolysis and inhibition of passive hemolysis were performed according to the published method [19].

P. mirabilis O28 antiserum (1 ml) was absorbed at 4°C for 1 h with 1 ml packed sheep red blood cells coated with the respective antigen (200 µg/0.2 ml). The titre of antibodies after absorption was evaluated using passive immunohemolysis.

NMR spectroscopy. ^{13}C-NMR spectra were run with a Bruker AM-300 instrument in D_2O at 60°C. ^{1}H-NMR spectra were obtained using a Bruker AM-500 instrument in D_2O at 30°C and 70°C. The spectra were run at pH 3 and 6 for the intact and *O*-deacetylated polysaccharide, respectively. Acetone was used as internal standard (δ_H 2.225, δ_C 31.45). One-dimensional total correlation spectroscopy (TOCSY), NOE, two-dimensional correlation spectroscopy (COSY), and rotating frame NOE spectroscopy (ROESY) were performed as described [20, 21].

Sugar and amino acid analysis. Polysaccharide was hydrolysed with 2 M trifluoroacetic acid (1 ml, 120°C, 2 h), and the hydrolysate was analysed using a Biotronik LC-2000 sugar analyser as described previously [20] and a Biotronik LC-4010 amino acid analyser under the conventional conditions.

For determination of absolute configurations of monosaccharides and amino acids, the polysaccharide was heated with 0.1 ml (*R*)-2-butanol and 0.02 ml acetyl chloride for 3 h at 100°C with occasional ultrasonication. The solution was evaporated and the residue conventionally acetylated and analysed by GLC on a capillary column (25 m×0.2 mm) coated with SE-54.

***O*-Deacetylation.** The O-specific polysaccharide (50 mg) was heated with 12% aqueous ammonia (3 ml, 60°C, 2 h), the solution was concentrated and freeze-dried to give an *O*-deacetylated polysaccharide (45 mg).

Partial acid hydrolysis. The *O*-deacetylated polysaccharide (1 mg) was *N*-acetylated with acetic anhydride in saturated aqueous sodium hydrogen carbonate, hydrolysed with 0.1 M hydrochloric acid (100°C, 16 h), reduced with sodium borohydride, methylated with methyl iodide in dimethylsulfoxide in the presence of solid sodium hydroxide [22], and analysed by GLC/MS in both chemical ionisation and electron impact mode.

RESULTS

Characterisation and composition of the O-specific polysaccharide. The O-specific polysaccharide was obtained by cleavage with diluted acetic acid of LPS of *P. mirabilis* O28, isolated from dry bacterial cells by the phenol/water procedure [14].

Acid hydrolysis of the polysaccharide liberated galactose and galacturonic acid which were identified using a sugar analyser. Analysis of the hydrolysate on an amino acid analyser revealed the presence of glucosamine, serine, and lysine in almost equal amounts. Absolute configurations of the monosaccharides (all D) and the amino acids (both L) were determined by GLC of acetylated glycosides [23] or of esters with (*R*)-2-butanol.

The ^{13}C-NMR spectrum of the O-specific polysaccharide contained signals for four anomeric carbons in the region 96–103 ppm, three carbons bearing nitrogen at 54.7, 55.8, and 57.3 ppm, three hydroxymethyl groups at 61.5 ppm and 63.1 ppm (2C), 15 other carbons bearing oxygen in the region 66–83 ppm, four methylene groups of lysine at 23.1, 27.5, 32.9, and 40.7 ppm (data confirmed by the attached-proton test), one *N*-acetyl and one *O*-acetyl group (CH_3 at 23.7 and 21.4 ppm, respectively), and six CO groups in the region 170–178 ppm.

The ^{1}H-NMR spectrum of the O-specific polysaccharide contained seven signals in the low-field region (4.4–5.8 ppm) which belong to four anomeric protons and, probably, to H5 of GalA, and to a proton at a carbon bearing an acetoxyl group. In addition, there were signals for the *O*-acetyl group at 2.08 ppm, the *N*-acetyl group at 1.97 ppm (both singlets), four methylene groups of lysine at 1.3–1.9 (6H) and at 3.02 ppm (2H), and other protons in the region 3.3–4.4 ppm.

These data showed that the O-specific polysaccharide has a tetrasaccharide repeating unit containing two residues of D-GalA and one residue each of D-galactose, D-GlcNAc, L-serine, and L-lysine as well as one *O*-acetyl group. The absence from the spectrum of low-field signals in the region 83–88 ppm characteristic for furanoses [24] proved that all sugar residues are pyranosides.

Treatment of the O-specific polysaccharide with aqueous ammonia resulted in an *O*-deacetylated polysaccharide. Its ^{13}C-NMR (Fig. 1) and ^{1}H-NMR spectra (Fig. 2) lacked the signals for the *O*-acetyl group. The signal at 5.80 ppm in the ^{1}H-NMR spectrum shifted upon *O*-deacetylation up-field to 4.505 ppm, and thus belonged to a proton deshielded by the acetoxyl group. *O*-Deacetylation also caused changes in the ^{13}C-NMR spectrum which are discussed below.

Structure of carbohydrate chain of the *O*-deacetylated polysaccharide. The 500-MHz ^{1}H-NMR spectrum of the *O*-deacetylated polysaccharide was completely assigned with the help of sequential, selective spin-decoupling, one-dimensional TOCSY, and two-dimensional COSY (Table 1). The signals for two α-linked GalA residues (units **A** and **C**), α-linked galactose (unit **B**), and β-linked GlcNAc (unit **D**) were recognised on the basis of the coupling constants and multiplicity of the corresponding signals.

In the two-dimensional ROESY spectrum of the *O*-deacetylated polysaccharide (Fig. 3), the presence of the correlation peak at 4.544/4.271 ppm showed the spatial proximity of H1 of GlcNAc (unit **D**) and H4 of one of GalA (unit **A**) and thus, proved the 1,4-linkage between these sugar units. In turn, unit **A** was linked to the residue of galactose (unit **B**) at position 4, as followed from the correlation of the resonances for H1 **A** (5.049 ppm) and H4 **B** (4.126 ppm). Another correlation peak for H1 **A** (5.049/3.85 ppm) indicated its spatial interaction with H6a, H6b **B** that can be observed in α-1,4-linked disaccharides

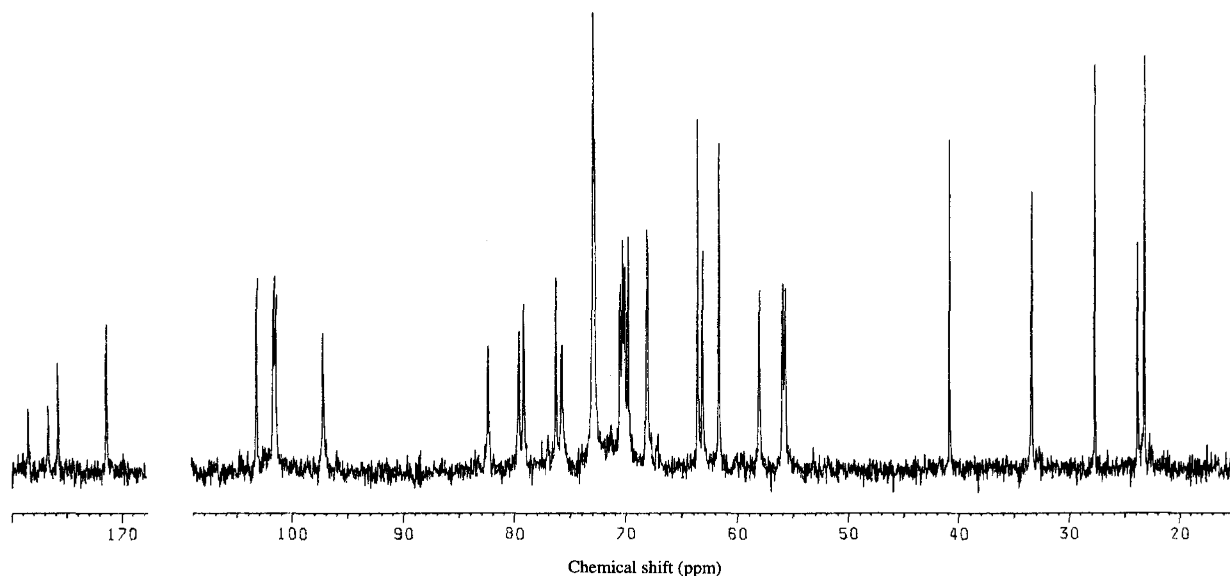


Fig. 1. 75-MHz ^{13}C-NMR spectrum of *P. mirabilis* O28 *O*-deacetylated polysaccharide.

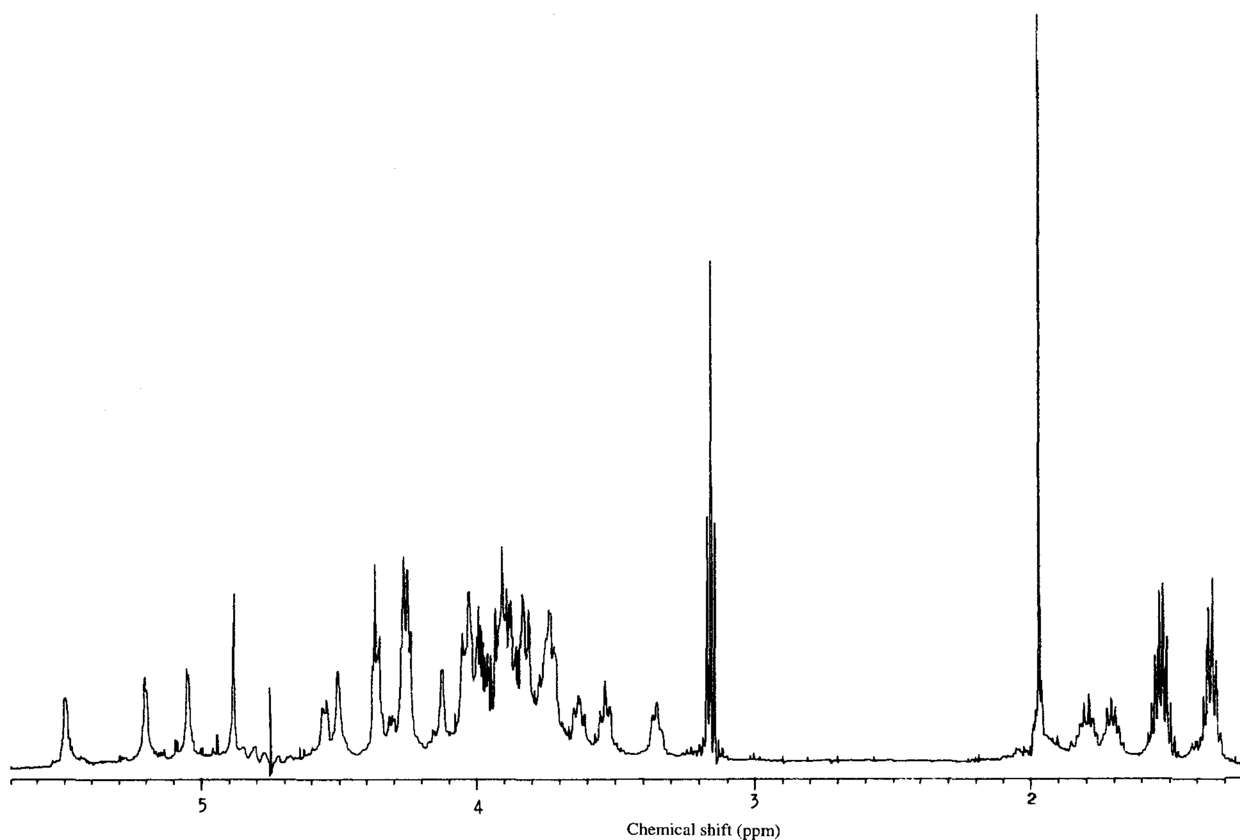


Fig. 2. 500-MHz ^{1}H-NMR spectrum of *P. mirabilis* O28 *O*-deacetylated polysaccharide.

with galactopyranose glycosylated by a sugar having the same absolute configuration [25].

Two correlation peaks (5.200/4.039 and 5.200/4.505 ppm) were observed also for H1 **B**. The correlating signals belonged to H3 and H4 of the second GalA (unit **C**) and proved the substitution of unit **C** by unit **B** at position 3. The NOE on the adjacent proton reflects the proximity of H1 **B** to H4 **C** characteristic for α1→3-linked disaccharides with the same absolute configuration of the constituent monosaccharides [25]. No NOE on H3 **C** would appear in the case of substitution of unit **C** at position 4. And, finally, the correlation of H1 **C** to H3 **D** (5.497/3.808 ppm) proved the 1,3-linkage between these two units.

Therefore, the *O*-deacetylated polysaccharide is linear and has the sequence of the monosaccharide residues **-A-B-C-D-** with units **A** and **B** substituted at position 4 and units **C** and **D** at position 3.

Table 1. Data of 500-MHz ^{1}H-NMR spectrum of *O*-deacetylated polysaccharide. Chemical shift for the *N*-acetyl group = 1.97 ppm.

Sugar unit	Chemical shift [coupling constant] for					
	H1	H2	H3	H4	H5	H6
	ppm [Hz]					
→4)GalAα (**A**)	5.049 [$J_{1,2}$ 3.6]	3.745 [$J_{2,3}$ 9.5]	4.046 [$J_{3,4}$ 3.5]	4.271 [$J_{4,5}$ <2]	4.880	
→4)Galα (**B**)	5.200 [$J_{1,2}$ 3.5]	3.885 [$J_{2,3}$ 9.8]	4.010 [$J_{3,4}$ 3.2]	4.126 [$J_{4,5}$ <2]	4.25[a] [$J_{5,6a}$ 4.5]	3.870[b] [$J_{5,6b}$ 4.8]
→3)GalAα (**C**)	5.497 [$J_{1,2}$ 3.4]	3.982 [$J_{2,3}$ 10]	4.039 [$J_{3,4}$ 3.3]	4.505 [$J_{4,5}$ <2]	4.354	
→3)GlcNAc (**D**)	4.544 [$J_{1,2}$ 8]	3.633 [$J_{2,3}$ 9.8]	3.808 [$J_{3,4}$ 9.5]	3.535 [$J_{4,5}$ 9.5]	3.351 [$J_{5,6a}$ 3.0]	3.726[c] [$J_{5,6b}$ 6.5]
Lys		4.255 [$J_{2,3}$ 6.4]	1.798[d] [$J_{3,4}$ 7.6]	1.341 [$J_{4,5}$ 7.6]	1.530 [$J_{5,6}$ 7.0]	3.159
Ser		4.373 [$J_{2,3a}$ 4.2]	3.940[e] [$J_{2,3b}$ 3.8]			

[a] Determined from the two-dimensional heteronuclear ^{13}C, ^{1}H COSY spectrum.
[b] H6a; H6b at 3.822 ppm [$J_{6a,6b}$ 12.0 Hz].
[c] H6a; H6b at 3.630 ppm [$J_{6a,6b}$ 12.0 Hz].
[d] H3a; H3b at 1.710 ppm.
[e] H3a; H3b at 3.892 ppm [$J_{3a,3b}$ 12.0 Hz].

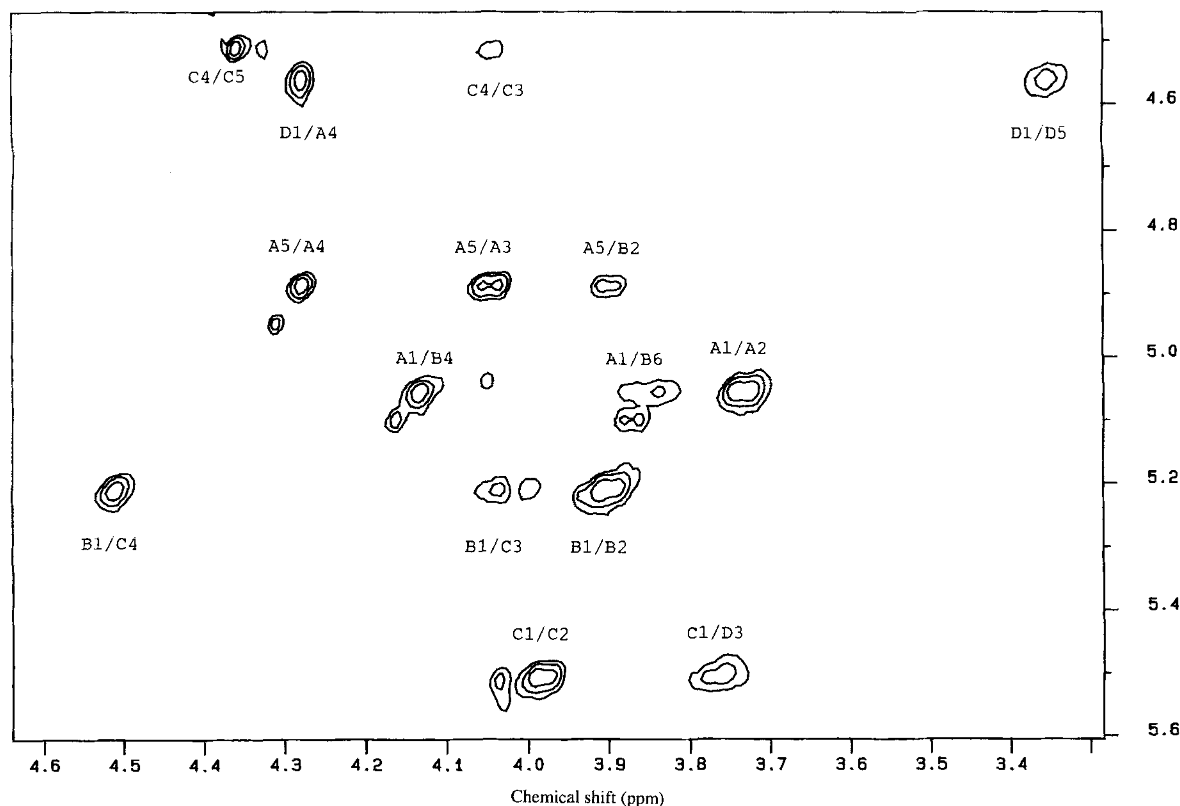


Fig. 3. Part of a two-dimensional 500-MHz ROESY spectrum of *P. mirabilis* O28 *O*-deacetylated polysaccharide.

Application of two-dimensional heteronuclear ^{13}C,^{1}H COSY provided complete assignment of the ^{13}C-NMR spectrum of the *O*-deacetylated polysaccharide (Table 2) which confirmed the NOE data. Marked downfield displacements greater than 5 ppm (α-effects of glycosylation) of the signals for C4 of units **A** and **B** and C3 of units **C** and **D**, as compared with their positions in the spectra of the corresponding unsubstituted monosaccharides [24], were evident of their glycosylation at the positions listed above.

The β-effects of glycosylation of unit **D** by unit **C** were found to be −2.3 ppm for C2 and +1.4 ppm for C4 that is characteristic for glycosylation of GlcNAc at position 3 by GalA having the same absolute configuration [26]. Thus, the analysis of the glycosylation effects on the ^{13}C chemical shifts and the

Table 2. Chemical shifts in 75-MHz ^{13}C-NMR spectra of polysaccharides. Chemical shift for the *N*-acetyl group 23.7 ppm (Me) and 175.5–175.7 ppm (CO), *O*-acetyl group 21.4 ppm (Me) and 174.0 ppm (CO).

Sugar unit		Chemical shift for					
		C1	C2	C3	C4	C5	C6
		ppm					
O-Deacetylated polysaccharide	→4)GalA*α* (**A**)	101.2	70.0	70.5	79.1	72.8	171.2
	→4)Gal*α* (**B**)	97.0	69.7	70.1	79.1	72.8	62.9
	→3)GalA*α* (**C**)	101.3	68.0	75.6	68.0	72.8	171.2
	→3)GlcNAc*β* (**D**)	103.0	55.7	82.5	72.6	76.2	61.5
	Lys	178.5	55.8	33.4	23.1	27.6	40.7
	Ser	176.4	57.9	63.4			
O-specific polysaccharide	→4)GalA*α* (**A**)	101.5	70.0	70.4	78.9	72.7	171.5
	→4)Gal*α* (**B**)	96.2	69.4	70.1	79.2	72.7	63.1
	→3)GalA*α* (**C**)	101.5	68.4	73.2	69.0	71.6	170.1
	→3)GlcNAc*β* (**D**)	103.0	55.8	82.9	72.7	76.1	61.5
	Lys	178.0[a]	54.7	32.9	23.1	27.5	40.7
	Ser	177.6[a]	57.3	63.1			

[a] Assignment could be interchanged.

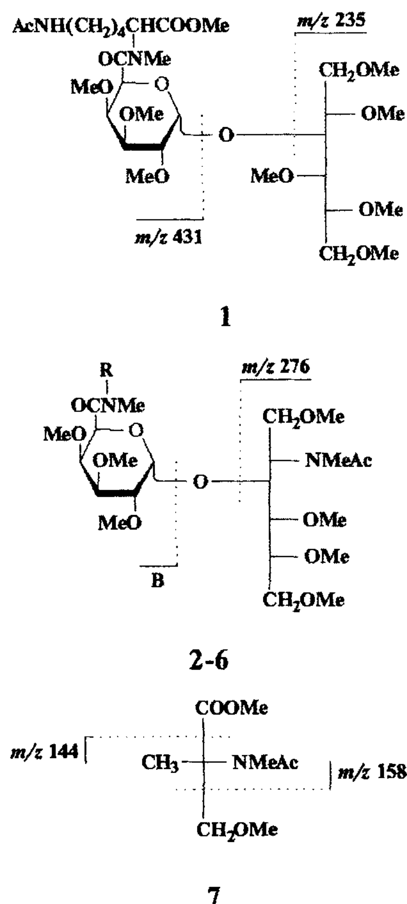
NOE data (see above) allowed independent determination of the relative absolute configuration of all constituent monosaccharides, which are in agreement with the results of GLC analysis of optically active glycosides with (*R*)-2-butanol.

Location of amino acids and the *O*-acetyl group; structure of the O-specific polysaccharide. The chemical shifts for C6 of GalA at 171.5 and 170.1 ppm (for units **A** and **C**, respectively) as well as for C1 of lysine and serine at 178.0 and 177.6 ppm in the ^{13}C-NMR spectrum of the O-specific polysaccharide indicated that the amino acids are amide-linked to the carboxyl group of GalA (see, for example, the published data [9, 10]). The positions of the signals for C2 and C6 of lysine at 54.7 and 40.7 ppm, respectively, are typical of acylation of this amino acid at the *α*-amino group [9, 10]. The positions of the signals for H5 of both GalA residues in the ^{1}H-NMR spectrum, unlike those in uronic acids with the free carboxyl group [27], did not significantly depend on the pD of the solution. In contrast, the signals for H2 of serine and lysine shifted markedly down-field by about 0.3 ppm upon change of pD from 7 to 2. These data confirmed amidation of both GalA residues by the amino acids, the latter having the free carboxyl group.

In the *O*-deacetylated polysaccharide, preirradiation of H5 **A** at 4.88 ppm caused significant NOEs on H3 and H4 of the same sugar residue and a small but observable NOE on H2 of lysine at 4.255 ppm. A similar experiment on unit **C** was performed with the O-specific polysaccharide because the signals for H5 **C** (4.46 ppm) and H2 of serine (4.34 ppm) are better separated than in the spectrum of the *O*-deacetylated polysaccharide. As a result of preirradiation of H5 **C,** a small NOE on H2 of serine was detected. Therefore, unit **A** carries lysine, whereas serine is attached to unit **C**.

This conclusion was confirmed by partial hydrolysis of the *O*-deacetylated and *N*-acetylated (at the free amino group of lysine) polysaccharide with 0.1 M hydrochloric acid at 100°C. Under these conditions the glycosidic linkages of galactose and GlcNAc were split selectively to give a mixture of disaccharides with amidated GalA derivative at the nonreducing end. After borohydride reduction, followed by methylation [22], a mixture of methylated glycosyl alditols were obtained which were studied by GLC/MS.

The only lysine-containing product found (**1**, M_r 682) corresponded to the fragment **A**→**B**. The presence in the electron impact mass spectrum of peaks with *m/z* 431 (glycosyl cation) and 235 corresponding to the residues of GalA(Lys) and galactitol, respectively, showed that GalA(Lys) is attached in the polysaccharide to galactose.



Instead of the expected serine-containing product **2** with M_r 640 and a peak of the glycosyl cation with *m/z* 348, a homologous compound **3** with M_r 654 and glycosyl cation with *m/z* 362 was revealed, which was derived by 2-*C*-methylation of the serine residue. Other identified products corresponding to the

Table 3. Characteristic ions derived from methylated compounds 2–6. The data of the chemical ionisation ($[M + H]^+$) and electron impact (glycosyl cation) mass spectra are given.

Compound	R	*m/z* for	
		$[M+H]^+$	glycosyl cation
2	$MeOCH_2CHCOOMe$	641	348
3	$MeOCH_2C(CH_3)COOMe$	655	362
4	$CH_2{=}CCOOMe$	609	316
5	$CH_3CH{=}CCOOMe$	623	330
6	$(CH_3)_2C{=}CCOOMe$	637	344

Table 4. Data of passive immunohemolysis. n.d., not determined.

Antigen (LPS-OH) of *P. mirabilis*	Reactivity (reciprocal titre) in passive immunohemolysis of antiserum against *P. mirabilis*		
	O28	S1959	R14
O28	5120	1 280	5 120
S1959	2560	10 240	10 240
R4/O28	10	n.d.	n.d.
R14/S1959	5120	10 240	10 240
R13/S1959	10	n.d.	n.d.
R110/S1959	10	n.d.	n.d.
R45/S1959	10	n.d.	n.d.

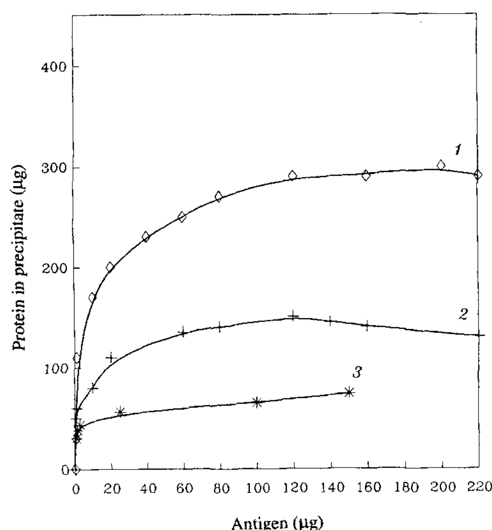


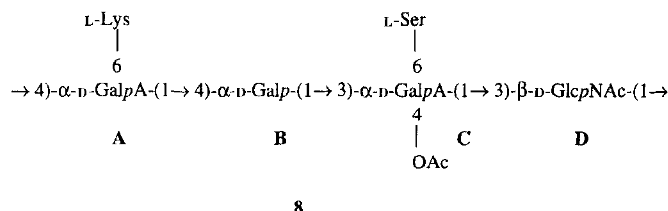
Fig. 4. Reactivity of *P. mirabilis* O28 antiserum with *P. mirabilis* O28 O-specific polysaccharide (1) and nonfractionated products of acid degradation of *P. mirabilis* O28 LPS (2) and of *P. mirabilis* R14 antiserum with *P. mirabilis* O28 O-specific polysaccharide (3) in quantitative precipitation test.

fragment **C**→**D** were a glycosyl alditol **4** with M_r 608, formed by elimination of methanol from **2**, and two minor homologous products with M_r 622 and 636, which were tentatively identified as *C*-methylation-elimination products **5** and **6** (Table 3). The presence in the electron impact mass spectra of the compounds **2–6** of the peak of the ion with *m/z* 276 corresponding to the residue of glucosaminitol confirmed that GalA(Ser) is attached in the polysaccharide to GlcNAc.

C-Methylation of serine under the applied conditions [22] was confirmed by a model experiment with *N*-acetyl-L-serine which afforded only one GLC-detectable product **7** with M_r 203 formed by 2-*C*-methylation. Similar by-products were obtained under the same conditions from the amide of GalA with threonine [10, 28].

As mentioned above, the signal at 4.505 ppm in the ^{1}H-NMR spectrum of the *O*-deacetylated polysaccharide shifted downfield to 5.80 ppm in the spectrum of the O-specific polysaccharide due to a strong deshielding effect of the acetoxyl group. Since this signal belongs to H4 of the 3-substituted GalA (unit **C**), the *O*-acetyl group is located at O4 of this residue. Such location was confirmed by displacements of the signals for C3, C4, and C5 of unit **C** from 75.6, 68.0, and 72.8 ppm in the ^{13}C-NMR spectrum of the *O*-deacetylated polysaccharide to 73.2, 69.0, and 71.6 ppm in the spectrum of the O-specific polysaccharide (Table 2), which are characteristic for α- and β-effects of *O*-acetylation of this unit at position 4 [29].

Therefore, based on these data, it was concluded that the O-specific polysaccharide of *P. mirabilis* O28 has the structure **8**.



Serological study. In study of the epitope specificity of the *P. mirabilis* O28 antigen, heterologous antigens (LPSs of *P. mirabilis* S1959 and a number of R-mutants) and the corresponding antisera were involved.

In a quantitative precipitation test, the shape of the precipitation curves of the reactions of the *P. mirabilis* O28 antiserum with nonfractionated products of acid degradation of the LPS and with the homologous O-specific polysaccharide as well as of the reaction of the *P. mirabilis* R14 antiserum with the O28 O-specific polysaccharide were similar (Fig. 4, curves 1–3), but the strongest reaction was observed for the O28 O-specific polysaccharide with the homologous antiserum (220 µg polysaccharide precipitated 290 µg antibodies from 100 µl nondiluted antiserum; Fig. 4, curve 1).

The ability of the *P. mirabilis* O28 antiserum to react with both homologous and heterologous antigens was demonstrated in passive immunohemolysis (Table 4). Marked reactions of the *P. mirabilis* O28 antiserum with the homologous alkali-treated LPS (LPS-OH) as well as with LPS-OH from *P. mirabilis* S1959 and R14 were observed. The antisera against *P. mirabilis* S1959 and R14 reacted similarly. There was no reaction between the O28 antiserum and LPS-OH of *P. mirabilis* R-mutants lacking the O-specific polysaccharide chain. Similar results were obtained in SDS/PAGE/Western blot (data not shown).

As expected, total absorption of the *P. mirabilis* O28 antiserum with LPS-OH of *P. mirabilis* O28 and S1959 was observed in the homologous test system and the heterologous test systems of *P. mirabilis* S1959 LPS-OH/*P. mirabilis* O28 antiserum and *P. mirabilis* R14 LPS-OH/*P. mirabilis* O28 antiserum (Table 5). Absorption with LPS-OH of *P. mirabilis* R14 resulted only in a decrease of the reactivity with the homologous antigen and the antigen of *P. mirabilis* S1959. These results suggested the pres-

Table 5. Data of passive immunohemolysis with absorbed *P. mirabilis* O28 antiserum.

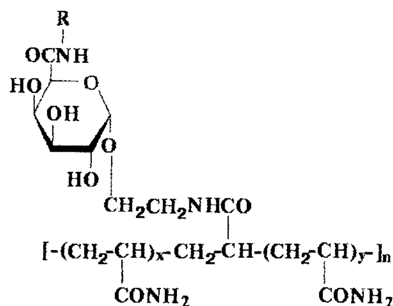
Antiserum absorbed with	Antigen (LPS-OH) of *P. mirabilis* used in passive immunohemolysis	Reciprocal titre
Sheep red blood cells	O28	5120
LPS-OH of *P. mirabilis*		
O28	O28	< 20
	S1959	< 20
	R14	< 20
S1959	O28	160
	S1959	< 20
	R14	< 20
R14	O28	2560
	S1959	1280
	R14	< 20

Table 6. Inhibition of passive immunohemolysis in test system *P. mirabilis* O28 LPS-OH/*P. mirabilis* O28 antiserum.

Inhibitor	Inhibitory dose
	ng
P. mirabilis	
O28 LPS	7.8
nonfractionated products of degradation of O28 LPS	3.9
O28 O-specific polysaccharide	1.9
O28 core oligosaccharide	>2000
S1959 LPS	7.8
R14 LPS	156.2
R110 LPS	>2000
R13 LPS	>2000
R4 LPS	>2000
R5 LPS	>2000
Synthetic antigens **9** containing	
D-GalA(D-Lys)	39.0
D-GalA(L-Lys)	78.1
D-GalA(L-Ala)	>5000
D-GalA(D-Ala)	>5000
D-GalA(L-Ser)	>5000
D-GalA(L-Thr)	>5000

ence of a common epitope in LPSs of *P. mirabilis* O28, S1959, and R14/1959.

For serological investigation of *Proteus* LPSs, related polyacrylamide-based synthetic antigens, containing amides of D-GalA with various amino acids (general structure **9**, where R is a residue of an amino acid linked via the α-amino group, see Table 6), have been synthesised [16]. The *P. mirabilis* LPSs, products of degradation of *P. mirabilis* O28 LPS, and the artificial antigens were tested as inhibitors of passive immunohemolysis in the homologous test system *P. mirabilis* O28 LPS-OH/ *P. mirabilis* O28 antiserum (Table 6). The reaction was strongly inhibited by LPS of *P. mirabilis* O28 and S1959 as well as by the O28 degradation products (except for the core oligosaccharide) and to a lower extent by *P. mirabilis* R14 LPS. LPSs of the R-mutant strains had no inhibiting activity. Of the synthetic glycopolymer antigens, positive results were observed only with those containing D-GalA(L-Lys) and D-GalA(D-Lys).



9

To confirm the immunodominant role of lysine, a diluted (1:20) sample of the *P. mirabilis* O28 antiserum was incubated with the synthetic antigens **9** or with *P. mirabilis* O28 LPS and then tested in passive immunohemolysis using sheep red blood cells coated with *P. mirabilis* O28 LPS-OH as antigen. As shown in Table 7, glycopolymers containing D-GalA(L-Lys) and D-GalA(D-Lys), but not D-GalA(L-Ser) inhibited the reactivity of the O28 antiserum.

Table 7. Data of passive immunohemolysis with *P. mirabilis* O28 LPS-OH and *P. mirabilis* O28 antiserum treated with synthetic antigens.

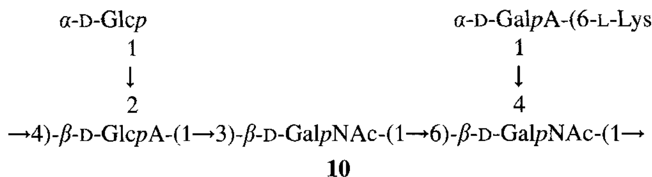
P. mirabilis O28 antiserum treated with	Reciprocal titre
P. mirabilis O28 LPS-OH	< 20
Synthetic antigens **9** containing	
D-GalA(D-Lys)	160
D-GalA(L-Lys)	320
D-GalA(L-Ser)	5120
D-GalA(L-Ala)	5120
Veronal buffer (control)	5120

DISCUSSION

The O-specific polysaccharide of *P. mirabilis* O28 has a unique structure **8** distinguished by the presence of two amides of D-galacturonic acid with amino acids (L-serine and L-lysine) in the tetrasaccharide repeating unit. One of them, D-GalA(L-Lys), has been earlier identified in the O-antigen of *P. mirabilis* S1959 [6, 7] and in LPS of *P. mirabilis* R14/S1959, which is a transient-like form corresponding to the T-form of *Salmonella* (Radziejewska-Lebrecht, J., unpublished data), while, to the best of our knowledge, D-GalA(L-Ser) has hitherto not been found in lipopolysaccharides. Uronic acids are common constituents of O-specific polysaccharides of *Proteus* [30] and some of them are amidated by L-amino acids; in addition to the amides listed above, these are D-GalA(L-Ala) [8, 9], D-GalA(L-Thr) [10], and D-GlcA(L-Lys) [8].

Serological study with synthetic glycopolymers **9** containing amides of D-GalA with various amino acids showed the importance of D-GalA(L-Lys) for the immunospecificity of the *P. mirabilis* O28 O-antigen, while no role of D-GalA(L-Ser) was revealed. Interestingly, the absolute configuration of lysine is apparently of no importance as the synthetic antigens containing amides of D-GalA with L-lysine and D-lysine were both good inhibitors (Tables 5 and 6). These data suggest that the immunodominant role in the corresponding epitope belongs to the $NH_2(CH_2)_4$ group of lysine rather than to the whole residue of

L-lysine or D-GalA(L-Lys). The presence of a common L-lysine-containing epitope seems to account also for the strong serological cross-reactions between LPSs of *P. mirabilis* O28 (structure **8**), S1959 (structure **10**), and R14/S1959 [the position of GalA(Lys) in this LPS remains unknown].



The authors thank Prof. Dr K. Kotelko for her current interest in this work and Mr H. Moll for help in GLC/MS analysis. This work was supported by grant 4 4401 91 02p (02) of the Sciences Research Committee (KBN, Poland) and by grant 93-03-5839 of the Russian Foundation in Fundamental Sciences.

REFERENCES

1. Rubin, R. H., Tolkoff-Rubin, N. E. & Cortan, R. S. (1986) Urinary tract infections, pyelonephritis and reflux nephropaty, in *The kidney* (Brenner, B. M. & Rector, F. C., eds) pp. 1085–1141, W. B. Sanders Co., Philadelphia.
2. Allison, C., Coleman, N., Jones, P. L. & Hughes, C. (1992) Ability of *Proteus mirabilis* to invade human urothelial cells is coupled to motility and swarming differentiation, *Infect. Immun. 60*, 4740–4746.
3. Loomes, L. M., Senior, B. W. & Kerr, M. A. (1992) Proteinases of *Proteus* spp.: Purification, properties, and detection in urine of infected patients, *Infect. Immun. 60*, 2267–2273.
4. Mobley, H. L. T. & Chippendale, G. R. (1990) Hemagglutinin, urease, and hemolysin production by *Proteus mirabilis* from clinical sources, *J. Infect. Dis. 161*, 525–530.
5. Penner, J. L. & Hennessy, J. N. (1980) Separate O-grouping schemes for serotyping clinical isolates of *Proteus vulgaris* and *Proteus mirabilis*, *J. Clin. Microbiol. 12*, 304–309.
6. Gromska, W. & Mayer, H. (1976) The linkage of lysine in the O-specific chains of *Proteus mirabilis* 1959, *Eur. J. Biochem. 62*, 391–399.
7. Kaca, W., Knirel, Y. A., Vinogradov, E. V. & Kotelko, K. (1987) Structure of the O-specific polysaccharide of *Proteus mirabilis* S1959, *Arch. Immunol. Ther. Exp. 35*, 431–437.
8. Vinogradov, E. V., Krajewska-Pietrasik, D., Kaca, W., Shashkov, A. S., Knirel, Y. A. & Kochetkov, N. K. (1989) Structure of *Proteus mirabilis* O27 O-specific polysaccharide containing amino acids and phosphoethanolamine, *Eur. J. Biochem. 185*, 645–650.
9. Vinogradov, E. V., Shashkov, A. S., Knirel, Y. A., Kochetkov, N. K., Sidorczyk, Z. & Swierzko, A. (1991) The structure of *Proteus penneri* strain 14 O-specific polysaccharide containing D- and L-alanine, *Carbohydr. Res. 219*, C1–C3.
10. Sidorczyk, Z., Swierzko, A., Knirel, Y. A., Vinogradov, E. V., Chernyak, A. Y., Kononov, L. O., Cedzynski, M., Rozalski, A., Kaca, W., Shashkov, A. S. & Kochetkov, N. K. (1995) Structure and epitope specificity of the O-antigen of *Proteus penneri* 12 (ATCC 33519) containing amide of D-galacturonic acid with L-threonine, *Eur. J. Biochem. 230*, 713–721.
11. Kotelko, K. (1986) *Proteus mirabilis*: Taxonomic position, pecularities of growth, components of the cell envelope, *Curr. Top. Microbiol. Immunol. 129*, 181–215.
13. Gromska, W., Kaca, W. & Kotelko, K. (1978) The role of lysine in the serological specificity of some *Proteus mirabilis* lipopolysaccharides, *Bull. Acad. Pol. Sci., Ser. Sci. Biol. 26*, 7–13.
14. Westphal, O. & Jann, K. (1965) Bacterial lipopolysaccharides: extraction with phenol-water and further application of the procedure, *Methods Carbohydr. Chem. 5*, 83–89.
15. Galanos, C., Lüderitz, O. & Westphal, O. (1969) A new method for the extraction of R lipopolysaccharides, *Eur. J. Biochem. 9*, 245–249.
16. Chernyak, A. Y., Kononov, L. O. & Kochetkov, N. K. (1994) Glycopolymers from synthetic fragments (amides of α-D-galacturonic acid with amino acids) of *Proteus* O-antigens, *J. Carbohydr. Chem. 13*, 383–396.
17. Schlecht, S. & Westphal, O. (1968) Über die Herstellung von Antiserum gegen die somatischen (O)-Antigene von *Salmonella*, III. Untersuchungen über prezipitierende Antiseren, *Zentralbl. Bakteriol. I Abt. Orig. 207*, 317.
18. Lowry, O. H., Roberts, N. R., Leiner, K. Y., Wu, M. L. & Farr, A. L. (1954) The quantitative histochemistry of brain, *J. Biol. Chem. 207*, 1–17.
19. Neter, E. (1956) Bacterial hemagglutination and hemolysis, *Bacterial Rev. 20*, 166–188.
20. Vinogradov, E. V., Sidorczyk, Z., Swierzko, A., Rozalski, A., Daeva, E. D., Shashkov, A. S., Knirel, Y. A. & Kochetkov, N. K. (1991) The structure of O-specific polysaccharide chain of *Proteus penneri* strain 16 lipopolysaccharide, *Eur. J. Biochem. 197*, 93–103.
21. Shashkov, A. S., Vinogradov, E. V., Knirel, Y. A., Nifant'ev, N. E., Kochetkov, N. K., Dabrowski, J., Kholodkova, E. V. & Stanislavsky, E. S. (1992) Structure of the O-specific polysaccharide of *Salmonella arizonae* O45, *Carbohydr. Res. 241*, 177–188.
22. Ciucanu, I. & Kerek, F. (1984) A simple and rapid method for the permethylation of carbohydrates, *Carbohydr. Res. 131*, 209–217.
23. Gerwig, G. J., Kamerling, J. P. & Vliegenthart, J. F. G. (1979) Determination of the D and L configuration of neutral monosaccharides by high-resolution capillary G. L. C., *Carbohydr. Res. 77*, 1–7.
24. Bock, K. & Pedersen, C. (1983) Carbon-13 nuclear magnetic resonance spectroscopy of monosaccharides, *Adv. Carbohydr. Chem. Biochem. 41*, 27–65.
25. Lipkind, G. M., Shashkov, A. S., Mamyan, S. S. & Kochetkov, N. K. (1989) The nuclear Overhauser effect and structural factors determining the conformations of disaccharide glycosides, *Carbohydr. Res. 181*, 1–12.
26. Shashkov, A. S., Lipkind, G. M., Knirel, Y. A. & Kochetkov, N. K. (1988) Stereochemical factors determining the effects of glycosylation on the ^{13}C chemical shifts in carbohydrates, *Magn. Reson. Chem. 26*, 735–747.
27. Vinogradov, E. V., Knirel, Y. A., Shashkov, A. S. & Kochetkov, N. K. (1987) Determination of the degree of amidation of 2-deoxy-2-formamido-D-galacturonic acid in O-specific polysaccharides of *Pseudomonas aeruginosa* O4 and related strains, *Carbohydr. Res. 170*, C1–C4.
28. Vinogradov, E. V., Knirel, Y. A., Kochetkov, N. K., Radziejewska-Lebrecht, J. & Kaca, W. (1993) Structural study of the O-antigens of *Proteus mirabilis* O28 and 3/6 containing amides of D-galacturonic acid with L-amino acids; β-elimination and *C*-methylation in L-serine and L-threonine in methylation analysis, *Bioorg. Khim. 19*, 1132–1136.
29. Jansson, P.-E., Kenne, L. & Schweda, E. J. (1987) Nuclear magnetic resonance and conformational studies on monoacetylated methyl D-gluco- and D-galacto-pyranosides, *J. Chem. Soc. Perkin Trans. 1*, 377–383.
30. Knirel, Y. A., Vinogradov, E. V., Shashkov, A. S., Sidorczyk, Z., Rozalski, A., Radziejewska-Lebrecht, J. & Kaca, W. (1993) Structural study of O-specific polysaccharides of *Proteus*, *J. Carbohydr. Chem. 12*, 379–414.