ELSEVIER

www.elsevier.nl/locate/carres

Carbohydrate Research 318 (1999) 186–192

CARBOHYDRATE RESEARCH

Note

Structure and cross-reactivity of the O-antigen of *Proteus vulgaris* O8

Andrei V. Perepelov [a], Dorota Babicka [b], Alexander S. Shashkov [a], Nikolay P. Arbatsky [a], Sof'ya N. Senchenkova [a], Antoni Rozalski [b], Yuriy A. Knirel [a,*]

[a] *N.D. Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, Leninsky Prospekt 47, R-117913 Moscow, Russia*

[b] *Institute of Microbiology and Immunology, University of Lodz, Banacha 12/16, PL-90-237 Lodz, Poland*

Received 14 January 1999; accepted 19 April 1999

Abstract

A high-molecular-mass O-specific polysaccharide was obtained by mild acid degradation of *Proteus vulgaris* O8 lipopolysaccharide followed by gel permeation chromatography. Studies of the polysaccharide by sugar and methylation analyses and ^{1}H and ^{13}C NMR spectroscopy, including 2D COSY, TOCSY, NOESY, and H-detected $^{1}H,^{13}C$ heteronuclear multiple-quantum coherence (HMQC) experiments, demonstrated the presence of a tetrasaccharide repeating unit having the following structure:

α-D-Gal*p*
1
↓
3
→3)-β-D-Glc*p*A-(1→4)-α-L-Fuc*p*NAc-(1→3)-α-D-Glc*p*NAc-(1→

The role of an epitope associated with the α-L-Fuc*p*NAc-(1 → 3)-D-Glc*p*NAc disaccharide in serological cross-reactivity of *P. vulgaris* O8 is discussed.

Keywords: Proteus vulgaris; Lipopolysaccharide; O-Specific polysaccharide structure; Serological cross-reactivity

1. Introduction

Bacteria of the genus *Proteus* are a common cause of urinary tract infections that can lead to severe complications, such as acute or chronic pyelonephritis and formation of bladder and kidney stones. Potential virulence factors of *Proteus* mediating the infectious processes are fimbriae, flagella, urease, proteases, hemolysins, invasiveness, capsular polysaccharide, and outer-membrane lipopolysaccharide (LPS) [1,2]. The structure of the polysaccharide chain of LPS (O-antigen) defines the serological O-specificity of the bac-

* Corresponding author. Tel.: +7-95-938-3613; fax: +7-95-135-5328.

E-mail address: knirel@ioc.ac.ru (Y.A. Knirel)

0008-6215/99/$ - see front matter
PII: S0008-6215(99)00101-9

teria. Based on the immunospecificity of the O-antigens, two species, *P. mirabilis* and *P. vulgaris*, were classified into 60 O-serogroups [3,4]. However, serological cross-reactivity of strains from different serogroups is not uncommon [5].

Structural and immunochemical investigations of *Proteus* O-antigens are necessary to understand the cross-reactivity of *Proteus* strains on the molecular level and to improve the serological classification of these clinically important bacteria. Structures of a number of O-antigens of *P. vulgaris*, *P. mirabilis* and *P. penneri* have been determined and correlated to the serological differentiation of the bacteria [5]. Now, we report the structure of a new O-specific polysaccharide of *P. vulgaris* O8 and the serological relatedness of this strain to *P. vulgaris* O12 and *P. mirabilis* O6.

The O-specific polysaccharide was obtained by mild acid degradation of LPS isolated from bacterial cells of *P. vulgaris* O8 by the phenol–water procedure [6], followed by gel permeation chromatography (GPC) on Sephadex G-50. Sugar analysis, after full acid hydrolysis of the polysaccharide, revealed Gal, GlcN and 2-amino-2,6-dideoxygalactose (FucN). Methanolysis of the polysaccharide, followed by GLC analysis of the derived acetylated methyl glycosides, resulted in the identification of GlcA. GLC analysis of acetylated glycosides with (*S*)-2-butanol and (*R*)-2-octanol revealed that Gal, GlcN and GlcA have the D configuration, whereas FucN has the L configuration.

The ^{13}C NMR spectrum of the polysaccharide was typical of a regular polymer (Fig. 1). It contained signals for four anomeric carbons at δ 99.6–105.3, two carbons bearing nitrogen (C-2 of GlcN and FucN) at δ 55.5 and 50.6, two $HOCH_2$–C groups (C-6 of GlcN and Gal) at δ 62.4 and 63.4, CH_3–C group (C-6 of FucN) at δ 16.7, COOH group (C-6 of GlcA) at 173.7, 12 other sugar ring carbons at δ 69.7–84.2, and two *N*-acetyl groups (CH_3 at δ 23.7 and 23.9, CO at δ 176.2 and 176.3). The ^1H NMR spectrum of the polysaccharide contained signals for four anomeric protons at δ 4.65–5.16, one signal for CH_3 group of FucN at 1.32, two *N*-acetyl groups at δ 2.03 and other signals at δ 3.58–4.46. Therefore, the polysaccharide has a tetrasaccharide repeating unit containing one residue each of D-galactose, 2-acetamido-2-deoxy-D-glucose, 2-acetamido-2,6-dideoxy-L-galactose, and D-glucuronic acid.

Methylation analysis of the polysaccharide using GLC–MS of partially methylated alditol acetates revealed terminal Gal, 3,4-disubstituted FucNAc, and 3-substituted GlcNAc. When the methylated polysaccharide was reduced with $LiBH_4$ prior to hydrolysis, 3,6-di-*O*-methylglucose was identified, which was evidently derived from 3-substituted GlcA.

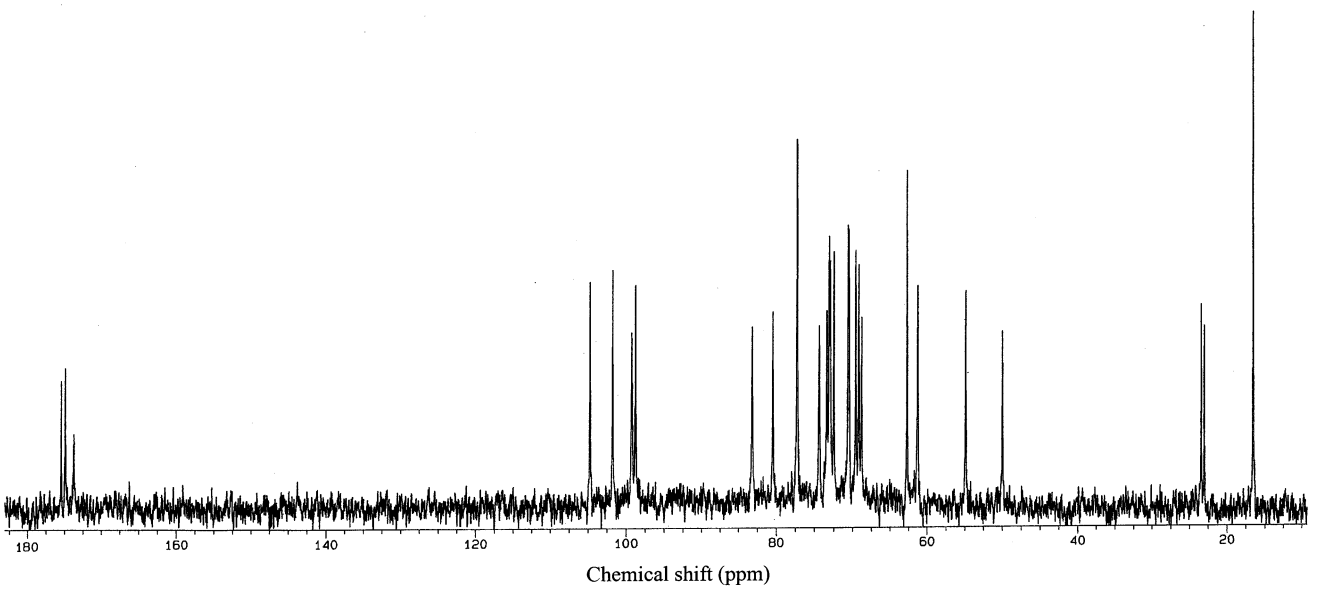

Fig. 1. 125 MHz ^{13}C NMR spectrum of the O-specific polysaccharide.

Table 1
^{1}H NMR data (δ, ppm) for the O-specific polysaccharide [a]

Sugar residue	Proton					
	H-1	H-2	H-3	H-4	H-5	H-6a,6b
→3)-α-D-Glc*p*NAc-(1→	5.16	4.12	3.90	3.62	4.08	3.85
→3)-β-D-Glc*p*A-(1→	4.65	3.58	3.63	3.83	3.83	
→4)-α-L-Fuc*p*NAc-(1→	5.04	4.44	4.03	4.12	4.46	1.32
α-D-Gal*p*-(1$^{\uparrow 3}$→	5.05	3.71	3.78	3.91	4.10	3.69

[a] The chemical shift for NAc is δ 2.03.

Therefore, the polysaccharide is branched, a FucNAc residue is at the branching point, the terminal position in the side chain is occupied by a Gal residue, and residues of GlcNAc and GlcA are monosubstituted at position 3.

The ^{1}H NMR spectrum of the polysaccharide was assigned using 2D COSY, TOCSY, NOESY, and H-detected ^{1}H,^{13}C HMQC experiments (Table 1). Spin systems of Gal, GlcNAc, FucNAc, and GlcA were identified based on the typical $^3J_{H,H}$ coupling constant values. GlcNAc and FucNAc were distinguished from GlcA and Gal by correlations in the HMQC spectrum of protons at carbons bearing nitrogen at δ 4.12 and 4.44 to the corresponding carbons at δ 55.5 and 50.6, respectively. The coupling constant value of $J_{1,2}$ 3–4 Hz indicated that residues of GlcNAc, FucNAc and Gal are α-linked, whereas the value of $J_{1,2}$ 8 Hz showed that GlcA is β-linked.

The TOCSY spectrum contained cross-peaks of H-1 with H-2–H-5 for GlcNAc and H-1 with H-2–H-4 for Gal and FucNAc, but only an H-1/H-2 cross-peak for GlcA. Assignment for GlcNAc was completed using the COSY spectrum, where a H-5/H-6 cross-peak was present at δ 4.08/3.85. The position of the Gal H-6 signal at δ 3.69 was found from the HMQC spectrum by its cross-peak with C-6 at δ 63.4. The Gal H-5 and FucNAc H-5 chemical shifts were determined from the COSY spectrum (Fig. 2), which displayed H-5/H-6 cross-peaks at δ 4.10/3.69 and 4.46/1.32, respectively. The positions of the signals for H-3 and H-4 of GlcA were found from the COSY spectrum, and that for H-5 from the NOESY spectrum which, as expected for a β-linked pyranoside, showed a strong H-1/H-5 cross-peak at δ 4.65/3.83. Likewise strong intraresidue H-1/H-2 cross-peaks at δ 5.16/4.12, 5.04/4.44 and 5.05/3.71 were observed in the NOESY spectrum for GlcNAc, FucNAc, and Gal, respectively, typical of α-linked pyranosides. The NOESY experiment revealed also the following strong interresidue cross-peaks between the transglycosidic protons: GlcNAc H-1/GlcA H-3, GlcA H-1/FucNAc H-4, FucNAc H-1/GlcNAc H-3 and Gal H-1/FucNAc H-3 at δ 5.16/3.63, 4.65/4.12, 5.04/3.90 and 5.05/4.03, respectively. These data demonstrated the full sequence and the modes of substitution of the monosaccharide residues in the polysaccharide.

The ^{13}C NMR spectrum of the polysaccharide was assigned using a 2D ^{1}H,^{13}C HMQC experiment (Table 2). The positions of the signals for C-2–C-6 of Gal at δ 70.4, 71.4, 71.5, 73.2 and 63.4, respectively, were close to those for the nonsubstituted α-galactopyranose [7]. Relatively low-field positions of the signals for C-3 of α-GlcNAc and β-GlcA at δ 78.3 and 84.2, as compared with their positions in the corresponding nonsubstituted monosaccharides at δ 71.74 and 76.53 [8], respectively, confirmed glycosylation of these sugar residues at O-3. Substitution of α-Fuc*p*NAc at O-3 and O-4 followed from downfield displacements of the signals for C-3 and C-4 to δ 75.3 and 81.1 (compare the chemical shifts of δ 68.7 and 72.4 for C-3 and C-4 of nonsubstituted α-Fuc*p*NAc [7], respectively). Therefore, the ^{13}C

chemical shift data were in full agreement with the NOESY and methylation analysis data.

On the basis of the data obtained, it was concluded that the O-specific polysaccharide of *P. vulgaris* O8 has the following structure:

α-D-Gal*p*
1
↓
3
→3)-β-D-Glc*p*A-(1→4)-α-L-Fuc*p*NAc -(1→3)-α-D-Glc*p*NAc-(1→

Proteus mirabilis O8

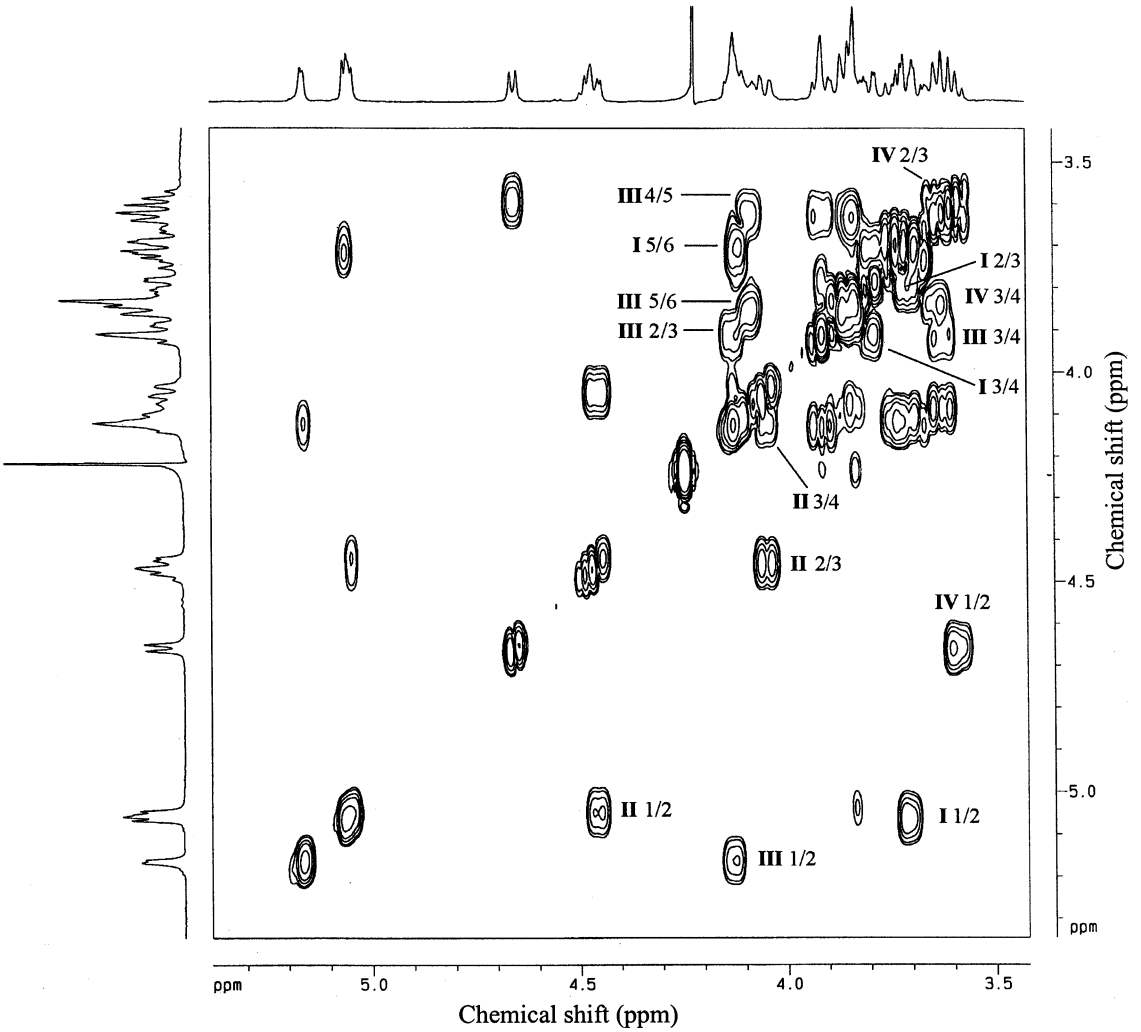

Fig. 2. Part of a 500 MHz 2D COSY spectrum of the O-specific polysaccharide. The corresponding parts of the ^{1}H NMR spectra are displayed along the vertical and horizontal axes. Arabic numerals refer to the atoms in sugar residues denoted by Roman numerals as follows: I, Gal; II, FucNAc; III, GlcNAc; IV, GlcA.

Table 2
^{13}C NMR data (δ, ppm) for the O-specific polysaccharide [a]

Sugar residue	Carbon					
	C-1	C-2	C-3	C-4	C-5	C-6
→3)-α-D-Glc*p*NAc-(1→	100.0	55.5	78.3	70.3	73.2	62.4
→3)-β-D-Glc*p*A-(1→	105.3	74.2	84.2	73.6	77.9	173.7
→4)-α-L-Fuc*p*NAc-(1→	99.6	50.6	75.3	81.1	69.7	16.7
α-D-Gal*p*-(1→	102.5	70.4	71.4	71.5	73.2	63.4

[a] The chemical shifts for NAc are δ 23.7, 23.9 (Me), 176.2 and 176.3 (CO).

LPSs of a number of *Proteus* strains with known structure of the O-antigen were tested with polyclonal rabbit anti-*P. vulgaris* O8 serum in serological assays. In a passive hemolysis test (PHT) and enzyme immunosorbent assay (EIA), anti-*P. vulgaris* O8 serum reacted with homologous LPS at a high titre of 1:51,200. From the heterologous LPSs, the strongest cross-reaction was observed between anti-*P. vulgaris* O8 serum and LPS of *P. mirabilis* O6 (the titres of the reaction were 1:3200 and 6400 in PHT and EIA, respectively). A marked cross-reaction was also demonstrated between anti-*P. mirabilis* O6 serum and *P. vulgaris* O8 LPS in EIA and PHT, respectively (the titre was 1:1600 in both assays). Another cross-reactive LPS of *P. vulgaris* O12 was less active and reacted at a titre of 1:800 with anti-*P. vulgaris* O8 serum in PHT only.

Cross-reactivity with heterologous O-antisera is not uncommon for *Proteus* strains and is due to the presence of common epitopes on LPSs [5]. Most often these epitopes are associated with the same or similar oligosaccharide fragments in the O-specific polysaccharide (e.g., see Ref. [9]). Structures of the cross-reactive O-antigens of *P. vulgaris* O12 and *P. mirabilis* O6 have been established (see below). They share with the O-antigen of *P. vulgaris* O8 an α-L-Fuc*p*NAc-(1→3)-β-D-Glc*p*NAc disaccharide fragment, which is important for manifesting of the immunospecificity of the O8 antigen. Remarkably, in all three O-antigens the α-L-Fuc*p*NAc residue is located at the branching point of the polysaccharide chain [10,11].

The role of an epitope associated with the α-L-Fuc*p*NAc-(1→3)-β-D-Glc*p*NAc disaccharide fragment in cross-reactivity of *Proteus* O-antigens has already been discussed [12]. A part of this epitope, a single →3)-β-D-Glc*p*NAc residue, was found to be sufficient to provide the cross-reactivity between *P. mirabilis* O6 and O23 [11]. A lower reactivity of anti-*P. vulgaris* O8 serum with LPSs of *P. mirabilis* O6 and *P. vulgaris* O12, as compared with the homologous LPS, was evidently accounted for by different structures of the remaining parts of the repeating units, which have different sizes and different sugar composition. The importance of the anomeric configuration of the D-GlcNAc residue, which is α in the homologous antigen and β in the heterologous antigens, cannot be excluded as well.

The same structure as determined in this work for the O-antigen of *P. vulgaris* O8 has been reported for the O-specific polysaccharide of a *Proteus* strain classified to serogroup O23 [13]. Later, a different structure was established for two strains of *P. mirabilis* O23 [11,14]. A serological study, which was undertaken by us to clarify this discrepancy, showed that the strain studied first was classified into serogroup O23 erroneously. Indeed, rabbit O-antiserum against this strain strongly reacted with LPS of *P. vulgaris* O8 and, vice versa, anti-*P. vulgaris* O8 O-serum strongly reacted with LPS of the mistakenly classified strain. None of the O-antisera reacted with LPSs of two strains of *Proteus* serogroup O23, *P. mirabilis* PrK 41/57 and *P. vulgaris* PrK 44/57, which were obtained from the Czech National Collection of Type Cultures. The structures of the O-antigens of these two strains are being investigated.

α-D-Glc*p*A
1
↓
3
→4)-α-L-Fuc*p*NAc-(1→3)-β-D-Glc*p*NAc-(1→

Proteus mirabilis O6

α-D-Glc*p*-(1→6)-α-D-Gal*p*NAc
1
↓
3
→6)-β-D-Glc*p*-(1→4)-α-L-Fuc*p*NAc-(1→3)-β-D-Glc*p*NAc-(1→3)-Gro-1-*P*-(O→

Proteus vulgaris O12 (authors' unpublished data)

2. Experimental

Bacterial strain, isolation and degradation of LPS.—*P. vulgaris* O8 (strain PrK 17/57) came from the Czech National Collection of Type Cultures (Institute of Epidemiology and Microbiology, Prague). The bacterium was grown as described [15]. LPS was isolated

from dried bacterial cells by phenol–water extraction [6] and purified by treatment with cold aq 50% CCl_3CO_2H, followed by dialysis of the supernatant. Delipidation of LPS (100 mg) was performed with 2% aq HOAc at 100 °C until lipid A precipitated out of solution [16]. The precipitate was removed by centrifugation (13,000*g*, 20 min), and the supernatant was fractionated on a column of Sephadex G-50 (Pharmacia, Sweden) in 0.05 M pyridinium acetate buffer pH 4.5, monitored by a Knauer differential refractometer (Germany). The yield of the high-molecular-mass O-specific polysaccharide was 27% of the LPS weight. Alkali-treated LPS was prepared by treatment of LPS with 0.25 M NaOMe in abs MeOH at 37 °C for 15 h [17], and the product was then dissolved in water and lyophilised.

Sugar analysis.—The polysaccharide was hydrolysed with 2 M CF_3COOH (120 °C, 2 h). Amino sugars were identified using a Biotronik LC-2000 amino acid analyser equipped with a column (0.4 × 22 cm) of Ostion LG AN B cation-exchange resin as described in Ref. [9]. Neutral sugars were identified using a Biotronik LC-2000 sugar analyser on a column (0.4 × 15 cm) of Dionex A × 8 anion-exchange resin using 0.5 M sodium borate buffer pH 8.0 at 65 °C. Uronic acids were analysed by GLC as acetylated methyl glycosides prepared by methanolysis of the polysaccharide with 1 M HCl in MeOH (80 °C, 16 h), followed by acetylation with a 1:1 pyridine–Ac_2O mixture (80 °C, 2 h). The absolute configurations of the monosaccharides were determined by GLC of acetylated (*S*)-2-butyl glycosides (for GlcN and GlcA) or (*R*)-2-octyl glycosides (for Gal and FucN) according to the published method [16,18], and modified as described in Ref. [19]. GLC was performed with a Hewlett–Packard model 5890 chromatograph equipped with an Ultra 2 capillary column using a temperature gradient of 160 °C (1 min) to 290 °C at 10 °C/min.

Methylation analysis.—The polysaccharide was methylated according to the Hakomori procedure [20], and the methylated polysaccharide was recovered by extraction with EtOAc. A part of it was hydrolysed as in sugar analysis, and partially methylated alditol acetates derived were identified by GLC–MS on a NERMAG R10-10L mass spectrometer (France) equipped with a DB-5 fused-silica capillary column using published data [21,22]. Another part was reduced with $LiBH_4$ in 70% aq 2-propanol (20 °C, 16 h), then hydrolysed and analysed as above.

NMR spectroscopy.—NMR spectra were recorded with a Bruker DRX-500 spectrometer for solutions in D_2O at 60 °C using internal acetone (δ_H 2.225, δ_C 31.45) as reference. Standard Bruker software (XWINNMR 1.2) was used to acquire and maintain the NMR data. A mixing time of 200 and 100 ms was used in 2D TOCSY and NOESY experiments, respectively.

Serological techniques.—Polyclonal rabbit anti-*P. vulgaris* O8 serum was prepared by immunisation of New Zealand white rabbits with heat-killed bacteria as described in Ref. [23]. PHT with alkali-treated LPS and EIA with LPS as antigen, were performed as described previously [9].

Acknowledgements

This work was supported by grants 99-04-48279 from the Russian Foundation for Basic Research and 4PO5A 140 14 from the Sciences Research Committee (KBN, Poland). We thank M. Wykrota and A. Ziolkowski for their excellent technical assistance.

References

[1] A. Rozalski, Z. Sidorczyk, K. Kotelko, *Microbiol. Mol. Biol. Rev.*, 61 (1997) 65–89.
[2] J.L. Penner, in A. Ballows, H.G. Trüper, W. Harder, H. Schleifer (Eds.), *The Genera Proteus, Providencia and Morganella*, Springer, Berlin, 1992, pp. 2849–2863.
[3] P. Larsson, *Methods Microbiol.*, 14 (1984) 187–214.
[4] J.L. Penner, C. Hennessy, *J. Clin. Microbiol.*, 12 (1980) 304–309.
[5] Y.A. Knirel, W. Kaca, A. Rozalski, Z. Sidorczyk, *Pol. J. Chem.*, 73 (1999) 895–907.
[6] O. Westphal, K. Jann, *Methods Carbohydr. Chem.*, 5 (1965) 83–91.
[7] G.M. Lipkind, A.S. Shashkov, Y.A. Knirel, E.V. Vinogradov, N.K. Kochetkov, *Carbohydr. Res.*, 175 (1988) 59–75.
[8] P.-E. Jansson, L. Kenne, G. Widmalm, *Carbohydr. Res.*, 188 (1989) 169–191.
[9] B. Bartodziejska, A.S. Shashkov, D. Babicka, A.V. Grachev, A. Torzewska, N.A. Paramonov, A.Y.

Chernyak, A. Rozalski, Y.A. Knirel, *Eur. J. Biochem.*, 256 (1998) 488–493.
[10] L.M. Beynon, A.J. Dumanski, R.J.C. McLean, L.L. MacLean, J.C. Richards, M.B. Perry, *J. Bacteriol.*, 174 (1992) 2172–2177.
[11] M. Cedzynski, A.S. Swierzko, A. Ziolkowski, A. Rozalski, W. Kaca, N.A. Paramonov, E.V. Vinogradov, Y.A. Knirel, *Microbiol. Immunol.*, 42 (1998) 7–14.
[12] Y.A. Knirel, E.V. Vinogradov, A.S. Shashkov, Z. Sidorczyk, A. Rozalski, I. Radziejewska-Lebrecht, W. Kaca, *J. Carbohydr. Chem.*, 12 (1993) 379–414.
[13] Y.A. Knirel, E.V. Vinogradov, A.S. Shashkov, Z. Sidorczyk, W. Kaca, A. Rozalski, K. Kotelko, N.K. Kochetkov, *Dokl. Akad. Nauk.*, 324 (1992) 333–338.
[14] D. Uhrín, V. Chandan, E. Altman, *Can. J. Chem.*, 73 (1995) 1600–1604.
[15] K. Kotelko, W. Gromska, M. Papierz, Z. Sidorczyk, D. Krajewska, K. Szer, *J. Hyg. Epidemiol. Microbiol. Immunol.*, 21 (1977) 271–284.
[16] K. Leontein, B. Lindberg, J. Lönngren, *Carbohydr. Res.*, 62 (1978) 359–362.
[17] I.R. Chester, G.W. Gray, S.G. Wilkinson, *Biochem. J.*, 126 (1972) 395–407.
[18] G.J. Gerwig, J.P. Kamerling, J.F.G. Vliegenthart, *Carbohydr. Res.*, 62 (1978) 349–357.
[19] A.S. Shashkov, S.N. Senchenkova, E.L. Nazarenko, V.A. Zubkov, N.M. Gorshkova, Y.A. Knirel, R.P. Gorshkova, *Carbohydr. Res.*, 303 (1997) 333–338.
[20] H.E. Conrad, *Methods Carbohydr. Chem.*, 6 (1972) 361–364.
[21] P.-E. Jansson, L. Kenne, H. Liedgren, B. Lindberg, J. Lönngren, *Chem. Commun. Univ. Stockholm*, 8 (1976) 1–75.
[22] G.O.H. Schwarzmann, R.W. Jeanloz, *Carbohydr. Res.*, 34 (1974) 161–168.
[23] B. Bartodziejska, A.S. Shashkov, A. Torzewska, A.V. Grachev, A. Ziolkowski, N.A. Paramonov, A. Rozalski, Y.A. Knirel, *Eur. J. Biochem.*, 259 (1998) 212–217.