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Structure of the O-Specific, Sialic Acid Containing Polysaccharide Chain and Its Linkage to the Core Region in Lipopolysaccharide from *Hafnia alvei* Strain 2 As Elucidated by Chemical Methods, Gas-Liquid Chromatography/Mass Spectrometry, and ^{1}H NMR Spectroscopy†

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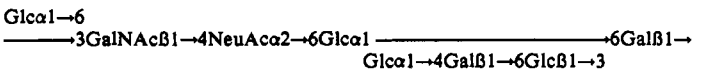
ABSTRACT: Mild acid hydrolysis of *Hafnia alvei* strain 2 lipopolysaccharide released no O-specific polysaccharide but instead gave a monomeric octasaccharide repeating unit with *N*-acetylneuraminic acid as the reducing terminus. In addition, a dimer of the octasaccharide repeating unit, and also a decasaccharide composed of a fragment of the O-specific polysaccharide chain and the core region, were obtained in minute amounts. On the basis of the sugar and methylation analyses, periodate oxidation, and ^{1}H NMR spectroscopy of the lipopolysaccharide hydrolytic products, the biological repeating unit of the O-specific polysaccharide was shown to be a branched octasaccharide:



The linkage between the O-specific polysaccharide chain and core region has also been determined and has yielded strong evidence that *N*-acetylneuraminic acid is an inherent lipopolysaccharide component. The lipopolysaccharide of *H. alvei* strain 2 is the first lipopolysaccharide reported to contain 4-substituted neuraminic acid in its O-specific polysaccharide region.

Hafnia alvei microorganisms form one of the lesser known enterobacterial genera, as far as structural and serological aspects of their O antigens are concerned. Preliminary chemical characterization of the lipopolysaccharides isolated from 33 strains of this genus showed that the lipopolysaccharide of strain 2 contains sialic acid as a component (Romanowska et al., 1988). The presence of sialic acid in lipopolysaccharides of some strains of *Salmonella*, *Escherichia coli*, and *Citrobacter* was reported earlier (Jann & Westphal, 1975), but to date the structure of their O-specific polysaccharides is unknown. The structures of capsular antigens

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isolated from *E. coli* K1 (McGuire & Binkley, 1964), K92 (Egan et al., 1977), and K9 (Dutton et al., 1987) and of the polysaccharides of *Neisseria meningitidis* serogroups Y and W-135 (Bhattacharjee et al., 1976) containing *N*-acetylneuraminic acid have been already established.

In the present paper the structural data on the O-specific polysaccharide and the type of linkage between the O-specific part and the core region in *H. alvei* strain 2 lipopolysaccharide are reported.

Materials and Methods

H. alvei strain 2 derived from the collection of the Institute of Immunology and Experimental Therapy (Wroclaw, Poland) was used in the experiments.

The growth of the bacteria in liquid medium, isolation and purification of the lipopolysaccharide, analysis of neutral sugars, hexosamines, and sialic acid, and SDS–polyacrylamide gel electrophoresis (SDS–PAGE)[1] were carried out as described previously (Romanowska et al., 1988).

Affinity chromatography on Sepharose 4B–serotonin was as described by Sturgeon and Sturgeon (1982). The affinity column was prepared by immobilizing serotonin (Sigma, 200 mg) on CNBr-activated Sepharose 4B (15 mL) in 0.1 M bicarbonate buffer, pH 8.6, for 2.5 h at room temperature. Unreacted, activated groups on the Sepharose were inactivated by an excess of aqueous ethanolamine. For examination, the column was washed with water and loaded with standard sialic acid (Sigma). The bound material was eluted with 0.5 M ammonium acetate.

Methylation was performed by the Hakomori procedure (1964) and the methylated product, after purification with Sep-Pak C18, was partly hydrolyzed (sample a) according to Stellner et al. (1973) and partly methanolized (1 M HCl in MeOH, 80 °C, 4 h) (sample b). Sample a was neutralized with AG3X-4A (COO^-), reduced with $NaBD_4$, and peracetylated, and sample b was dried with a nitrogen stream and peracetylated. Then both samples were analyzed by GLC/MS.

Periodate oxidation of the oligosaccharide fraction (20 mg) was carried out with 0.1 M $NaIO_4$ (2 mL) at 4 °C for 48 h. The excess of periodate was destroyed by adding 0.3 mL of ethylene glycol. The oxidized product was reduced with 60 mg of $NaBH_4$ at room temperature, neutralized with CH_3C-OOH, and desalted on a Bio-Gel P-4 column.

Gas–liquid chromatography/mass spectrometry analysis was carried out with a Hewlett-Packard 5995 A system using a OV-101 glass capillary column (0.2 mm × 12 m) and a temperature program of 120–240 °C, 3 °C/min.

Proton Nuclear Magnetic Resonance Spectroscopy. For ^1H NMR measurements the samples were repeatedly exchanged with D_2O, with intermediate lyophilization. The monomer (fraction 2-S1) was then dissolved in 0.35 mL of D_2O containing a trace of acetone, which was used as an internal reference (δ 2.225). The dimer (fraction 1) and the decasaccharide contained in fraction 1a were examined in a 90% D_2O + 10% $(CD_3)_2CO$-d_6 solution. ^1H NMR spectra

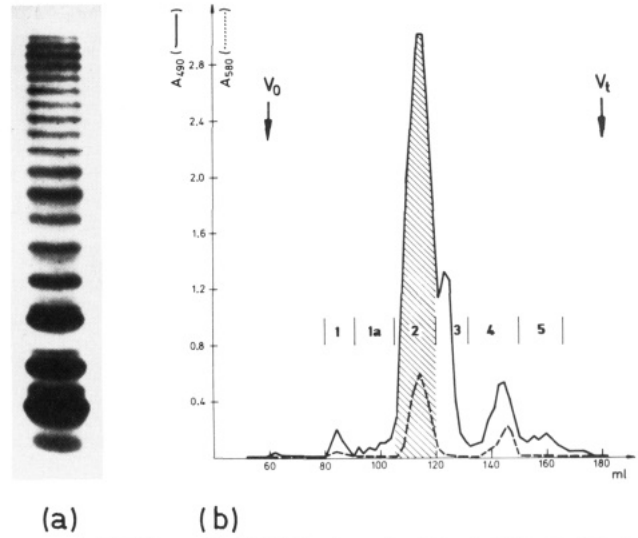


FIGURE 1: (a) Silver-stained SDS–polyacrylamide gel of *H. alvei* strain 2 lipopolysaccharide; (b) fractionation of carbohydrate material isolated from *H. alvei* strain 2 lipopolysaccharide after acetic acid hydrolysis on a Bio-Gel P-4 column (1.6 × 100 cm) equilibrated with pyridine/acetic acid buffer, pH 5.6. The flow rate was 2 mL/30 min. The absorbance was measured at 490 nm for phenol/sulfuric acid reaction and at 580 nm for the resorcinol reaction characteristic of sialic acid.

were obtained at 303 K with a Bruker AM-500 spectrometer operating at 500 MHz. The one-dimensional (1D) spectrum of the monomer was recorded at 600 MHz (courtesy of Bruker Analytische Messtechnik, Rheinstetten, Germany). The 1D total correlation [TOCSY (Braunschweiler & Ernst, 1983) or homonuclear Hartmann–Hahn (HOHAHA)] spectroscopy experiments were performed as described by Subramanian and Bax (1987), with variable mixing time during accumulation; the DANTE pulse sequence (Morris & Freeman, 1978) was used for selective excitation. Digital resolution was 0.16 Hz/point. Theoretical 1D spectra of the constituent sugar residues were simulated and iterated by using the Bruker PANIC program. The 1D nuclear Overhauser enhancement (NOE) spectra were measured according to Wagner and Wüthrich (1979).

Two-dimensional (2D) ^1H shift-correlated (COSY), long-range (LR) and triple-quantum-filtered (TQF) COSY, and relayed, double-relayed, and triple-relayed coherence transfer (RCT) spectra were obtained by using Bruker standard software [for theoretical and methodological aspects, see Ernst et al., (1987)]. Experimental details are given in figure legends (see supplementary material).

Rotating-frame 2D NOE (ROESY) spectra were recorded in the phase-sensitive mode by using the method of time-proportional phase incrementation (TPPI) (Marion & Wüthrich, 1983). The pulse sequence proposed by Rance (1987) was used, the mixing time being composed of a train of short (ca. 20°) pulses (Kessler et al., 1987), sandwiched by two *z*-filters. The *rf* carrier frequency was placed about 1 kHz downfield relative to the center of proton resonances during the spin–lock time and at the center during evolution and acquisition. The effective field for spin locking was 2.5 kHz. The mixing time was 200 ms, and the delay for the *z*-filters was 3 ms. A total of 512 free induction decays (FID), 32 scans each for the monomer and 64 scans for the decasaccharide, were accumulated, with relaxation delays of 1.5 s between scans. The time-domain spectra were zero-filled, multiplied by a squared cosine bell function, and Fourier-transformed to give a final resolution of 2.9 Hz/point in both dimensions.

[1] Abbreviations: NeuAc, *N*-acetylneuraminic acid; Glc, D-glucose; Gal, D-galactose, GalNAc, *N*-acetyl-D-galactosamine; Hep, L-glycero-D-mannoheptose; dOclA, 3-deoxy-D-octulosonic acid; SDS–PAGE, SDS–polyacrylamide gel electrophoresis, GLC/MS, gas–liquid chromatography/mass spectrometry; NMR, nuclear magnetic resonance; 1D and 2D, one and two dimensional; TOCSY, total correlation spectroscopy; HOHAHA, homonuclear Hartmann–Hahn spectroscopy; COSY, correlation spectroscopy; TQF, triple quantum filtered; RCT, relayed coherence transfer; NOE, nuclear Overhauser enhancement; ROESY; rotating-frame NOE spectroscopy.

Table I: Sugar Analysis of the Oligosaccharide Fractions Isolated from *H. alvei* Strain 2 Lipopolysaccharide

fraction	sugar components (molar ratio)				
	D-Glc	D-Gal	D-GalNAc	NeuAc	LD-Hep
1	4.0	1.7	0.9	1.0	tr[a]
1a	5.0	1.8	–[b]	–	2.8
2-S1	4.0	1.8	0.9	1.0	tr
2-S2	2.0	–	–	–	2.7

[a] tr, component present in trace amounts. [b] –, component absent.

Table II: Methylation Analysis of the Oligosaccharide Fractions Isolated from *H. alvei* Strain 2 Lipopolysaccharide

methylated sugar[a]	fractions (molar ratio)		
	1	1a	2-S1
2,3,4,6-Me_4Glc	4.7	3.0	3.0
2,4,6-Me_3Glc	tr[b]	tr	tr
2,3,6-Me_3Gal	2.0	1.0	1.0
2,3,4-Me_3Glc	2.7	1.0	1.0
2,4-Me_2Glc	–[c]	0.6	–
2,4-Me_2Gal	1.7	0.7	0.8
4-MeGalNAc	1.8	–	0.9
1,2,7,8,9-Me_5Neu5Ac	2.0	–	1.0
2,3,4,6,7-Me_5Hep	tr	1.0	tr
2,4,6,7-Me_4Hep	tr	tr	tr
2,4,6-Me_3Hep	tr	0.6	tr

[a] 2,3,4,6-Me_4Glc = 2,3,4,6-tetra-*O*-methylglucose, etc. [b] tr, component present in trace amounts. [c] –, component absent.

RESULTS

Isolation and Characterization of the Carbohydrate Material of H. alvei Strain 2 Lipopolysaccharide. The lipopolysaccharide of strain 2 analyzed by SDS–PAGE showed a high molecular weight ladderlike pattern proving its smooth character (Figure 1a) (Romanowska et al., 1988).

The lipopolysaccharide (100 mg), delipidated by acid hydrolysis (1% CH_3COOH, 100 °C, 0.5 h), released a water-soluble carbohydrate portion and lipid sediment. The carbohydrate material was then separated by gel filtration on a Bio-Gel P-4 column into five oligosaccharide fractions (Figure 1b). There was no high molecular weight polysaccharide fraction. The major oligosaccharide fraction 2 (39 mg) containing sialic acid was submitted to affinity chromatography on a Sepharose 4B–serotonin column. It was divided into two subfractions: 2-S1 and 2-S2. Fraction 2-S1 (28 mg) was eluted from the column with water; fraction 2-S2 (10.3 mg) was retained and then eluted with 0.5 M ammonium acetate. Fractions 1 (2 mg) and 1a (1.4 mg) were rechromatographed on a Bio-Gel P-4 column.

The sugar analysis of the oligosaccharide fractions (1, 1a, 2-S1, and 2-S2) showed fractions 1 and 2-S1 to contain the same sugar components: glucose, galactose, *N*-acetylgalactosamine, and sialic acid (NeuAc) (Table I). Both of these fractions originate from O-specific polysaccharide. Fraction 2-S2, composed mainly of glucose, heptose, and 3-deoxyoctulosonic acid, showed core character. Fraction 1a is an intermediate one, being composed of some O-specific as well as core sugars.

Methylation Analysis and Periodate Oxidation of the Oligosaccharide Fractions. The results of the methylation analysis are shown in Table II. Fraction 2-S1 contained three terminal glucoses, 4-substituted galactose, 6-substituted glucose, 3,6-disubstituted galactose, 3,6-disubstituted *N*-acetylgalactosamine, and 4-substituted *N*-acetylneuraminic acid. The composition of fraction 1 corresponds to a dimer of fraction 2-S1, since it yielded the same methylated derivatives

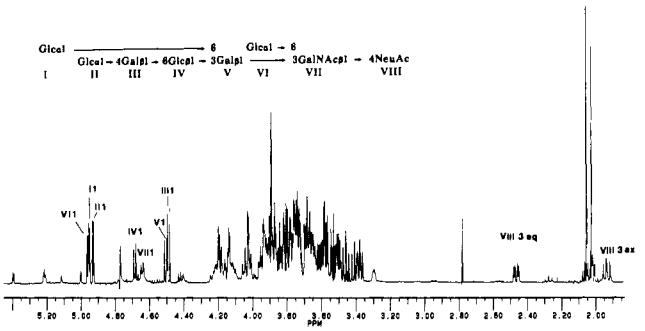


FIGURE 2: Resolution-enhanced 600-MHz ^1H NMR spectrum of the monomer (fraction 2-S1) from *H. alvei* strain 2 in D_2O at 303 K. Arabic numerals refer to the protons in the sugar residue denoted by the Roman numeral.

plus one more 6-substituted glucose in place of one terminal glucose in fraction 2-S1. Fraction 1a contained three terminal glucoses, 4-substituted galactose, 6-substituted glucose, 3,6-disubstituted glucose and galactose, and terminal and 3,7-disubstituted heptoses.

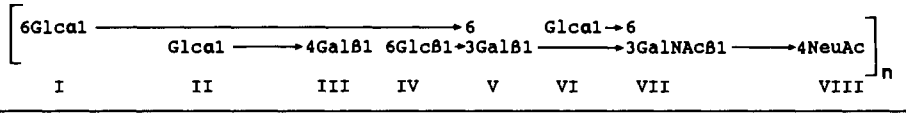
All fractions showed the presence of trace amounts of 3-substituted glucose and heptose, obviously derived from contamination by the core.

During periodate oxidation of fractions 1 and 2-S1 all glucoses and one of two galactoses were destroyed, but the other galactose and *N*-acetylgalactosamine residues were resistant to oxidation. This result is in agreement with the linkage positions of the sugars as determined by methylation analysis.

^1H NMR Spectroscopy. The 600-MHz spectrum of the monomer (fraction 2-S1) from *H. alvei* strain 2 (Figure 2) exhibits in the anomeric proton region four doublet signals of β sugar residues ($^3H_{1,2} \approx 7.8$ Hz) and three doublets of α residues ($^3J_{1,2} \approx 3.8$ Hz). The quasi triplet at 1.940 ppm and the double doublet at 2.475 correspond to H3ax and H3eq of a neuraminic acid residue. All these signals have an approximately equal integral intensity; hence, the repeating unit of the polysaccharide chain consists of eight residues. Besides, there are several weak signals of admixtures from other, incompletely separated fractions. Due to the variety of new NMR methods developed recently [for reviews, see Dabrowski (1987, 1989) and Homans et al. (1987)], the assignments can be obtained in many different ways, the choice of an optimum strategy often being a matter of convenience under the given circumstances of the particular NMR laboratory. Here, we used 1D TOCSY–HOHAHA spectra and 2D COSY, LR-COSY, and TQF-COSY spectra for the identification of the constituent sugar residues and 2D ROESY spectra for determining their sequence and glycosylation sites.

Although the H1 resonances of the three α sugar units are only a few hertz apart, good individual HOHAHA subspectra of these component units could be obtained by a selective excitation of these resonances by using the DANTE pulse sequence (Davis & Bax, 1985). Two of these subspectra look almost identical (Figure 3a,b), but the H2, H3, H6a, and H6b chemical shifts differ clearly (Table III). Since signals of all of the six nonanomeric protons occur simultaneously in each spectrum, some way of assigning the individual signals was required. H2 was identified by the H1/H2 connectivity in the COSY spectrum (supplementary material, Figure 1) and H3 by the converging slopes of the H2 and H3 multiplets in the HOHAHA spectrum; thus, H4, which shows the same coupling pattern as H3, was identified by elimination. The vicinal coupling constants can be read from these multiplets with sufficient accuracy and show that these sugar residues have

Table III: ^{1}H NMR Data[a] for the Octasaccharide Monomer (Fraction 2-S1) and Dimer (Fraction 1) Obtained from the O-Specific Polysaccharide from *H. alvei* Strain 2

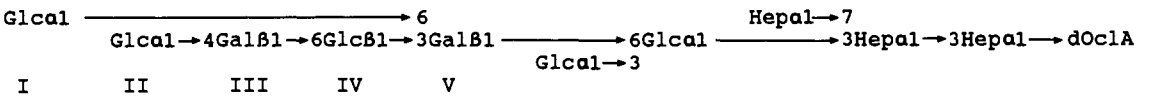


$n = 1$	I	II	III	IV	V	VI	VII	VIII
H1	4.956	4.935	4.492	4.695	4.511	4.965	4.651	
H2	3.578	3.537	3.578	3.367	3.669	3.576	3.904	
H3	3.684	3.746	3.732	3.505	3.812	3.701	3.944	1.940, 2.475
H4	3.410	3.461	4.032	3.53	4.200	3.387	4.139	4.135
H5	3.656	4.154	3.763	3.580	3.895	3.656	3.93	4.015
H6a	3.746	3.773	3.827	3.875	3.92	3.770	3.685	4.068
H6b	3.881	3.813	3.875	4.193	3.92	3.879	3.94	

$n = 2$[b]	I′	I	II′	II	III′, III	IV′, IV	V′, V	VI′, VI	VII′, VII	VIII′	VIII
H1	4.962	4.995	4.947	4.985	4.501	4.705	4.526	4.969	4.663		
H2	3.592	3.592	3.546	3.565	3.606	3.397	3.699	3.592	3.96		
H3	3.710	3.820	3.770	3.775	3.744	3.525	3.832	3.717	3.96	1.790, 2.972	1.923, 2.457
H4	3.427	3.386	3.480	3.484	4.045	3.54	4.215	3.370	4.155	3.810	4.118
H5	3.68	3.795	4.172	4.175	3.76	3.603	3.915	3.67		3.915	4.033
H6a	3.76	3.795	3.79	3.79	3.857	3.876	3.93	3.76			
H6b	3.90	3.925	3.81	3.81	3.920	4.210	3.93	3.90			

[a] Chemical shifts were obtained in D_2O solution at 303 K relative to acetone set equal to 2.225 ppm. [b] Primed Roman numerals refer to the octasaccharide fragment at the nonreducing end of the chain.

Table IV: ^{1}H NMR Data[a] for the Decasaccharide Comprising Segments of the O-Specific[b] and Core Moieties (Fraction 1a) of the Lipopolysaccharide from *H. alvei* Strain 2



H1	4.965	4.948	4.503	4.705	4.527	5.415	5.232	4.985	5.135	5.220
H2	3.580	3.545	3.602	3.398	3.702	3.567	3.675	3.998	4.362	4.065
H3	3.715	3.775	3.742	3.529	3.837	3.780	4.010			
H4	3.424	3.482	4.05	3.53	4.203	3.464	4.068			
H5	3.682	4.175	3.855	3.60	3.91	4.040	4.06			
H6A						3.77	3.96			
H6B						3.922	4.06			

[a] Chemical shifts were obtained in a 90% D_2O + 10% $(CD_3)_2CO$ solution at 303 K relative to acetone set equal to 2.225 ppm. [b] For numbering of the sugar residues, cf. formula in Table III and Figure 2.

a gluco configuration ($^3J_{2,3} \approx {}^3J_{3,4} \approx {}^3J_{4,5} \approx 9.5$ Hz). Finally, H5 was distinguished from H6a and H6b by its complex coupling pattern resulting from coupling with three neighboring protons. The subspectrum of the third of these α sugar residues (Figure 3c) is similar, except for the extremely low-field-shifted H5 resonance, which is characteristic of the Glcα1–4Galβ disaccharide segment (Lipkind et al., 1988), and the very weak coupling of H5 with the methylene protons, which points to a special conformation of the hydroxymethyl group. The remarkable H1 high-field shift for the terminal 1–6 (I and VI) and 1–4 (II) α-linked glucose units ($\delta < 5.0$; see Table III), which contrasts with δ 5.21–5.515 found for 1–2- (Romanowska et al., 1986) and 1–3- (Table IV) linked ones, can be attributed to the shielding exerted by their aglycons. Thus, 1–6-linked aglycons were found to cause an ~0.1–0.4 ppm high-field shift of the glycon H1 resonances, as compared with 1–2- (De Bruyn et al., 1975) or 1–3-linked ones (De Bruyn et al., 1975; Dabrowski et al., 1982, 1984; Vliegenthart et al., 1983). A 4-linked galactose aglycon causes a similar high-field shift of the H1 α-Glc resonance, as observed for the Glcα–4Galβ1–OMe/Glcα1–3Galβ1–OMe pair of methyl glycosides (4.99 vs 5.14 ppm) (Lipkind et al., 1988), and for the Glcα1–4Galβ segment of a polysaccharide (4.915 ppm) (Di Fabio et al., 1989).

Of the four β sugar residues, three have the galacto configuration, as shown by their $^3J_{2,3} \approx 8$ Hz, $^3J_{3,4} \approx 3$ Hz, and $^3J_{4,5} \approx 1$ Hz coupling constants. Although magnitude-mode COSY spectra provide only approximate values of coupling constants, this accuracy is sufficient for establishing relative configurations at ring carbons, since one need only distinguish between "large" (≈7–10 Hz) and "small" (≈1–4 Hz) J values (Koerner et al., 1987; Dabrowski, 1987, 1989). In our experience, galactoses and galactosamines can practically always be recognized by the characteristic flattened shape of their H3/H4 COSY cross-peaks (supplementary material, Figures 1 and 2), this shape being determined by the sum of active (a) and passive (p) coupling constants $^3J_{3,4}$ (a) + $^3J_{2,3}$ (p) ≈ 13 Hz in one dimension ($F2$) and $^3J_{3,4}$ (a) + $^3J_{4,5}$ (p) ≈ 4 Hz in the other ($F1$). The small values of the sums $^3J_{3,4} + {}^3J_{4,5}$ were confirmed by the HOHAHA spectra (Figure 3d–f). On the other hand, the very small $^3J_{4,5}$ coupling constant (≈1 Hz), which is notorious for halting magnetization transfer (Da-

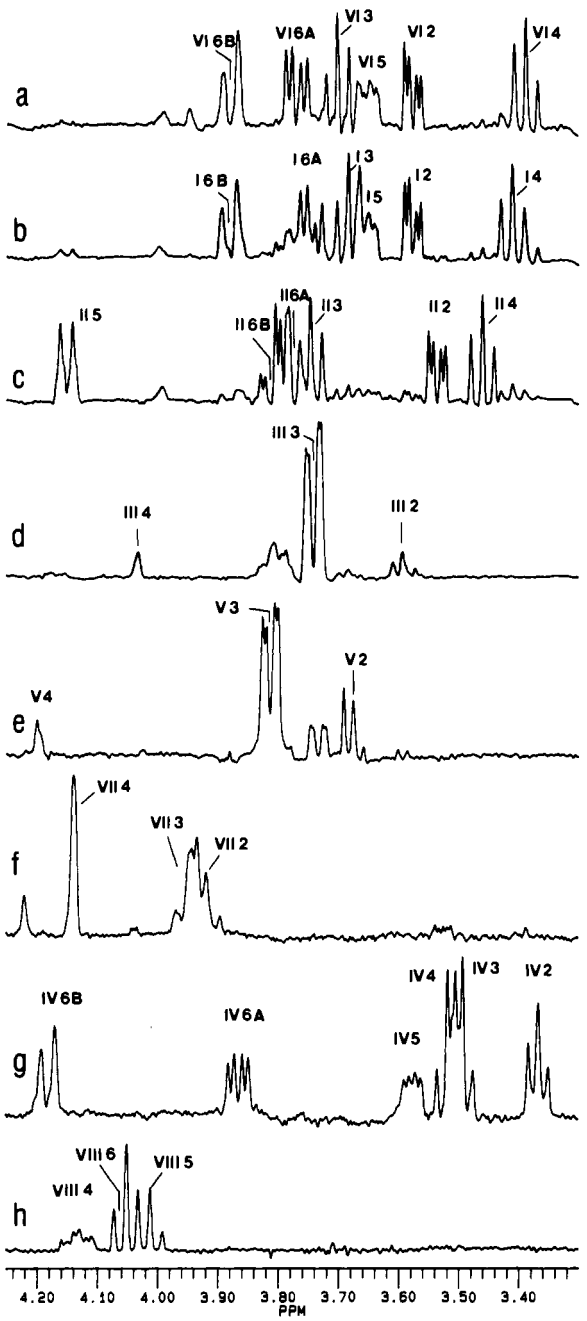


FIGURE 3: One-dimensional 500-MHz TOCSY–HOHAHA spectra of the eight sugar residues of the monomer (fraction 2-S1) from *H. alvei* strain 2, obtained with semiselective excitation of the corresponding anomeric proton resonances (a–g), or the H3eq resonance of the neuraminic acid residue (f). For the formula, see Figure 2 and Table III.

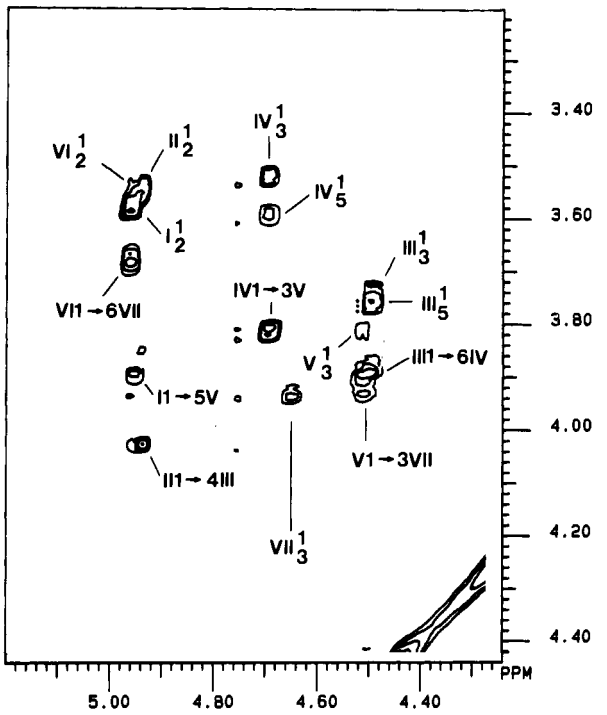


FIGURE 4: Anomeric region of the 2D 500-MHz rotating-frame NOE spectrum (ROESY) of the monomer (for the formula, see Figure 2 and Table III). For intraresidue NOE cross-peaks, the horizontal (*F*2) and vertical (*F*1) axes give the chemical shifts of the protons designated by the superscript and subscript, respectively; interresidue NOE connectivities are indicated by arrows.

browski, 1987, 1989), rendered assignment of the H5, H6a, and H6b resonances difficult. However, the H4 resonances clearly verified by the HOHAHA spectra enabled us to establish the H4/H5 connectivities in the COSY spectrum optimized for small coupling constants (LR-COSY, supplementary material, Figure 2). Once the H5 chemical shifts are known, the connectivities with H6a and H6b can be traced in the same LR-COSY spectrum or in the conventional COSY spectrum, depending on the relevant coupling constants. In addition, a TQF spectrum (not shown), which, in principle, leaves only signals of triads of mutually coupled protons (each with each, as H5, H6a, and H6b), are also very useful in tracing these connectivities. GalNAc was distinguished from the Gal residues by the strong low-field shift of its H2 resonance (Table III).

The identity of the fourth of the β sugar residues could not be established by analyzing the coupling constants determined by inspection, because the tightly coupled H3 and H4 resonances (Table III) exhibited a second-order multiplet (Figure 3g). However, the typical high-field position of the H2 resonance (<3.34 ppm) leaves no doubt that this unit is a glucose, and the almost identical H1 and H2 chemical shifts found for a similarly linked glucose residue in the Glcβ1–3Galβ1–OMe disaccharide (Lipkind et al., 1988) confirm this conclusion. Finally, spectrum simulation showed the relevant coupling constants to correspond to a glucose ($^3J_{3,4}$ = 8.6 Hz and $^3J_{4,5}$ = 9.7 Hz).

NeuAc resonances were assigned by HOHAHA (Figure 3h). No attempt was made to follow magnetization transfer beyond H6, where it was halted because of the extremely small H6/H7 coupling constant.

The sequence and linkage analysis was performed with the aid of a ROESY spectrum. The contour plot of this spectrum (Figure 4) clearly shows transglycosidic through-space connectivities between the anomeric and the corresponding aglyconic protons for five of the seven glycosidic bonds (III1→4III, III1→6IV, IV1→3V, V1→3VII, and VI1→6VII). The NOE response for the VIII1→4VIII proton pair, which confirms the →4NeuAc glycosylation site proved by methylation analysis, was weak and could only be seen in a cross section through the 2D matrix (not shown). It should be noticed, however, that *intra*residue NOEs for the GalNAc-VII synaxial H1/H3 and H1/H5 pairs (both at ca. 3.93 ppm) are also very weak, this being explainable by a short transverse relaxation time T_2 for the anomeric GalNAc proton (evidenced by its broadened signal in the 1D spectrum, Figure 2). The second uncertain dipolar transglycosidic connectivity refers to the I1→6V linkage. A cross-peak at the I1/5V coordinate is clearly visible, but since C5 cannot be a site of glycosylation, it can be inferred that the linkage is 1→6. The conformation, however, is such that H1 of Glc-I is distant from the methylene

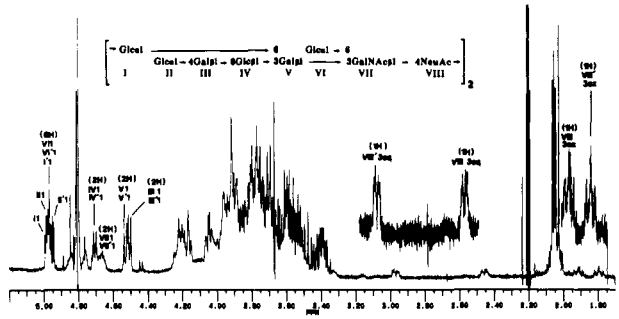
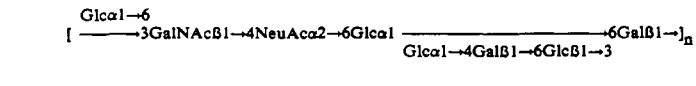


FIGURE 5: Resolution-enhanced 500-MHz ^1H NMR spectrum of the dimer (fraction 1) from *H. alvei* strain 2 in D_2O at 303 K. Labeling is as in Figure 2, but dashed Roman numerals refer to the octasaccharide fragment at the nonreducing end of the chain.

protons of Gal-V yet comes close to its H5; neither of the formally possible alternative linkages 1→4 or 1→2 would allow such a proximity.

The dimer (fraction 1) was investigated applying the same techniques and, in addition, the multistep RCT and 1D NOE spectroscopy. In the 1D overall spectrum, the H1 signals for the III, IV, V, and VII residues have a two-proton integral intensity each, and those for residues I, II, and VI have a total six-proton intensity but are split into several, not fully resolved components (Figure 5). At the same time, new H3ax and H3eq signals (one-proton intensity each) for a NeuAc residue that is substituted at the "reducing" end occur at 1.790 and 2.972 ppm [cf. Dutton et al., (1987)], along with the but slightly shifted NeuAc signals found for the monomer (1.923 vs 1.940 and 2.457 vs 2.475 ppm). Small shifts of the same order of magnitude (vs the shifts obtained for the monomer), which are observed for most of the resonances in the spectrum, can be accounted for by solvent effects, since 10% acetone-d_6 was added to the solution of the dimer for better field-frequency lock stability (Bock et al., 1988) during long-time measurements. When searching for the sialylated residue, one can exclude units III–VII because not only their H1 but also the H2–H6 resonances are undifferentiated (Table III); i.e., sialylation-induced shifts (Koerner et al., 1983; Berman et al., 1988; Strecker et al., 1989) are not operating. Although each of the two remaining α-Glc units I and II shows two sets of resonances, it is obviously the former that is sialylated, as follows from the strong NOE response for H6 of Glc-I when irradiating H3ax of NeuAc-VIII′ (not shown). The rather surprising differentiation of H1–H3 resonances for Glc-II and -II′ would then imply that the two trisaccharide side chains are bent toward the main chain, where units II and II′ approach moieties exerting different shielding.

Fraction 1a turned out to contain a decasaccharide composed of a pentasaccharide segment of the O-specific chain, glycosidically linked to a pentasaccharide segment of the core region (see formula in Table IV). The chemical shifts for the I–IV residues of the former segment are virtually identical with those found for these residues in the "nonreducing" part of the dimer examined in the same solvent (90% D_2O + 10% acetone-d_6), and the chemical shifts for residue V differ slightly due to the shielding by a different aglycon. Although it was not attempted in this work to determine the whole structure of the core oligosaccharide, the two core Glc residues were fully assigned and the link between these two regions was unequivocally established by ROESY. Consequently, the repeating unit of the O-specific polysaccharide has the following structure:



Discussion

H. alvei strain 2 lipopolysaccharide yielded a high molecular weight banding pattern on SDS–PAGE analysis, giving evidence for the presence of as many as 18 O-specific repeating units within the structure. However, during the usual hydrolytic procedure applied for the separation of the carbohydrate and lipid moieties of the lipopolysaccharide, the O-specific chain was split off almost completely, to give a monomeric repeating unit octasaccharide. This was a result of the lability of the ketosidic linkages of the NeuAc component of the O-specific chain under the conditions used. The monomeric octasaccharide that was formed was terminated by a NeuAc residue at the reducing end.

Due to the presence, among the hydrolytic products, of minute amounts of a dimer of the octasaccharide, as well as a decasaccharide composed of a fragment of the repeating unit and of the core region, it was possible to determine the type of linkage between the monomeric units and also between the O-specific chain and the core region. Consequently, we have been able to establish the structure of the biological repeating unit of *H. alvei* strain 2 lipopolysaccharide. This structure is unusual because for the first time NeuAc has been found to be a component of the O-specific chain of a lipopolysaccharide. Moreover, an octasaccharide repeating unit of a lipopolysaccharide has not, to the best of our knowledge, been previously reported.

Acknowledgments

We thank Dr. Anna Romanowska for the SDS–PAGE analysis of *H. alvei* strain 2 lipopolysaccharide.

Supplementary Material Available

Two figures showing 2D COSY and long-range COSY spectra of the monomer (fraction 2-S1) from *Hafnia alvei* strain 2 (2 pages). Ordering information is given on any current masthead page.

Registry No. NeuAc, 131-48-6; *H. alvei* octasaccharide, 132910-42-0.

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DNA-Containing Liposomes as a Model for the Study of Cell Membrane Permeation by Anthracycline Derivatives†

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ABSTRACT: The uptake of anthracycline derivatives into large unilamellar vesicles (LUV) in response to a driven force provided by DNA encapsulated inside the LUV has been investigated. Four anthracyclines have been used: adriamycin, 4′-*O*-tetrahydropyranyladriamycin (THP-ADR), daunorubicin (DNR), and carminomycin. No quenching of the drug fluorescence is observed through interaction of the drugs with the lipidic bilayer. Rapid quenching of drug fluorescence occurs when drugs intercalate between the base pairs of DNA. The kinetics of the decay of anthracycline fluorescence in the presence of DNA-containing liposomes can thus be used to follow the diffusion of the drug through the membrane. The initial rates of uptake, as a function of pH, and lipid bilayer permeability coefficients have been calculated for the neutral forms of THP-ADR and DNR. This system suggests that anthracycline may gain access to cells by passive diffusion of the neutral form of the drug under the action of a driven force provided by DNA in the nucleus.

The anthracycline antibiotic adriamycin (ADR)[1] is one of the most potent anticancer drugs in clinical use. It is active against a wide range of malignancies, including sarcomas, carcinomas, melanomas, leukemias, and lymphomas (Arcamone, 1981). Other anthracycline derivatives such as daunorubicin (DNR), THP-adriamycin (THP-ADR), and carminomycin (CAR) also have outstanding antitumor activity. One of the major obstacles of chemotherapy is that, after repeated treatments, cellular resistance to the drug appears. A particular phenotype of resistant cells, called multidrug resistance (MDR), has been recognized and encompasses a broad pattern of resistance to anticancer drugs derived from natural products (Bradley & al., 1988; Kessel, 1988). A common feature of MDR cells is a net decrease intracellular accumulation of drug that has been ascribed to an increased efflux pump mechanism (Dano, 1973; Inaba & Johnson, 1977; Skovsgaard, 1978; Riordan & Ling, 1985) and associated with the overexpression of an integral membrane glycoprotein, the P-glycoprotein (Juliano & Ling, 1976; Beck et al. 1979; Riordan & Ling, 1979). It has been proposed that P-glycoprotein may function as an energy-dependent drug efflux pump.

The determination of the precise role of P-glycoprotein involves the determination and the comparison of the mechanism of drug uptake and release by resistant and sensitive cells. We have recently developed a new spectrofluorometric method based on the observation that the fluorescence of an

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[1] ADR, adriamycin; THP-ADR, 4′-*O*-tetrahydropyranyladriamycin; CAR, carminomycin; DNR, daunorubicin; LUV, large unilamellar vesicles; PC, egg phosphatidylcholine; PA, egg phosphatidic acid; PS, phosphatidylserine; CHOL, cholesterol; FCCP, carbonyl cyanide [*p*-(trifluoromethoxy)phenyl]hydrazone.

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