ELSEVIER

Carbohydrate Research 269 (1995) 125–138

CARBOHYDRATE RESEARCH

Structural studies of the O-specific chain and a core hexasaccharide of *Hafnia alvei* strain 1192 lipopolysaccharide

Wojciech Jachymek [a], Carl Petersson [b], Anne Helander [c], Lennart Kenne [b,*], Czeslaw Lugowski [a], Tomasz Niedziela [a]

[a] *L. Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Science, Czerska 12, 53-114 Wroclaw, Poland*

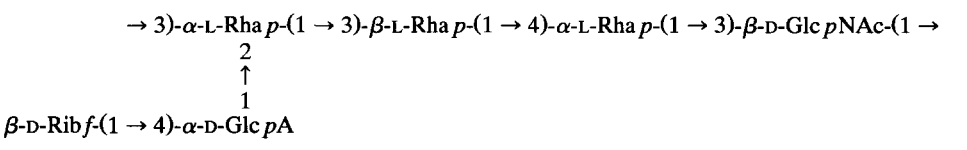
[b] *Department of Chemistry, Swedish University of Agricultural Sciences, P.O. Box 7015, S-750 07 Uppsala, Sweden*

[c] *Swedish NMR Centre, P.O. Box 17035, S-104 06 Stockholm, Sweden*

Received 22 April 1994; accepted 20 October 1994

Abstract

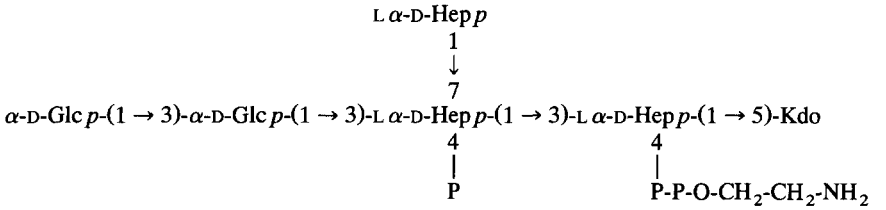
The structure of the O-specific side-chain and a core hexasaccharide of the *Hafnia alvei* strain 1192 lipopolysaccharide has been investigated. Methylation analysis, NMR spectroscopy, MALDI-TOF spectrometry, and various specific chemical degradations were the principal methods used. It is concluded that the polysaccharide is composed of hexasaccharide repeating-units having the following structure which is partially *O*-acetylated in the 2-position of the → 4)-α-D-Glc*p*A-(1 → (70%) and on different positions of the L-Rha residues (50%).



* Corresponding author.

SSDI 0008-6215(94)00348-3

The core hexasaccharide was found to have the following structure:



Keywords: Hafnia alvei; Lipopolysaccharides; Structure; O-Antigen; Core oligosaccharide

1. Introduction

Hafnia alvei is a typical member of Enterobacteriaceae. Bacteria of this species are Gram-negative, motile, peritrichously flagellated, rarely encapsulated rods. *H. alvei* is an opportunistic pathogen found in some incidences of nosocomial infections, and cases of septicemia caused by these bacteria have also been reported. It is frequently encountered in pathological specimens that are in most cases found in mixed cultures and the isolation of *H. alvei* from some incidences of gastroenteritis has also been reported. The serotyping scheme of *H. alvei* includes 39 O-serotypes and some of these show cross-reactivities with the O-antigens of certain strains of *Salmonella, Escherichia coli, Enterobacter cloacae,* and *Citrobacter freundii* [1]. Preliminary chemical characterisation of lipopolysaccharides isolated from 33 strains of this genus has been reported [2]. Recently the structures of the O-specific polysaccharides from *H. alvei* strains ATCC 13337, 2, 38, 39, 1187, 1191, 1205, and 1211 have been elucidated [3–9].

The detailed structure of the core region of any *H. alvei* lipopolysaccharide (LPS) has not been determined yet, except for some data concerning a core hexasaccharide in strain 2 [5] and a core trisaccharide in strains 32 and 1192 [10] and strains 2 and 1211 [11]. Since the core region as well as the O-specific polysaccharide (PS) plays an important role in bacterial physiology and interaction with the host, it was of current interest to characterise these parts of the *H. alvei* strain 1192 endotoxin.

2. Results and discussion

The LPS of *H. alvei*, isolated by conventional methods [12] and purified on Sepharose 2B [13], showed smooth character in SDS/PAGE analysis [14] with a pattern that indicated different lengths of the O-polysaccharide chains. The O-specific polysaccharide (PS) and core oligosaccharide (OS) were liberated by mild acidic hydrolysis and isolated by gel filtration on Bio-Gel P-10. The PS was eluted in four peaks which had the same structure but of different molecular mass. In addition to PS, three fractions with lower molecular mass components were obtained. One fraction contained both O-antigen and core sugars, the next fraction a core oligosaccharide and the last fraction a trisaccharide which has been analysed earlier [10]. Sugar analysis of the PS gave

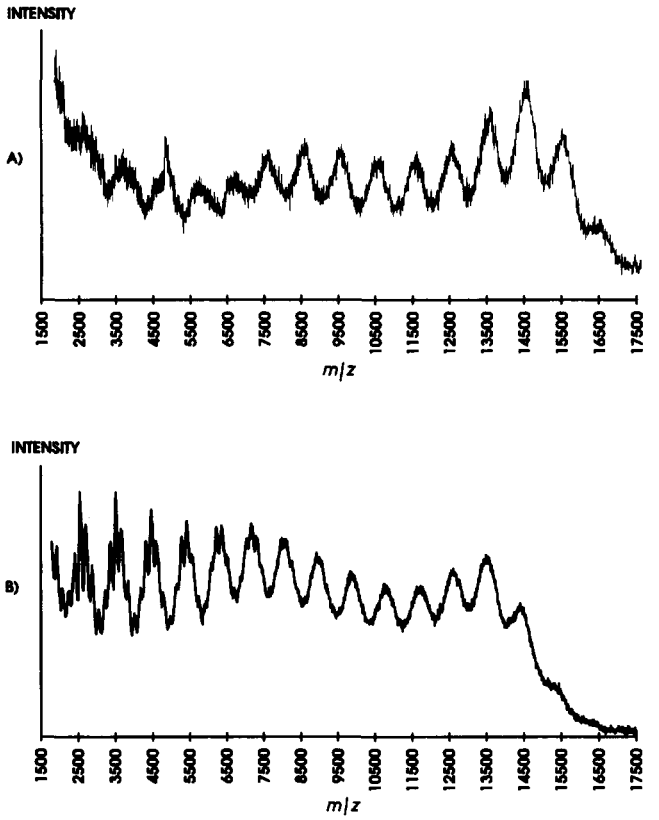


Fig. 1. MALDI-TOF spectra of (A) native PS and (B) *O*-deacetylated PS.

L-rhamnose, D-ribose, and D-glucosamine in the relative proportions 3.0:0.7:0.9, determined as their alditol acetates on GLC–MS. A five-proton spin-system of the PS in the ^{1}H NMR spectrum indicated the presence of a uronic acid residue. The PS was therefore carboxyl-reduced and sugar analysis of this material gave in addition to the other sugars a relative proportion of 0.9 of D-glucose. The absolute configurations of the sugars were determined as devised by Gerwig et al. [15,16]. The matrix-assisted-laser desorption ionisation (MALDI) spectrum of the O-specific PS (negative mode) (Fig. 1), using a time-of-flight (TOF) instrument, showed clusters of ions up to m/z 15521, indicating the presence of a large number of components with different molecular weights. The difference of 998 between the main components indicated the size of the repeating unit. However, the molecular weight of a hexasaccharide repeating unit, consisting of 3 Rha, 1 Rib, 1 GlcNAc, and 1 GlcA, is lower (949). The presence of *O*-acetyl groups, on average ca. 1.2 per repeating unit, can explain this difference. This was supported by a MALDI-TOF spectrum (Fig. 1) of *O*-deacetylated PS which showed clusters of ions with a difference corresponding to 949. The methylation analysis of the PS (Table 1) showed the presence of terminal ribofuranose, 3-substituted rhamnose, 4-substituted

Table 1
Methylation analysis of native and modified O-specific polysaccharide (PS) from *Hafnia alvei* strain 1192 lipopolysaccharide

Sugar [a]	t_R [b]	Molar ratio				
		A [c]	B	C	D	E
1,4,6-Sug	0.41	- [d]	-	-	-	0.52
2,3,5-Rib	0.54	0.56	0.59	0.62	-	0.45
3,4-Rha	0.89	-	-	-	-	0.81
2,3-Rha	0.90	1.12	1.06	1.14	-	0.96
2,4-Rha	0.92	1.00	1.00	1.00	2.00	1.00
4-Rha	1.09	0.60	0.71	0.78	-	-
2,3,6-Glc-*6d* [e]	1.24	-	-	0.94	-	-
2,3-Glc-*6d* [f]	1.47	-	0.93	-	-	0.96
3,4,6-GlcNAc	1.67	-	-	-	1.09	-
4,6-GlcNAc	1.90	0.89	0.80	0.78	-	-

[a] 1,4,6-Sug = 1,4,6-tri-*O*-methyl-2,5-anhydromannitol, etc.
[b] Retention time for the corresponding alditol acetate, relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol, on an HP-1 glass capillary column at 150–270°C, 8°/min.
[c] **A**, Original PS; **B**, methylated and ester-reduced PS; **C**, carboxyl-reduced PS; **D**, Smith-degraded PS; **E**, methylated and ester-reduced oligosaccharide obtained by *N*-deacetylation and deamination of PS.
[d] A dash (-) indicates that the component is not present.
[e] 6,6-Dideuterio-2,3,6-tri-*O*-methyl-D-glucose.
[f] 6,6-Dideuterio-2,3-di-*O*-methyl-D-glucose.

rhamnose, 2,3-disubstituted rhamnose, and 3-substituted *N*-acetylglucosamine. In the methylation analysis of carboxyl-reduced PS, using $NaBD_4$, a 4-substituted glucose-*6,6-*d_2 was also present. In PS that was first methylated and then reduced with 'Superdeuteride' [$LiB(C_2H_5)_3D$], 2,3-di-*O*-methylglucose-*6,6-*d_2 was produced instead of the 2,3,6-tri-*O*-methylglucose derivative.

The ^{1}H NMR spectrum of *O*-deacetylated PS (Figs. 2 and 3) contained signals for six anomeric protons at δ 5.08 ($J_{1,2} < 2$ Hz), 4.96 ($J_{1,2}$ 3 Hz), 4.93 ($J_{1,2} < 2$ Hz), 4.84 ($J_{1,2} < 2$ Hz), 4.75 ($J_{1,2}$ 7.5 Hz), and 4.72 ($J_{1,2} < 2$ Hz) (Table 2) supporting a hexasaccharide repeating unit. However, the anomeric region of the spectrum was rather complex because the signal at δ 4.93 had lower intensity (65%) and two of the signals (δ 5.08 and 4.96) showed a splitting with signals also at δ 5.10 and 4.99, respectively. This indicates that there is a nonstoichiometric amount of one sugar residue and this influences the chemical shifts of two other signals. The ^{13}C NMR spectrum showed the presence of six signals for anomeric carbons at δ 108.8, 103.2, 101.6, 101.0, 100.4, and 98.1. The H,C-correlations and the $^1J_{C,H}$ values obtained from HMQC experiments showed that three sugars had the α configuration ($^1J_{C,H} \geq 170$ Hz for signals at δ 5.08, 4.96, and 4.84) and two the β configuration ($^1J_{C,H} \approx 160$ Hz for signals at δ 4.75 and 4.72). The type of sugar and the anomeric configuration could be determined from the coupling constants and the chemical shifts. The sugar with the anomeric proton signal at δ 4.93 was assigned as a β-ribofuranose residue from the characteristic ^{13}C chemical shifts [17] (Table 2). Since the ^{1}H NMR spectrum is complex the assignments of the major signals and spin systems were made by 2D TOCSY and HMQC experiments (Fig.

Table 2
^{1}H and ^{13}C chemical shifts (ppm) for the sugar residues in the *O*-deacetylated O-specific polysaccharide (PS) of *Hafnia alvei* strain 1192 lipopolysaccharide and the ^{1}H chemical shifts for the acetylated sugar residues in the native PS

Residue	H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5/C-5	H-6/C-6
I β-D-Rib*f*-(1 →	4.93 108.8	4.14 75.5	4.37 70.5	4.01 82.8	3.66; 3.89 62.1	
II → 4)-α-D-Glc*p*A-(1 →	4.96 98.1	3.58 70.5	3.75 71.0	3.50 80.5	4.48 71.5	175.0
III → 2,3)-α-L-Rha*p*-(1 →	5.08 100.4	4.24 76.8	3.97 79.8	3.55 71.5	4.03 68.0	1.23 17.2
IV → 3)-β-L-Rha*p*-(1 →	4.72 101.0	4.10 71.1	3.66 81.5	3.45 72.0 [a]	3.45 73.0 [a]	1.30 17.4
V → 4)-α-L-Rha*p*-(1 →	4.84 101.6	3.88 70.5	3.84 69.7	3.54 83.3	4.05 68.0	1.27 17.2
VI → 3)-β-D-Glc*p*NAc-(1 →	4.75 103.2	3.82 56.5	3.68 81.8	3.40 69.5	3.41 76.5	3.72; 3.86 61.0
→ 4)-2-*O*-Ac-α-D-Glc*p*A-(1 → [b]	5.26	4.70	3.99	3.63	[c]	

[a] The assignment could be reversed.
[b] These residues are from the native PS
[c] Not determined

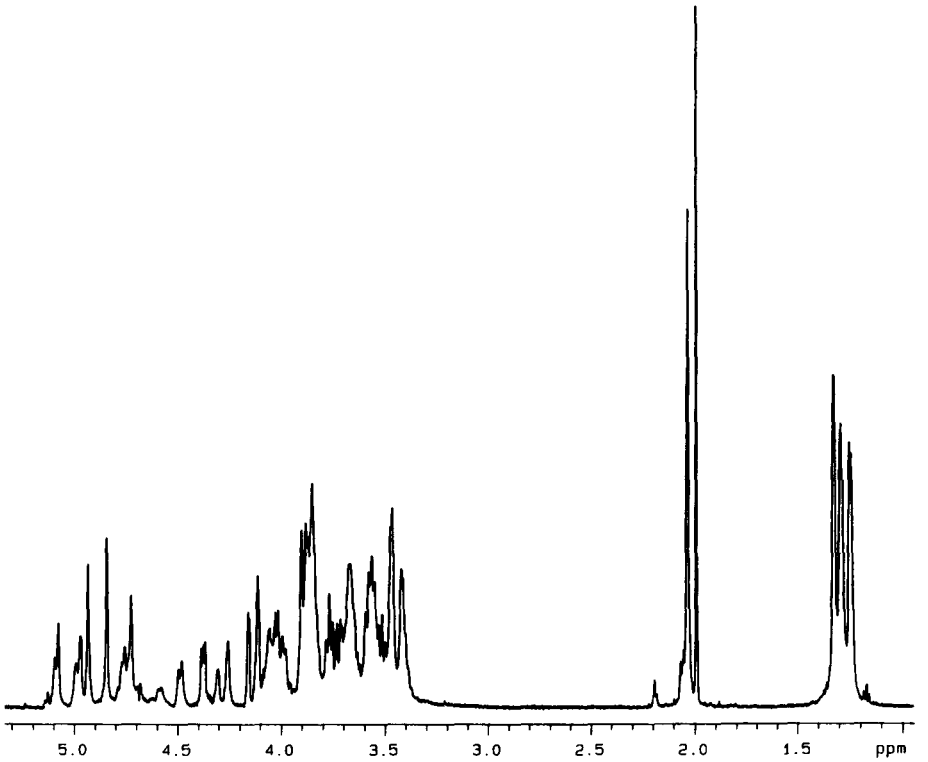


Fig. 2. The 600-MHz ^{1}H NMR spectrum of the *O*-deacetylated polysaccharide.

3). Thus, in agreement with data from the methylation analysis and the MALDI-TOF spectra, the repeating unit consists of two α-L-Rha, one β-L-Rha, one β-D-GlcNAc, and one α-D-GlcA as pyranosyl residues and a β-ribofuranosyl residue.

In order to obtain information on the sequence of the sugars in the repeating unit, the PS was subjected to a Smith degradation [18] and the products were separated on Bio-Gel P-2. The main product was eluted in the trisaccharide region and methylation analysis (Table 1) of this material showed the presence of terminal glucosamine and 3-substituted rhamnose in the molar ratio 1:2. ^{1}H NMR spectra also showed signals from a deoxyerythritol group in addition to those from the three sugar residues. By NOE difference experiments, connectivities between the sugars were established. Interresidue NOEs, observed as increased signal intensities, were found between H-1 of β-D-GlcNAc-(1 → and H-3 of → 3)-α-L-Rha-(1 → (δ 4.77/3.92), H-1 of → 3)-α-L-Rha-(1 → and H-3 of → 3)-β-L-Rha-(1 → (δ 5.09/3.64), and H-1 of → 3)-β-L-Rha-(1 → and H-2 of → 2)-4-deoxy-L-erythritol (δ 4.78/3.64), giving the following structure of the tetrasaccharide:

β-D-Glc*p*NAc-(1 → 3)-α-L-Rha*p*-(1 → 3)-β-L-Rha*p*-(1 → 2)-4-deoxy-L-erythritol

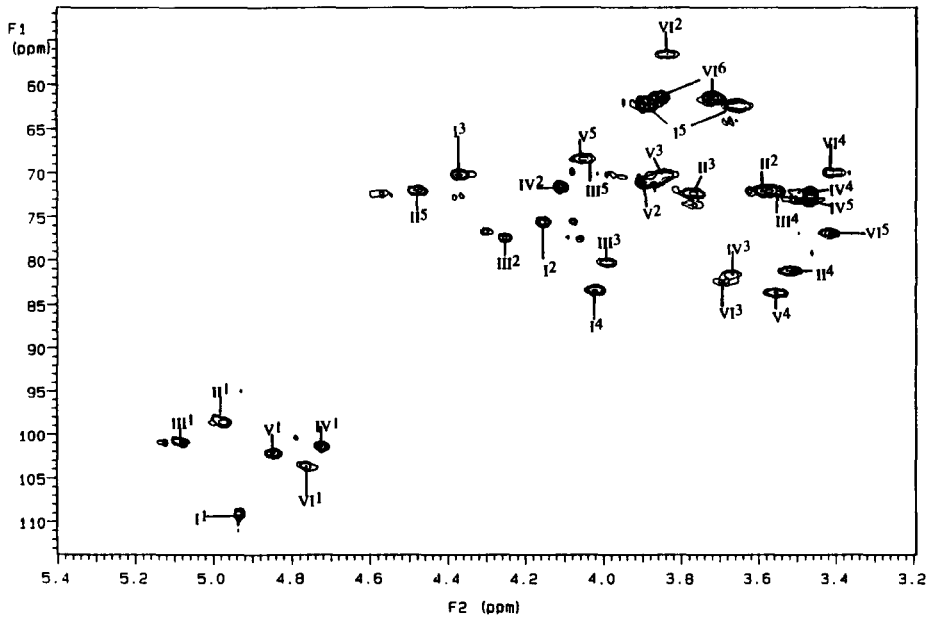


Fig. 3. Part of the 600-MHz ^{1}H-decoupled HMQC spectrum of the *O*-deacetylated polysaccharide.

This is in agreement with the methylation analysis data of native PS, and suggests the sequence:

→ 3)-β-D-Glc*p*NAc-(1 → 3)-α-L-Rha*p*-(1 → 3)-β-L-Rha*p*-(1 → 4)-α-L-Rha*p*-(1 →

as part of the repeating unit.

To obtain the position of the side-chain in the repeating unit, the PS was *N*-deacetylated and then depolymerised by deamination of the glucosamine residues. The 2,5-anhydromannose formed was reduced and the oligosaccharide subjected to methylation analysis involving reduction of the methyl ester (Table 1). This showed the presence of terminal ribfuranose, 3-substituted 2,5-anhydromannitol, 2-substituted rhamnose, 3-substituted rhamnose, 4-substituted rhamnose, and 4-substituted glucose-*6,6-d*$_2$. Thus the 2,3-branched rhamnose residue was converted into a 2-linked rhamnose residue when the glycosidic bond of the glucosamine residue was cleaved in the deamination reaction. This showed that the rhamnose substituted with the GlcNAc residue in the 3-position is the branched sugar in the native PS.

The sequence was also corroborated by NOESY experiments showing the connectivities between the sugar residues. Interresidue NOEs, observed as NOESY cross-peaks, were found between H-1 of → 3)-β-D-GlcNAc-(1 → and H-3 of → 2,3)-α-L-Rha-(1 → (δ 4.75/3.97), H-1 of → 2,3)-α-L-Rha-(1 → and H-3 of → 3)-β-L-Rha-(1 → (δ 5.08/3.66), H-1 of → 3)-β-L-Rha-(1 → and H-4 of → 4)-α-L-Rha-(1 → (δ 4.72/3.54), H-1 of → 4)-α-L-Rha-(1 → and H-3 of → 3)-β-D-GlcNAc-(1 → (δ 4.84/3.68), H-1 of β-D-Rib-(1 → and H-4 of → 4)-α-D-GlcA-(1 → (δ 4.93/3.50), and H-1 of → 4)-α-D-GlcA-(1 → and H-2 of the 2-linked α-L-Rha-(1 → (δ 4.96/4.24). The NOE

results are in agreement with data from methylation analyses and the structures of the oligosaccharides obtained by chemical degradations.

From the MALDI-TOF spectra of the native and the *O*-deacetylated PS it is evident that the O-specific chains, released by the 1% acetic acid treatment, consist of up to 15 repeating units. The results thus suggest the following structure for the repeating unit of the *H. alvei* 1192 O-specific side-chain:

$$[\rightarrow 3)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow 3)\text{-}\beta\text{-L-Rha}p\text{-}(1\rightarrow 4)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow 3)\text{-}\beta\text{-D-Glc}p\text{NAc-}(1\rightarrow]_{n\leq 15}$$

$$\begin{array}{l} \qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad 2 \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad \uparrow \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad 1 \\ \beta\text{-D-Rib}f\text{-}(1\rightarrow 4)\text{-}\alpha\text{-D-Glc}p\text{A} \end{array}$$

Comparison of ^{1}H NMR spectral data for native and *O*-deacetylated polysaccharide showed that the the *O*-acetyl groups were located at the 2-position of the $\rightarrow 4)$-α-D-Glc*p*A-(1 $\rightarrow$ and on different positions of the L-Rha residues (Table 2). The shifts of the signals, induced by the *O*-acetyl groups, were in accordance with published data for *O*-acetylated monosaccharades [19]. According to the intensities of the cross-peaks in the COSY spectrum ca. 70% of the $\rightarrow 4)$-α-D-Glc*p*A-(1 $\rightarrow$ residues and 50% of a L-Rha residue were acetylated.

The peaks in the MALDI-TOF spectrum are split, indicating some heterogeneity in the PS. This heterogeneity of the material was also observed as split signals in the anomeric region of the ^{1}H NMR spectrum and could be caused by incomplete substitution by the ribofuranosyl group in the polysaccharide released from the lipopolysaccharide by mild acid hydrolysis. This furanosyl linkage is known to be very sensitive to acid treatment.

Structure analysis of core oligosaccharide, OS.—Enzymatic and chemical analysis of the core oligosaccharide (Fr. III) gave D-glucose, L-*glycero*-D-*manno*-heptose (LDHep), Kdo, phosphorus, and ethanolamine in the molar ratios 2:1:1:2:1. Methylation analysis (Table 3) showed terminal glucose, terminal heptose, and 3-substituted glucose in equimolar amounts whereas the derivative of Kdo was not detected. The core OS was first dephosphorylated and then keto- and carboxyl-reduced. Sugar analysis of this material showed the presence of glucose, heptose, and 3-deoxyoctitol in the molar ratios 2:3:1, and methylation analysis the presence of terminal glucose and heptose, 3-substituted glucose, 3-substituted heptose, 3,7-disubstituted heptose, and 5-substituted 3-deoxyoctitol residues.

The ^{1}H NMR spectrum (Fig. 4) showed some heterogeneity of the OS and contained major signals for five anomeric protons at δ 5.37, 5.20, 5.19, 5.10, and 4.99 (Table 4) and for two deoxy protons at δ 1.91 and 2.26, indicating that the core OS consists of five sugars in addition to Kdo. The sugars with the anomeric proton signals at δ 5.37 and 5.19, respectively, were shown to be of the *gluco* configuration as only couplings of $\sim$ 10 Hz between ring protons were observed in the COSY spectra. The spin-systems with signals for anomeric protons at δ 5.20, 5.10, and 4.99 all showed small $^3J_{\text{H-1,H-2}}$ and $^3J_{\text{H-2,H-3}}$ values and were assigned to the heptose residues. The $^1J_{\text{C-1,H-1}}$ values ($>$ 170 Hz) obtained by HMQC experiments showed that the OS contained α-D-glucopyranosyl and L α-D-heptopyranosyl residues.

To obtain sequence information on the sugars in the core hexasaccharide, dephosphorylated OS was oxidised with sodium metaperiodate, reduced with sodium borodeu-

Table 3
Methylation analysis of original and modified core oligosaccharide (OS) from *Hafnia alvei* strain 1192 lipopolysaccharide

Sugar [a]	t_R [b]	Molar ratio				
		A [c]	B	C	D	E
2,3,4,6-Glc	1.00	1.00	1.00	1.00	- [d]	1.00
2,3,4,6-Man	1.02	-	-	-	1.05	-
2,4,6-Glc	1.21	1.05	0.92	-	-	1.19
2,4,6-Man-*6d* [e]	1.25	-	-	1.02	1.00	-
1,2,4,6,7,8-Kdo-ol [f]	1.26	-	0.52	-	-	-
2,3,4,6,7-Hep	1.40	0.94	0.92	-	-	0.93
2,4,6,7-Hep	1.62	-	0.98	0.96	-	-
4-Et-2,6,7-Hep [g]	1.64	-	-	-	-	0.44
2,4,6-Hep	1.90	-	0.90	-	-	-
4-Et-2,6-Hep [h]	1.92	-	-	-	-	0.76

[a] 2,3,4,6-Glc = 2,3,4,6-tetra-*O*-methyl-D-glucose, etc.
[b] Retention time for the corresponding alditol acetate, relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol, on an HP-1 glass capillary column at 150–270°C, 8°/min.
[c] **A**, original OS; **B**, dephosphorylated and keto- and carboxyl-reduced OS; **C**, dephosphorylated and Smith-degraded OS; **D**, dephosphorylated and twice Smith-degraded OS; **E**, methylated, dephosphorylated, and ethylated OS.
[d] A dash (-) indicates that the component is not present.
[e] 6-Deuterio-2,4,6-tri-*O*-methyl-D-mannose.
[f] Keto- and carboxyl-reduced Kdo.
[g] 4-*O*-Ethyl-2,6,7-tri-*O*-methyl-L,D-heptose.
[h] 4-*O*-Ethyl-2,6-di-*O*-methyl-L,D-heptose.

teride, and hydrolysed with 2% acetic acid. Methylation analysis of this Smith-degraded OS gave terminal glucose, 3-substituted 6-deuteriomannose, and 3-substituted heptose. The Smith-degraded OS was further oxidised with sodium metaperiodate, reduced with sodium borohydride, and hydrolysed with acetic acid. Methylation analysis of the twice Smith-degraded material gave terminal mannose and 3-substituted 6-deuteriomannose.

To obtain further sequence information, the core OS was analysed by a NOESY experiment. Interresidue NOEs, observed as cross-peaks, were found between H-1 of α-D-Glc-(1 → and H-3 of → 3)-α-D-Glc-(1 → (δ 5.37/3.94), H-1 of → 3)-α-D-Glc-(1 → and H-3 of → 3,7)-L α-D-Hep-(1 → (δ 5.19/4.11), H-1 of → 3,7)-L α-D-Hep-(1 → and H-3 of → 3)-L α-D-Hep-(1 → (δ 5.10/4.06), H-1 of → 3)-L α-D-Hep-(1 → and H-5 of → 5)-Kdo (δ 5.20/4.16), and between H-1 of L α-D-Hep (δ 4.99) and a proton giving a signal at δ 3.74, probably a non-assigned H-7.

The substitution positions of the phosphate groups in the core OS were determined by a methylation analysis involving methylation, removal of the phosphate groups using 48% HF, and then ethylation of the formed hydroxy groups. By this procedure the hydroxyls carrying phosphate groups in the native oligosaccharide were labelled with *O*-ethyl groups. GLC–MS analysis gave, in addition to terminal glucose, terminal heptose, 3-substituted glucose, and 5-substituted 3-deoxyoctitol, 3-substituted heptose with a 4-*O*-ethyl group and 3,7-disubstituted heptose with a 4-*O*-ethyl group. The phosphate substitution pattern was revealed from an inverse detected H,P-correlation

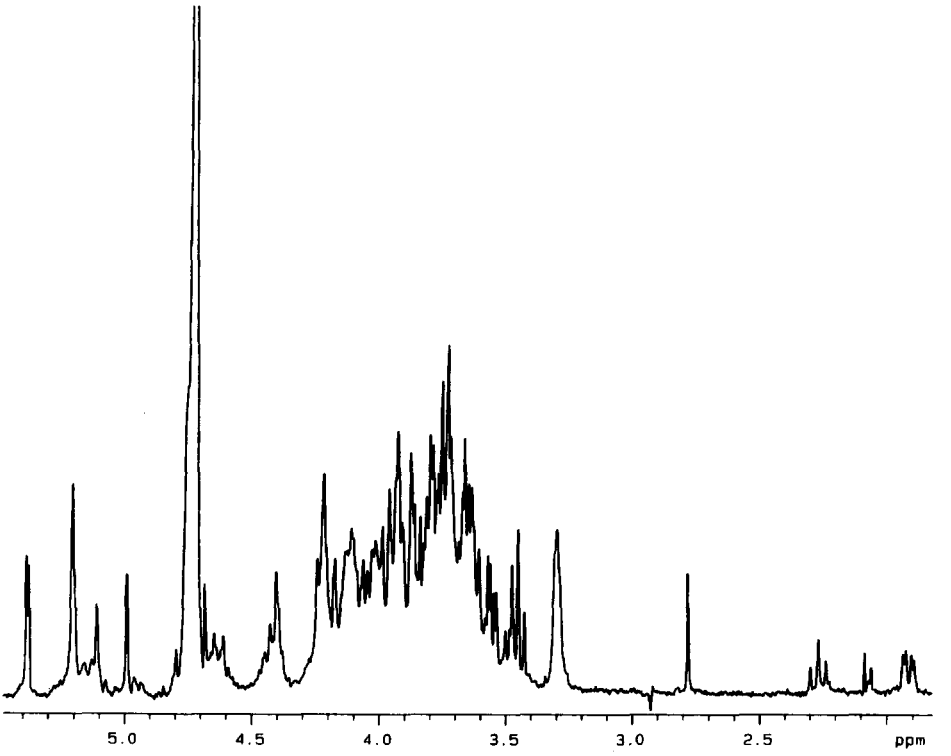


Fig. 4. The 400-MHz ^{1}H NMR spectrum of the core hexasaccharide.

experiment (HMQC). This experiment and the ^{1}H NMR spectrum showed that the major part of the → 3)-Hep-(1 → residue, with the anomeric proton signal at δ 5.20, was substituted in the 4-position with a pyrophosphorylethanolamine group. This was evident

Table 4
^{1}H and ^{13}C chemical shifts (ppm) of some signals for the sugar residues in the core oligosaccharide (OS) of *Hafnia alvei* strain 1192 lipopolysaccharide

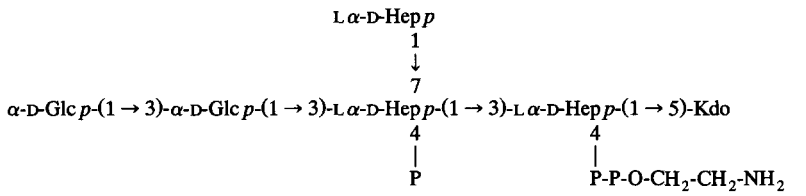
Residue	H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5/C-5
α-D-Glc*p*-(1 →	5.37 100.8	3.55 73.4	3.74 74.3	3.44 71.4	
→ 3)-α-D-Glc*p*-(1 →	5.19 101.3	3.65 72.0	3.94 81.0		
→ 3,7)-L α-D-Hep*p*-(1 → [a]	5.10 104.5	4.39 71.6	4.11 79.8	4.36 71.9	
L α-D-Hep*p*-(1 →	4.99 101.2	3.91 71.1	3.86 72.4		
→ 3)-L α-D-Hep*p*-(1 → [b]	5.20 103.4	3.99 73.0	4.06 80.8	4.64 73.2	4.22 70.4
→ 5)-Kdo			1.91, 2.26 35.1	4.12 67.2	4.16 74.4
-P-P-EtNH$_2$	4.20	3.30			

[a] This sugar residue is substituted with a phosphate group in the 4-position. The ^{31}P NMR chemical shift is δ 1.2.

[b] This sugar residue is substituted with a pyrophosphorylethanolamine (80%) or a phosphorylethanolamine (20%) group in the 4-position. The ^{31}P NMR chemical shifts are δ −10.4 and −11.1 for the former and δ 0.8 for the latter.

from phosphorus signals at δ −10.4 and −11.1 for pyrophosphate groups with P,H-connectivities to H-4 of the → 3)-Hep-(1 → and H-1 of the ethanolamine residue, respectively. The → 3,7)-Hep-(1 → residue was substituted by a phosphate group in the 4-position. This was evident from a phosphorus signal at δ 1.2 with a P,H-connectivity to H-4 of the sugar residue.

The results from sugar and methylation analysis together with NMR spectral data suggest the following structure of the core hexasaccharide:



3. Experimental

General methods.—GLC–MS was carried out 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. Gel permeation chromatography was performed on a column (100 × 1.6 cm) of Bio-Gel P-10 and on a column (100 × 1.6 cm) of Bio-Gel P-2 equilibrated with pyridine–AcOH buffer (pH 5.6). Eluates were monitored with a Knauer differential refractometer and all fractions checked by ^{1}H NMR spectroscopy. Matrix-assisted laser-desorption mass spectrometry was performed in the negative mode and with ca. 10 μJ energy of the laser beam on an LDI-1700XS time-of-flight instrument using 2,5-dihydroxybenzoic acid as matrix.

NMR spectroscopy.—NMR spectra were obtained for D_2O solutions at 35°C on Varian Unity 500 and 600 and Varian VXR-400 spectrometers, using sodium 3-trimethylsilylpropionate-d_4 (δ_H 0.00) and acetone (δ_C 31.00) as internal references and 85% phosphoric acid (δ_P 0.00) as external reference. The signals were assigned by one- and two-dimensional experiments (dq-COSY, relayed COSY, double relayed COSY, TOCSY, NOESY, and HMQC). The delay time in the relayed COSY experiments was 100 ms. The mixing times used in the NOESY experiments were 200 and 600 ms, and in the TOCSY experiments 30, 60, and 90 ms. The HMQC experiments were performed both with and without carbon decoupling.

Preparation of LPS, PS, and core OS.—*H. alvei* strain 1192 was obtained from the collection of the Pasteur Institute (Paris). LPS was prepared by phenol–water extraction of bacterial cells and purified by column chromatography on Sepharose 2B [13]. LPS was analysed by SDS-PAGE [14] and the LPS bands were detected by silver staining [20]. LPS was degraded by mild acid hydrolysis using aq 1% AcOH at 100°C for 30–45 min, and the solution was cooled and centrifuged. The supernatant solution was fractionated on a column (100 × 1.6 cm) of Bio-Gel P-10. PS (Fraction I) was separated from the core OS (Fractions II, III, and IV). All fractions were collected separately and freeze-dried. Further purification of the lower molecular weight core OS fractions (III

and IV) obtained from the Bio-Gel P-10 column was achieved by chromatography on Bio-Gel P-2. The main components were eluted in the hexa- and tri-saccharide regions, respectively.

Sugar and methylation analyses.—A solution of the sample (~ 0.5 mg) in 2 M aq CF_3CO_2H (1.0 mL) was kept in a closed vial at 120°C for 2 h. The sugars in the hydrolysate were then converted into alditol acetates by conventional methods. The absolute configurations of the sugars were determined as described by Gerwig et al. [15,16] using (+)-2-butanol for glycosylation.

Methylations were performed according to the method of Hakomori [21]. Products were recovered by reversed-phase chromatography on Sep-Pak C_{18} cartridges [22]. The permethylated material was hydrolysed with 2 M aq CF_3CO_2H at 120°C for 2 h. The partially methylated sugars in the hydrolysate were then converted into alditol acetates by conventional methods. Reduction of ester groups with 'Superdeuteride' [$LiB(C_2H_5)_3D$] after methylation of polysaccharide was carried out according to Bhat et al. [23].

O-*Deacetylation of PS.*—PS (10 mg) was treated with aq 12% NH_3 (2 mL) at room temperature for 16 h whereafter the solution was freeze-dried. The product was analysed by ^1H NMR spectroscopy.

Carboxyl reduction of PS.—PS (10 mg) was dissolved in water (1.5 mL) and treated with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (50 mg) at pH 4.75 and then with $NaBH_4$ (60 mg) at 37°C for 16 h according to the method of Taylor et al. [24]. The excess of $NaBH_4$ was destroyed by adding aq 50% AcOH and the sample was dialysed against distilled water and freeze-dried (8 mg).

Smith degradation of PS.—PS (20 mg) was oxidised with 0.1 M $NaIO_4$ (pH 5.0, 2 mL) at 4°C for 3 days in the dark. The oxidised sample was reduced with $NaBH_4$ (50 mg) at room temperature whereafter the mixture was acidified (pH 6.0) by adding aq 50% AcOH. Reduced PS was purified on a column of Bio-Gel P-2 and freeze-dried. The product was degraded by mild acid hydrolysis using aq 2% AcOH at 100°C for 1.5 h and the Smith-degraded PS was purified on a column of Bio-Gel P-2, yielding the oligosaccharide product (8 mg) after freeze-drying.

N-*Deacetylation and deamination of PS.*—PS (20 mg) was dissolved in absolute hydrazine (5 mL) and kept at 85°C for 8 days. The hydrazine was removed in vacuum over H_2SO_4 and the product purified on a column of Bio-Gel P-10 and freeze-dried. The product (16 mg) was analysed by ^1H NMR spectroscopy. *N*-Deacetylated PS (15 mg) was dissolved in water (1.5 mL), and a solution of aq 5% $NaNO_2$ (2.5 mL) and aq 33% AcOH (2.5 mL) was added. The solution was kept at room temperature for 45 min, then treated with Dowex 50 (H^+) resin and freeze-dried. The product was dissolved in water (2 mL) and reduced with $NaBH_4$ at room temperature for 2 h. The excess of $NaBH_4$ was destroyed with Dowex 50 (H^+) resin and H_3BO_3 removed as $(MeO)_3B$ by codistillation with MeOH. The residue was purified on a column of Bio-Gel P-2. Part of the main component (7 mg) was used for methylation analysis involving reduction of the methyl ester group. The derived partially methylated alditol acetates were analysed by GLC–MS.

Dephosphorylation of core OS.—Crude core OS (Fraction III from the fractionation on Bio-Gel P-10) (30 mg) was incubated with 48% hydrofluoric acid (3 mL) at 4°C for

3 days with stirring. The acid was evaporated in a stream of nitrogen at room temperature, the residue dissolved in water (5 mL), and the solution freeze-dried. The product was purified by column chromatography on Bio-Gel P-10 and freeze-dried.

Complete reduction (ketone and carboxyl groups) of the Kdo residue in core OS.—Dephosphorylated core OS (5 mg) was dissolved in water (1 mL) and reduced with $NaBH_4$ (20 mg) at room temperature for 16 h. For carboxyl reduction the residue in water (1.5 mL) was treated with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (50 mg) at pH 4.75 and then with $NaBH_4$ (60 mg) at 37°C for 16 h according to the method of Taylor et al. [24]. The excess of $NaBH_4$ was destroyed by adding AG50W-X8 (H^+) resin and the H_3BO_3 removed as $(MeO)_3B$. The reduced OS was additionally purified by column chromatography on Bio-Gel P-2 and freeze-dried.

Smith degradation of the core OS.— Dephosphorylated core OS (20 mg) was oxidised with 0.1 M $NaIO_4$ (pH 5.0, 2 mL) at 4°C for 3 days in the dark. The oxidised sample was reduced with $NaBD_4$ (60 mg) for 16 h at room temperature. The mixture was acidified (pH 6.0) by adding aq 50% AcOH, and reduced OS was purified on a column of Bio-Gel P-2 and freeze-dried. The product was degraded by mild hydrolysis with aq 2% AcOH at 100°C for 1.5 h and the Smith-degraded OS was purified on a column of Bio-Gel P-2 yielding a product (9 mg) after freeze-drying. The Smith-degraded material (8 mg) was additionaly oxidised in 0.1 M $NaIO_4$ (pH 5.0, 1 mL) at 4°C for 3 days in the dark, reduced with $NaBH_4$ (30 mg), and processed as described above for the first Smith degradation. Part of the twice Smith-degraded oligosaccharide (4 mg) was used for methylation analysis.

Localisation of the phosphate groups in core OS.—Core OS (2 mg) was methylated and purified as described above and then incubated with 48% hydrofluoric acid (1 mL). The product was alkylated using EtI as described, then hydrolysed, the sugars were converted into alditol acetates, and these were analysed by GLC–MS.

Acknowledgements

This work was supported by grants from the Swedish Natural Science Research Council and the Swedish Council of Foresty and Agricultural Research. A scholarship from the University supporting the collaboration between the Swedish and Polish groups is acknowledged.

References

[1] R. Sakazaki and K. Tamura, *The Prokaryotes*, Vol. III, Springer-Verlag, New York, 1992, pp 2817–2821.
[2] A. Romanowska, E. Katzenellenbogen, M. Kulakowska, A. Gamian, D. Witkowska, M. Mulczyk, and E. Romanowska, *Microbiol. Immunol.*, 47 (1988) 151–156.
[3] A. Gamian, E. Romanowska, H.J. Opferkuch, M. Hauck, and J. Dabrowski, *Eur. J. Biochem.*, 186 (1989) 611–620.
[4] 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.
[5] A. Gamian, E. Romanowska, U. Dabrowski, and J. Dabrowski, *Biochemistry*, 30 (1991) 5032–5038.

[6] E. Katzenellenbogen, E. Romanowska, U. Dabrowski, and J. Dabrowski, *Eur. J. Biochem.*, 200 (1991) 401–407.
[7] E. Katzenellenbogen, E. Romanowska, D. Witkowska, and A.S. Shashkov, *Carbohydr. Res.*, 231 (1992) 51–54.
[8] E. Katzenellenbogen, E. Romanowska, N.A. Kocharova, Y.A. Knirel, A.S. Shashkov, and N.K. Kochetkov, *Carbohydr. Res.*, 231 (1992) 249–260.
[9] A. Gamian, E. Romanowska, U. Dabrowski, and J. Dabrowski, *Eur. J. Biochem.*, 213 (1993) 1255–1260.
[10] W. Jachymek, C. Lugowski, E. Romanowska, D. Witkowska, C. Petersson, and L. Kenne, *Carbohydr. Res.*, 251 (1994) 327–330.
[11] E. Katzenellenbogen, A. Gamian, E. Romanowska, U. Dabrowski, and J. Dabrowski, *Biochem. Biophys. Res. Commun.*, 194 (1993) 1058–1064.
[12] O. Westphal and K. Jann, *Methods Carbohydr. Chem.*, 5 (1965) 83–89.
[13] E. Romanowska, *Anal. Biochem.*, 33 (1970) 383–389.
[14] U.K. Laemli, *Nature (London)*, 227 (1970) 680–685.
[15] G.J. Gerwig, J.P. Kamerling, and J.F.G. Vliegenthart, *Carbohydr. Res.*, 62 (1978) 349–357.
[16] G.J. Gerwig, J.P. Kamerling, and J.F.G. Vliegenthart, *Carbohydr. Res.*, 77 (1979) 1–7.
[17] P.A.J. Gorin and M. Mazurek, *Can. J. Chem.*, 53 (1975) 1212–1223.
[18] I.J. Goldstein, G.W. Hay, B.A. Lewis, and F. Smith, *Methods Carbohydr. Chem.*, 5 (1965) 361–370.
[19] P.-E. Jansson, L. Kenne, and E. Schweda, *J. Chem. Soc., Perkin Trans. 1*, (1987) 377–383.
[20] C.M. Tsai and C.E. Frasch, *Anal. Biochem.*, 119 (1982) 115–119.
[21] S. Hakomori, *J. Biochem. (Tokyo)*, 55 (1964) 205–208.
[22] T.J. Waeghe, A.G. Darvill, M. McNeil, and P. Albersheim, *Carbohydr. Res.*, 123 (1983) 281–304.
[23] U.R. Bhat, B.S. Krishnaiah, and R.W. Carlsson, *Carbohydr. Res.*, 220 (1991) 219–227.
[24] R.L. Taylor, J.E. Shively, and H.E. Conrad, *Methods Carbohydr. Chem.*, 7 (1976) 149–151.