ELSEVIER

Carbohydrate Research 266 (1995) 221–228

CARBOHYDRATE RESEARCH

Lipopolysaccharide core region of *Hafnia alvei*: structure elucidation using chemical methods, gas chromatography–mass spectrometry, and NMR spectroscopy ☆

Andrzej Gamian [a], Ewa Katzenellenbogen [a,*], Elżbieta Romanowska [a], Ursula Dabrowski [b], Janusz Dabrowski [b]

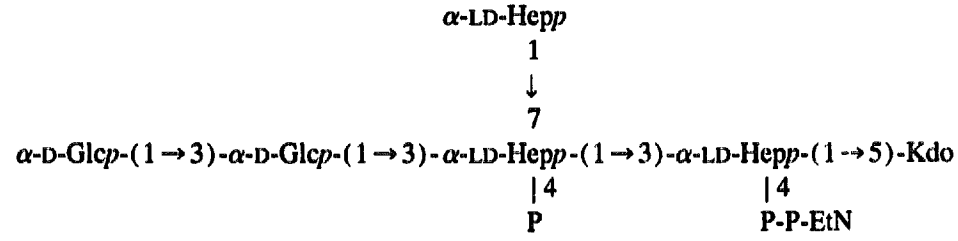
[a] *Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, ul. Czerska 12, PL-53-114 Wroclaw, Poland*

[b] *Max-Planck-Institut fur Medizinische Forschung, Heidelberg, Germany*

Received 5 April 1994; accepted 2 August 1994

Abstract

Sugar and methylation analysis with the use of gas chromatography–mass spectrometry and ^1H NMR spectroscopy proved that the core oligosaccharides isolated from lipopolysaccharides of eight *Hafnia alvei* strains have the identical hexasaccharide skeleton. However, ^1H,^{31}P heterocorrelated spectra showed that the phosphorylation pattern is not the same. The branched heptose for the ATCC 13337, 1187, 2, 1191, 1196, 1220, and 481L strains is phosphorylated as in the following formula, where P = –O–P(O)(O$^-$)$_2$ and P-PEtN = [–O–P(O)(O$^-$)]$_2$–O(CH$_2$)$_2$NH$_3^+$



☆ LDHep, L-*glycero*-D-*manno*-heptose; Kdo, 3-deoxy-D-*manno*-octulosonic acid; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; 1D and 2D, one- and two-dimensional; COSY, correlated spectroscopy; NOE, nuclear Overhauser effect; FABMS, fast atom bombardment-mass spectrometry

* Corresponding author.

SSDI 0008-6215(94)00266-5

A different phosphorylation pattern was found for the 1211 strain, where the branched heptose residue is 6-substituted by a monophosphorylethanolamine group, $\cdots \rightarrow 3(\rightarrow 7)(PEtN \rightarrow 6)$-α-LD-Hep*p*-$(1 \rightarrow 3)\cdots$, where $PEtN = -O-P(O)(O^-)-O(CH_2)_2NH_3^+$.

Keywords: Lipopolysaccharide ; Core oligosaccharide; *Hafnia alvei*; NMR

1. Introduction

The core structures of lipopolysaccharides of *Enterobacteriaceae* have been described for *Salmonella*, *Escherichia coli* [1], and *Shigella* [2–4]. Five distinct core regions: R_a, R_1, R_2, R_3, and R_4 are present in these genera [5]. Recently, we reported four novel core types which occur in *Citrobacter* lipopolysaccharides [6–9].

Our immunochemical studies have been extended to the lipopolysaccharides of *Hafnia alvei* genus and the structures of O-specific polysaccharides of strains ATCC 13337, 1187, 39, 2, 1211, 38, 1205, and 1191 have been elucidated [10–16]. The aim of the present work was to complete the structural data on core oligosaccharides isolated from lipopolysaccharides of eight *H. alvei* strains: ATCC 13337, 1187, 2, 1211, 1191, 1196, 1220, and 481L.

2. Experimental

Materials.—*Hafnia alvei* standard strain ATCC 13337 and strains 1187, 1191, 1196, 1211, and 1220 were derived from the collection of the Pasteur Institute (Paris), whereas strains 2 and 481L came from the collection of the Institute of Immunology and Experimental Therapy (Wroclaw).

The preparation of lipopolysaccharide, its mild acid hydrolysis, and fractionation of the carbohydrate material by means of gel permeation chromatography on Bio-Gel P-4 were described previously [6,7]. Core oligosaccharides were rechromatographed on columns of Bio-Gel P-4 or Bio-Gel P-2. The core oligosaccharide of strain 2 was further purified by use of affinity chromatography on Sepharose 4B-serotonin [12].

Analytical procedures.—The determination of sugar components in the form of alditol acetates, ethanolamine as free amino groups, phosphorus, 3-deoxy-octulosonic acid as well as methylation analysis were performed as reported earlier [3,6,7]. Dephosphorylation was carried out by treatment with 48% HF at 4°C for 3 days. The absolute configuration of sugar components was determined as reported previously [3]. The complete reduction of the carbonyl (with $NaBH_4$) and carboxyl (with EDC–$NaBH_4$) groups of the 3-deoxyoctulosonic acid residue in the core oligosaccharide was performed as described in Ref. [8]. High-voltage electrophoresis was performed using Whatman 1 paper, in the buffer 5:2:43 pyridine–acetic acid, pH 5.3. Electrophoregrams were run at 3.3 kV (45 V/cm) for 45 min and stained with ninhydrin.

Gas–liquid chromatography–mass spectrometry analysis was performed with a Hewlett–Packard 5971A system using an HP-1 glass capillary column (0.2 mm × 12 m) and a temperature program of 150 → 270°C at 8°C/min.

^1H and ^{31}P NMR.—The samples were exchanged twice with ^2H_2O with intermediate lyophilization, and then dissolved in 0.4 mL ^2H_2O containing a trace of acetone, the signal of which was set at 2.225 ppm as reference. The ^1H and ^{31}P NMR spectra were recorded on a Bruker AM 500 spectrometer operating at 500 and 202 MHz, respectively. Bruker standard software was applied for two-dimensional (2D) COSY and $^1H,^{31}P$ inverse heterocorrelated spectra. For COSY, 80 transients were accumulated, and the time-domain data matrix containing 1K × 256 points was multiplied before Fourier transformation by the sine-bell function and zero-filled, twice in t_1 and once in t_2, to give a digital resolution of 2.9 Hz in the frequency domain. For the heterocorrelated spectra, 96 transients were accumulated for each of the 256 experiments, the size of the time domain data matrix being 4K × 256 points. Before Fourier transformation, the matrix was zero-filled in the t_1-dimension and multiplied by a square sine-bell window function shifted by $\pi/4$ and $\pi/2$ in t_2 and t_1, respectively. The digital resolution in the frequency domain was 1.2 and 19.7 Hz per point for ^1H and ^{31}P, respectively. The one-dimensional (1D) TOCSY (total correlation spectroscopy) spectra and the 2D ROESY (rotating frame NOE) spectrum were recorded in the same way as reported previously [12].

3. Results and discussion

Isolation and chemical analysis of core oligosaccharides.—The lipopolysaccharide of each of the examined strains released a water-soluble carbohydrate portion after acid hydrolysis (1% AcOH, 100°C, 30–60 min). This was separated on a Bio-Gel P4 column (1.8 × 90 cm) into 4–5 fractions. Typically, fraction P_1 (50–70 mL) contained the polymeric O-specific polysaccharide described earlier [10–15]; fraction P_2 or P_3 (110–130 mL) contained the core oligosaccharide; fraction P_4 (140 mL) contained a trisaccharide of core origin, found only for strains 2 and 1211. The latter was recently found to be composed of galactose, heptose, and 3-deoxyoctulosonic acid [17]. Fraction P_5 contained free 3-deoxyoctulosonic acid. To improve their purity, the core oligosaccharide fractions were rechromatographed on a Bio-Gel P-2 column.

Sugar analysis of the core oligosaccharide fraction of all eight strains showed the same composition: two D-glucoses, three LD-heptoses, and Kdo. Apart from the sugar components, the oligosaccharides also contained phosphorus (5–6.5%) and ethanolamine (3–6%), which are characteristic of the core region of lipopolysaccharides.

Methylation analysis.—The core oligosaccharides of the eight strains were methylated before and after dephosphorylation, and after double (carbonyl–carboxyl) reduction and dephosphorylation. These results show that the dephosphorylated oligosaccharides are identical (Table 1). All the oligosaccharides examined contain terminal glucose and heptose, and 3-substituted glucose. Small amounts of 3,7-disubstituted heptose and traces of 3-substituted heptose were observed in intact samples because of some dephosphorylation during the course of methylation. 5-Substituted 3-deoxyoctulosonic acid was identified in the double-reduced and dephosphorylated core oligosaccharide.

The phosphorylated carbon atoms of the heptosyl residues were identified for the standard strain (ATCC 13337) core oligosaccharide. This oligosaccharide was methylated, dephosphorylated (48% HF at 4°C for 3 days), and then remethylated with deuterated methyl

Table 1
Methylation analysis of the core oligosaccharide[a] of *Hafnia alvei* strains before and after chemical degradation

Methylated sugar	t_R	*H. alvei* core oligosaccharide (molar ratio)		
		Intact	Dephosphorylated	Double-reduced
2,3,4,6-Me_4Glc	1.00	1.0	1.0	1.0
2,4,6-Me_3Glc	1.21	1.0	1.0	0.9
2,3,4,6,7-Me_5Hep	1.40	0.8	1.0	0.8
2,4,6,7-Me_4Hep	1.62	tr	0.9	0.8
2,4,6-Me_3Hep	1.90	0.2	1.0	1.0
1,2,4,6,7,8-Me_6Kdo	1.26	–	–	0.7

The core oligosaccharides of eight strains studied proved to be identical in methylation analysis; 2,3,4,6-Me_4Glc = 2,3,4,6-tetra-*O*-methyl-D-glucose, etc.; 1,2,4,6,7,8-Me_6Kdo = 1,2,4,6,7,8-hexa-*O*-methyl-5-acetyl-3-deoxyoctitol; t_R, retention time for the corresponding alditol acetate relative to that of 1,5-diacetyl tetra-*O*-methyl-D-glucitol (t_R 1.00) on an HP-1 glass capillary column at 150–270°C at 8°C/min; (–) indicates component not present; tr, trace amount.

iodide (C^2H_3I). The identification of 4-*O*-($^2H_3$)methyl-2,6,7-tri-*O*-methyl-1,3,5-tri-*O*-acetyl-heptitol and 4-*O*-($^2H_3$)methyl-2,6-di-*O*-methyl-1,3,5,7-tetra-*O*-acetyl-heptitol showed that the 3-substituted and 3,7-disubstituted heptose residues were originally both phosphorylated at C-4. The complete incorporation of deuteriomethyl groups at C-4 during the methylation analysis indicated stoichiometric phosphorylation of two heptose residues in the native oligosaccharide.

For the core oligosaccharide of strain 2, the localization and structure of the phosphorus-containing substituent of the unbranched heptose was established with the aid of the β-elimination reaction. The oligosaccharide was oxidized with periodate (0.1 M $NaIO_4$, 4°C, 48 h), and then treated with alkali (0.025 M NaOH, 37°C, 30 min). Diphosphorylethanolamine was identified in the mixture using high-voltage electrophoresis. The result of β-elimination showed that, in the core of strain 2, diphosphorylethanolamine substitutes the unbranched heptose at C-4.

The branched heptose of the *H. alvei* core of strain 2 is substituted only by phosphoryl, as proved by FABMS of the core oligosaccharide [18].

As will be shown in the next section, these partial results on the phosphorylation patterns cannot be extended to all of the eight core oligosaccharides.

NMR spectroscopy.—The oligosaccharide skeleton was identical for all the hexasaccharides investigated, but the phosphorylation pattern in the case of *H. alvei* strain 1211 was different from the remaining seven strains of this genus.

Full assignments of proton resonances for the strain 1211 core hexasaccharide (Table 2) were derived from 2D COSY and 1D TOCSY spectra, and sequence and linkage analysis were performed with the aid of a ROESY spectrum. The two glucose residues have an α configuration ($^3J_{1,2} \approx 3.9$). The α and β anomers of mannose-configured heptoses cannot be discriminated by coupling constants as these were small for both; however, the absence of the intraresidue H-1–H-3 and H-1–H-5 NOEs shows that H-1 is equatorial, i.e., the configuration is α, as expected for the core heptoses. The assignments for the remaining seven hexasaccharides were obtained in a similar manner, but linkage analysis was dispensed

Table 2
^{1}H chemical shifts for the core oligosaccharide of *H. alvei* strain 1211 [a]

Proton	Chemical shifts (ppm) in residue					
	V α-D-Glc*p*- (1→3)-	IV α-D-Glc*p*- (1→3)-	VI [α-LD-Hep*p*- (1→7)]	III α-LD-Hep*p*- (1→3)- 6PEtN [c]	II α-LD-Hep*p*- (1→5)- 4P-PEtN [d]	I Kdo [b]
H-1	5.371	5.306	4.975	5.138	5.207	
H-2	3.561	3.665	3.954	4.408	4.047	
H-3	3.764	3.931	3.904	4.05	4.114	1.921; 2.278
H-4	3.447	3.66	3.85	4.04	4.639	4.139
H-5	4.024	3.886	3.64	3.780	4.248	4.190
H-6	3.784	3.769	4.040	4.775	4.115	3.839
H-6′	3.844	3.956				
H-7			3.675	3.643	3.737	3.699
H-7′			3.748	3.894	3.737	
H-8						3.514
H-8′						3.902

[a] Chemical shifts for ^2H_2O solution are given relative to acetone set equal to 2.225 ppm at 307 K;
[b] Kdo = 3-deoxyoctulosonic acid;
[c] PEtN = –O–P(O)(O$^-$)–O(CH$_2$)$_2$NH$_3^+$; methylene proton signals at 3.31 and 4.18 ppm;
[d] P-PEtN = [–O–P(O)(O$^-$)]$_2$–O(CH$_2$)$_2$NH$_3^+$; methylene proton signals at 3.30 and 4.22 ppm.

Table 3
^{1}H chemical shifts for the core oligosaccharide of *H. alvei* strain ATCC 13337 [a]

Proton	Chemical shifts (ppm) in residue					
	V α-D-Glc*p*- (1→3)-	IV α-D-Glc*p*- (1→3)-	VI [α-LD-Hep*p*- (1→7)]	III α-LD-Hep*p*- (1→3)- 4P [c]	II α-LD-Hep*p*- (1→5)- 4P-PEtN [d]	I Kdo [b]
H-1	5.388	5.209	5.005	5.115	5.214	
H-2	3.556	3.659	3.934	4.404	4.015	
H-3	3.757	3.976	3.88	4.121	4.081	1.917; 2.280
H-4	3.458	3.675	3.859	4.423	4.634	4.125
H-5	4.019	3.94	3.625	3.808	4.233	4.185
H-6	3.782	3.795	4.050	4.241	4.113	3.820
H-6′	3.895	3.94				
H-7			3.647	3.595	3.731	3.696
H-7′			3.740	3.776	3.731	
H-8						3.481
H-8′						3.952

[a] Chemical shifts for ^2H_2O solution are given relative to acetone set equal to 2.225 ppm at 305 K;
[b] Kdo = 3-deoxyoctulosonic acid;
[c] P = –O–P(O)(O$^-$)$_2$;
[d] P-PEtN = [–O–P(O)(O$^-$)]$_2$–O(CH$_2$)$_2$NH$_3^+$; methylene proton signals at 3.301 and 4.219 ppm.

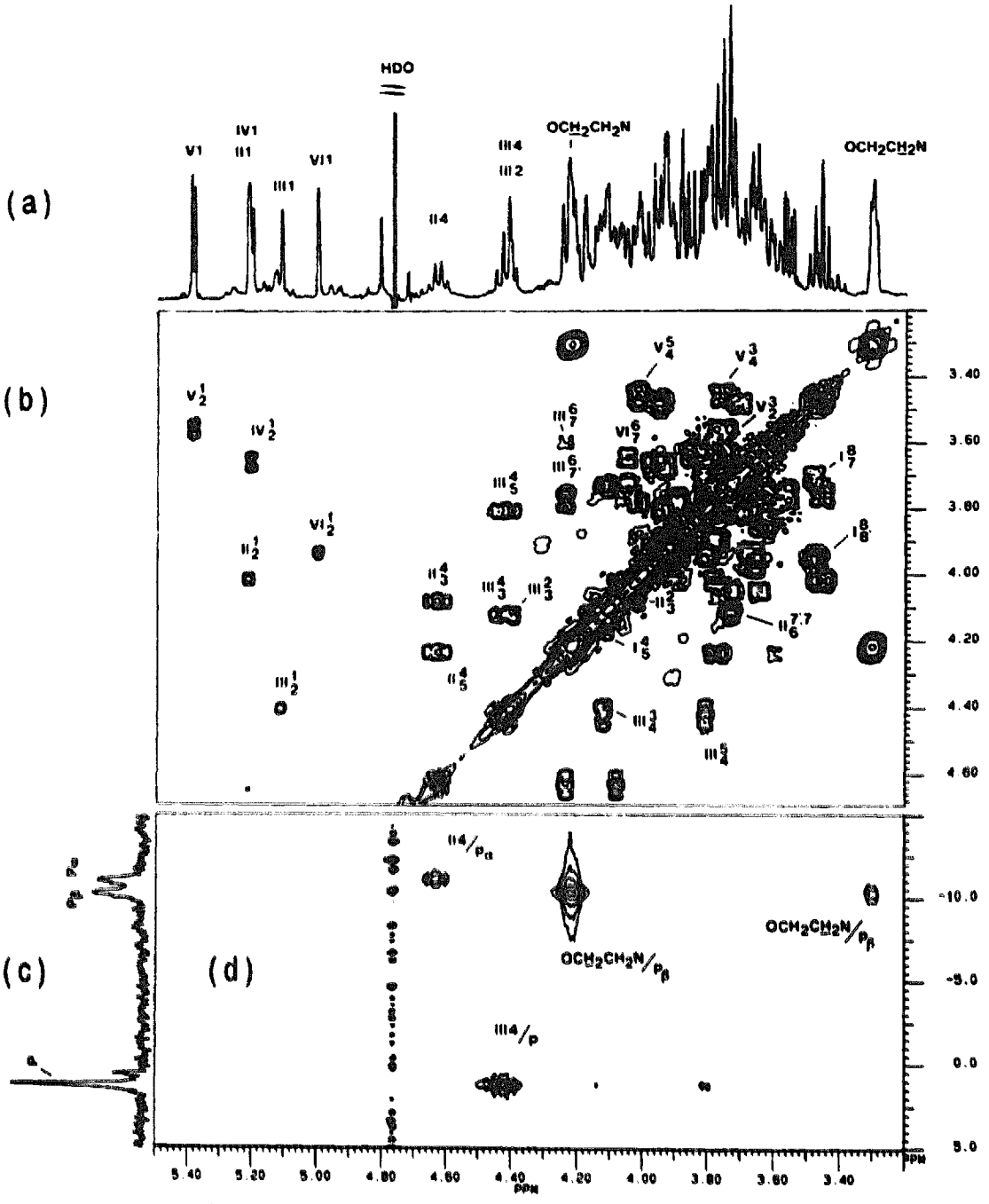


Fig. 1. Partial 500-MHz ^{1}H NMR and 202-MHz ^{31}P NMR spectra of the core oligosaccharide from *H. alvei* strain ATCC 13337. (a) 1D ^{1}H NMR spectrum. Roman numerals denote the sugar residues, and arabic numerals refer to the protons in these residues (for formula and labelling, see Table 3); OCH_2CH_2N indicates the methylene protons of the diphosphoryl-ethanolamino substituent. (b) 2D COSY spectrum. Chemical shifts of the protons to which the superscript and subscript refer should be read on the horizontal and vertical axes, respectively. (c) 1D ^{31}P NMR spectrum. P_α and P_β indicate the phosphorus atoms in the diphosphoryl-ethanolamino substituent, $-O-P_\alpha(O)(O^-)-O-P_\beta(O)(O^-)-O(CH_2)_2NH_3^+$, and P denotes the phosphorus atom of the monophosphate group, $-P(O)(O^-)_2$. (d) Part of the 500-MHz $^{1}H,^{31}P$ inverse correlated spectrum. The ordinate of each correlation contour defines the chemical shift of the phosphorus atom and the abcissa corresponds to the chemical shift of the proton coupled with this phosphorus.

with, in view of the similarity of the results of the methylation analysis obtained for all eight oligosaccharides (Table 1). The chemical shifts for the ATCC 13337 hexasaccharide, as a representative of this group of seven core oligosaccharides, are presented in Table 3 (cf. Fig. 1). Almost all of these chemical shifts are very similar to those obtained for *H. alvei* 1211 (Table 2), differing at most by several hundredths of a ppm, but the Hep-III H-4 and H-6 chemical shifts differ by several tenths of a ppm. The branched Hep-III residue of the 1211 hexasaccharide must be C-6 phosphorylated, since its H-6 resonance is shifted downfield by 0.534 ppm as compared with the same resonance for the ATCC 13337 hexasaccharide. Conversely, it is the H-4 resonance of Hep-III of the ATCC 13337 hexasaccharide which is shifted downfield (by 0.383 ppm), hence C-4 must be phosphorylated in this core. The almost identical extreme low-field position of the H-4 resonance for the unbranched Hep-II residues of both of these saccharides (4.639 and 4.634 ppm) shows both of them to be phosphorylated at C-4.

^{31}P NMR spectra and 2D heterocorrelated $^{1}H,^{31}P$ spectra confirmed the phosphorylation sites indicated by the ^{1}H NMR data and, in addition, enabled us to determine the nature and distribution of the phosphorus-containing substituents. Mono- and di-phosphoryl (pyrophosphoryl) groups can be unambiguously distinguished by ^{31}P chemical shifts (~ 0 and ~ -10 ppm, respectively [19]). The 1D ^{31}P NMR spectrum of the ATCC 13337 hexasaccharide (Fig. 1c) shows signals in both these regions, viz., at 1.2, -10.3, and -11.1 ppm. In the heterocorrelated spectrum (Fig. 1d), the -11.1 ppm resonance correlates with the II H-4 resonance, thus identifying both the attachment site in the Hep-II residue and the α phosphorus atom signal, P_α, of the pyrophosphoryl substituent. The P_β chemical shift at -10.3 ppm correlates with two chemical shifts (3.301 and 4.219 ppm) assigned in the proton spectra (Figs. 1a and b) to the protons of the ethanolamino group [20], hence the structure of the whole substituent at C-4 of the Hep-II residue is $-O-P_\alpha(O)(O^-)-O-P_\beta(O)(O^-)-O(CH_2)_2NH_3^+$. The phosphorus resonance at 1.2 ppm shows a cross-peak at the chemical shift of the Hep-III H-4, and two weak cross-peaks due to long-range coupling to the neighbouring H-3 and H-5. Since no coupling to ethanolamino protons is manifested, the monophosphoryl substituent at C-4 of the Hep-III residue must be an unsubstituted phosphate group, $-P(O)(O^-)_2$. Identical results have been obtained for the hexasaccharides from the strains 1187, 2, 1191, 1196, 1220, and 481L. The strain 1211 hexasaccharide is exceptional. While the type of phosphorylation of its Hep-II residue is the same as just described for the other strains, Hep-III is phosphorylated at C-6 by a monophosphorylethanolamino substituent, $-O-P(O)(O^-)-O(CH_2)_2NH_3^+$. This follows from the heterocorrelated spectrum (not shown), where the resonance of a monophosphoryl substituent at 0.25 ppm exhibits a connectivity with H-6 of the Hep-III residue and with two further resonances at 3.31 and 4.18 ppm, corresponding to ethanolamine methylene protons.

Recently, serological studies were performed on lipopolysaccharides of *Hafnia alvei* using immunoelectrophoresis and immunoblotting [21]. The results obtained proved that a common lipopolysaccharide core region exists in *Hafnia* genus. This conclusion is in complete agreement with the structural data presented above.

Acknowledgements

This work was supported by grant No. 406249101 from the Committee of Scientific Studies (KBN) to A.G., E.K., and E.R.

References

[1] P.E. Jansson, A.A. Lindberg, B. Lindberg, and R. Wollin, *Eur. J. Biochem.*, 115 (1981) 571–577.
[2] P.E. Jansson, B. Lindberg, A.A. Lindberg, and R. Wollin, *Carbohydr. Res.*, 68 (1979) 385–389.
[3] E. Katzenellenbogen and E. Romanowska, *Eur. J. Biochem.*, 113 (1980) 205–211.
[4] A. Gamian and E. Romanowska, *Eur. J. Biochem.*, 129 (1982) 105–109.
[5] O. Holst and H. Brade, *Bacterial Endotoxic Lipopolysaccharides*, CRC Press, Boca Raton, FL, Vol. 1, 1992, pp 135–170.
[6] E. Romanowska, A. Gamian, and J. Dabrowski, *Eur. J. Biochem.*, 161 (1986) 557–564.
[7] E. Romanowska, A. Gamian, C. Lugowski, A Romanowska, J. Dabrowski, M. Hauck, H.J. Opferkuch, and C.W. von der Lieth, *Biochemistry*, 27 (1988) 4153–4161.
[8] A. Gamian and E. Romanowska, *Carbohydr. Res.*, 198 (1990) 381–383.
[9] E. Katzenellenbogen, A. Gamian, E. Romanowska, U. Dabrowski, and J. Dabrowski, *Eur. J. Biochem.*, 196 (1991) 197–201.
[10] E. Katzenellenbogen, E. Romanowska, N.A. Kocharova, A.S. Shashkov, G.M. Lipkind, Y.A. Knirel, and N.K. Kochetkov, *Carbohydr. Res.*, 203 (1990) 219–227.
[11] A. Gamian, E. Romanowska, H.J. Opferkuch, M. Hauck, and J. Dabrowski, *Eur. J. Biochem.*, 186 (1989) 611–620.
[12] A. Gamian, E. Romanowska, U. Dabrowski, and J. Dabrowski, *Biochemistry*, 30 (1991) 5032–5038.
[13] E. Katzenellenbogen, E. Romanowska, U. Dabrowski, and J. Dabrowski, *Eur. J. Biochem.*, 200 (1991) 401–407.
[14] E. Katzenellenbogen, E. Romanowska, D. Witkowska, and A.S. Shashkov, *Carbohydr. Res.*, 231 (1992) 51–54.
[15] E. Katzenellenbogen, E. Romanowska, N.A. Kocharova, Y.A. Knirel, A.S. Shashkov, and N.K. Kochetkov, *Carbohydr. Res.*, 231 (1992) 249–260.
[16] A. Gamian, E. Romanowska, U. Dabrowski, and J. Dabrowski, *Eur. J. Biochem.*, 213 (1993) 1255–1260.
[17] E. Katzenellenbogen, A. Gamian, E. Romanowska, U. Dabrowski, and J. Dabrowski, *Biochem. Biophys. Res. Commun.*, 194 (1993) 1058–1064.
[18] A. Gamian, J. Ulrich, J. Defaye, M. Mieszala, E. Romanowska, in preparation.
[19] I.D. Campbell and C.M. Dobson, *Methods Biochem. Anal.*, 25 (1979) 1–133.
[20] U. Dabrowski, J. Dabrowski, F. Helling and H. Wiegandt, *J. Biol. Chem.*, 265 (1990) 9737–9743.
[21] C. Lugowski, W. Jachymek, T. Niedziela, A. Romanowska, D. Witkowska, E. Romanowska, *FEMS Immunol. Med. Microbiol.*, in press.