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# Structural study and serological characterisation of the O-specific polysaccharide of *Hafnia alvei* PCM 1185, another *Hafnia* O-antigen that contains 3,6-dideoxy-3-[(*R*)-3-hydroxybutyramido]-D-glucose

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**Abstract**

The O-specific polysaccharide of *H. alvei* strain PCM 1185 contains D-glucose, D-glucuronic acid, 2-acetamido-2-deoxy-D-glucose, and 3,6-dideoxy-3-[(*R*)-3-hydroxybutyramido]-D-glucose (Qui3NAcyl) in the ratios 2:1:1:1, as well as *O*-acetyl groups. On the basis of sugar and methylation analyses of the polysaccharide before and after chemical modifications (*O*-deacetylation, carboxyl reduction, Smith degradation), as well as $^{1}H$ and $^{13}C$ NMR spectroscopy, including 1D sequential, selective spin-decoupling, 2D homonuclear, and $^{13}C,^{1}H$ heteronuclear correlation spectroscopy (COSY), and 2D rotating-frame NOE spectroscopy, it was found that the polysaccharide has a pentasaccharide repeating unit with the following structure:

α-D-Glc*p*
1
↓
4
→4)-β-D-Glc*p*A-(1→3)-α-D-Glc*p*NAc-(1→2)-β-D-Qui*p*3NAcyl-(1→6)-α-D-Glc*p*-(1→

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with *O*-acetyl groups present in nonstoichiometric amounts, mainly at position 2 of GlcA and position 6 of GlcNAc or lateral Glc. Serological study showed that *H. alvei* strain PCM 1185 can be placed in a new serotype D and that an *O*-acetyl group can be a part of its epitope. © 1996 Elsevier Science Ltd.

*Keywords: Hafnia alvei*; O-Antigen; Lipopolysaccharide; Enterobacteria; NMR spectroscopy

## 1. Introduction

This work is a part of our systematic studies of *Hafnia alvei* lipopolysaccharides (LPSs). A common hexasaccharide core structure [1,2] and a structure of a core-like trisaccharide present in several strains [3,4] have been reported. Structures of over ten O-specific polysaccharides have been elucidated ([4–8] and references therein), most of which are neutral or, more often, acidic hexosaminoglycans. Some of the O-antigens contain an (*R*)-3-hydroxybutyryl group [9], which acylates the amino group of 2-amino-2-deoxy-D-glucose, 3-amino-3,6-dideoxy-D-glucose, or 3-amino-3,6-dideoxy-D-galactose, and serological studies of them have been carried out [10].

We now report the structure of an acidic O-specific polysaccharide chain of *H. alvei* strain PCM 1185 LPS and an extension of the serological studies to this new (*R*)-3-hydroxybutyryl-containing O-antigen.

## 2. Results and discussion

*Isolation and compositional analysis.*—The LPS was isolated from dried bacteria by phenol–water extraction [11] in a yield of 3.4% and hydrolysed with dilute acetic acid. The mixture of poly- and oligo-saccharides obtained was fractionated by GPC on Sephadex G-50 to give an O-specific polysaccharide (PS) in a yield of about half of the total amount of the material eluted from the column.

Sugar analysis of the PS and carboxyl-reduced PS by GLC–MS of the derived alditol acetates revealed the presence of glucose, 2-amino-2-deoxyglucose, and a 3,6-dideoxy-3-(3-hydroxybutyramido)hexose in the molar ratios 2:0.7:0.9 and 3:1.1:1.1, respectively. The last component was indistinguishable by PC and GLC–MS data from 3,6-dideoxy-3-[(*R*)-3-hydroxybutyramido]-D-glucose (D-Qui3NAcyl) from *H. alvei* 1216 PS [5] and differed from the corresponding D-*galacto* isomer from *H. alvei* 1211 PS [12]. The *gluco* configuration of the sugar moiety was confirmed by NMR data (see below). Analysis of the hydrolysate of the PS using a sugar analyser confirmed the presence of glucuronic acid.

Colorimetric assays [5] showed that the PS contains D-glucose (20%), GlcA (14%), *O*-acetyl groups (1 μmol/mg), and (*R*)-3-hydroxybutyric acid (0.8 μmol/mg). After carboxyl reduction of the PS, the content of D-glucose increased to 30%, thus showing that GlcA is D. The D configuration of GlcN and Qui3N was determined by the method of Gerwig et al. [13].

The $^{13}C$ NMR spectrum of the PS (Fig. 1a) contained a number of signals with different integral intensities, probably due to nonstoichiometric *O*-acetylation. In fact,

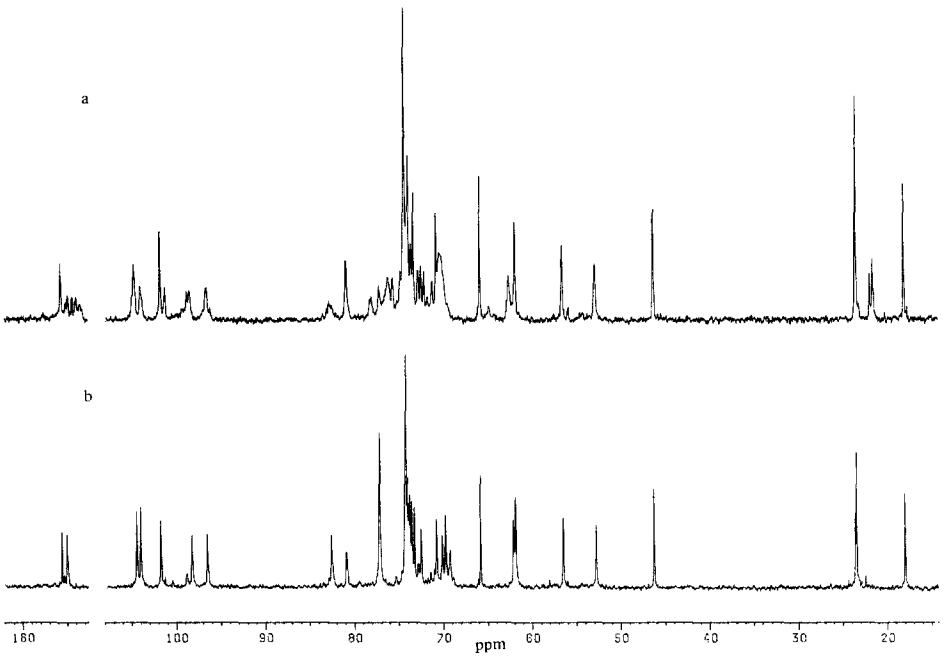



Fig. 1. 75-MHz $^{13}C$ NMR spectrum of the intact (a) and *O*-deacetylated (b) polysaccharide at pD 5.

the spectrum contained signals for *O*-acetyl groups at δ 21.6 and 21.9 with much lower intensities than, for example, the C-6 signal of Qui3NAcyl at δ 18.1.

After *O*-deacetylation of the PS with aqueous ammonia, the $^{13}C$ NMR spectrum (Fig. 1b) was typical of a regular polymer having a pentasaccharide repeating unit. It contained signals for five anomeric carbons in the region δ 96.5–104.4, three $CH_2O$ groups (C-6 of GlcN and two Glc residues), of which two are nonsubstituted (δ 61.6 and 62.1) and the third is substituted (δ 69.2, data of attached-proton test [14]), a methyl group (C-6 of Qui3N) at δ 18.0, a carboxyl group (C-6 of GlcA) at δ 175.0, two carbons bearing nitrogen (C-2 of GlcN and C-3 of Qui3N) at δ 52.8 and 56.5, and other sugar ring carbons in the region δ 69.7–82.6, as well as an *N*-(3-hydroxybutyryl) and an *N*-acetyl group ($CH_3$ at δ 23.4 and 23.6, CO at δ 175.0 and 175.6, C-2 and C-3 of the 3-hydroxybutyryl group at δ 46.3 and 65.8, respectively). When the pD of a PS solution in $D_2O$ was changed from 5 to 1, the C-6 signal of GlcA shifted from δ 175.0 to 172.5, thus indicating that the carboxyl group is free.

The $^1H$ NMR spectrum of the *O*-deacetylated PS also contained signals for five anomeric protons in the region δ 4.3–5.5, a methyl group of Qui3N at δ 1.25 (d, 3 H, $J_{5,6}$ 6 Hz, H-6), a 3-hydroxybutyryl group at δ 1.16 (d, 3 H, $J_{3,4}$ 6 Hz, H-4) and 2.36 (m, 2 H, H-2), and an *N*-acetyl group at δ 1.96 (s, 3 H).

Therefore, the PS includes D-glucose, 2-acetamido-2-deoxy-D-glucose, D-glucuronic acid, and 3,6-dideoxy-3-[(*R*)-3-hydroxybutyramido]-D-glucose in the ratios 2:1:1:1.

*Structural study.*—Methylation and GLC–MS analysis of the derived methylated alditol acetates revealed the substitution pattern of the monosaccharide residues in the

Table 1
Data of methylation analysis

| Methylated sugar | $t_R$ [a] | Molar ratio | | |
|---|---|---|---|---|
| | | PS | Carboxyl-reduced PS | Oligosaccharide **1** |
| 2,3,4,6-MeGlc | 1.00 | 0.83 | 0.77 | |
| 2,3,6-MeGlc | 1.24 | | 0.80 | |
| 2,3,4-MeGlc | 1.28 | 0.21 | 0.24 | |
| 2,3-MeGlc | 1.48 | 1.00 | 1.00 | |
| 3,4,6-MeGlcNMeAc | 1.67 | | | 1.10 |
| 4,6-MeGlcNMeAc | 1.90 | 0.29 | 0.20 | |
| 4-MeQui3NMeAcyl [b] | 2.07 | 0.25 | 0.26 | 1.00 |

[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).
[b] Acyl stands for 3-methoxybutyryl.

PS (Table 1). There are present terminal and 4,6-disubstituted glucose residues, 3-substituted GlcNAc, 2-substituted Qui3NAcyl, and 4-substituted GlcA, the last conclusion being based on the identification of 2,3,6-tri-*O*-methylglucose in analysis of carboxyl-reduced PS. A certain amount of 2,3,4-tri-*O*-methylglucose derived from both the PS and carboxyl-reduced PS may be accounted for by incomplete removal of the *O*-acetyl group from the position 6 of the lateral glucose residue during methylation (see below).

For further analysis, the $^1$H NMR spectrum of the *O*-deacetylated PS was assigned using sequential, selective spin-decoupling, 2D shift-correlated spectroscopy (COSY), and H,H-relayed COSY (Table 2). Relatively large coupling constants $J_{2,3}$, $J_{3,4}$, and $J_{4,5}$ 9–10 Hz, determined from the spectrum, were typical of the *trans*-diaxial orientation of the neighbouring pyranoid ring protons [15] in agreement with the *gluco* configuration of the five constituent monosaccharides. The spin-systems of GlcA and Qui3NAcyl were identified on the basis of the appearance of the H-5 signal, which was a doublet ($J_{4,5}$ 9 Hz) and a double quartet ($J_{4,5}$ 9 Hz, $J_{5,6}$ 6 Hz), respectively. The

Table 2
$^1$H NMR chemical shifts for the *O*-deacetylated polysaccharide at pD 5 ($\delta$ in ppm) [a]

| Sugar residue | H-1 | H-2 | H-3 | H-4 | H-5 | H-6a | H-6b |
|---|---|---|---|---|---|---|---|
| → 4)-β-D-Glc*p*A-(1 → | 4.34 | 3.30 | 3.70 | 3.68 | 3.75 | | |
| → 3)-α-D-Glc*p*NAc-(1 → | 5.44 | 4.07 | 3.48 | 3.44 | 3.53 | 3.7–3.8 | |
| → 2)-β-D-Qui*p*3NAcyl-(1 → | 4.65 | 3.45 | 3.90 | 3.17 | 3.48 | 1.25 | |
| → 4)-α-D-Glc*p*-(1 → <br> 6 <br> ↑ | 5.42 | 3.41 | 3.88 | 3.29 | 3.86 | 4.06 | 3.67 |
| α-D-Glc*p*-(1 → | 5.07 | 3.47 | 3.66 | 3.34 | 3.68 | 3.7–3.8 | |

[a] The chemical shift for NAc is $\delta$ 1.96; the values for H-2, H-3, and H-4 of the 3-hydroxybutyryl group are $\delta$ 2.36, 4.14, and 1.16, respectively.

Table 3
$^{13}C$ NMR chemical shifts for the *O*-deacetylated polysaccharide at pD 5 ($\delta$ in ppm) [a]

| Sugar residue | C-1 | C-2 | C-3 | C-4 | C-5 | C-6 |
|---|---|---|---|---|---|---|
| → 4)-β-D-Glc*p*A-(1 → | 104.0 | 73.8 | 77.2 | 77.2 | 77.2 | 175.0 |
| → 3)-α-D-Glc*p*NAc-(1 → | 96.5 | 52.8 | 82.6 | 69.7 | 73.5 | 61.6 [b] |
| → 2)-β-D-Qui *p*3NAcyl-(1 → | 104.4 | 74.2 | 56.5 | 74.0 | 74.2 | 18.0 |
| → 4)-α-D-Glc*p*-(1 → 6 ↑ | 98.2 | 72.5 | 73.9 | 80.9 | 70.1 | 69.2 |
| α-D-Glc*p*-(1 → | 101.7 | 73.2 | 74.2 | 70.7 | 73.7 | 62.1 |

[a] The chemical shifts for NAc are $\delta$ 23.6 ($CH_3$) and 175.6 (CO); the values for C-1, C-2, C-3, and C-4 of the 3-hydroxybutyryl group are $\delta$ 175.0, 46.3, 65.8, and 23.4, respectively.
[b] Assignment could be interchanged.

spin-system of GlcNAc was distinguished from those of two glucose residues ($Glc^I$ and $Glc^{II}$) by a relatively lower-field position of the H-2 signal at $\delta$ 4.07 (cf. the positions of the H-2 signals of $Glc^I$ and $Glc^{II}$ at $\delta$ 3.41 and 3.47, respectively).

Assignments of the spin-systems for the amino sugars were confirmed by the positions of the signals for C-2 of GlcNAc and C-3 of Qui3NAcyl in the region of carbons bearing nitrogen at $\delta$ 52.8 and 56.5, respectively. This followed from the assignment of the $^{13}C$ NMR spectrum of the *O*-deacetylated PS (Table 3) which, with the $^1H$ NMR spectrum assigned, was performed using $^{13}C,^1H$ heteronuclear COSY. In the $^{13}C$ NMR of the PS, the signals for C-2 of Qui3NAcyl, C-3 of GlcNAc, C-4 of GlcA, and C-4 and C-6 of $Glc^I$ at 74.2, 82.6, 77.2, 80.9, and 69.2, respectively, shifted downfield, as compared with their positions in the spectra of the corresponding nonsubstituted monosaccharides [5,16]. This corroborated the substitution pattern of the PS determined by methylation analysis and showed that $Glc^I$ is 4,6-disubstituted.

As judged by $J_{1,2}$ coupling constants, GlcA and Qui3NAcyl are β-linked ($J_{1,2}$ 8 Hz), while the three other sugars are α-linked ($J_{1,2}$ 3.5–4 Hz) [15]. These data were consistent with the $^1J_{C\text{-}1,H\text{-}1}$ coupling constants [17] determined from the gated-decoupling $^{13}C$ NMR spectrum of the *O*-deacetylated PS, which were relatively low (ca. 163 Hz) for Qui3NAcyl and GlcA (C-1 signals at $\delta$ 104.4 and 104.0) and relatively high (170–172 Hz) for $Glc^I$, $Glc^{II}$, and GlcNAc (C-1 signals at $\delta$ 98.2, 101.7, and 96.5, respectively).

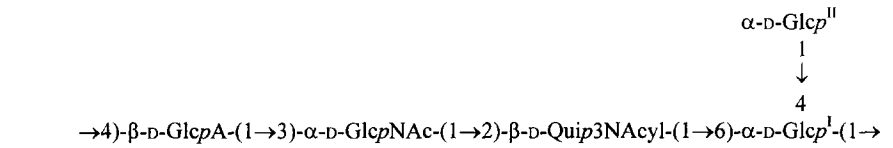
Smith degradation of the *O*-deacetylated PS resulted in destruction of glucose and GlcA to give an oligosaccharide-tetritol (**1**) which contained GlcNAc, Qui3NAcyl, and erythritol (Ery-ol) derived from $Glc^I$ in the molar ratios 1:0.8:0.4, as determined by GLC of alditol acetates. Methylation analysis of **1** (Table 1) revealed that GlcNAc occupies the terminal end and, therefore, **1** has the structure of α-D-GlcNAc-(1 → 2)-β-D-Qui3NAcyl-(1 → 1)-L-Ery-ol.

The full sugar sequence in the repeating unit was determined using a 2D rotating-frame NOE spectroscopy (ROESY) experiment with the *O*-deacetylated PS. In addition to

intraresidue cross-peaks (H-1,H-2 for α-linked sugars and H-1,H-2, H-1,H-3, and H-1,H-5 for β-linked sugars), the following interresidue cross-peaks between transglycosidic and some neighbouring protons were observed: GlcA H-1,GlcNAc H-2,3 at δ 4.34/4.07 and 4.34/3.48, GlcNAc H-1,Qui3NAcyl H-1,2 at δ 5.44/4.65 and 5.44/3.45, Qui3NAcyl H-1,$Glc^{I}$ H-6b at 4.65/3.67, and $Glc^{I}$ H-1,GlcA H-4 at δ 5.42/3.68. These correlations allowed unambiguous determination of the sugar sequence in the main chain of the polysaccharide as shown below.

$Glc^{II}$ H-1 exhibited two cross-peaks with $Glc^{I}$ H-4,6b at δ 5.07/3.29 and 5.07/3.67. Since position 6 of $Glc^{I}$ is occupied by Qui3NAcyl, this pointed to the attachment of $Glc^{II}$ at position 4 of $Glc^{I}$, and, thus, the cross-peak $Glc^{II}$ H-1,$Glc^{I}$ H-6b is accounted for by the spatial proximity of the two protons in the 1,6-linked disaccharide unit [18].

Therefore, on the basis of the chemical and NMR spectroscopic data obtained, it was concluded that the *O*-deacetylated PS has the following structure:




Comparison of the $^{13}C$ NMR spectra of the PS and *O*-deacetylated PS showed that *O*-deacetylation resulted in the disappearance of the signals at δ 101.2 and 64.9, and a significant increase of the signals at δ 104.0 and 62.1 which belonged to C-1 of GlcA and a $CH_2OH$ group (C-6) of a 6-nonsubstituted sugar residue, respectively (Table 3). These displacements suggested [19] that the *O*-acetyl groups are attached nonstoichiometrically at position 2 of GlcA and position 6 of $Glc^{II}$ or GlcNAc. However, the presence of an additional, minor *O*-acetyl group at another position cannot be excluded. As judged by the ratios of the intensities of the $^{13}C$ signals belonging to an *O*-acetylated sugar unit and the corresponding non-*O*-acetylated counterpart, the degree of *O*-acetylation varied from one batch of PS to another and may reach 50% at each of the main sites.

The *O*-acetylation pattern thus determined was consistent with the results of periodate oxidation of the sample of PS with the highest content of the *O*-acetyl groups, which destroyed the total glucose but only half of GlcA, the content of which decreased from 14 to 7.5%.

*Serological study.*—In SDS-PAGE, the LPS of *H. alvei* PCM 1185 showed a ladder-like pattern of slowly migrating bands typical of smooth-type LPS with high molecular weight O-specific polysaccharide chains having different lengths. Fast-migrating bands also present in the gel corresponded to rough-type LPS lacking any polysaccharide chain but containing a core oligosaccharide.

SDS-PAGE and immunoblotting experiments were carried out using *H. alvei* 1185 LPS and anti-*H. alvei* 1185 serum. This serum reacted mainly with slowly migrating LPS fractions having polysaccharide chains of different length and, thus, contained mainly antibodies directed against the O-specific polysaccharide.

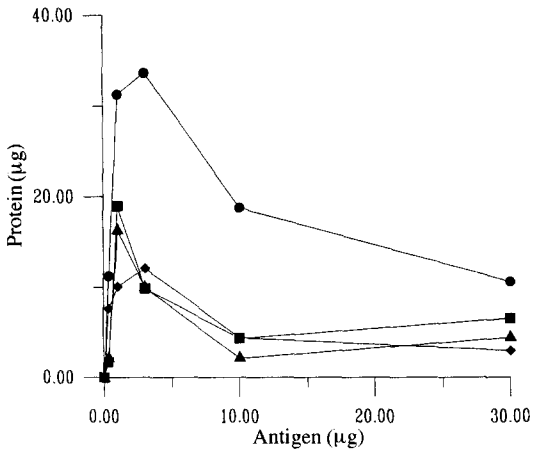



Fig. 2. Quantitative precipitation of the anti-*H. alvei* PCM 1185 serum with the homologous O-specific polysaccharide (PS) (●), *O*-deacetylated PS (▲), partially deglucosylated *O*-deacetylated PS (■), and carboxyl-reduced *O*-deacetylated PS (◆).

In the double immunodiffusion test, precipitation was only observed between anti-*H. alvei* 1185 serum and the homologous LPS, while none of the LPSs isolated from *H. alvei* ATCC 13337 and PCM strains 2, 1188, 1196, 1211, 1216, and 1221 reacted with this antiserum independent of whether the O-antigen contained an (*R*)-3-hydroxybutyryl group or not [10].

Therefore, *H. alvei* PCM 1185 is serologically related to none of the existing serotypes A–C and, thus, represents a new serotype D.

Quantitative immunoprecipitation with anti-*H. alvei* 1185 serum (Fig. 2) showed that *O*-deacetylation decreased significantly the serological activity of the PS and thus suggested that an *O*-acetyl group (or groups) may participate in an epitope. Neither carboxyl reduction nor partial removal of lateral $Glc^{II}$, using gentle, selective periodate oxidation, influenced significantly the residual reactivity of the *O*-deacetylated PS with the anti-*H. alvei* 1185 serum. This showed that the groups affected are not involved in any epitope.

## 3. Experimental

*General methods.*—GPC was performed on columns of Sephadex G-50 (2 × 100 cm), Bio-Gel P-4 (1.6 × 100 cm), and Sephadex G-10 (0.8 × 20 cm) in pyridinium acetate buffer (pH 5.6) and monitored by the phenol–$H_2SO_4$ reaction [20]. PC was performed on Whatman No. 1 paper using 6:4:3 1-butanol–pyridine–water and the alkaline silver nitrate reagent for detection of sugars. GLC–MS was performed with a Hewlett–Packard 5971A chromatograph equipped with an HP-1 glass capillary column (12 m × 0.2 mm) 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 60 °C with acetone ($\delta_H$ 2.225, $\delta_C$ 31.45) as internal standard. Selective spin-decoupling was carried out by a modified method [21]. 2D NMR experiments were performed using standard Bruker software; a mixing time of 0.2 s was used in a ROESY experiment.

*Bacterial strain and isolation of lipopolysaccharide and O-specific polysaccharide.*—*H. alvei* PCM 1185 derived from the Pasteur Institute Collection (Paris) was cultivated in a liquid medium [22]. LPS of *H. alvei* 1185 was isolated from dry bacterial mass by phenol–water extraction [11] and purified from nucleic acids as described [23]. LPSs of other strains used in this work were from the L. Hirszfeld Institute (Wrocław).

To obtain the O-specific polysaccharide (PS), LPS was hydrolysed with aq 1% HOAc for 1 h at 100 °C and a water-soluble material was fractionated by GPC on Sephadex G-50 to give the PS and a core oligosaccharide in yields of 47 and 28%, respectively, of the total amount of the material eluted from the column.

*Sugar and methylation analysis.*—The PS, or a chemically modified PS, was hydrolysed with 10 M HCl for 30 min at 80 °C, and monosaccharides obtained were converted conventionally into alditol acetates and analysed by GLC–MS; Qui3NAcyl, $t_R$ 1.30; Fuc3NAcyl from *H. alvei* PCM 1211, $t_R$ 1.29. In the same hydrolysate, Qui3NAcyl was additionally identified by PC, $R_{Rha}$ 1.13; Fuc3NAcyl, $R_{Rha}$ 1.06. GlcA was analysed by anion-exchange chromatography using a Biotronik LC-2000 sugar analyser as described [24].

The absolute configuration of glucose and GlcA was determined after hydrolysis of the PS and carboxyl-reduced PS with 2 M $CF_3CO_2H$ for 2 h at 120 °C, using D-glucose oxidase, and that of 3-hydroxybutyric acid after hydrolysis with 4 M HCl (100 °C, 2.5 h), using (*R*)-3-hydroxybutrate dehydrogenase as described [5]. The absolute configuration of GlcN and Qui3N was established by GLC of acetylated glycosides with (±)-2-butanol and (+)-2-butanol [13] after hydrolysis with 4 M HCl (100 °C, 18 h); D-Qui3N from the O-specific polysaccharide of *H. alvei* 1216 [5] was used as the authentic sample.

Methylation was performed according to the Hakomori method [25], and the permethylated material was hydrolysed with 10 M HCl for 30 min at 80 °C. The resulting partially methylated monosaccharides were converted conventionally into alditol acetates and analysed by GLC–MS.

*Chemical modifications and Smith degradation.*—*O*-Deacetylation was carried out with aq 12% ammonia at room temperature overnight. Carboxyl reduction was performed by the method of Taylor et al. [26].

*O*-Deacetylated PS (15 mg) was oxidised with 0.1 M $NaIO_4$ (1 mL) for 48 h at 4 °C; after adding ethylene glycol (0.1 mL), the product was reduced with $NaBH_4$ (50 mg) overnight, neutralised with concd HOAc, and desalted by GPC on Sephadex G-10. To ensure the complete oxidation the procedure was repeated using periodate treatment for 24 h. Hydrolysis of the oxidised PS was carried out with 0.5 M $CF_3CO_2H$ for 48 h at 20 °C, and oligosaccharide-tetritol **1** (6.5 mg) was isolated by fractionation on Bio-Gel P-4.

For partial deglucosylation, *O*-deacetylated PS was oxidised with 0.008 M $NaIO_4$ at 4 °C for 45 min; after adding ethylene glycol, borohydride reduction, and dialysis, the product was hydrolysed with 0.5 M $CF_3CO_2H$ for 24 h at 20 °C, dialysed, and lyophilised.

*SDS-PAGE and serological methods.*—SDS-PAGE was performed by the method of Laemmli [27] with some modifications [9].

Anti-*H. alvei* 1185 serum was obtained according to Romanowska et al. [28].

Double immunodiffusion [29] was carried out as described previously. For immunoblotting [10,30,31], the LPS after separation in SDS-PAGE was transblotted from the gel into nitrocellulose. The transblot was incubated with anti-*H. alvei* 1185 serum (primary antibody), washed with Tris-buffered saline, and incubated with horseradish peroxidase conjugate with goat anti-rabbit IgG (secondary antibody); the immunoblot was visualised by staining with 4-chloro-1-naphthol in the presence of $H_2O_2$. The quantitative precipitation test was performed essentially by the published method [32]. The amount of protein in the precipitate was estimated by the method of Lowry et al. [33].

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