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Structure of the O-specific polysaccharide of *Hafnia alvei* PCM 1222 containing 2-aminoethyl phosphate

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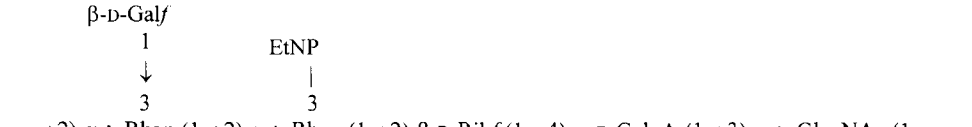
Abstract

The O-specific polysaccharide of *H. alvei* strain PCM 1222 has a branched hexasaccharide repeating unit containing D-galactose, L-rhamnose, D-ribose, D-galacturonic acid, and 2-acetamido-2-deoxy-D-glucose in the ratios 1:2:1:1:1, as well as 2-aminoethyl phosphate (EtNP) and *O*-acetyl groups in nonstoichiometric amounts. The polysaccharide was modified by carboxyl reduction, *O*-deacetylation, and dephosphorylation with 48% hydrofluoric acid, the last reaction being accompanied by removal of the lateral residue of β-galactofuranose. The modified polysaccharides were studied by methylation analysis and ^{1}H and ^{13}C NMR spectroscopy, including 2D correlation spectroscopy (COSY), H-detected $^{1}H,^{13}C$ and $^{1}H,^{31}P$ heteronuclear multiple-quantum coherence (HMQC), 1D NOE, 2D rotating-frame NOE spectroscopy (ROESY), and 2D combined total correlation spectroscopy (TOCSY) and ROESY (TORO). The following structure of the *O*-deacetylated polysaccharide was established:



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In different batches of the polysaccharide, the content of EtNP varied from 0.35 to 0.55 and that of the *O*-acetyl groups from 0.05 to 0.4 per repeating unit. It was tentatively suggested that the *O*-acetyl group is located at position 4 of a rhamnosyl residue. © 1996 Elsevier Science Ltd.

Keywords: *Hafnia alvei*; O-Antigen; Bacterial polysaccharide, structure; Lipopolysaccharide; Enterobacteria; 2-Aminoethyl phosphate

1. Introduction

In this paper we continue our structural studies of *Hafnia alvei* O-antigens. *H. alvei* is an opportunistic human pathogen found in some occurrences of nosocomial infections. According to the serological classification [1] this species includes 39 O-serotypes.

Recently, the structures of a number of O-specific polysaccharide chains of *H. alvei* lipopolysaccharides have been elucidated ([2–7] and references cited in [3]). Most of them contain sugar components uncommon for O-antigens, such as *N*-acetylneuraminic acid, glycerol phosphate, arabinitol phosphate, 3-amino-3,6-dideoxyhexoses, and 4-amino-4,6-dideoxyhexoses, as well as the (*R*)-3-hydroxybutyryl group.

We now report the structure of the *H. alvei* PCM 1222 O-specific polysaccharide that contains 2-aminoethyl phosphate.

2. Results and discussion

The lipopolysaccharide was isolated in 1.5% yield from dry bacterial mass by phenol–water extraction [8] followed by GPC on Sepharose 2B [9]. Mild acid degradation of the lipopolysaccharide followed by fractionation of a mixture of poly- and oligo-saccharides obtained by GPC on Sephadex G-50 afforded the O-specific polysaccharide (PS-I) in a yield of ca. 30% of the total amount of the material eluted from the column.

Sugar analysis by PC and GLC of alditol acetates after hydrolysis with 10 M hydrochloric acid (80 °C, 30 min) of PS-I and carboxyl-reduced PS-I (PS-II) revealed the presence of ribose, rhamnose, galactose, and GlcN; the molar ratios Rib + Rha (one peak in GLC on an HP-1 column):Gal:GlcN were 1.7:1.0:1.0 and 1.8:2.0:0.8, respectively. In addition, 2-aminoethyl phosphate (EtNP) was identified by PC after hydrolysis with 2 M trifluoroacetic acid (120 °C, 2 h).

Using colorimetric methods, the following components were quantified in PS-I: galactose 12.5% [10], ribose 14% [11], rhamnose 23% [12], GalA 9% [13], *O*-acetyl groups 0.36 μM/mg [14], phosphate 0.55 μM/mg [15], and free amino groups 0.5 μM/mg [16].

As determined with D-galactose oxidase [10], the content of galactose increased from 12.5% in PS-I to 23.5% in PS-II, thus confirming the D configuration of both Gal and GalA. The D configuration of GlcN was determined with hexokinase in the presence of ATP [17]; phosphorylation of GlcN was checked by PC and GLC–MS. The D configura-

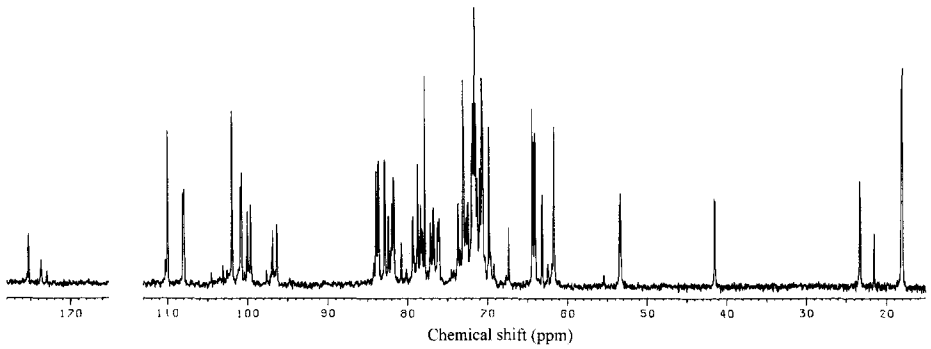


Fig. 1. 75-MHz ^{13}C NMR spectrum of the O-specific polysaccharide (PS-I).

tion of ribose and the L configuration of rhamnose were established by GLC of acetylated (*S*)-2-octyl glycosides [18].

The ^{31}P NMR spectrum of PS-I contained a signal for a phosphodiester group at δ 0.13.

The ^{13}C NMR spectrum of PS-I contained a number of signals with different intensities (Fig. 1); in particular, four of the six signals in the resonance region of anomeric carbons (δ 96–110) were split into two components. This reflected a heterogeneity of PS-I that could be due to nonstoichiometric *O*-acetylation and phosphorylation. In fact, in the spectrum there were signals for OAc (CH_3 at δ 21.5) and EtNP (CH_2O and CH_2N at δ 63.2 and 41.3 coupled to phosphorus, $J_{C,P}$ 4.1 and 6.6 Hz, respectively; cf. published data in [19]), which had much lower intensities than, for example, signals for NAc at δ 23.3. Judging from the ^{13}C NMR spectrum, the *O*-deacetylated polysaccharide (PS-III), like the initial PS-I, lacked strict regularity.

Methylation analysis of PS-I (Table 1) demonstrated the presence of nonsubstituted Gal*f*, 2-substituted Rib*f* and Rha*p*, 2,3-disubstituted Rha*p*, and 3-substituted Glc*p*NAc as the main components of the repeating unit. Therefore, PS-I is branched with a rhamnosyl residue at the branching point and Gal*f* attached as the lateral sugar of the

Table 1
Data of methylation analysis

Methylated sugar	t_R [a]	Molar ratio			
		PS-I	PS-II	PS-IV	OS
3,5-MeRib	0.75	0.71	0.52	1.0	1.0
3,4-MeRha	0.90	1.00	1.00	2.1	2.6
2,4-MeRha	0.92	0.14	0.09		
2,3,5,6-MeGal	1.01	1.07	0.80		
4-MeRha	1.09	1.10	0.45		
2,3,6-MeGal	1.22		1.40		
4,6-MeGlcNMeAc	1.90	0.68	0.30	0.4	0.7

[a] Retention time in GLC of the corresponding alditol acetate referenced to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol (2,3,4,6-MeGlc).

side chain. A small amount of 3-substituted Rha*p* was also present; this may have originated from the terminal nonreducing repeating unit where the rhamnosyl residue corresponding to the branching point in the internal repeating units has no substituent at position 2.

In methylation analysis of PS-II, in addition to the partially methylated sugars derived from PS-I, 2,3,6-tri-*O*-methylgalactose was identified, which originated from carboxyl-reduced GalA. Therefore, GalA in PS-I is substituted at position 4.

Dephosphorylation of PS-III with 48% hydrofluoric acid followed by fractionation on Bio-Gel P-2 resulted in a modified polysaccharide (PS-IV), an oligosaccharide product (OS), and a low molecular weight fraction. Both PS-IV and OS contained ribose, rhamnose (molar ratio ca. 1:2), GlcN, and GalA; thus, PS-IV has a pentasaccharide repeating unit. The low molecular weight fraction included mainly free galactose, 2-aminoethanol, and phosphate, as well as trace amounts of ribose and rhamnose. These data showed that dephosphorylation of PS-III was accompanied by complete cleavage of Gal*f* and that neither EtNP nor Gal*f* were part of the main polysaccharide chain. The ease of removal of the galactosyl group during dephosphorylation of PS-III with 48% hydrofluoric acid is clearly accounted for by its furanoid form.

Methylation analysis of PS-IV (Table 1) demonstrated that the ribosyl and both rhamnosyl residues are 2-substituted and that GlcNAc is 3-substituted; hence, PS-IV is

Table 2
^{1}H NMR chemical shifts (δ in ppm) [a]

Sugar residue	H-1	H-2	H-3	H-4	H-5 (H-5a)	H-6a (H-5b)	H-6b
Dephosphorylated polysaccharide (PS-IV)							
→ 2)-α-L-Rha*p*^{I}-(1 →	5.03	4.08	3.92	3.56	3.78	1.32	
→ 2)-α-L-Rha*p*^{II}-(1 →	5.12	4.07	3.91	3.48	3.71	1.32	
→ 2)-β-D-Rib*f*-(1 →	5.39	4.20	4.26	4.00	3.64	3.79	
→ 4)-α-D-Gal*p*A-(1 →	5.33	3.88	4.00	4.38	4.38		
→ 3)-α-D-Glc*p*NAc-(1 →	5.01	4.07	3.92	3.74	4.08	3.86	3.80
O-Deacetylated polysaccharide (PS-III)							
→ 2)-α-L-Rha *p*^{I}-(1 →	5.05	4.17	3.99	3.73	3.77	1.34 [b]	
3 ↑	(5.12)	(4.14)	(4.02)	(3.75)	(3.77)	(1.34) [b]	
→ 2)-α-L-Rha*p*^{II}-(1 →	5.11	4.08	3.92	3.48	3.77	1.36 [b]	
	(5.11)	(4.27)	(4.33)	(3.61)	(3.79)	(1.36) [b]	
→ 2)-β-D-Rib*f*-(1 →	5.39	4.20	4.31	3.98	3.67	3.80	
	(5.42)	(4.21)	(4.31)	(3.98)	(3.67)	(3.80)	
→ 4)-α-D-Gal*p*A-(1 →	5.30	3.89	3.97	4.35	4.19		
	(5.31)	(3.89)	(3.97)	(4.35)	(4.19)		
→ 3)-α-D-Glc*p*NAc-(1 →	5.03	4.02	3.96	3.75	4.03	3.87	3.83
	(5.06)	(4.06)	(3.06)	(3.75)	(4.03)	(3.87)	
β-D-Gal*f*-(1 →	5.27	4.16	4.12	4.03	3.86	3.74	3.70
	(5.27)	(4.16)	(4.12)	(4.03)	(3.86)	(3.74)	(3.70)

[a] Chemical shifts for the repeating unit substituted by EtNP are given in parentheses. Chemical shifts for EtNP are δ 4.15 (CH_2O) and 3.30 (CH_2N); the chemical shift for NAc is δ 2.00.
[b] Assignment could be interchanged.

Table 3
^{13}C NMR chemical shifts (δ in ppm) [a]

Sugar residue	C-1	C-2	C-3	C-4	C-5	C-6
Dephosphorylated polysaccharide (PS-IV)						
→ 2)-α-L-Rha*p*^{I}-(1 →	100.3	77.0	70.7	73.4	70.7	17.9 [b]
→ 2)-α-L-Rha*p*^{II}-(1 →	100.7	79.4	71.2	73.5	70.5	18.0 [b]
→ 2)-β-D-Rib*f*-(1 →	108.0	81.9	71.5	83.9	63.9	
→ 4)-α-D-Gal*p*A-(1 →	101.9	69.8	70.7	78.7	72.0	173.9
→ 3)-α-D-Glc*p*NAc-(1 →	97.0	53.3	81.6	71.5	73.0	61.6
O-Deacetylated polysaccharide (PS-III)						
→ 2)-α-L-Rha*p*^{I}-(1 →	99.8	76.3	77.0	72.9	71.0	18.0
3 ↑	(99.3)	(75.5)	(76.7)	(73.0)	(71.0)	(18.0)
→ 2)-α-L-Rha*p*^{II}-(1 → [c]	100.8	79.2	71.3	73.7	70.5	18.0
	(100.7)	(78.1) [c]	(76.3)	(72.7)	(70.5)	(18.0)
→ 2)-β-D-Rib*f*-(1 →	107.7	82.2	71.3	84.0	63.4	
	(107.8)	(82.5)	(71.2)	(84.0)	(63.4)	
→ 4)-α-D-Gal*p*A-(1 →	101.8	69.9	71.2	79.0	73.0	175.0
	(101.8)	(69.9)	(71.2)	(79.0)	(73.0)	(175.0)
→ 3)-α-D-Glc*p*NAc-(1 →	96.5	53.4	81.1	71.6	73.0	61.7
	(95.8)	(53.3)	(81.0)	(71.6)	(73.0)	(61.7)
β-D-Gal*f*-(1 →	109.9	82.8	77.8	83.7	71.7	64.1
	(109.9)	(82.7)	(77.8)	(83.6)	(71.7)	(64.4)

[a] Chemical shifts for the repeating units substituted by EtNP are given in parentheses. Chemical shifts for EtNP are δ 63.2 (CH_2O, $^{2}J_{P,C}$ 4.1 Hz) and 41.3 (CH_2N, $^{3}J_{P,C}$ 6.6 Hz), and for NAc 23.2–23.3 ppm (CH_3) and 175.2–175.3 (CO).
[b] Assignment could be interchanged.
[c] $^{3}J_{P,C}$ 4.5 Hz.

linear. Methylation analysis of OS gave similar results, thus suggesting that OS is an oligosaccharide with GalA at the nonreducing end. Comparison of methylation analysis data for PS-I and PS-IV led to the conclusion that Gal*f* and EtNP in PS-I are attached at position 3 of rhamnosyl residues.

An attempt to confirm the location of EtNP by methylation of PS-I with methyl iodide followed by dephosphorylation with 48% hydrofluoric acid and remethylation with [$^{2}H_3$]methyl iodide resulted in partial removal of Gal*f* during dephosphorylation and in partial depolymerisation, probably both during dephosphorylation and the second methylation.

PS-IV had the ^{1}H and ^{13}C NMR spectra typical of a regular polymer (Tables 2 and 3). The ^{13}C NMR spectrum of PS-IV contained signals for five anomeric carbons in the region δ 97–108, two C–CH_2OH groups (C-6 of GlcNAc and C-5 of Rib) at δ 61.6 and 63.9, two C–CH_3 groups (C-6 of Rha) at δ 17.9 and 18.0, C–COOH (C-6 of GalA) at δ 173.9, one carbon bearing nitrogen at δ 53.3, other sugar ring carbons in the region δ 69.8–83.9, and one *N*-acetyl group (CH_3 at δ 23.2, CO at δ 175.3). The ^{1}H NMR spectrum of PS-IV contained, inter alia, signals for five anomeric protons in the region δ 5.01–5.39, CH_3 (H-6) of Rha at δ 1.32 (both d, $J_{5,6}$ 6 Hz), and CH_3 of NAc at δ 2.00 (s). In the spectra there were no more signals for EtNP.

The coupling constant values $^{1}J_{C\text{-}1,H\text{-}1}$ were determined from the gated-decoupling ^{13}C NMR spectrum of PS-IV. These were in the region 170.7–173.6 Hz for four of five anomeric carbons (signals at δ 97.0–101.9) that pointed to the pyranoid form and α linkage of the corresponding monosaccharide units [20]. The coupling constant value $^{1}J_{C\text{-}1,H\text{-}1}$ 178.6 Hz and the chemical shift δ 108.0 for the fifth signal indicated that it belonged to β-linked Rib*f* [21,22].

The ^{1}H NMR spectrum of PS-IV was assigned using sequential, selective spin-decoupling, 2D COSY, and H,H-relayed COSY (Table 2), and the corresponding spin-systems of the five monosaccharide residues were identified on the basis of the coupling constant values. In particular, the values $J_{1,2} < 2$, $J_{2,3}$ 5, $J_{3,4}$ 7.5, $J_{4,5a}$ 4, and $J_{4,5b}$ 6 Hz for ribose confirmed this sugar residue to be β-furanoid [23].

For linkage and sequence analysis, 1D NOE experiments with sequential, selective preirradiation of anomeric protons were carried out in the difference mode. Preirradiation of H-1 of one of the rhamnosyl residues (Rha*p*^{II}) at δ 5.12 caused a strong interresidue NOE on H-2 of Rib*f* at δ 4.20. Likewise, strong NOEs were observed on H-4 of Gal*p*A at δ 4.38 and H-3 of Glc*p*NAc at δ 3.92 on preirradiation of H-1 of Rib*f* at δ 5.39 and H-1 of Gal*p*A at δ 5.33, respectively. Finally, joint preirradiation of H-1 of Glc*p*NAc at δ 5.01 and H-1 of the second rhamnosyl residue (Rha*p*^{I}) at δ 5.03 resulted in NOEs on H-2 of Rha*p*^{I} and Rha*p*^{II} at δ 4.07–4.08. In addition to the NOEs listed above, intraresidue NOEs appeared on H-2 on preirradiation of H-1 of the same α-linked sugar residue, while no intraresidue NOE was observed on preirradiation of H-1 of β-Rib*f*.

These data allowed determination of the following structure of PS-IV:

→2)-α-L-Rha*p*^{I}-(1→2)-α-L-Rha*p*^{II}-(1→2)-β-D-Rib*f*-(1→4)-α-D-Gal*p*A-(1→3)-α-D-Glc*p*NAc-(1→

PS-IV

This structure is in agreement with the methylation analysis data (see above) and with the results of the assignment of the ^{13}C NMR spectrum of PS-IV (Table 3), which, with the ^{1}H NMR spectrum assigned, was performed using an H-detected decoupled $^{1}H,^{13}C$ HMQC experiment. In particular, the substitution pattern was confirmed by the relatively low-field positions of the signals for C-2 of Rha*p*^{I}, Rha*p*^{II}, and Rib*f*, C-4 of Gal*p*A, and C-3 of Glc*p*NAc at δ 77.0, 79.4, 81.9, 78.7, and 81.6, respectively, as compared with their positions in the spectra of the corresponding nonsubstituted monosaccharides [20].

The ^{1}H NMR spectrum of PS-III contained ten signals for anomeric protons in the region δ 5.03–5.42. The intensities of four of these signals at δ 5.42, 5.31, 5.12, and 5.06 were less than the intensity of six other signals at δ 5.39, 5.30, 5.27, 5.11, 5.05, and 5.03. The less intense signals were shown to belong to repeating units substituted by EtNP, and the other signals to nonsubstituted repeating units or to sugars in the substituted units not influenced by phosphorylation. Five of the six more intense signals

had chemical shifts and coupling constants similar to those of the anomeric proton signals in the ^{1}H NMR spectrum of PS-IV (Table 2). The sixth signal at δ 5.27 ($J_{1,2}$ 2 Hz), which was absent from the spectrum of PS-IV, belonged to Gal*f*.

The ^{1}H NMR spectrum of PS-III was assigned using 2D COSY, one-step relayed COSY, and rotating frame NOE spectroscopy (ROESY), and sequential, selective spin decoupling performed in the difference mode was applied for determination of $^3J_{H,H}$ coupling constants (Table 2).

The coupling constant values $J_{1,2}$ 2, $J_{2,3}$ 3, $J_{3,4}$ 6, $J_{4,5}$ 4, $J_{5,6a}$ 4, and $J_{5,6b}$ 7 Hz confirmed that the sixth sugar residue in PS-III is β-Gal*f* (cf. published data in [24]). This was the only sugar residue that exhibited one series of signals in the spectrum, while the signals for the five remaining sugar residues were split into two series. The largest difference in the chemical shifts in the two series was observed for the signal of H-3 of Rha*p*^{II}, which shifted downfield to δ 4.33 (i.e., by 0.41 ppm) in the phosphorylated repeating unit. This suggests that EtNP is located at position 3 of Rha*p*^{II}.

This suggestion was confirmed by 2D H-detected ^{1}H,^{31}P HMQC experiments. The experiment optimised for the coupling constant $J_{H,P}$ 5 Hz revealed cross-peaks at δ_H/δ_P 4.15/0.13 (strong) and 3.30/0.13 (weak), which were due to correlation of phosphorus to CH_2O and CH_2N of the 2-aminoethyl group, respectively. Optimisation for the coupling constant $J_{H,P}$ 10 Hz resulted in the same cross-peak at δ_H/δ_P 4.15/0.13 (now weak) and an additional, expected cross-peak at δ_H/δ_P 4.33/0.13 (strong) due to correlation of phosphorus to H-3 of Rha*p*^{II}.

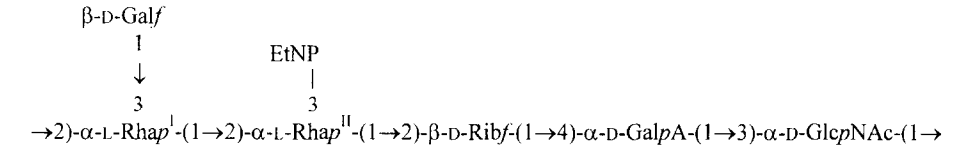
A ROESY experiment with PS-III revealed the same interresidue correlations between the main-chain monosaccharides in both phosphorylated (minor) and nonphosphorylated (major) repeating units as observed for PS-IV. Additional cross-peaks H-1 Rha*p*^{II}/H-5 Rha*p*^{I} at δ 5.11/3.77 and H-1 Rib*f*/H-5 Rha*p*^{II} at δ 5.39/3.77 and 5.42/3.79, which were not clearly observed in the 1D NOE spectra of PS-III, are to be expected for the (1 → 2)-linked trisaccharide fragment α-L-Rha*p*^{I}-(1 → 2)-α-L-Rha*p*^{II}-(1 → 2)-β-D-Rib*f* (e.g., [25]). The presence of strong cross-peaks H-1 Gal*f*/H-3 Rha*p*^{I} at δ 5.27/3.99 in the nonphosphorylated and 5.27/4.02 in the phosphorylated repeating units proved that Gal*f* is attached at position 3 of Rha*p*^{I}.

The sequence of the monosaccharide residues in PS-III was further confirmed by a 2D hybrid experiment combining TOCSY and ROESY (TORO [26]). The correlations between the anomeric protons appeared as a result of stepwise transfer of H-1 magnetisation by the TOCSY mechanism to other protons of the same sugar residue and by the ROESY mechanism to H-1 of the glycosylating sugar residue. In accordance with the structure proposed, the spectrum displayed H-1/H-1 cross-peaks for Rha*p*^{I}/Rha*p*^{II}, Rha*p*^{I}/Gal*f*, Rha*p*^{II}/Rib*f*, Rib*f*/Gal*p*A, Gal*p*A/Glc*p*NAc, and Glc*p*NAc/Rha*p*^{I} correlations in both phosphorylated and nonphosphorylated repeating units.

The ^{13}C NMR spectrum of PS-III was assigned using H-detected ^{1}H,^{13}C HMQC and relayed HMQC experiments (Table 3). Downfield displacements of the signals for C-2 and C-3 for Rha*p*^{I}, C-2 of Rha*p*^{II} and Rib*f*, C-4 of Gal*p*A, and C-3 of Glc*p* by 4–9 ppm, as compared with their positions in the spectra of the corresponding nonsubstituted monosaccharides [20], corroborated the substitution pattern determined by methylation analysis and ROESY (see above). A coupling constant $^3J_{P,C}$ of 4.5 Hz observed in the spectrum for the signal C-2 of Rha*p*^{II} and displacements of the signals for C-3

(downfield, by 5.0 ppm), C-2 and C-4 (both upfield, by 1.0–1.1 ppm) of Rhap^{II} in the phosphorylated repeating unit confirmed nonstoichiometric phosphorylation of Rhap^{II} at position 3.

Therefore, on the basis of the data obtained, it was concluded that PS-III has the following structure:



PS-III

As judged by the relative intensities of the signals for phosphorylated and nonphosphorylated repeating units in the ^{13}C NMR spectra of PS-III, the content of EtNP in the two batches studied of the O-specific polysaccharide varied from 0.35 to 0.55 per repeating unit.

Comparison of the ^{13}C NMR spectra of PS-III and PS-I showed that the latter contained a signal at δ 67.3 absent from the former spectrum. This signal could be assigned to C-5 of Rhap^{I} or Rhap^{II} shifted upfield due to a β-effect of *O*-acetylation at position 4 [27] and thus the site of attachment of the *O*-acetyl group could be tentatively determined as O-4 of a rhamnosyl residue. As judged by the ratios of the intensities of the signals in the ^{13}C NMR spectrum of PS-I, the content of the *O*-acetyl groups was ca. 0.4 per repeating unit. In other batches studied this varied down to < 0.1 per repeating unit, as determined by the colorimetric method [14].

As with most other O-antigens of *H. alvei*, the O-specific polysaccharide of strain 1222 is acidic. In addition to D-galacturonic acid, it contains a much less common acidic component, namely, 2-aminoethyl phosphate, which has not been found hitherto in O-antigens of bacteria but in some *Proteus* strains [19,28,29].

3. Experimental

Chromatography and mass spectrometry.—GPC was performed on a column (2 × 100 cm) of Sephadex G-50 or Bio-Gel P-2 and on a column (1.6 × 80 cm) of TSK HW-40 (S) with monitoring by the phenol–H_2SO_4 reaction or using a Knauer differential refractometer, respectively. GLC was carried out using a Hewlett–Packard 5890 instrument equipped with a glass capillary column (25 m × 0.2 mm) of Ultra 2 stationary phase. GLC–MS was performed with a Hewlett–Packard Model 5971 A chromatograph using an HP-1 glass capillary column (12 m × 0.2 mm) and a temperature program of 150 → 270 °C at 8 °C/min. PC was performed on Whatman No. 1 paper using a system of 6:4:3 1-butanol–pyridine–water and the alkaline $AgNO_3$ spray reagent for detection of monosaccharides.

NMR spectroscopy.—NMR experiments were performed for solutions in D_2O with acetone (δ_H 2.225, δ_C 31.45) as internal standard or aq 85% H_3PO_4 (δ_P 0) as external standard. ^{13}C NMR and H-detected ^1H,^{31}P HMQC spectra were recorded with a Bruker AM-300 spectrometer at 60 °C. ^1H NMR, 1D NOE, 2D COSY, and relayed COSY experiments with PS-III were carried out using a Bruker WM-250 instrument at 60 °C. H-detected ^1H,^{13}C HMQC experiments with PS-III and PS-IV, and ^1H NMR and other 2D NMR experiments with PS-II were performed on a Bruker AMX-400 instrument at 40 °C. Selective spin-decoupling was carried out by a modified method [30]. Two-dimensional NMR experiments were performed using standard Bruker software. A mixing time of 0.2 s was used in the ROESY experiment; times of 0.15 s and 0.1 s were used for TOCSY and ROESY, respectively, in the combined TORO experiment.

Isolation of lipopolysaccharide and O-specific polysaccharide.—H. alvei PCM 1222 derived from the Pasteur Institute Collection (Paris) was cultivated in a liquid medium [31]. Lipopolysaccharide was isolated from dry bacterial mass as described [8,9] and hydrolysed with aq 1% AcOH for 1.5–2 h at 100 °C. A water-soluble portion was fractionated on Sephadex G-50 to give the O-specific polysaccharide (PS-I).

Compositional and methylation analyses.—The content of different constituents was determined using colorimetric methods applied directly to PS-I [12–16] or after hydrolysis with 2 M CF_3CO_2H for 2 h at 120 °C [10,11]. For PC, GLC, and the hexokinase test, hydrolysis was performed under the same conditions or with 10 M HCl for 30 min at 80 °C.

Methylation was carried out by the procedure of Conrad [32] (for PS-I) or Gunnarson [33] (for PS-II, PS-IV, and OS). Methylated products were hydrolysed with 2 M CF_3CO_2H for 2 h at 120 °C, conventionally reduced with $NaBH_4$, acetylated, and analysed by GLC–MS using published data [34].

Chemical modifications.—O-Deacetylation was carried out with aq 12% ammonia at room temperature overnight. Carboxyl reduction was performed by the method of Taylor et al. [35]. Dephosphorylation was performed with aq 48% HF for 48 h at 4 °C, HF was removed in a vacuum desiccator over KOH or in a stream of N_2, and the products were fractionated by GPC on TSK HW-40 or Bio-Gel P-2.

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