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Structure of the O-specific polysaccharide of *Hafnia alvei* 1204 containing 3,6-dideoxy-3-formamido-D-glucose

Ewa Katzenellenbogen [a], Elzbieta Romanowska [a], Nina A. Kocharova [b], Aleksander S. Shashkov [b], Yuriy A. Knirel [b,*], Nikolay K. Kochetkov [b]

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

[b] *N.D. Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, Leninsky Prospekt 47, Moscow 117913, Russian Federation*

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Abstract

The O-specific polysaccharide of *Hafnia alvei* strain 1204 has a hexasaccharide repeating unit containing D-mannose, D-glucuronic acid, 2-acetamido-2-deoxy-D-glucose, 2-acetamido-2-deoxy-D-galactose, and 3,6-dideoxy-3-formamido-D-glucose (Qui3NFo) in the ratios 2:1:1:1:1 as well as *O*-acetyl groups. On the basis of methylation analysis of the intact, carboxyl-reduced, and Smith-degraded polysaccharide as well as 1D and 2D NMR spectroscopy, including 1D total correlation spectroscopy, 1D NOE spectroscopy, 2D homonuclear shift-correlated spectroscopy (COSY), and $^{13}C,^{1}H$ heteronuclear COSY, the following structure of the *O*-deacetylated polysaccharide was established:

→3)-α-D-Man*p*-(1→2)-α-D-Man*p*-(1→3)-β-D-Glc*p*NAc-(1→

→2)-β-D-Qui*p*3NFo-(1→3)-α-D-Gal*p*NAc-(1→4)-α-D-Glc*p*A-(1→

Location of the *N*-formyl group, occurring as two stereoisomers in the ratio ~3:1, was determined by an NOE on H-3 Qui3N arising on pre-irradiation of HCO of the minor (*E*) isomer.

* Corresponding author.

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The *O*-acetyl groups are attached in nonstoichiometric amounts at position 3 of GlcA and position 6 of a mannose residue or GlcNAc.

Keywords: O-Antigen; Lipopolysaccharide; Enterobacteria; NMR spectroscopy; Formyl group

1. Introduction

The enteric bacterium *Hafnia alvei* is an opportunistic pathogen associated with nosocomal infections [1]. Recently, the structures of the O-specific polysaccharide chains of lipopolysaccharides of a number of serologically different strains of this species have been established (refs [2–6] and references cited in ref. [2]). We now report the elucidation of the structure of the O-specific polysaccharide of *H. alvei* strain 1204.

2. Results and discussion

The lipopolysaccharide of *H. alvei* 1204 was isolated in a yield of 2.5% from dried bacterial cells by phenol–water extraction [7] followed by GPC on Sepharose 2B [8]. The O-specific polysaccharide was obtained by mild degradation of the lipopolysaccharide followed by GPC on Sephadex G-50.

Sugar analysis of the polysaccharide by GLC-MS of the derived alditol acetates revealed the presence of mannose, glucose, 2-amino-2-deoxyglucose, 2-amino-2-deoxygalactose, and 3-amino-3,6-dideoxyhexose in the ratios 2.2:0.4:1:0.84:0.32. Of these sugars, glucose is a common component of *Hafnia* lipopolysaccharides [9] and is probably derived from the core oligosaccharide. HVPE and a colorimetric assay [10] showed the presence of glucuronic acid (13%). The D configuration of hexoses, hexosamines, and glucuronic acid (after carboxyl-reduction [11]) was determined by enzymatic methods as described previously [2]. The 6-deoxyamino sugar was identified by PC as 3-amino-3,6-dideoxyglucose (Qui3N, R_{Glc} 1.29); its D configuration was proven by analysis of the ^{13}C NMR spectrum of the polysaccharide (see below). The polysaccharide was found to be *O*-acetylated with the content of *O*-acetyl groups 4.7% as determined by the colorimetric method [12].

The ^{13}C NMR spectrum of the polysaccharide [Fig. 1(a)] contained a number of signals with different integral intensities, typical of a polymer with a masked regularity, for example, caused by nonstoichiometric *O*-acetylation. This conclusion was confirmed by different intensities of the signals for the *O*-acetyl groups at δ 21.6 and 21.8. The ^{13}C NMR spectrum of the polysaccharide *O*-deacetylated with aqueous ammonia [Fig. 1(b)] indicated a lesser degree of irregularity but, together with the major signals, still contained a series of minor signals. This was due to the occurrence of an *N*-formyl group attached to Qui3N as two stereoisomers (see below).

Analysis of the main series of signals in the ^{13}C NMR spectrum showed that the *O*-deacetylated polysaccharide had a hexasaccharide repeating unit (there were six signals for anomeric carbons in the region δ 98–104) containing one 6-deoxy sugar (Qui3N, a signal for C-6 at δ 18.3), one uronic acid (a signal for C-6 in the region δ

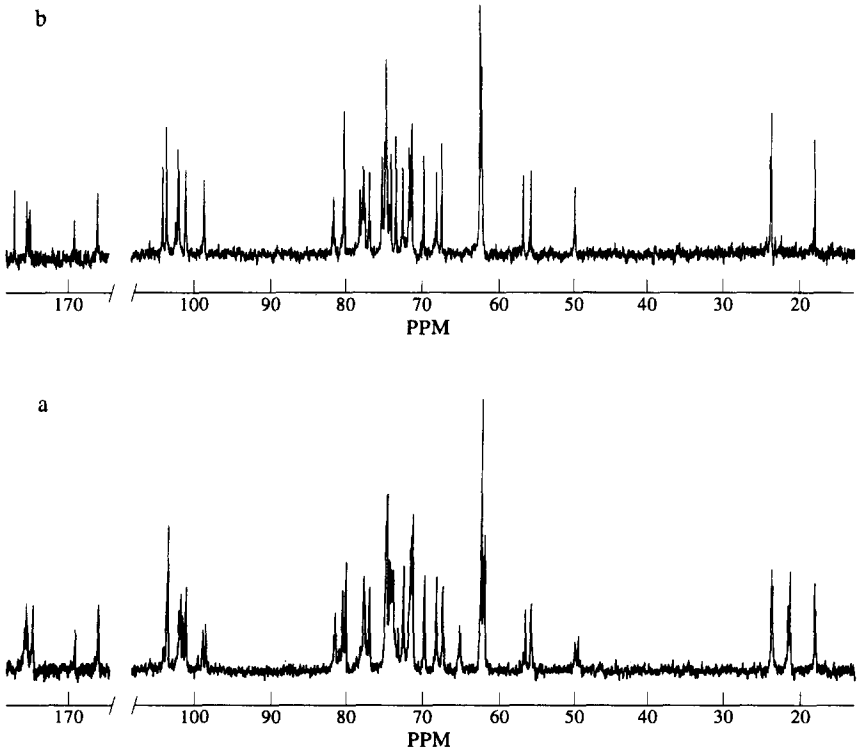

Fig. 1. 75-MHz ^{13}C NMR spectrum of the intact (a) and *O*-deacetylated (b) polysaccharide.

174.7–175.2), and three amino sugars (signals for carbons bearing nitrogen at δ 49.8, 55.7, and 56.7). Two of the amino sugars were *N*-acetylated [signals at δ 23.6 and 23.9 (Me) and in the region δ 174.7–175.2 (CO)], while the third one carried an *N*-formyl group (signals for HCO at δ 165.9 and 168.9 belonging to the (*Z*) and (*E*) isomer, respectively; cf. the published data [13,14]).

Accordingly, in the ^1H NMR spectrum of the *O*-deacetylated polysaccharide (Fig. 2) there were present two series of signals. The main series contained signals for six anomeric protons in the region δ 4.6–5.5, the CH_3–C group (H-6) of Qui3N at δ 1.29 (d, $J_{5,6}$ Hz), and two *N*-acetyl groups at δ 2.02 and 2.09. In accordance with the occurrence of two isomers, the *N*-formyl group gave two signals at δ 8.25 and 7.93 with the ratio of the integral intensities $\sim$ 3:1 (cf. the published data [14,15]).

Therefore, the repeating unit of the polysaccharide contains two mannose residues and one residue of each of GlcA, GlcN, GalN, and Qui3N as well as two *N*-acetyl groups, one *N*-formyl group, and *O*-acetyl groups. As judged by the attached-proton test data [16], all CH_2O groups in the *O*-deacetylated polysaccharide resonate in the region δ 62–63 and, hence, none of the hexoses and hexosamines is glycosylated at position 6.

The ^1H NMR spectrum of the *O*-deacetylated polysaccharide was assigned using sequential, selective spin-decoupling, 1D total-correlated spectroscopy (TOCSY-

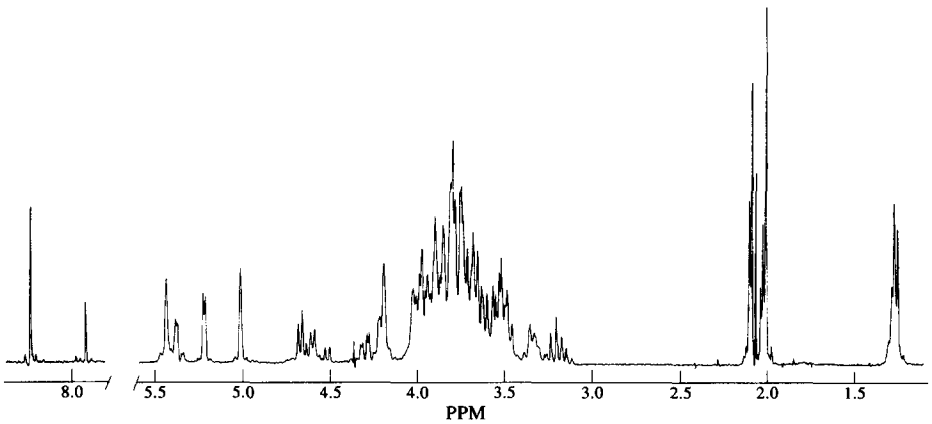

Fig. 2. 250-MHz ^{1}H NMR spectrum of *O*-deacetylated polysaccharide.

HOHAHA [17]) with sequential, selective excitation of the anomeric protons, 2D shift-correlated spectroscopy (COSY), and H,H-relayed COSY (Table 1). As judged by the coupling constant values $J_{1,2}$ 7–8 Hz, GlcN (unit **A**) and Qui3N (unit **B**) are β-linked, while GalN (unit **C**) and GlcA (unit **D**), characterised by the coupling constant value $J_{1,2}$ 4 Hz, are α-linked [18]. The absence of NOE on H-3 and H-5 on preirradiation of H-1 of the same sugar residue showed that both mannose residues (units **E** and **F**) are α-linked.

Analysis of NOE arising on preirradiation of HCO was used for determining the location of the *N*-formyl group. While no NOE was observed on preirradiation of the major isomer at δ 8.25, preirradiation of the minor isomer at δ 7.93 caused a significant NOE on H-3 of Qui3N, which gave a signal at δ 3.38 also belonging to the minor series. Therefore, the formyl group is attached to the amino group of Qui3N that is in accord with the presence of two series of signals for this residue in the ^{1}H NMR

Table 1
^{1}H NMR chemical shifts for the *O*-deacetylated polysaccharide (δ in ppm) [a]

Sugar unit	H-1	H-2	H-3	H-4	H-5	H-6
→ 3)-β-D-Glc*p*NAc-(1 → (**A**)	4.61 [b]	3.72	3.67	3.49	3.34	
→ 2)-β-D-Qui*p*3NFo-(1 → (**B**)						
(*Z*) isomer	4.69	3.64	3.97	3.20	3.55	1.28
(*E*) isomer	4.67	3.58	3.38	3.15	3.50	1.29
→ 3)-α-D-Gal*p*NAc-(1 → (**C**)	5.38	4.27	4.01	4.20	4.02	
→ 4)-α-D-Glc*p*A-(1 → (**D**)	5.22	3.61	3.95	3.76	4.15	
→ 3)-α-D-Man*p*-(1 → (**E**)	5.02	4.20	3.93	3.90	3.51	
→ 2)-α-D-Man*p*-(1 → (**F**)	5.45	4.03	3.82	3.71	3.80	

[a] The chemical shifts for NAc are δ 2.02–2.11 and for the *N*-formyl group δ 8.25 and 7.93 for the (*Z*) and (*E*) isomer, respectively.

[b] In the repeating units with the (*Z*) configuration of the formyl group; δ 4.52 in the units with the (*E*) configuration.

Table 2
^{13}C NMR chemical shifts for the *O*-deacetylated polysaccharide (δ in ppm) [a]

Sugar unit	C-1	C-2	C-3	C-4	C-5	C-6
→ 3)-β-D-Glc*p*NAc-(1 → **(A)**	101.7 [b]	55.7	81.4	72.4	76.7	62.1 [c]
→ 2)-β-D-Qui*p*3NFo-(1 → **(B)**						
(*Z*) isomer	103.8	77.5	56.7	74.7	73.9	18.3
(*E*) isomer	103.8	77.3	56.7	74.1	73.9	18.3
→ 3)-α-D-Gal*p*NAc-(1 → **(C)**	98.4	49.8	77.9	71.2	71.5	62.4 [c]
→ 4)-α-D-Glc*p*A-(1 → **(D)**	101.7	73.3	75.1	77.5	74.5	
→ 3)-α-D-Man*p*-(1 → **(E)**	103.3	69.6	80.0	67.3	74.6	62.2 [c]
→ 2)-α-D-Man*p*-(1 → **(F)**	100.8	79.9	71.3	68.0	74.5	62.2 [c]

[a] The chemical shifts for the methyl group of NAc are δ 23.6 and 23.9, for the carbonyl groups of NAc and the carboxyl group of GlcA are δ 174.7, 175.0, and 175.2, and for the *N*-formyl group are δ 165.9 and 168.9 for the (*Z*) and (*E*) isomer, respectively.
[b] In the repeating units with the (*Z*) configuration of the formyl group; δ 102.1 in the units with the (*E*) configuration.
[c] Assignment could be interchanged.

spectrum (Table 1). The NOE experiment allowed also assignment of the signals at δ 8.25 and 7.93 to the (*Z*) and (*E*) isomer, respectively, since only in the latter is the spatial contact between HCO and H-3 of Qui3N possible. Consequently, GlcN and GalN are *N*-acetylated.

Linkage and sequence analysis of the *O*-deacetylated polysaccharide was performed using 1D rotating-frame NOE spectroscopy (ROESY [19]) with sequential, selective excitation of the anomeric protons. The following correlations between the transglycosidic protons were observed: H-1 **A** at δ 4.61/H-2 **B** at δ 3.64 (main series), H-1 **B** at δ 4.69/H-3 **C**, H-1 **C**/H-4 **D**, H-1**D**/H-3 **E**, H-1 **E**/H-2 **F**, and H-1 **F**/H-3 **A**. These data suggested that the *O*-deacetylated polysaccharide is linear and has the following structure:

→ 3)-α-D-Man*p*-(1 → 2)-α-D-Man*p*-(1 → 3)-β-D-Glc*p*NAc-(1 →
E **F** **A**

→ 2)-β-D-Qui*p*3NFo-(1 → 3)-α-D-Gal*p*NAc-(1 → 4)-α-D-Glc*p*A-(1 →
B **C** **D**

This structure is in agreement with the ^{13}C NMR spectrum of the *O*-deacetylated polysaccharide which was assigned using 2D heteronuclear ^{13}C,^{1}H COSY (Table 2). In particular, low-field displacements of the signals for H-2 of units **B** and **F**, H-3 of units **A**, **C**, and **E**, and H-4 of unit **D**, as compared with their positions in the spectra of the corresponding nonsubstituted monosaccharides [20], are caused by glycosylation and confirmed the substitution pattern determined by NOE spectroscopy.

As compared with the corresponding free monosaccharide, in the β-(1 → 3)-linked disaccharide fragment **B** → **C** the signal for C-1 of unit **B** is shifted downfield by ~ 7 ppm which is typical of the same absolute configuration of the constituent monosaccharides, that is of the D configuration of Qui3N (this shift would be < 4 ppm in the case of the L configuration of unit **B** [21]).

Table 3
Methylation analysis data

Methylated sugar	t_R [a]	Molar ratio [b]		
		Intact polysaccharide	Carboxyl-reduced polysaccharide	Oligosaccharide **1**
3,4,6-Me_3-Man	1.21	1.40	1.00	n.d.
2,4,6-Me_3-Man	1.24	0.75	0.90	n.d
2,3,6-Me_3-Glc	1.24	n.d.	1.00	n.d.
4-Me-Qui3N	1.58	1.05	0.80	0.5
3,4,6-Me_3-GlcN	1.66	n.d.	n.d.	1.00
4,6-Me_2-GlcN	1.90	1.00	1.00	n.d.
4,6-Me_2-GalN	1.92	0.72	0.80	0.73

[a] Retention time of the corresponding alditol acetate relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-glucitol.
[b] n.d., not detected.

Comparison of the ^{13}C NMR spectra of the intact and *O*-deacetylated polysaccharide showed that in the former the intensities of the signals for C-2,3,4 of GlcA significantly decreased, most likely due to their shift from δ 73.3, 75.1, and 77.5 in the spectrum of the *O*-deacetylated polysaccharide to the regions δ 71.2–71.6, 76.8–77.6, and 73.8–74.7, respectively. These displacements were caused by partial *O*-acetylation and suggested [22] that one of the *O*-acetyl groups is attached at position 3 of GlcA. In addition, a signal for one of the hydroxymethyl groups was shifted downfield to δ 65.1 thus indicating the attachment of the second *O*-acetyl group at position 6 of a mannose or a hexosamine residue. The signal for C-5 of GalNAc did not change its position at δ 71.5 and, hence, a mannose residue or GlcNAc is nonquantitatively *O*-acetylated at position 6. As judged by the ratios of the signals belonging to the *O*-acetylated and the corresponding non-*O*-acetylated sugar unit, the degree of *O*-acetylation varied from one batch of the polysaccharide to another but may exceed 50% at each position.

The structure of the polysaccharide thus established was confirmed by chemical methods.

Methylation of the intact polysaccharide [23] followed by GLC-MS analysis of the derived alditol acetates led to identification of 3,4,6-tri-*O*-methylmannose, 2,4,6-tri-*O*-methylmannose, 3,6-dideoxy-4-*O*-methyl-3-methylaminoglucose, 2-deoxy-4,6-di-*O*-methyl-2-methylaminoglucose, and 2-deoxy-4,6-di-*O*-methyl-2-methylaminogalactose (Table 3). Therefore, one of the mannose residues, GlcN, and GalN are substituted at position 3, while the second mannose residue and Qui3N are substituted at position 2. When the carboxyl-reduced [11] polysaccharide was applied to methylation analysis, 2,3,6-tri-*O*-methylglucose was additionally identified, giving evidence of substitution of GlcA at position 4.

Periodate oxidation of the *O*-deacetylated polysaccharide destroyed half of the mannose and almost all GlcA, while other components of the repeating unit were unaffected. In the intact polysaccharide about half of the GlcA was stable which is in accord with partial *O*-acetylation of this sugar unit at position 3. Mild acid hydrolysis of the oxidised *O*-deacetylated polysaccharide with aq 2% acetic acid (100°C, 2 h) was complicated by the increased stability of the glycosidic linkage of the oxidised GlcA and

unexpected stability of the linkage of the oxidised 2-substituted mannose residue. To overcome this difficulty, Smith degradation was carried out with the carboxyl-reduced *O*-deacetylated polysaccharide and the hydrolysis was prolonged to 6 h. As a result, oligosaccharide-alditol **1** was obtained which contained all three amino sugars present in the polysaccharide together with erythritol (Ery-ol). Methylation analysis of **1** (Table 3) resulted in identification of 3,6-dideoxy-4-*O*-methyl-3-methylaminoglucose (from the 2-substituted Qui3N), 2-deoxy-3,4,6-di-*O*-methyl-2-methylaminoglucose (from the terminal GlcN), and 2-deoxy-4,6-di-*O*-methyl-2-methylaminogalactose (from the 3-substituted GalN). These data and the results of the ^{1}H NMR study, including 1D NOE spectroscopy carried out as described above for the *O*-deacetylated polysaccharide, showed that **1** has the following structure

$$\beta\text{-D-Glc}p\text{NAc-}(1 \rightarrow 2)\text{-}\beta\text{-D-Qui}p\text{3NFo-}(1 \rightarrow 3)\text{-}\alpha\text{-D-Gal}p\text{NAc-}(1 \rightarrow 2)\text{-Ery-ol}$$

which is consistent with the structure of the repeating unit of the polysaccharide.

It is worthy of note that, like many other studied strains of *H. alvei* [2–4,6,24,25], strain 1204 produces an acidic O-specific polysaccharide. *N*-Acyl derivatives of Qui3N and other rarely occurring 6-deoxyamino sugars are components of some other O-antigens of *Hafnia* [2,25,26] but, to the best of our knowledge, the *N*-formyl derivative of Qui3N identified in the strain 1204 has not been found hitherto in bacterial polysaccharides.

3. Experimental

General methods.—GPC was performed on a column (2 × 100 cm) of Sephadex G-50 or 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. PC was performed on Whatman No. 1 paper using a system of 1-butanol–pyridine–water (6:4:3, v/v); substances were detected on paper with the alkaline silver nitrate reagent. GLC-MS was performed with a Hewlett–Packard Model 5971 A chromatograph equipped with a glass capillary column (12 m × 0.2 mm) of HP-1 as stationary phase using a temperature program of 150–270°C at 8°C/min. The ^{1}H and ^{13}C NMR spectra were recorded with a Bruker WM-250 and a Bruker AM-300 spectrometer for solutions in D_2O at 70°C with acetone (δ_H 2.225, δ_C 31.45) as internal standard.

Bacterial strain and isolation of lipopolysaccharide and O-specific polysaccharide. —*H. alvei* strain 1204 was derived from the Collection of the Pasteur Institute (Paris). Cultivation of the bacterium in a liquid medium [27] and isolation of the lipopolysaccharide were performed as described previously [7,8]. The lipopolysaccharide was degraded with aq 1% AcOH (100°C, 1 h), and the water-soluble portion was fractionated by GPC on Sephadex G-50 to give the O-specific polysaccharide in the yield 33.6% of the total amount of the material eluted from the column.

Sugar and methylation analysis.—Hydrolysis was performed with 2 M trifluoroacetic acid (120°C, 2 h) and monosaccharides were conventionally converted into alditol

acetates and analysed by GLC-MS. Methylation was done by the Hakomori method [23]. The methylated products were hydrolysed with 10 M hydrochloric acid (80°C, 30 min), and partially methylated monosaccharides were analysed by GLC-MS as alditol acetates.

O-Deacetylation.—The polysaccharide was treated with aq 12% ammonia at room temperature overnight.

Carboxyl reduction.—The polysaccharide was treated by the method of Taylor et al. [11].

Smith degradation.—The *O*-deacetylated carboxyl-reduced polysaccharide (32 mg) was oxidised with 0.1 M $NaIO_4$ (4°C, 48 h, in the dark), ethylene glycol was added, the mixture was reduced with an excess of $NaBH_4$ (2 h), neutralised with concd AcOH, desalted on TSK HW-40, and hydrolysed with aq 2% AcOH (100°C, 2 h), and the product was isolated by GPC on TSK HW-40 and additionally hydrolysed for 6 h under the same conditions to give oligosaccharide **1** (7 mg).

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