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Structure of the O-specific polysaccharide, containing a glycerol phosphate substituent, of *Hafnia alvei* strain 1220 lipopolysaccharide

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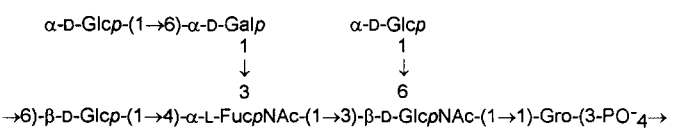
Abstract

The O-specific polysaccharide of the lipopolysaccharide produced by *Hafnia alvei* strain 1220 contained D-glucose, D-galactose, *N*-acetyl-D-glucosamine, *N*-acetyl-L-fucosamine (2-acetamido-2,6-dideoxy-L-galactose), glycerol, and phosphate. It was proved by composition and methylation analyses, Smith degradation, dephosphorylation, and one- and two-dimensional ^{1}H NMR spectroscopy to be a teichoic acid-like polymer with a branched hexasaccharide repeating unit of the following structure:



Keywords: *Hafnia alvei*; Lipopolysaccharide; O-Specific polysaccharide structure

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1. Introduction

Hafnia alvei, a member of the enterobacterial family, occurs most often in nosocomial infections causing enteric disorder, urinary tract infections, septicaemia, and meningitis [1].

The chemical characterization of lipopolysaccharides isolated from the *H. alvei* group was carried out in 1988 [2]. The structures of the O-specific polysaccharides of nine *H. alvei* strains have already been elucidated [3–10]. Their repeating units range from a disaccharide [7] for strain 38 to a sialic acid-containing octasaccharide from strain 2 [5]. As a part of a systematic programme, we now present the structure of the O-specific polysaccharide of *H. alvei* strain 1220.

2. Experimental

Materials.—*H. alvei* strain 1220, obtained from the collection of the Pasteur Institute (Paris), was grown in liquid medium, as described previously [11].

Lipopolysaccharide (LPS) was isolated from the dry bacterial cells by phenol–water extraction followed by purification on a Sepharose 2B column (100 × 2 cm) [12]. O-Specific polysaccharide (PS) obtained after mild acid hydrolysis of the LPS (aq 1% AcOH, 100 °C, 1 h) was purified by means of gel permeation chromatography on a Sephadex G-50 column (100 × 2 cm) in pyridine–AcOH buffer (pH 5.75) monitored by the phenol–H_2SO_4 reaction [13]. The yield of the lipopolysaccharide and the O-specific polysaccharide amounted to 4.5 and 1.1%, respectively, of the dry bacteria.

Analytical methods.—Sugar and phosphorus determinations, paper chromatography, and dephosphorylation were carried out as reported earlier [3,9]. Glycerol was identified in the hydrolyzate of the dephosphorylated PS (2 M CF_3CO_2H, 120 °C, 2 h) by paper chromatography using 1-butanol–pyridine–water (6:4:3, by volume) as irrigant and HIO_4 and benzidine reagents as detector, and by GLC–MS analysis as the alditol acetates.

The absolute configurations of the sugar components (glucose, galactose, and glucosamine) were established as D by using the enzymes glucose oxidase, galactose oxidase, and hexokinase [3]. The configuration of 2-amino-2,6-dideoxygalactose (fucosamine) was determined as L by GLC according to the method [14] using (*R*)- and (*S*)-2-butanol.

Methylation analysis was performed by the Hakomori method [15] and the methylated product was hydrolyzed with 2 M CF_3CO_2H at 120 °C for 2 h or with 10 M HCl at 80 °C for 30 min.

Periodate oxidation and Smith degradation; the PS (5.7 mg) dissolved in 0.1 M $NaIO_4$ (0.6 mL) was kept for 3 days at 4 °C. Then ethylene glycol (0.025 mL) and $NaBH_4$ (25 mg) were added successively to the mixture which was kept overnight at room temperature. The mixture was neutralized with aq 50% AcOH to pH 6, and desalted on a Bio-Gel P-2 column (100 × 2 cm). The degraded product was treated with aq 2% AcOH at 100 °C for 1 h, freeze-dried to yield 3.8 mg, and then purified on a Bio-Gel P-2 column.

GLC–MS analysis was performed 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 samples were exchanged twice with D_2O, with intermediate lyophilization, and then dissolved in D_2O (0.4 mL) containing a trace of acetone, whose signal was set at δ 2.225 as reference. The ^{1}H and ^{31}P NMR spectra were recorded on a Bruker AM 500 spectrometer operating at 500 and 202 MHz, respectively. The ^{13}C DEPT measurement (distortionless enhancement by polarization transfer) was carried out on an AM 360 spectrometer at 90.5 MHz. Bruker standard software was applied for two-dimensional (2D) COSY, TOCSY (total correlation spectroscopy), and $^{1}H,^{31}P$ inversely heterocorrelated spectra. For the heterocorrelated spectra, 80 transients were accumulated for each of the 256 experiments, the size of the time domain data matrix being 4K × 256 points. Before Fourier transformation, the matrix was zero-filled in the t_1 dimension and multiplied by a square sine-bell window function shifted by $\pi/4$ and $\pi/2$ in t_2 and t_1, respectively. The digital resolution in the frequency domain was 1.5 and 19.7 Hz per point for ^{1}H and ^{31}P, respectively. The one-dimensional (1D) TOCSY spectra and the 2D ROESY (rotating frame NOE) were recorded in the same way as reported previously [5].

3. Results and discussion

Isolation and chemical analysis of the O-specific polysaccharide.—The lipopolysaccharide of *H. alvei* 1220, which derived from a wild strain, had smooth character in SDS/PAGE analysis [2]. The mild acid degradation of the LPS afforded the O-specific polysaccharide (P_1 fraction) which was separated on a Sephadex G-50 column from the lower molecular weight carbohydrate fractions P_2, P_3, and P_4. The intermediate P_2 fraction proved to be mainly galactan-like. The P_3 fraction was identified as core oligosaccharide of the typical structure existing in the *Hafnia* genus [16], and the P_4 fraction contained free 3-deoxyoctulosonic acid. The sugar analysis of the O-specific polysaccharide (PS) showed that it contains D-glucose, D-galactose, *N*-acetyl-D-glucosamine, and *N*-acetyl-L-fucosamine in molar ratios 3:1.6:1:0.9. Moreover, the presence of glycerol and phosphorus (1 mol P/mol GlcNAc) was observed.

To dephosphorylate and depolymerize, the polysaccharide was treated with 48% HF (4 °C, 3 days). The mixture was freed from HF by evaporation with nitrogen and purified on a Bio-Gel P-2 column. This yielded phosphate-free oligosaccharide monomer as the major product, but small amounts (10–15%) of the monomer still contained phosphoryl group.

During periodate oxidation of the polysaccharide, all hexoses were oxidized except *N*-acetylfucosamine and *N*-acetylglucosamine which were resistant to the oxidation. After Smith degradation the oxidized product was purified on a Bio-Gel P-2 column and then submitted to methylation analysis together with the native and dephosphorylated polysaccharides.

Methylation analysis.—Methylation analysis of the polysaccharide and the oligosaccharide monomer (Table 1) revealed the presence of terminal glucoses (two in the PS,

Table 1
Methylation analysis of *H. alvei* strain 1220 O-specific polysaccharide before and after chemical degradation

Methylated sugar [a]	t_R [b]	Molar ratio		
		Polysaccharide		Oligosaccharide monomer
		Native	Smith degraded	
2,3,4,6-Me_4Glc	1.00	2.2	– [c]	3.0
2,4,6-Me_3Gal	1.25	0.3	–	–
2,3,4-Me_3Glc	1.27	–	–	≤ 0.2
2,3,4-Me_3Gal	1.35	1.3	–	1.0
3,4-Me_2-2-NMeAc-2,6-ddGal	1.49	–	1.0	–
2-NMeAc-2,6-ddGal	1.71	0.8	–	0.9
4,6-Me_2GlcNAc	1.90	–	1.0	–
4-MeGlcNAc	2.15	0.8	–	0.9

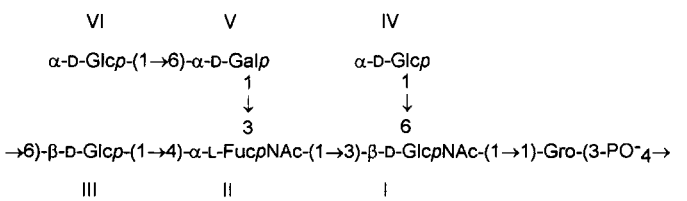
[a] 2,3,4,6-Me_4Glc = 2,3,4,6-tetra-*O*-methyl-D-glucose, etc.; dd = dideoxy.
[b] t_R = Retention time for the corresponding alditol acetate relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol (t_R = 1.00) on an HP-1 glass capillary column at 150–270 °C (8 °C/min).
[c] – Indicates component not present.

and three in the monomer), 6-substituted galactose, 3,4-disubstituted *N*-acetylfucosamine, and 3,6-disubstituted *N*-acetylglucosamine. Small amounts of 6-substituted glucose found in the monomer originated from its phosphorylated portion. In the original polysaccharide this glucose residue was not accessible during methylation analysis because of its participation in phosphodiester linkage.

The main Smith-degraded product proved to be a disaccharide composed of terminal *N*-acetylfucosamine and 3-substituted *N*-acetylglucosamine (Table 1).

The sugar analysis of the polysaccharide showed the presence of somewhat higher amounts of galactose. In the methylation analysis of the PS, a non-stoichiometric quantity of 3-substituted galactose as well as an increased amount of 6-substituted galactose were also observed. These additional amounts of galactose found in the PS are derived from galactan contamination of fraction P_2. After depolymerization of the PS, the resulting oligosaccharide monomer contained no extra amounts of galactose.

NMR spectroscopy.—In order to simplify and condense the presentation of the NMR data, the numbering of the sugar residues (Roman numerals) and protons of each residue (Arabic numerals), which follow from the entire assignment procedure, will be used here in advance.



The glycerol and six sugar residues present were identified with the aid of 2D COSY, 2D TOCSY, 1D TOCSY, and ^{13}C DEPT spectra. The sequences were established with

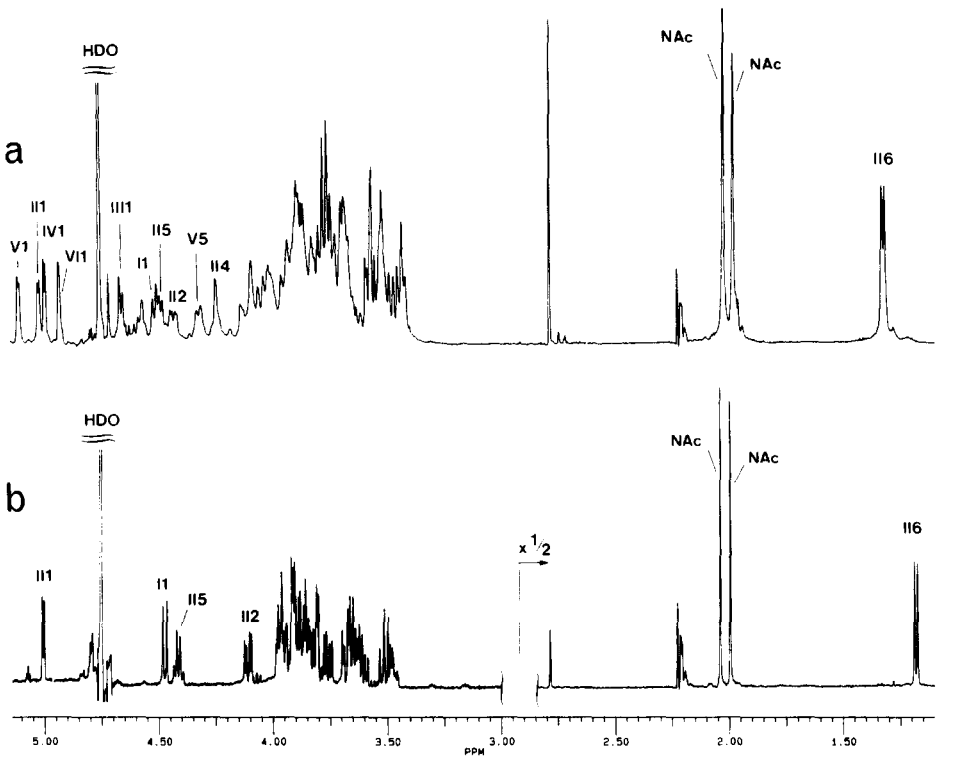


Fig. 1. 1D ^{1}H NMR spectra. (a) Spectrum of the O-specific polysaccharide of *H. alvei* strain 1220. Roman numerals denote the sugar residues, and arabic numerals refer to the protons in these residues (for the formula and labelling, see Table 2. (b) Spectrum of the α-L-Fuc*p*NAc-(1 → 3)-β-D-Glc*p*NAc-(1 → 1)-glycerol disaccharide derivative isolated after the Smith degradation of the polysaccharide.

the use of 1D ROESY and {^{31}P}^{1}H-correlated spectra. The results are summarized in Table 2.

In the low-field region of the 1D ^{1}H NMR spectrum (Fig. 1a), four of the anomeric proton signals correspond to α residues II, IV, V, and VI ($^3J_{1,2} \leq 3.5$ Hz). Of the two remaining anomeric proton signals, one is deformed by the nearby water signal, and the other is overlapped with the II H-5 signal, but both of them were observed as undistorted doublets in the 1D TOCSY spectra generated by a semi-selective excitation of the I H-5 and the degenerate III H-3,4,5 resonances. The $^3J_{1,2}$ coupling constants of 8.2 Hz for I and 7.6 Hz for III show both these residues to have a β configuration.

The spectrum in Fig. 1a also exhibits two three-proton singlets at δ 1.99 and 2.02, characteristic of *N*-acetyl groups, and a three-proton doublet ($^3J = 6.3$ Hz) at δ 1.316, obviously attributable to a methyl group of a deoxy sugar.

Clearly, two of the α sugars (IV and VI) have a *gluco* configuration, since the vicinal coupling constants for the H-2/H-3, H-3/H-4, and H-4/H-5 proton pairs, obtained from the 1D TOCSY spectra (Fig. 2a and d), are within 9–10 Hz limits. The excitation of the H-1 proton of the third α sugar at δ 5.101 (V) produced a TOCSY

Table 2
Chemical shifts [a] for the O-specific polysaccharide of *H. alvei* strain 1220

Proton	Chemical shift (δ) in residue						
	VI D-Glc $p\alpha$ 1 →	V 6-D-Gal $p\alpha$ 1 →	→ 3-Gro-1 → PO_4 →	III 6-D-Glc $p\beta$ 1 →	II 4-L-Fuc pNAc α 1 → 3	IV D-Glc $p\alpha$ 1 → 6	I 3-D-Glc pNAc β 1 →
H-1	4.925	5.101	3.83; 3.89	4.655	5.010	4.988	4.507
H-2	3.574	3.682	4.00	3.425	4.428	3.578	3.894
H-3	3.560	3.915	3.66; 3.95	3.52	4.044	3.775	3.72
H-4	3.465	4.086		3.52	4.240	3.427	3.72
H-5	3.805	4.322		3.52	4.480	3.76	3.69
H-6a	3.76	3.689		4.01	1.316	3.76	3.84
H-6b	3.876	3.880		4.09	1.316	3.88	3.94

[a] Chemical shifts for a D_2O solution are given relative to acetone (δ 2.225), measured at 305 K.

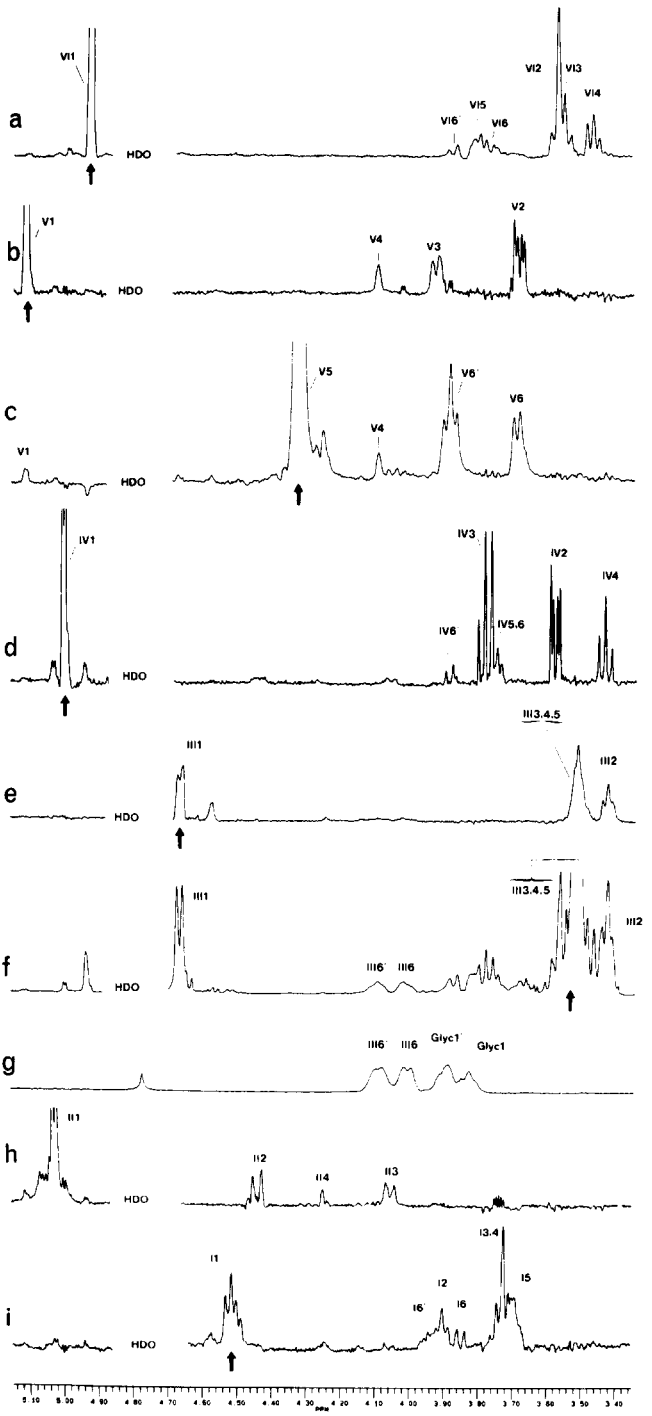
spectrum that contained only the H-2, H-3, and H-4 signals (Fig. 2b), that is, magnetization transfer was stopped at the C-4–C-5 bond — a well-known indication [17] of a very small $^3J_{4,5}$ coupling constant, and hence of a *galacto* configuration of this residue. The connectivity path was also limited to these protons in the 2D TOCSY spectrum. In order to complete the assignments for this sugar unit, the gap to H-5 was bridged by the ROESY connectivity for the H-4/H-5 *gauche* and H-3/H-5 synaxial protons, and a TOCSY spectrum was generated by excitation at the H-5 frequency thus found, to yield the H-6a and H-6b signals (Fig. 2c). The *galacto* configuration of the α unit V is convincingly confirmed by the strong low-field shifts of its V H-2–H-5 resonances, as compared with those found for the α-glucose units IV and VI. Indeed, these shifts are explainable [18,19] by the synaxial position of the V HO-4 group relative to V H-2, the antiperiplanar position of this group relative to V H-3 and V H-5, and the equatorial orientation of V H-4 vs. the axial one of IV H-4 and VI H-4.

In the case of the fourth α-sugar unit (II), the connectivity track leading from H-1 breaks off at H-4 both in the COSY and 2D TOCSY spectrum (Fig. 2h), pointing to a *galacto* configuration, as with unit V. The low-field shifts of the II H-2–H-5 protons (all ≥ 4 ppm) also confirm the *galacto* configuration, as in the case of V. Again, the H-4/H-5 bottleneck was passed in a 2D ROESY experiment in that both the H-4/H-5 *gauche* and the H-3/H-5 synaxial connectivities were found, and H-5 was then shown in the same 2D TOCSY and COSY spectra to be scalarly coupled to a methyl group. Thus, unit II has the skeleton of a fucose. However, judging by the extremely strong low-field shift of the II H-2 resonance, as compared with that found for typical fucoses (4.428 vs. $\sim$ 3.8 ppm [20,21]), one of the NAc groups mentioned above must be located at C-2 of this residue.

The H-1/H-2 correlation for the β unit III was found in the 2D COSY spectrum and confirmed in the 1D TOCSY spectrum generated by excitation of the III H-1 resonance (Fig. 2e). The latter spectrum also displayed the degenerate H-3, H-4, and H-5, but no H-6a, H-6b resonances, probably because of ineffective magnetization transfer due to small $^3J_{5,6a}$ and $^3J_{5,6b}$ coupling constants; the same refers to the cross-section drawn at the III H-1 coordinate in the 2D TOCSY spectrum. However, the III H-5/H-6a and III H-5/H-6b connectivities are manifest both in the 1D TOCSY spectrum obtained with excitation of III H-5 (Fig. 2f), and in the cross-section through the III H-5 coordinate in the 2D TOCSY spectrum. Unfortunately, due to the degeneracy of the H-3–H-5 resonances, neither the $^3J_{3,4}$ nor $^3J_{4,5}$ coupling constants can be extracted from the spectra; hence, the stereochemistry at C-3 and C-4 cannot be determined. However, since three glucose residues were found with the aid of chemical methods (Table 1), and unit I will be shown to be a glucosamine residue, unit III must be a glucose residue.

The H-1 resonance of the β unit I is correlated in the 2D COSY spectrum with H-2, and this correlation is extended in the 2D TOCSY spectrum to the nearly degenerate H-3, H-4, and H-5 resonances, which are difficult to find in COSY due to multiple overlap. The H-6a,6-b signals were assigned in a 1D TOCSY spectrum taken with a longer mixing time (Fig. 2i). Similarly as with unit II, the strong low-field shift of the I H-2 resonance, compared to that of III H-2, shows that unit I contains the second of the NAc groups seen in Fig. 1a, that is, it is a glucosamine residue (see Table 1).

The assignments for the amino sugar residues I and II are unequivocally confirmed


a
VI1
HDO
VI6'
VI5
VI6
VI2
VI3
VI4
b
V1
HDO
V4
V3
V2
c
V1
HDO
V5
V4
V6'
V6
d
IV1
HDO
IV6
IV3
IV5,6
IV2
IV4
e
III1
HDO
III3,4,5
III2
f
III1
HDO
III6'
III6
III3,4,5
III2
g
III6'
III6
Glyc1'
Glyc1
h
II1
HDO
II2
II4
II3
i
I1
HDO
I6'
I2
I6
I3,4
I5

by the basic 1D and correlated 2D COSY spectra of the disaccharide isolated after Smith degradation of the polysaccharide (see Fig. 1b for the 1D spectrum, and Table 1).

Along with the proton signals of the six hexopyranose residues, the TOCSY spectrum exhibited signals of the spin system of a glycerol residue (Table 2).

The sequences and linkage sites have been established with the aid of ROESY and $^{1}H/^{31}P$ correlation spectroscopy. The ROEs for the corresponding transglycosidic anomeric/aglyconic proton pairs were found for the VI(1 → 6)V(1 → 3)II, III(1 → 4)II, II(1 → 3)I, and IV(1 → 6)I sequences. GlcNAc-I H-1 showed a correlation with the H-3a,3b protons of glycerol. The final details of sequence follow from the $\{^{31}P\}^{1}H$-heterocorrelated spectrum (Fig. 2g). The ^{31}P resonance at 1.63 ppm correlates with two pairs of methylene proton resonances: one at 3.83 and 3.89 ppm, which corresponds to H-1a and H-1b of glycerol, and the other at 4.01 and 4.09 ppm, attributed to H-6a and H-6b of β-glucose residue III.

Although the available amount of polysaccharide was not sufficient for a $^{1}H/^{13}C$-correlated spectrum to be recorded, a ^{13}C DEPT spectrum confirmed several of the structural features established by the above analysis. Most informative are the sugar and glycerol methylene carbon signals, which are easily recognizable owing to their negative phase. They are assigned as follows: terminal Glc-IV and Glc-VI at 61.36 and 61.42 ppm (cf. [22]); PO_4 → 6Glc-III at 65.06 ppm (cf. PO_4 → 6GalNAc at 65.31 ppm [23], PO_4 → 6GlcNAc at 65.02 ppm [24], and PO_4 → 6Gal at 66.27 ppm [25]); PO_4 → 1Gro at 66.24 ppm (cf. 68.68 ppm in [25]); Gal-V at 66.85 ppm (cf. [22]); Glc NAc-I at 67.14 ppm (cf. 68.92 in [26]); and GlcNAc-I → 3Gro at 72.05 ppm (cf. GalNAc → Gro at 70.44 in [24]). The two acetamido groups are confirmed by the NAc-substituted ring-carbon signals at 49.86 and 56.36 ppm, and the methyl signals at 22.92 and 23.15 ppm. The FucNAc-II 6-methyl signal occurs at 16.16 ppm (cf. 16.17 ppm for Fuc3NAcyl in [26]).

The O-specific polysaccharide described in this paper shows a teichoic-acid character, like the polysaccharides of *H. alvei* strains 1205 and 1191 [8,9]. All of them contain alditol phosphate.

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Fig. 2. (a–f,i) 500-MHz TOCSY-HOHAHA spectra of the subunit sugar residues from the PS of *H. alvei* strain 1220 obtained with semiselective excitation of the corresponding anomeric proton resonances (a, b, d, e, and i) or the H-5 signals of residues V (c) and III (f). (h) 2D TOCSY cross-section (row) at w_1 corresponding to the anomeric proton chemical shift of residue II. (g) Cross-section of the $^{1}H,^{31}P$-correlated spectrum at w_1 corresponding to the PO_4^- signal. For the formula and labelling, see Table 2.

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