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Note

Structure of the lipopolysaccharide core region of *Hafnia alvei* strains 1185 and 1204

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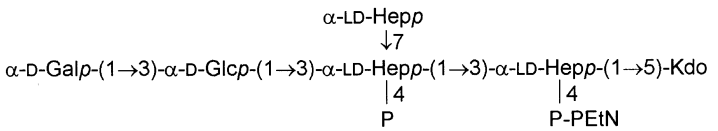
Abstract

Sugar and methylation analyses using gas chromatography/mass spectrometry and NMR spectroscopy proved that the core oligosaccharides of *Hafnia alvei* strains 1185 and 1204 have the following formula:

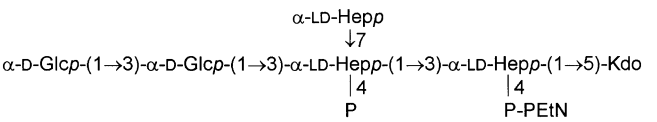


where Kdo = 3-deoxy-oct-2-ulosonic acid and P–PEtN = diphosphorylethanolamine. The structure shown above is a slight modification of the typical core region of *H. alvei* lipopolysaccharides. The difference refers to one sugar only: terminal galactose is present in the core of strains of 1185 and 1204, while terminal glucose in the typical core.

Keywords: LPS core; Heptose; O-Specific polysaccharides

1. Introduction

In the studies on carbohydrate moieties of *Hafnia alvei* lipopolysaccharides comprising over 20 strains, the structures of their O-specific polysaccharides (see Ref. [1] and references cited therein) and core oligosaccharides [2,3] were elucidated. It was found that in the entire *Hafnia* genus a prevailing structure of lipopolysaccharide core region is a hexasaccharide composed as follows [2]:



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Recently, in three *H. alvei* strains (23, 1222 and 39) non-typical core regions were described [3]. The core oligosaccharides of strains 23 and 1222 have the same structure as *Escherichia coli* R4 core region, and the core oligosaccharide of strain 39 has the structure of *Salmonella* Ra core. In this paper we report on a new variant of *Hafnia alvei* core region identified in strains 1185 and 1204.

The water-soluble carbohydrate portion obtained from LPS after acid hydrolysis was separated on Sephadex G-50 column into four fractions. Fraction 3 (200–250 mL) contained core oligosaccharide, which was a subject of the present studies. To improve its purity, the core oligosaccharide fraction was rechromatographed on Bio-Gel P-2 column. Sugar analysis of the core oligosaccharides of strains 1185 and 1204 showed the same composition: one D-galactose, one D-glucose, three L-glycero-D-manno-heptoses and Kdo. Apart from the sugar components, the oligosaccharides contained phosphorus (5.5–6%) and ethanolamine (4–6%), typical components of LPS core region.

The core oligosaccharides of both *H. alvei* strains were submitted to various chemical modifications: (1) dephosphorylation, (2) double (carbonyl–carboxyl) reduction and dephosphorylation, and (3) double reduction, dephosphorylation and Smith degradation (periodate oxidation followed by $NaBH_4$ reduction). In order to reduce carbonyl and carboxyl groups of 3-deoxy-octulosonic acid residue which form at the terminal reducing end, the double reduction of the core oligosaccharide was used. To localize the branch point in the heptose region, the oligosaccharide was submitted twice to Smith degradation. In the first degradation, sodium borohydride and, in the second, sodium borodeuteride was used for reduction. The intact core oligosaccharides and those after the modifications were methylated. The results of methylation analysis (Table 1) showed that oligosaccharides of both strains contained terminal galactose, terminal heptose and 3-substituted glucose. Trace amounts of 3,7-di-substituted and 3-substituted heptoses were present in the intact samples because of some dephosphorylation during the course of methylation. The 5-substituted 3-deoxyoctitol was determined in double reduced and dephosphorylated core oligosaccharide. The confirmation of the last result was obtained by methylation analysis of the material double reduced, dephosphorylated and Smith degraded. Terminal glucose (obtained from 3-substituted glucose), 3-substituted mannose (from 3-substituted heptose), 3-substituted heptose (from 3,7-disubstituted heptose) and 4-substituted 2-deoxyribitol (from 5-substituted 3-deoxyoctulosonic acid) were identified in this material.

Table 1
Methylation analysis of the core oligosaccharide [a] isolated from *H. alvei* strains 1185 and 1204 before and after chemical degradations

Methylated sugar	t_R	*H. alvei* core oligosaccharide (molar ratio)			
		Intact	Dephosphorylated	Double-reduced	Double-reduced Smith-degraded
2,3,4,6-Me_4Glc	1.00				1.0
2,3,4,6-Me_4Gal	1.05	0.9	0.9	0.8	
2,4,6-Me_3Glc	1.21	1.0	1.0	0.9	
2,4,6-Me_3Man	1.23				0.8
2,3,4,6,7-Me_5Hep	1.40	0.9	1.0	0.8	
2,4,6,7-Me_4Hep	1.62	tr	0.9	0.8	0.8
2,4,6-Me_3Hep	1.90	tr	1.0	1.0	
1,2,4,6,7,8-Me_6Kdo	1.26			0.7	
1,3,5-2dMe_3Rib	0.65				0.4

[a] The core oligosaccharide of strains 1185 and 1204 proved to be identical in methylation analysis; 1,2,4,6,7,8-Me_6Kdo = 1,2,4,6,7,8-hexa-*O*-methyl-5-*O*-acetyl-3-deoxyoctitol; 1,3,5-2dMe_3Rib = 1,3,5-tri-*O*-methyl-4-*O*-acetyl-2-deoxyribitol; t_R, retention time for the corresponding alditol acetate relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol (t_R 1.00); a blank cell indicates component not present; tr, trace amount.

Table 2
^{1}H chemical shifts for the core oligosaccharide of *H. alvei* strain 1204

Proton	Chemical shifts (ppm) in residue					
	V α-D-Gal*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 [a]	**II** α-LD-Hep*p*-(1→5)-4P-PEtN [b]	**I** Kdo
H-1	5.46	5.22	5.01	5.12	5.23	
H-2	3.82	3.66	3.94	4.41	4.04	
H-3	3.91	3.99	3.88	4.12	4.09	1.92; 2.28
H-4	4.01	3.69	3.88	4.43	4.62	4.13
H-5		3.94	3.63	3.81	4.23	4.19
H-6			4.05	4.24	4.10	
H-6′			4.05	4.24	4.10	
H-7					3.77	
H-7′					3.77	
OCH_2					4.21	
CH_2N					3.30	

[a] P = –O–P(O)(O$^-$)$_2$.
[b] P–PEtN = [–O–P(O)(O$^-$)]$_2$–O(CH$_2$)$_2$NH$_3^+$.

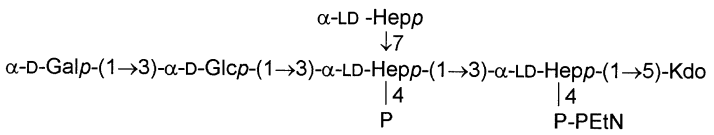
To determine the phosphorylated oxygen atoms of the heptosyl residues of core oligosaccharide of *H. alvei* strain 1185, it was methylated, dephosphorylated and then remethylated with deuterated methyl iodide (C^2H_3I). The identification of 4-*O*-trideuteriomethyl-2,6,7-tri-*O*-methyl-1,3,5-tri-*O*-acetyl-heptitol and 4-*O*-trideuteriomethyl-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 both originally phosphorylated at O-4. The complete incorporation of deuteriomethyl groups at O-4 during the methylation procedure indicated stoichiometric phosphorylation of two heptose residues in native oligosaccharide.

NMR spectroscopy.—Partial assignments of proton resonances for the core hexasaccharide from strain 1204 were derived from 2D COSY and TOCSY spectra starting from the resonances unequivocally identified by their chemical shifts (Table 2). A part of double-quantum filtered COSY spectrum of original core oligosaccharide from *H. alvei* strain 1204 is shown on Fig. 1. The anomeric configuration and the type of sugar residue **V** have been determined on the basis of scalar coupling constants as an α-galactose. A value of $^3J_{H\text{-}1,H\text{-}2} = 3.8$ Hz is typical for α configuration (H_{ax}–H_{eq}) of this residue. In case of β-Gal its diaxial coupling constant $^3J_{H\text{-}1,H\text{-}2} > 7$ Hz, like it is for the coupling constant $^3J_{H\text{-}2,H\text{-}3} = 10.6$ Hz. The coupling constant of $^3J_{H\text{-}3,H\text{-}4} = 2.5$ Hz indicates again on the mutual configuration H_{ax}–H_{eq}, what means that H-4 is the equatorial proton giving the evidence that sugar residue **V** is a galactose. The connection **V** α-Gal-(1→3)-**IV** α-Glc was determined on the basis of the correlation at NOESY spectrum. Strong correlation **V** H-1/**IV** H-3 and weak correlation **V** H-1/**IV** H-2 or **V** H-1/**IV** H-4 have been observed. The chemical shifts of residues, except galactose, were the same as for proton signals in the the typical core oligosaccharide of standard strain of *H. alvei* ATCC 13337 published previously [2].

The ^{31}P spectrum of original core sample from *H. alvei* 1185 revealed, besides the expected signals with chemical shifts at 1.20, −10.34 and −11.16 ppm, also few minor signals present in the region of signals of phosphate residues, namely at 2.45, 0.68 and 0.56 ppm. The sample of the dephosphorylated core oligosaccharide possessed a signal at 1.34 ppm what means that the dephosphorylation of this sample was not complete. This indicates that the original native sample from 1185 strain is a heterogeneous material regarding to the minor substitutions with phosphate residues. The heterogeneity of the 1185 core

material could be due to the migration of phosphate substituents. The majority of NMR experiments has been performed on the more homogenous core oligosaccharide from strain 1204. Its ^{31}P spectrum contained three expected signals of chemical shifts at 1.20, −10.41 and −11.20 ppm. On the correlation spectrum $^{1}H-^{31}P$ HMQC (Fig. 2) the expected correlations were seen: 4.43/1.20 (**III** Hep H-4/P), 4.63/−11.20 (**II** Hep H-4/Pα) and 4.21/−10.41 (CH_2/Pβ).

On the basis of the data obtained, it was concluded that the core oligosaccharides isolated from strains 1185 and 1204 have the identical hexasaccharide structure of the following formula:



The oligosaccharide presented above has a slightly modified structure of the typical core oligosaccharide of *H. alvei* (see Section 1). They both differ in one structural element only: terminal galactose in the core of strains 1185 and 1204 is present in the place of terminal glucose of the typical core.

2. Experimental

Materials.—*H. alvei* strains PCM 1185 and PCM 1204 were derived from the collection of Pasteur Institute (Paris). The preparation of lipopolysaccharide (LPS), its mild hydrolysis (300 mg/30 mL 1% AcOH, 100 °C, 1 h) and fractionation of the carbohydrate material us-

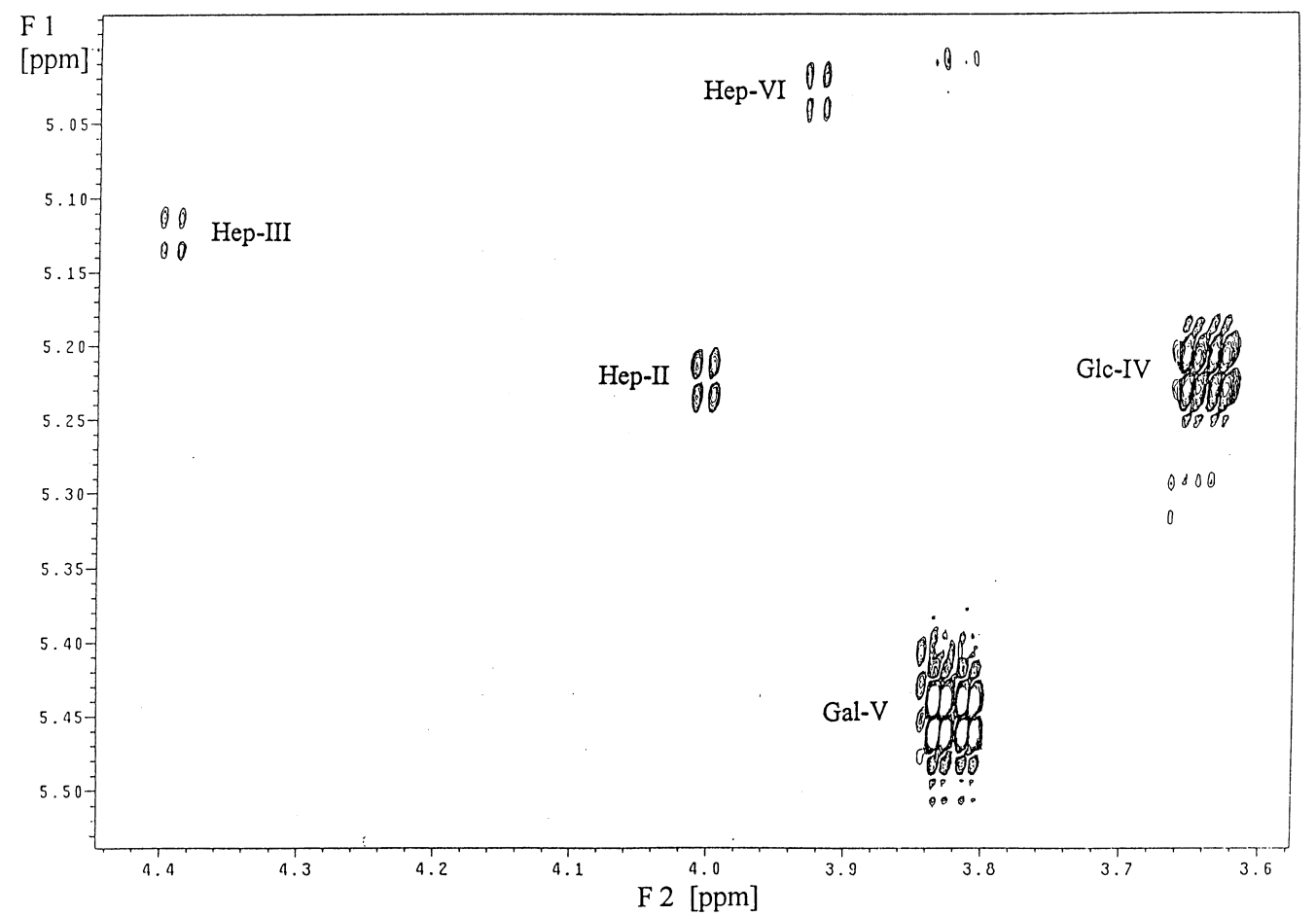


Fig. 1. Part of the double-quantum filtered COSY spectrum of the core oligosaccharide from *H. alvei* strain 1204 displaying H-1–H-2 cross-peaks corresponding to the correlations of H-1 and H-2 protons on the F1 and F2 axis, respectively.

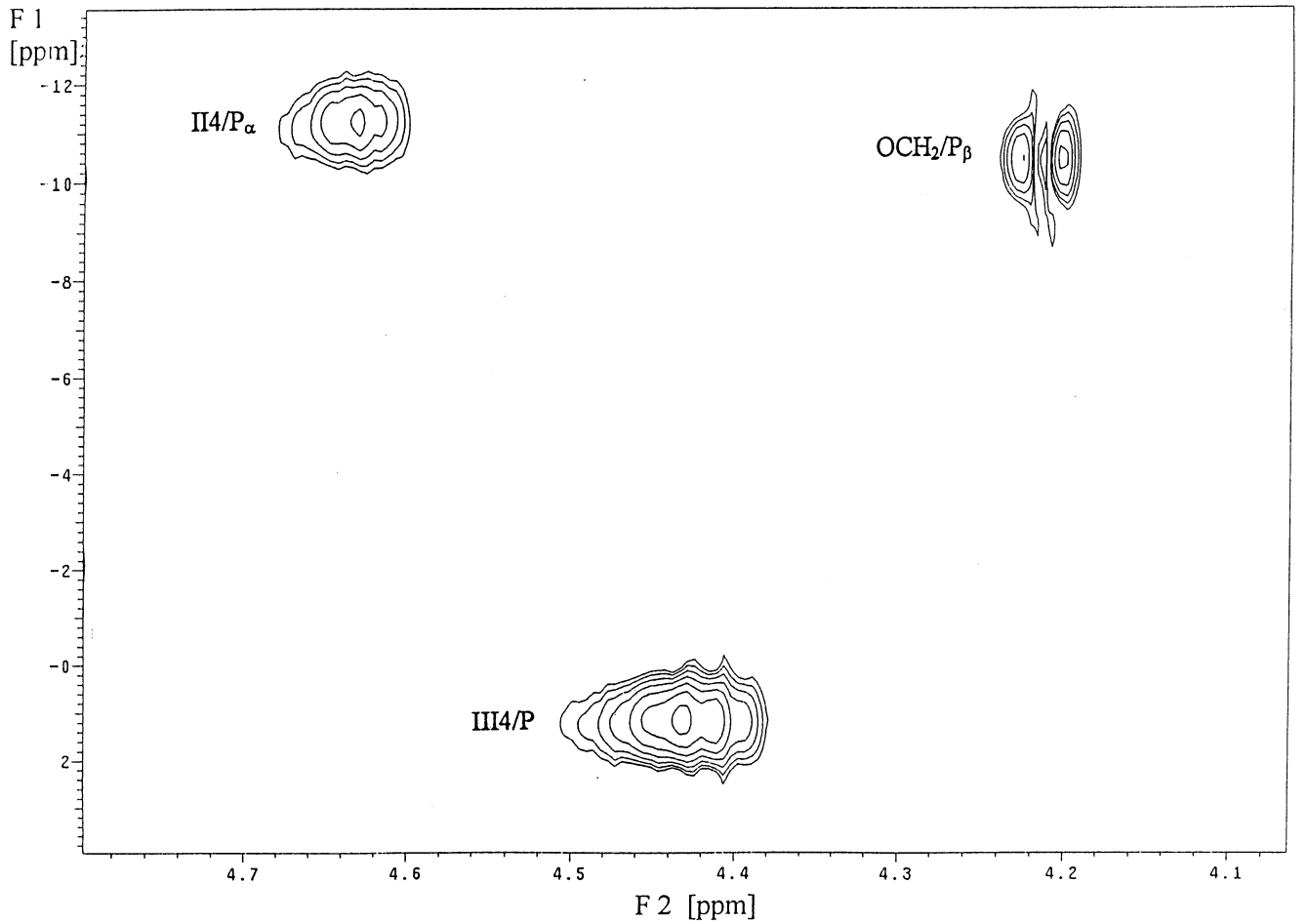


Fig. 2. $^{1}H-^{31}P$ HMQC spectrum of the core oligosaccharide from *H. alvei* strain 1204. ^{31}P chemical shifts are on the F1 axis and those of ^{1}H on the F2 axis.

ing permeation chromatography on Sephadex G-50 (2 × 100 cm) were described previously [4,5]. Core oligosaccharides were rechromatographed on columns of Bio-Gel P-2 (1.6 × 100 cm).

Analytical procedures.—The determination of sugar components in the form of alditol acetates by GLC–MS [6], ethanolamine as free amino groups, phosphorus, 3-deoxy octulosonic acid were carried out as reported earlier [7–9]. Methylation was performed according to Gunnarson' s procedure [10]. Dephosphorylation was carried out by treatment with 48% HF at 4 °C for 3 days. The absolute configuration of sugar components was determined enzymically using D-glucose and D-galactose oxidases [7]. The complete reduction of the carbonyl (with $NaBH_4$) and carboxyl (with EDC–$NaBH_4$) groups of the 3-deoxy-octulosonic acid residue in the core oligosaccharide as well as Smith degradation of the double-reduced core oligosaccharide were performed as described in [11].

NMR spectroscopy.—The samples were exchanged with $^{2}H_2O$, lyophilized and dissolved in 0.5 mL of $^{2}H_2O$. All spectra were recorded on a Varian Unity-Plus 500 MHz spectrometer operating at 500 and 202.6 MHz for ^{1}H and ^{31}P, respectively. All spectra were acquired at 298 K using hypercomplex mode to provide quadrature detection in F1 [12]. The following acquisition parameters were used for double-quantum filtered COSY [13], clean-TOCSY [14] and NOESY [15] spectra: spectral width 3300 Hz, acquisition time 0.45 s, water presaturation 1.7 s, t_1 dimension 256 complex points, number of transients 32. Mixing times for clean-TOCSY and NOESY were 160 and 200 ms, respectively. $^{1}H-^{31}P$ HMQC spectrum [16] was recorded using water presaturation 1.5 s, sweep width (^{1}H) 4500 Hz,

acquisition time (^{1}H) 0.6 s, sweep width (^{31}P) 4400 Hz, $t_{1,max} = 0.12$ s. Multiple-quantum delay was tuned to $J_{H,P} = 10$ Hz. Spectra were referenced to external Me_4Si (^{1}H) and external 85% H_3PO_4 (^{31}P).

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