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Structure of the *Hafnia alvei* strain PCM 1188 O-specific polysaccharide

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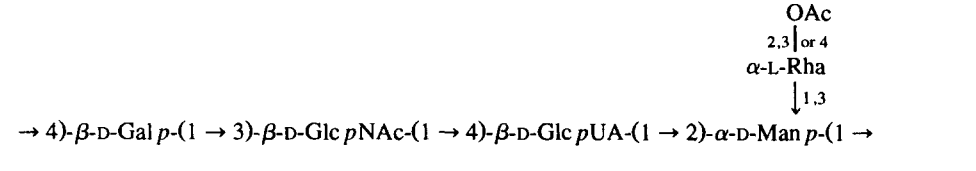
Abstract

The lipopolysaccharide was extracted from cells of *Hafnia alvei* PCM 1188 strain and, after mild acid hydrolysis, the O-specific polysaccharide isolated and characterized. On the basis of sugar and methylation analysis, FAB mass spectrometry and NMR spectroscopy of the polysaccharide and oligosaccharides obtained after Smith degradation, or solvolysis with anhydrous hydrogen fluoride, the repeating unit of the O-specific polysaccharide was shown to be the pentasaccharide:



Abbreviations: LPS, lipopolysaccharide; FABMS, fast-atom bombardment mass-spectrometry; LSIMS, liquid secondary-ion mass-spectrometry; Hex, hexose; HexNAc, 2-acetamido-2-deoxyhexose; HexUA, hexuronic acid; EDC, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride

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Keywords: Lipopolysaccharide; *Hafnia alvei*; Endotoxin; LPS; O-Antigen

1. Introduction

Hafnia alvei microorganisms are among the less known *Enterobacteriaceae* although they are found in soil, water, and dairy products. These bacteria have also been reported as opportunistic agents in hospital infections [1] and as a cause of diarrhoea in humans [2]. According to the serological classification of Sakazaki [1], 68 O-antigens and 34 H-antigens are present in the *H. alvei* genus. Immunochemical studies on lipopolysaccharides of this genus include more than 30 strains [3]. Recently, the structures of ten O-specific polysaccharides of *H. alvei* strains were elucidated [4–10], and uncommon monosaccharide subunits were found such as sialic acid, 3-amino-3,6-dideoxy- and 4-amino-4,6-dideoxy-hexoses, glycerol and pentitol phosphates, ribose, and 3-D-hydroxybutyric acid. In addition, D-glucuronic acid has been reported in strains 1216 and 1204 [11,12]. We described now the structure of the O-antigen of *H. alvei* strain PCM 1188, which also contains D-glucuronic acid and acetylated L-rhamnose as a branch, confirming the diversity of the genus.

2. Results and discussion

The lipopolysaccharide of *H. alvei* PCM 1188, analyzed by polyacrylamide gel electrophoresis in the presence of SDS, showed a high molecular weight ladderlike pattern proving its smooth character [3]. In reaction with homologous rabbit antiserum, it did not show cross reaction with other *H. alvei* lipopolysaccharides, which means that this strain represents a single defined *H. alvei* O-serotype. Mild acetic acid hydrolysis of LPS released a lipid sediment, and a water-soluble carbohydrate portion which was separated into five fractions by gel filtration on a Bio-Gel P-4 column. The high molecular weight fraction is the O-specific polysaccharide, whereas other fractions arise from the core related oligosaccharides which are not the subject of the present work.

Results of the sugar analysis of the polysaccharide are shown in Table 1. The *gluco* configuration for the hexuronic acid was inferred from high voltage paper electrophore-

Table 1
Sugar analysis (molar ratio) of *H. alvei* 1188 O-specific polysaccharide and its degradation products

Sugar component	Polysaccharide			
	Original	Carboxyl reduced	Hydrolysis/ hexokinase	$NaIO_4$ oxidized
Tetrose	–	–	–	0.8
L-Rha	1.2	1.0	0.7	0.4
D-Man	0.9	0.9	0.1	1.0
D-Glc	0.1	0.8	0.1	0.1
D-Gal	1.0	1.2	1.0	–
D-GlcN	0.9	0.6	0.3	1.0
D-GlcUA	1.0	0.1	n.d.	0.3
L,D-Hep	< 0.1	< 0.1	0.1	–

Table 2
Methylation analysis (molar ratio) of *H. alvei* 1188 O-specific polysaccharide and its degradation products

Methylated sugar	Polysaccharide			Oligosaccharide	
	Original	Carboxyl reduced	Prehm methylation	Smith degradation A	Smith degradation B
2,3,4-Me_3-Rha	1.0	1.1	0.1	0.7	–
2,3-Me_2-Rha	–	–	0.6	–	–
3,4-Me_2-Rha	–	–		–	–
2,4-Me_2-Rha	–	–	0.4	–	–
3,4,6-Me_3-Man	–	–	–	0.1	1.0
2,3,6-Me_3-Gal	1.0	1.0	0.8	–	–
2,3,6-Me_3-Glc	–	0.8	–	–	–
4,6-Me_2-Man	0.8	1.1	1.1	0.7	–
3,4,6-Me_3-GlcN(Me)Ac	–	–	–	1.0	1.0
4,6-Me_2-GlcN(Me)Ac	0.5	0.3	0.2	–	–

sis and by conversion to glucitol hexaacetate with lithium borohydride and subsequent acetylation. Mannose and 2-amino-2-deoxyglucose were shown to have the D-configuration by reaction with hexokinase which phosphorylates specifically the D-isomers. Galactose was oxidized with D-galactose oxidase (15% of galactose content), whereas after carboxyl reduction of the glucuronic acid with EDC, the resulting glucose was oxidized by D-glucose oxidase (10% of glucose detected). The L-configuration was assigned to rhamnose on the basis of the GLC analysis of the acetylated chiral octyl glycoside derivatives [13] (Table 1).

When the polysaccharide was treated with sodium periodate, the galactose constituent was completely oxidized, but only 60% of the rhamnose was oxidized while, after de-*O*-acetylation of the polysaccharide, it was completely oxidized. This implies the location of *O*-acetyl groups on the rhamnose unit.

Methylation analysis of the polysaccharide is shown in Table 2. In order to locate the *O*-acetyl substitution, the permethylated polysaccharide resulting from methylation according to Prehm [14] was hydrolyzed, reduced with sodium borodeuteride and acetylated. Analysis by GLC-MS of the methylated products showed the presence of two peaks of rhamnose derivatives in the proportion 0.34:0.63. The first peak was identified as 1,3,5-tri-*O*-acetyl-2,4-di-*O*-methyl-L-rhamnitol. The second peak showed both typical fragmentations for 1,4,5-tri-*O*-acetyl-2,3-di-*O*-methyl-L-rhamnitol (*m/z* 118, 162, 203, 247) and for 1,2,5-tri-*O*-acetyl-3,4-di-*O*-methyl-L-rhamnitol (*m/z* 131, 190, 234). The intensity of ion fragments of 2,3-Me_3-Rha was about twice that of the ions derived from 3,4-Me_2-Rha, indicating the proportion of O-4 to O-2 substituted rhamnoses as 2:1. The results indicate the presence, in the polysaccharide, of three differently *O*-acetylated rhamnose residues. The O-3-substituted rhamnose accounts for 37% of the total acetylated rhamnose. These results are in good agreement with the periodate oxidation experiments which show that, in the original polysaccharide, only 60% of the rhamnose content was oxidized as should be the case when the *O*-acetyl group occupies the O-2 or O-4 position. Rhamnose units resistant to periodate oxidation (~ 40%) have to be

substituted with an *O*-acetyl group at O-3. The detected monosubstitution of rhamnose units at the O-2, O-3 and O-4 positions, in ~ 20, 40 and 40% ratio, respectively, may be due to the migration of the *O*-acetyl group.

Smith degradation of the periodate oxidized polysaccharide resulted, after gel filtration on Bio-Gel P-4, in two oligosaccharides A and B in almost equal quantities. The results from methylation analysis of oligosaccharide A (Table 2) suggested that, in the polysaccharide, the periodate sensitive galactose residue substitutes the 2-amino-2-deoxyglucose unit at O-3. Applied to oligosaccharide B, the same approach showed the presence of terminal 2-amino-2-deoxyglucose and a O-2-linked mannose (Table 2), which means that, in the original polysaccharide, rhamnose is linked to O-3 of the mannose residue. Smith degradation of the de-*O*-acetylated, periodate oxidized polysaccharide resulted in only oligosaccharide B. Further analysis by FABMS and NMR spectroscopy confirmed the presence of hexuronic acid in oligosaccharides A and B. These results indicate that the linkage between periodate oxidized glucuronic acid and mannose was not cleaved during the Smith degradation. Solvolysis of the polysaccharide with liquid HF at −30°C for 20 min, followed by separation of the solvolysis products by gel filtration on Bio-Gel P-4 column, resulted in Fractions 1–9. The oligosaccharide Fractions 6–9 were analyzed further. When the time of solvolysis was prolonged to 50 min, Fraction 8 was essentially obtained, beside a minor proportion of Fraction 9.

The FAB mass spectra of underivatized oligosaccharide B showed deprotonated $[M - H]^-$ and protonated $[M + H]^+$ quasimolecular ions at m/z 662 in negative mode and m/z 664 in positive mode, respectively, in accordance with a composition Hex, HexNAc, HexUA, tetritol. The positive FAB mass spectrum of the peracetylated oligosaccharide B had both protonated and cationized quasimolecular ions of almost equal abundance (Fig. 1) at m/z 1126 $[M + H]^+$ and m/z 1148 $[M + Na]^+$. These ions have 11 acetyl groups, as inferred by comparing acetylated and deuterioacetylated homologous ions. The structure of this oligosaccharide was deduced from the B_i ions (Domon and Costello nomenclature [15]) in the spectrum shown in Fig. 1. A terminal nonreducing HexNAc (B_1 ion at m/z 330, 3 acetyl groups) is linked to a hexuronic

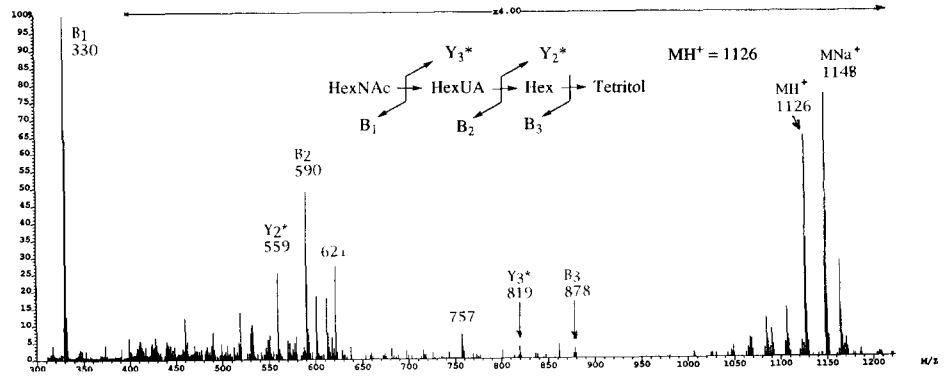


Fig. 1. Positive FAB mass spectrum and fragmentation pattern for peracetylated oligosaccharide B resulting from Smith degradation of the periodate oxidized O-specific polysaccharide.

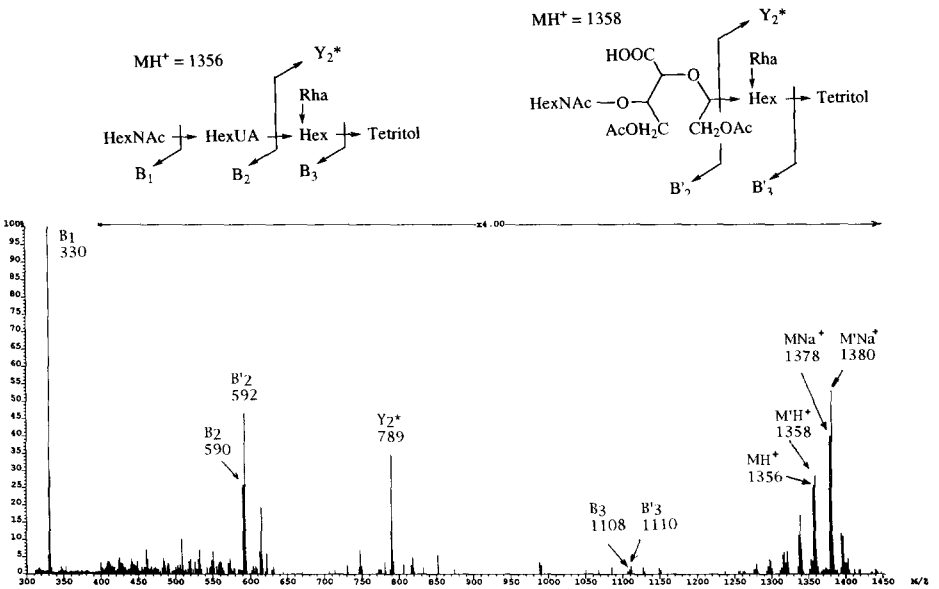
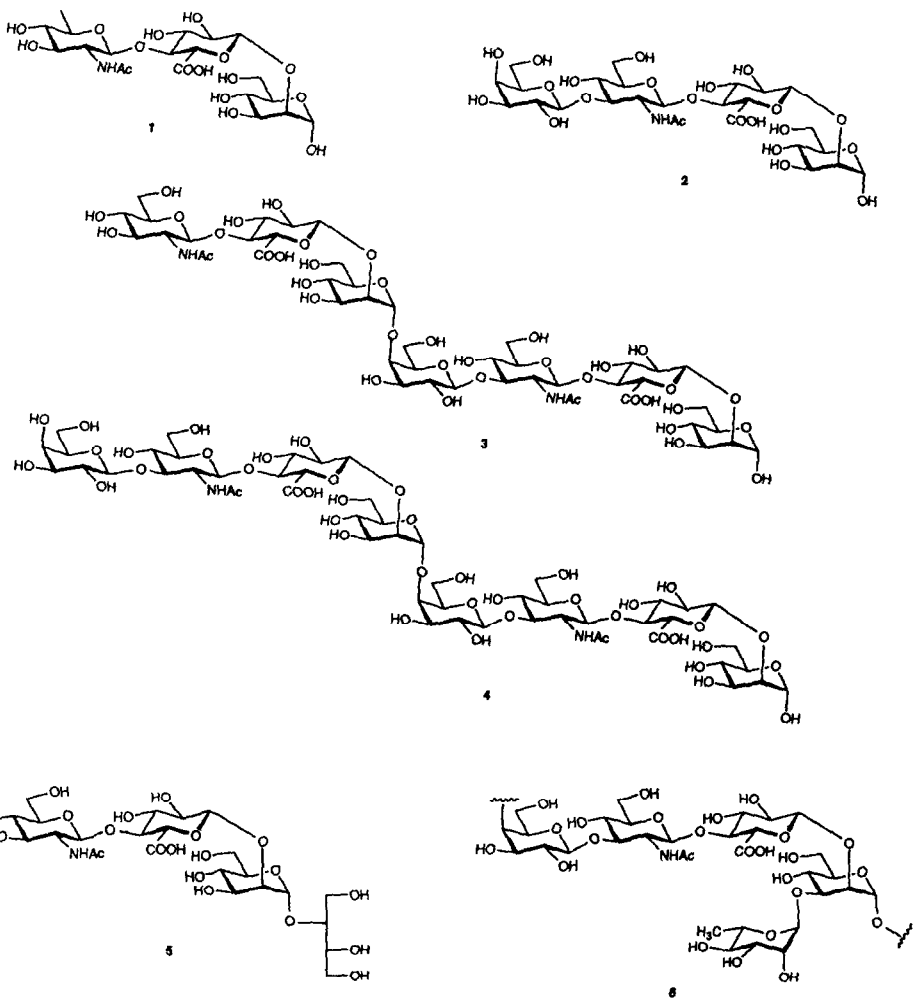


Fig. 2. Positive mass spectrum and fragmentation pattern for peracetylated oligosaccharide A resulting from Smith degradation of the periodate oxidized O-specific polysaccharide.

acid (HexUA) residue, as shown from the B_2 fragment at m/z 590 (5 acetyl groups). Although of low abundance, the next B_3 fragment is seen at m/z 878, with a hexose residue being added. This sequence is confirmed with two Y_i^* ions (the asterisk indicates Y_i ions cationized with sodium): Y_2^* at m/z 559 with six acetyl groups and Y_3^* at m/z 819 with eight acetyl groups. The sequence of this oligosaccharide is shown in Fig. 1. The rhamnose containing oligosaccharide A was also peracetylated and its positive FAB mass spectrum is shown in Fig. 2. Four major peaks can be distinctly seen in the molecular ion region corresponding respectively to protonated (m/z 1356 and 1358) and cationized (m/z 1378 and 1380) quasimolecular ions. This indicates the presence of a mixture of two pentasaccharides with a sequence analogous to fraction B, comprising rhamnose connected to a mannose residue. A B_2 ion is found at m/z 590 (HexNAc → HexUA) and is clearly associated with another B'_2 ion (m/z 592) corresponding to an open chain hexuronic acid. Terminal nonreducing HexNAc is still observed at m/z 330, as for fraction B. The two B_3 ions at m/z 1110 (and 1108) are also of low intensities. Identical ends for these two pentasaccharides are confirmed by ions at m/z 789 (Y_2^* ions), which include rhamnose. Absence of the ion m/z 559 confirms the external position of the rhamnose residue. The sequence of these two pentasaccharides is shown in Fig. 2.

Other oligosaccharides were obtained by partial depolymerization of the polysaccharide with anhydrous HF. The tri- (**1**) and tetra-saccharide (**2**) structures for oligosaccharides in Fractions 9 (minor) and 8 (major), respectively, correspond to the expected labilities in anhydrous HF of the glycosidic bonds present in the starting polysaccharide, i.e., 6-deoxyhexose > α-hexose > β-hexose > 2-amino-2-deoxyhexose, uronic acid [16].

Using shorter times for the fluorolysis (see Experimental), higher oligosaccharides were obtained from which the hepta- and octa-saccharides **3** and **4** (Fractions 7 and 6, respectively) were isolated (m/z 1268 and 1430, respectively, for $[M + Na]^+$ quasi-molecular ions).



The main Fraction 8, peracetylated and studied by FABMS in positive mode, was shown to be a mixture of a tetrasaccharide (m/z 1248, $[M + H]^+$ for the sodium salt of the uronic acid) and the corresponding glycosyl fluoride (m/z 1208, $[M + H]^+$). Their sequence, as deduced from the B_i ions which comprises an ion B_1 of a terminal non reducing hexose at m/z 331 followed by HexNAc with a B_2 ion at m/z 618 and by HexUA with a B_3 ion at m/z 900, is then as follows:

Table 3
Selected ^{1}H and ^{13}C NMR [a] data (500 and 125.7 MHz, respectively, D_2O; δ, ppm; J, Hz) for oligosaccharides **1**–**5** and de-*O*-acetylated polysaccharide **6**

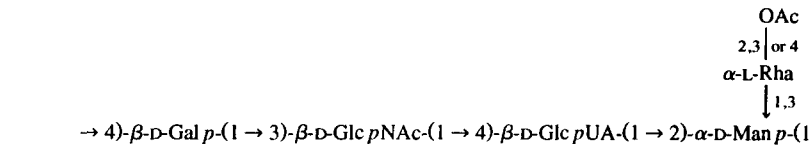
Compound/residue	H-1	$J_{H-1,H-2}$	C-1	C-2	C-3	C-4	C-5	C-6	$J_{C-1,H-1}$
1									
β-D-Glc*p*NAc-(1→	4.51	8.4	101.0	55.4	73.5	69.7	75.8	60.5	163.3
4)-β-D-Glc*p*UA-(1→	4.55	8.6	101.7	72.1	73.6	79.9	68.9	175.1	163.7
2)-α-D-Man*p*	5.25	1.8	92.0	78.4	69.3	66.9	73.8	60.0	167.8
2									
β-D-Gal*p*-(1→	4.42	8.0	103.5	70.6	72.5	68.5	75.2	61.0	162.9
3)-β-D-Glc*p*NAc-(1→	4.55	8.2	100.8	54.4	82.2	68.5	75.3	60.5	165.7
4)-β-D-Glc*p*UA-(1→	4.59	8.0	101.7	72.1	73.5	79.9	68.9	175.0	162.9
2)-α-D-Man*p*	5.29	1.8	92.0	78.4	69.3	66.9	73.5	60.4	170.0
3									
4)-β-D-Gal*p*-(1→	4.42	8.0	103.6	70.4		77.3	74.7		162.4
3)-β-D-Glc*p*NAc-(→	5.52	8.4	100.8	54.3	82.4	68.4	75.8		164.6
(and terminal)	4.47	8.3				68.3	75.5		
4)-β-D-Glc*p*UA-(1→	4.56	8.1	101.7			79.9		175.0	162.2
			101.6						162.2
2)-α-D-Man*p*-(1→	4.92	1.7	99.3	77.5	69.5	66.4			170.0
reducing α-D-Man*p*	5.25	1.6	92.0	78.4	69.3	66.9			170.5
4									
4)-β-D-Gal*p*-(1→	4.42	8.0	103.6	70.5		77.2	74.7		162.4
(and terminal)	4.40	8.0	103.4	70.4		68.4			
3)-β-D-Glc*p*NAc-(1→	4.50	8.4	100.7	54.3	82.4	68.3	75.3		164.6
	4.48	8.4			82.8		75.2		
2)-α-D-Man*p*-(1→	4.91	1.7	99.3	77.3	69.5	66.4		170.0	
reducing α-D-Man*p*	5.24	1.6	91.9	78.3	69.3	66.8		170.5	
5									
β-D-Glc*p*NAc-(1→	4.48	7.8	100.6	55.0	72.6	69.3	75.5	60.5	163.8
4)-β-D-Glc*p*UA-(1→	4.51	8.3	101.3	71.8	73.2	79.6	69.1	175.1	163.2
2)-α-D-Man*p*-(1→	5.07	2.0	99.0	77.3	69.3	66.4	73.3	60.8	170.0
2)-D-Threitol	60.1	79.3	70.7	61.9					
6									
4)-β-D-Gal*p*-(1→			103.9			77.2	75.3		162.9
3)-β-D-Glc*p*NAc-(1→			100.9	54.2	82.7		74.8		160.0
4)-β-D-Glc*p*UA-(1→			103.0		80.3			175.2	163.5
2,3)-α-D-Man*p*-(1→			99.3	77.2	76.8	64.4			170.3
α-L-Rha*p*-(→			99.7					16.7	170.0

[a] Tentative assignments.

$$\text{Hex} \rightarrow \text{HexNAc} \rightarrow \text{HexUA} \rightarrow \text{Hex-OAc}_4(\text{or-OAc}_3,\text{F})$$
$$B_1 \mid \qquad B_2 \mid \qquad B_3 \mid$$

Further confirmation for the sequence and anomeric configuration of the sugar units were obtained from comparative NMR analysis of the oligosaccharides and polysaccharide, especially from the $J_{H-1,H-2}$ and $J_{H-1,C-1}$ values. The spectroscopic data are presented in Table 3.

From the above results, the structure of the repeating unit of the *H. alvei* strain PCM 1188 O-specific polysaccharide is then as follows:



Although glucuronic acid is a common component of the lipopolysaccharides of the strain 1188 and strains 1216 and 1204, the serological cross-reactivity was not expressed in rocket immunoelectrophoresis and passive haemagglutination (unpublished results) in this group. This observation indicates that glucuronic acid is not an inmunodominant part of their epitopes.

3. Experimental

Isolation of the lipopolysaccharide and O-specific polysaccharide from the bacterial strain.—*Hafnia alvei* strain PCM 1188, derived from the collection of the Pasteur Institute (Paris), was used in the experiments. Bacteria were cultivated in liquid medium with aeration at 37°C for 24 h, then harvested and freeze-dried. The lipopolysaccharide was isolated by phenol–water extraction [17] and purified on a Sepharose 2B column as described [3,7]. The LPS (200 mg) was hydrolyzed with acetic acid (1%, 20 mL) for 45 min at 100°C and following the centrifugation of the precipitated lipid A, the supernatant was freeze-dried and then fractionated on a Bio-Gel P-4 column (1.6 × 100 cm) in aq pyridinium acetate buffer (0.05 M, pH 5.6) monitored by refractometry. A typical yield of the lipopolysaccharide and the O-specific polysaccharide was 2.2% and 0.8%, respectively, of the dry bacterial mass.

Analytical methods.—Total hydrolysis of oligo- and poly-saccharide samples was made with M HCl for 4 h at 100°C, followed by evaporation by a stream of N_2. After reduction with $NaBH_4$ and acetylation with acetic anhydride–pyridine, the hexosamines and neutral sugars were analyzed by GLC using a Varian 2000 gas chromatograph fitted to a glass OV225 column. Separation was done at 180°C for hexitol acetates, or 220°C for hexosamine derivatives. Carboxyl reduction of the polysaccharide was performed according to Taylor et al. [18]. The uronic acid content was determined by the method of Blumenkrantz and Asboe-Hansen [19], and *O*-acetyl groups according to Hestrin [20]. De-*O*-acetylation of the polysaccharide was performed in aq 12% NH_4OH at room temperature overnight; then the solution was evaporated and lyophilized. Paper chromatography was carried out on Whatman 1 paper with 6:4:3 *n*-butanol–pyridine–water, using alkaline $AgNO_3$ reagent for detection. Electrophoresis for uronic acid characterization was performed on Whatman 1 paper at 3 kV intensity for 45 min, followed by detection with the same reagent. Sugar analysis was performed after carboxyl reduction with $LiBH_4$ [21].

Methylation analysis.—Methylation was performed by the Hakomori procedure [22], and the products were purified using Sep Pak C18 cartridges. The methylated product was hydrolyzed with 2 M trifluoroacetic acid at 120°C for 2 h, then reduced with $NaBD_4$ and acetylated for GLC-MS analysis. In order to establish the position of *O*-acetyl groups in the polysaccharide, the methylation was carried out according to Prehm [14].

A sample of the polysaccharide (1 mg) in trimethyl phosphate (0.9 mL) was treated with 2,6-di-(*tert*-butyl)pyridine (0.15 mL) and methyl trifluoromethanesulphonate (0.15 mL) for 2 h at 50°C in an ultrasonic bath. The methylated product was further treated as described above. GLC-MS analysis was performed with a Hewlett–Packard 5971 A system using a HP-1 glass capillary column (0.2 mm × 12 m) and a temperature programming of 8°C/min from 150 to 270°C and 70 eV ionization potential.

Periodate oxidation and Smith degradation.—The polysaccharide, or de-*O*-acetylated polysaccharide sample (20 mg), was oxidized with 0.1 M $NaIO_4$ (2 mL) at 4°C for 48 h. The excess of periodate was destroyed by adding 0.1 mL of ethylene glycol. The oxidized product was reduced with 50 mg of $NaBH_4$ at 4°C, neutralized with AcOH, dialyzed against water and freeze-dried. This procedure was repeated with 24 h of oxidation. For mild acid Smith degradation, the sample was treated with 0.5 M trifluoroacetic acid (2 mL) at room temperature for 42 h, then evaporated and purified on Bio-Gel P-4 column.

Partial solvolysis with anhydrous hydrogen fluoride.—The polysaccharide (10–27 mg), in a teflon vial, was dissolved in anhydrous HF (5 mL) and kept at −30°C for either 20 or 50 min. The reaction was stopped by adding ether (10 mL). The precipitated product was dissolved in water (2 mL), heated for 3 h at 90°C to hydrolyze glycosyl fluorides, and fractionated on Bio-Gel P-4 column using pyridinium acetate pH 5.6 as eluent. HPLC analyses of oligosaccharides were done on a Waters apparatus equipped with a M600E pump and a M996 photodiode array detector, both controlled by a Millennium 2010 software. TSK G2500PWXL (7.5 × 250 mm), or Nucleosil C18 (6.2 × 250 mm) columns, were run in the isocratic mode with 1% AcOH in water at 1 mL/min.

NMR spectroscopy.—For NMR measurements, the samples were repeatedly treated with D_2O, with intermediate lyophilization, and then dissolved in D_2O (0.3 mL), containing a trace of acetone which was used as internal reference. ^1H (and ^{13}C) NMR spectra were recorded at 500 (125.7) MHz with a Bruker AM-500 instrument. Assignments of ^{13}C NMR spectra were assisted by DEPT experiments and literature data for the corresponding sugar residues analogously glycosylated [23–25].

FAB mass spectrometry.—FABMS was carried out on a Fisons-VG type ZAB-SEQ, double-focusing mass spectrometer working at 8 kV accelerating voltage. The LSIMS ion source was equipped with a Cs ion gun, giving a beam of 2 μA/35 keV for positive and negative mode. Thioglycerol, or a 1:1 thioglycerol–glycerol mixture, was used as the liquid matrix in the positive mode, and triethanolamine in the negative mode, for samples dissolved in MeOH. Peracetylation and per(deuterioacetylation) of oligosaccharides were done as described [26].

Determination of the absolute configuration of the monosaccharides.—The O-specific polysaccharide (1 mg) was hydrolyzed with 2 M trifluoroacetic acid for 2 h at 120°C to release sugar residues. The sample was treated with D-galactose oxidase [27]. Rhamnose was isolated from the hydrolysate by paper chromatography and its configuration was determined by GLC of the acetylated, derived (*S*)-(+)-2-octyl glycoside [13]. For the determination of the configuration of glucuronic acid, the carboxyl reduced polysaccharide (1 mg) was hydrolyzed as above and the glucose content was determined by D-glucose oxidase [28]. In order to determine the absolute configuration of mannose and

glucosamine, the polysaccharide was hydrolyzed with 10 M HCl for 30 min at 80°C, then treated with hexokinase in the presence of ATP. Complete phosphorylation of mannose and 2-acetamido-2-deoxyglucose was achieved as monitored by paper chromatography.

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