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The structure of the O-specific polysaccharide of *Hafnia alvei* strain 1216

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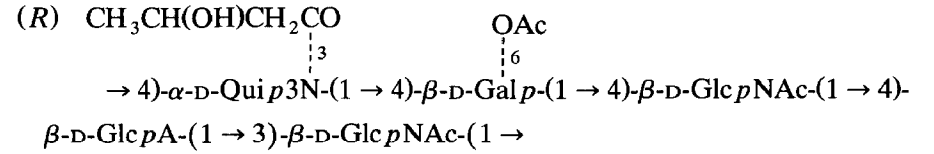
Abstract

The O-specific polysaccharide of *Hafnia alvei* strain 1216 is composed of D-galactose, D-glucuronic acid, 2-acetamido-2-deoxy-D-glucose, 3,6-dideoxy-3-[(*R*)-3-hydroxybutyramido]-D-glucose, and *O*-acetyl groups in the ratios 1:1:2:1:1. On the basis of sugar and methylation analyses of the intact and chemically degraded (*O*-deacetylated, carboxyl-reduced, Smith-degraded) polysaccharide and ^{1}H and ^{13}C NMR spectroscopy, including 2D shift-correlated (COSY, relayed COSY, ^{13}C, ^{1}H-COSY) and 1D NOE spectroscopy, it was concluded that the O-antigen is built up of linear pentasaccharide units having the following structure:



1. Introduction

Recently [1–6], the O-specific polysaccharide chains of lipopolysaccharides isolated from a number of *Hafnia alvei* strains have been structurally elucidated

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and found to be acidic or neutral hexosaminoglycans. We describe now the structure of the O-antigen of *H. alvei* strain 1216 containing D-glucuronic acid and 3-amino-3,6-dideoxy-D-glucose *N*-acylated by (*R*)-3-hydroxybutyric acid.

2. Results and discussion

The lipopolysaccharide was isolated from dry bacterial cells of *H. alvei* 1216 by extraction with phenol–water [7] and purified by gel-permeation chromatography on Sepharose 2B [8]. Hydrolysis of the lipopolysaccharide with aq 1% acetic acid gave an acidic O-specific polysaccharide purified on Sephadex G-50.

Hydrolysis of the polysaccharide with 10 M hydrochloric acid at 80°C followed by GLC–MS analysis of the derived alditol acetates revealed the presence of galactose, 2-amino-2-deoxyglucose, and 3,6-dideoxy-3-[(*R*)-3-hydroxybutyramido] hexose in the molar ratios 1.0:2.1:0.9, respectively. When hydrolysis was performed with 2 M trifluoroacetic acid at 120°C, glucosamine was not released completely, 3-hydroxybutyryl groups were partially split off, and 3-amino-3,6-dideoxyhexose appeared (molar ratios of the components: galactose, glucosamine, 3-acylamino-3,6-dideoxyhexose, and 3-amino-3,6-dideoxyhexose = 1.00:0.87:0.29 :0.26, respectively).

Using colorimetric methods [9,10], the polysaccharide was found to contain hexuronic acid (18%) and *O*-acetyl groups (0.94 μM/mg). Carboxyl-reduction [11] led to a decrease in the content of uronic acid from 18 to 2%. Monosaccharide analysis of the carboxyl-reduced polysaccharide, including hydrolysis with 10 M hydrochloric acid, led to the identification of the neutral and amino sugars listed above and additionally glucose clearly derived from the glucuronic acid (molar ratios of galactose, glucosamine, 3-acylamino-3,6-dideoxyhexose, and glucose were 1.0:2.2:1.0:1.0, respectively). Glucuronic acid was identified also by high-voltage electrophoresis after hydrolysis of the polysaccharide with 2 M trifluoroacetic acid.

The D configuration of glucose (derived from glucuronic acid), galactose, and glucosamine and the (*R*) configuration of the 3-hydroxybutyric acid were proved by treatment of hydrolysates of the polysaccharides with the appropriate enzymes: D-glucose oxidase [12], D-galactose oxidase [13], hexokinase [14], and (*R*)-3-hydroxybutyrate dehydrogenase [15], respectively. The mass spectrum of the 3,6-dideoxy-3-[(*R*)-3-hydroxybutyramido]hexose was identical to that of the same monosaccharide derivative with the D-*galacto* configuration isolated from the O-antigen of *H. alvei* 1211 [4] but, judging from the GLC and paper chromatography data, these two sugars were different. The D-*gluco* configuration of the sugar from *H. alvei* 1216 was determined by ^{1}H and ^{13}C NMR spectroscopy (see below). In summary, the sugar analyses showed that the repeating unit of the O-antigen contains residues of D-galactose, D-glucuronic acid, D-glucosamine, and 3-acylamino-3,6-dideoxyhexose in the ratios 1:1:2:1.

The ^{13}C NMR spectrum (Fig. 1, Table 1) confirmed that the polysaccharide has a pentasaccharide repeating unit since there were signals for five anomeric carbons at 104.8, 104.2, 102.1, 102.1, and 100.5 ppm. The spectrum contained *inter alia* the

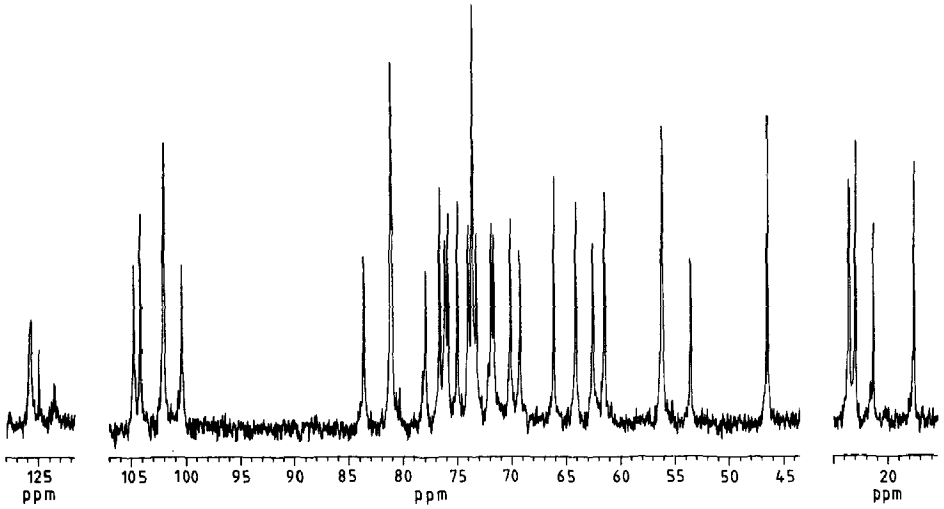


Fig. 1. ^{13}C NMR spectrum of the O-specific polysaccharide.

signals for three carbons bearing nitrogen (C-2 of two GlcN and C-3 of Qui3N) at 56.2, 56.2, and 53.6 ppm, one CH_3C (C-6 of Qui3N) at 17.6 ppm, and one COOH (C-6 of GlcA) at 173.6 ppm. The attached-proton test [16] revealed the signals for CH_2OH (C-6 of Gal and two GlcN) at 64.2, 62.6, and 61.5 ppm, and, hence, none of the monosaccharides was 6-substituted (C-6 of a 6-substituted monosaccharide would resonate [17,18] at 66–72 ppm). The spectrum indicated also the presence of

Table 1
Chemical shifts in the ^{13}C NMR spectra (δ in ppm) [a]

Unit [b]		C-1	C-2	C-3	C-4	C-5	C-6
O-specific polysaccharide [c]							
→4)-α-D-Qui*p*3NAcyl-(1→	(A)	100.5	71.7	53.6	81.1	69.3	17.6
→4)-β-D-Gal*p*-(1→	(B)	104.8	72.0	73.3	78.0	74.0	64.2
→4)-β-D-Glc*p*NAc-(1→	(C)	102.1	56.2	73.7	81.2	75.9	61.5
→4)-β-D-Glc*p*A-(1→	(D)	104.2	73.7	75.0	81.2	76.2	173.6
→3)-β-D-Glc*p*NAc-(1→	(E)	102.1	56.2	83.7	70.2	76.7	62.6
O-Deacetylated polysaccharide [d]							
→4)-α-D-Qui*p*3NAcyl-(1→	(A)	100.3	71.7	53.6	81.2	69.2	17.6
→4)-β-D-Gal*p*-(1→	(B)	104.6	72.1	73.4	77.8	76.7	61.4
→4)-β-D-Glc*p*NAc-(1→	(C)	101.9	56.3	73.8	80.2	76.1	61.4
→4)-β-D-Glc*p*A-(1→	(D)	104.3	73.8	75.0	81.2	77.9	175.3
→3)-β-D-Glc*p*NAc-(1→	(E)	102.2	56.3	83.5	70.2	76.7	62.5

[a] Additional signals: OAc at 21.4 (Me) and 174.9 ppm (CO); NAc at 23.5–23.7 (Me) and 175.9–176.0 ppm (CO); C-1,2,3,4 of 3-hydroxybutyryl group at 175.7–176.0, 46.6, 66.2, and 23.0 ppm, respectively.
[b] Acyl = (*R*)-3-hydroxybutyryl.
[c] Measurements at pD 3.
[d] Measurements at pD 6.

two *N*-acetyl groups (Me at 23.5 and 23.6 ppm), one *O*-acetyl group (Me at 21.4 ppm), and one *N*-(3-hydroxybutyryl) group (the signals for C-2,3,4 at 46.6, 66.2, and 23.0 ppm, respectively, cf. the published data [19]). The ^{13}C NMR spectrum of the polysaccharide *O*-deacetylated with aqueous ammonia [6] (Table 1) lacked the signal for the *O*-acetyl group.

The polysaccharide was therefore built up of pentasaccharide repeating units each containing one residue of D-galactose, D-glucuronic acid, and 3,6-dideoxy-3-[(*R*)-3-hydroxybutyramido]-D-glucose, two residues of 2-acetamido-2-deoxy-D-glucose, and an *O*-acetyl group in stoichiometric or nearly stoichiometric amount.

Methylation and GLC–MS analysis of the derived partially methylated alditol acetates were applied for linkage analysis of the intact and carboxyl-reduced polysaccharides. The results are given in Table 2, and the mass spectrum and fragmentation pattern of the derivative of 3,6-dideoxy-3-[(*R*)-3-hydroxybutyramido]-D-glucose are shown in Fig. 2. From the data obtained, it was concluded that the polysaccharide was linear, one of the GlcNAc residues was substituted at position 3, and other constituent monosaccharides were substituted at position 4.

The coupling constants $^{1}J_{C,H}$ of 160–164 Hz, determined from the gated-decoupling ^{13}C NMR spectrum of the polysaccharide, for four signals of the anomeric carbons at 102.1–104.8 ppm and 172 Hz for the fifth one at 100.5 ppm showed [20] that all sugar residues are pyranoid, four of them are β-linked and one α-linked.

The ^{1}H NMR spectrum of the *O*-deacetylated polysaccharide (Table 3) confirmed the presence of a pentasaccharide repeating unit, having signals for five anomeric protons in the region 4.46–4.86 ppm. Four of the sugar residues are of β configuration ($^{3}J_{1,2}$ 7.5 Hz) and the fifth one of α configuration ($^{3}J_{1,2}$ 4 Hz). This

Table 2
Data of methylation analysis [a]

Methylated sugar	t_R [b]	Molar ratio						
		Polysaccharide Intact			Carboxyl-reduced	Oligosaccharide OS-I	OS-II	OS-III
		A	*B*	*C*	*A*	*B*		
2,3,6Me$_3$Gal	1.23	1.0	1.0	1.0	1.0	– [c]	–	–
2,3,6Me$_3$Glc	1.25	–	–	–	0.86	–	–	–
2MeQui3NMeAc	1.55	–	tr [d]	0.3	tr	0.2	0.19	0.2
3,4,6Me$_3$GlcNMeAc	1.68	–	–	–	–	1.0	1.0	1.0
3,6Me$_2$GlcNMeAc	1.84	1.0	0.8	1.2	1.1	0.3	–	–
4,6Me$_2$GlcNMeAc	1.93	0.54	0.23	1.0	0.76	0.6	0.4	0.1
2MeQui3NMeAcyl [e]	2.05	0.65	0.95	–	0.68	1.0	0.53	0.95

[a] Methylated poly- and oligo-saccharides were hydrolysed with 10 M HCl at 80°C for 30 min (*A*), 2 M CF_3CO_2H at 120°C for 2 h (*B*), or according to Stellner et al. [30] (*C*).

[b] t_R, retention time of the corresponding alditol acetate relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol.

[c] -, Component not present.

[d] t_r, Trace amount,

[e] Acyl, 3-methoxybutyryl.

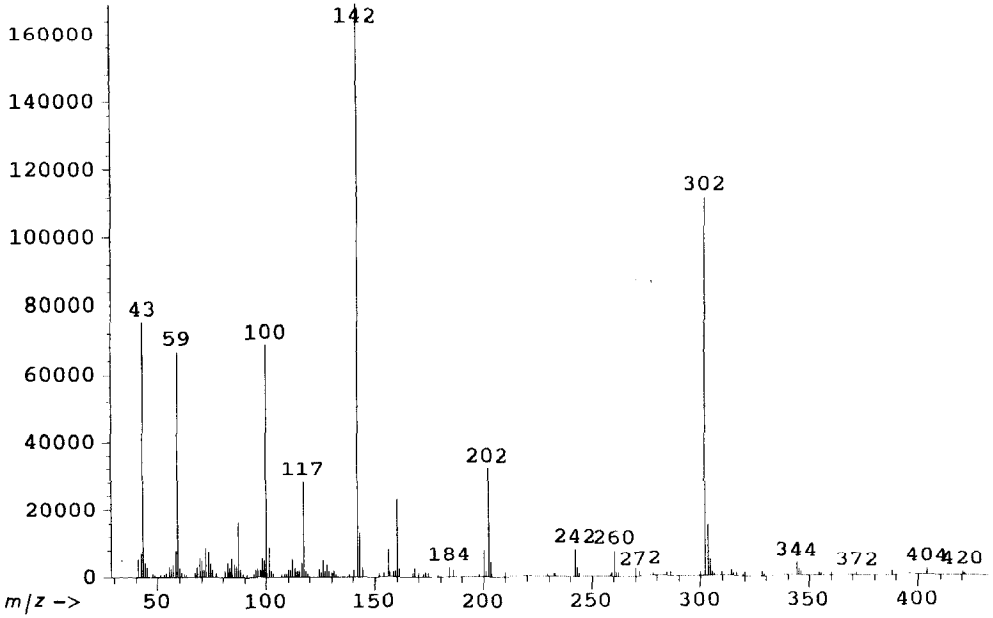


Fig. 2. Mass spectrum and fragmentation pattern of 1,4,5-tri-*O*-acetyl-3,6-dideoxy-3-[3-methoxy-*N*-methylbutyramido)-2-*O*-methylglucitol.

spectrum was assigned with the aid of sequential, selective spin-decoupling, 2D homonuclear shift-correlated spectroscopy (COSY), and COSY with one- and two-step relayed coherence transfer (COSYRCT). Some uncertainties arising from coincidence of the signals for two or more neighbouring protons of the same sugar residue were removed by measurement of the spectrum at two different pD values (for assignment of the signals for H-4,5 of the uronic acid), by using NOE spectroscopy (for assignment of the signals for H-3,5 of the β-linked sugar residues), and heteronuclear $^{13}C/^{1}H$ COSY spectroscopy (Fig. 3). The latter procedure also allowed assignment of the ^{13}C NMR spectrum of the polysaccharide (Table 1).

On the basis of the $^{3}J_{H,H}$ coupling constants determined from the ^{1}H NMR spectrum, the α-linked sugar was identified as Qui3N (unit **A**), its *gluco* configuration was proved, and the spin systems for the β-linked monosaccharides [Gal (unit **B**), GlcA (unit **D**), and two GlcN (units **C** and **E**)] were isolated. The two GlcN units were distinguished from GlcA on the basis of the correlation of their H-2 resonances in the $^{13}C/^{1}H$ COSY spectrum with the C-2 resonances at 56.3 ppm, i.e., in the region of carbons bearing nitrogen.

The following interresidue NOE were observed on sequential, selective preirradiation of each of the anomeric protons: H-1 **A**/H-4,6a,6b, **B**; H-1 **B**/H-3 or H-4 **C**; H-1 **C**/H-4 **D**; H-1 **D**/H-3 **E**; and H-1 **E**/H-4 **A**. These data indicated that the polysaccharide was linear and revealed the sequence –**A**–**B**–**C**–**D**–**E**– with units **A**, **B**, and **D** substituted at position 4, unit **E** at position 3, and unit **C** at position 3 or

Table 3
^{1}H NMR data for *O*-deacetylated polysaccharide at pD 6 (δ in ppm, J in Hz) [a]

H-1	H-2	H-3	H-4	H-5	H-6
→ 4)-α-D-Qui*p*3NAcyl [b]-(1 → (unit **A**)					
4.86	3.65	4.06	3.43	4.23	1.22
$J_{1,2}$4	$J_{2,3}$11	$J_{3,4}$11	$J_{4,5}$11	$J_{5,6}$6	
→ 4)-β-D-Gal*p*-(1 → (unit **B**)					
4.50	3.65	3.73	4.02	3.77	3.77 [c]
$J_{1,2}$7.5	$J_{2,3}$10	$J_{3,4}$3	$J_{4,5}$ < 2		
→ 4)-β-D-Glc*p*NAc-(1 → (unit **C**)					
4.60	3.7	3.7	3.7	3.63	3.86 [d]
$J_{1,2}$7.5				$J_{5,6a}$3	$J_{5,6b}$ < 3
→ 4)-β-D-Glc*p*A-(1 → (unit **D**)					
4.46	3.37	3.58	3.77 [e]	3.77 [f]	
$J_{1,2}$7.5	$J_{2,3}$9	$J_{3,4}$9			
→ 3)-β-D-Glc*p*NAc-(1 → (unit **E**)					
4.56	3.71	3.79	3.44	3.44	3.76 [g]
$J_{1,2}$7.5	$J_{2,3}$9	$J_{3,4}$9		$J_{5,6a}$6	$J_{5,6b}$ < 2
N-(3-Hydroxybutyryl)					
	2.46 [h]	4.23	1.24		
	$J_{2a,3}$6	$J_{2b,3}$7	$J_{3,4}$6		

[a] Additional signals: NAc at 2.04 and 2.01 ppm (both s).
[b] Acyl, (*R*)-3-hydroxybutyryl.
[c] H-6a; H-6b at 3.82 ppm.
[d] H-6a; H-6b at 4.00 ppm, $J_{6a,6b}$ 12Hz.
[e] 3.83 ppm at pD 2; $J_{4,5}$ 9 Hz.
[f] 4.02 ppm at pD 2.
[g] H-6a; H-6b at 3.96 ppm, $J_{6a,6b}$ 12 Hz.
[h] H-2a; H-2b at 2.52 ppm, $J_{2a,2b}$ 14 Hz.

4. Although H-1 **A** exhibited NOEs with both H-4 and H-6a,6b **B**, the site of glycosylation was HO-4 since none of the constituent monosaccharides was substituted at position 6 (*vide infra*). The appearance of the NOE on H-6a,6b **B** is typical [21] of a 4-substituted galactose residue and proved that the α-linked units **A** and **B** have the same absolute configuration, i.e., the D configuration of Qui3N.

The low-field displacements of the signals for transglycosidic carbons in the ^{13}C NMR spectrum of the *O*-deacetylated polysaccharide (Table 1), as compared with the data for the corresponding free monosaccharides [17,22], were due to the α-effects of glycosylation. They confirmed the substitution patterns of units **A**, **B**, **D**, and **E**, and proved unit **C** to be substituted at position 4. Indeed, substitution of unit **C** at position 3 would result [16] in the appearance of a signal for C-3 near 83.5 ppm instead of the observed signal for C-4 at 80.2 ppm. The β-effect of glycosylation of −1 ppm on C-4 of unit **A** again showed [23] that Qui3N had the same absolute configuration as the attached β-D-GlcN and confirmed that it was D. The NOE and the ^{13}C NMR data were in full agreement with the results of methylation analysis described above.

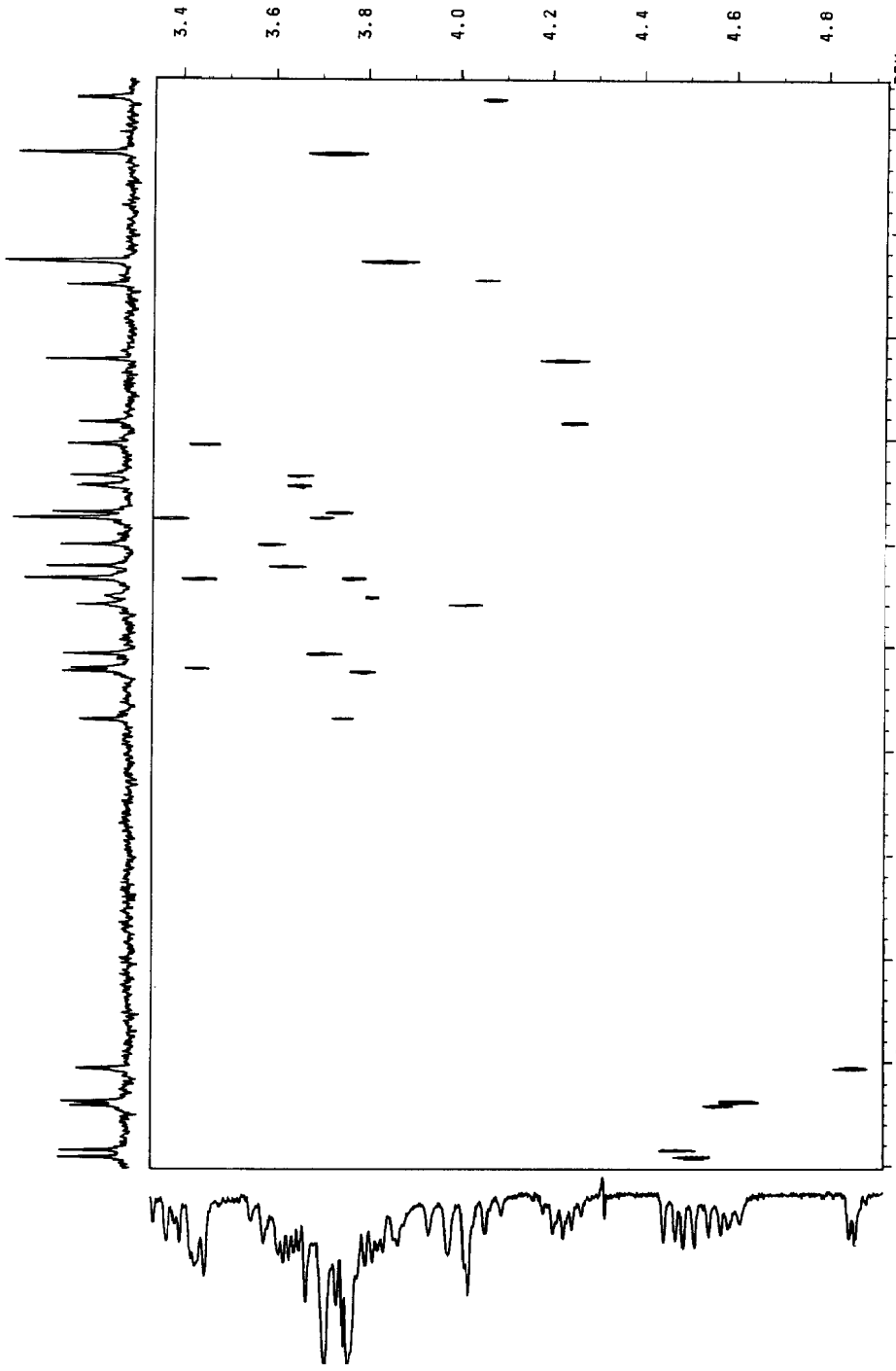
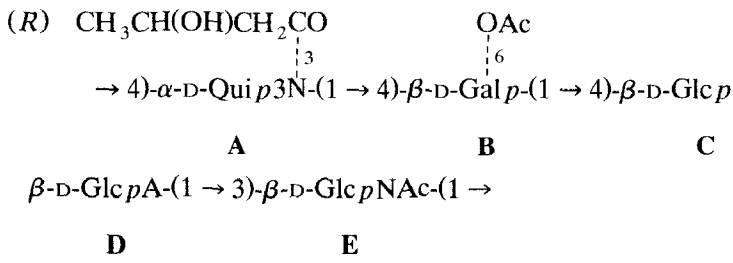


Fig. 3. Two-dimensional $^{13}C/^{1}H$ COSY spectrum of the *O*-deacetylated polysaccharide. The corresponding one-dimensional ^{1}H and ^{13}C NMR spectra are displayed along the F_1 and F_2 axes, respectively.

The sequence of the monosaccharide units was confirmed by modified Smith degradation of the *O*-deacetylated polysaccharide (hydrolysis with 0.5 M trifluoroacetic acid at room temperature) and fractionation on Bio-Gel P-2. According to the data of sugar and methylation analysis (Table 2), the last-eluted product (OS-III) was the expected di-*N*-acyl derivative of the bioside GlcN(1 → 4)Qui3N(1 → 2)Thr-ol. The source of OS-II, which was the main degradation product and contained 4-substituted Qui3N, one terminal GlcN, and one 3-substituted GlcN residue, may be accounted for by incomplete oxidation of 4-substituted GlcA or/and incomplete hydrolytic cleavage of its glycosidic linkage. The first-eluted product (OS-I) seemed to be a higher oligosaccharide.

Comparison of the ^{13}C NMR spectra of the *O*-deacetylated and intact polysaccharides (Table 1) showed displacements of the signals for C-5,6 of unit **B** from 76.7 and 61.4 ppm to 74.0 and 64.2 ppm, respectively. These displacements corresponded to the β- and α-effects of *O*-acetylation [24] and were indicative of location of the *O*-acetyl group at position 6 of the galactose residue (unit **B**). As judged by consideration of the corresponding molecular model, another shift (from 80.2 to 81.2 ppm) observed for C-4 of the neighbouring unit **C** may be due to spatial interaction of H-4 of unit **C** with the *O*-acetyl group at HO-6 of unit **B**.

Therefore, on the basis of the data obtained, it was concluded that the O-specific polysaccharide of *H. alvei* strain 1216 has the following structure:



3. Experimental

General methods.—Gel-permeation chromatography was carried out on columns (2 × 100 cm) of Sephadex G-50, Sephadex G-25, and Bio-Gel P-2, and monitored by the phenol–H_2SO_4 reaction [25]. Paper chromatography was performed on Whatman No. 1 paper, using 6:4:3 1-butanol–pyridine–water. High-voltage electrophoresis was run using the same paper in a pyridine–acetic acid buffer (pH 5.3) at 3000 V (50 V/cm) for 30 min. Substances were detected on paper with the alkaline $AgNO_3$ reagent. GLC–MS was carried out with a Hewlett–Packard 5971 A 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.

NMR spectroscopy.—The ^1H NMR spectra were recorded with a Bruker WM-250 instrument for solutions in D_2O at 70°C. The ^{13}C NMR spectra were recorded with a Bruker AM-300 instrument for solutions in D_2O at 60°C. Acetone was used

as an internal standard, δ_H 2.23 ppm, δ_C 31.45 ppm. Selective spin decoupling and 1D NOE spectra were obtained as described [26]. 2D homonuclear shift-correlated spectra (COSY and COSYRCT) and heteronuclear $^{13}C/^{1}H$ COSY spectrum (XHCORRD) were measured as described previously [6].

Bacterial strain, growth, and isolation of lipopolysaccharide and O-specific polysaccharide.—*H. alvei* strain 1216 was derived from the collection of the Pasteur Institute (Paris) and cultivated in the liquid medium as described previously [27]. The lipopolysaccharide (yield, 2.8%) was isolated as described [7,8], then degraded with aq 1% acetic acid at 100°C for 40 min, and the water-soluble portion was separated on Sephadex G-50 to give an O-specific polysaccharide and a core oligosaccharide fraction with yields of 41.2 and 30.6%, respectively, of the total amount of the material eluted from the column.

Sugar analysis.—The polysaccharide was hydrolysed with 2 M CF_3CO_2H (120°C, 2 h) or 10 M HCl (80°C, 30 min), and monosaccharides were analysed by GLC–MS as alditol acetates [28]. The mass-spectral data for 3-(3-acetoxybutyramido)-1,2,4,5-tetra-*O*-acetyl-3,6-dideoxyglucitol (*m/z*): 316, 302, 256, 242, 196, 136, 129, 128, 122, 114. Hydrolysis for determination of the absolute configurations of GlcN and 3-hydroxybutyric acid was done with 4 M HCl (100°C, 18 h) and 2 M HCl (100°C, 2.5 h), respectively.

Methylation analysis.—Methylation was done by the Hakomori method [29]. Methylated polysaccharide and oligosaccharides were hydrolysed with 2 M CF_3CO_2H (120°C, 2 h), 10 M HCl (80°C, 30 min), or according to Stellner et al. [30], and partially methylated monosaccharides were conventionally converted into alditol acetates and analysed by GLC–MS.

O-Deacetylation.—The O-specific polysaccharide (60 mg) was treated with aq 12% ammonia at room temperature overnight, and evaporation of ammonia followed by lyophilization then afforded the *O*-deacetylated polysaccharide (50 mg).

Carboxyl-reduction.—The O-specific polysaccharide (10 mg) in water (2 mL) was subsequently treated at room temperature with 1-(3-dimethylaminopropyl-3-ethylcarbodiimide hydrochloride (100 mg, 2 h at pH 4.75) and $NaBH_4$ (200 mg, overnight). The mixture was neutralised with aq 50% AcOH and dialysed against distilled water (3 × 10 L) to give the carboxyl-reduced polysaccharide (9.8 mg).

Smith degradation.—*O*-Deacetylated polysaccharide (16 mg) was oxidised with 0.1 M $NaIO_4$ (1.6 mL, 4°C, 72 h), ethylene glycol (0.064 mL) was added, the mixture was reduced with $NaBH_4$ (64 mg, overnight), neutralised with aq 50% AcOH, and desalted on Sephadex G-25, the modified polysaccharide (14 mg) was hydrolysed with 0.5 M CF_3CO_2H (room temperature, 42 h), the acid was removed by co-evaporation with methanol, and the hydrolysate was fractionated on Bio-Gel P-2 to yield OS-I (1.3 mg), OS-II (7.4 mg), and OS-III (1.8 mg).

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