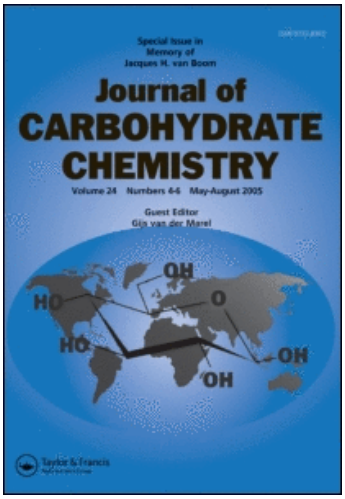


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# STRUCTURE OF THE O-SPECIFIC POLYSACCHARIDE OF *HAFNIA ALVEI* 23 HAVING AN OLIGOSACCHARIDE-PHOSPHATE REPEATING UNIT[1]

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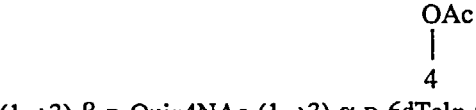
## ABSTRACT

Lipopolysaccharide (LPS) of *Hafnia alvei* 23 has an acid-labile O-specific polysaccharide (OPS) with a pentasaccharide-phosphate repeating unit containing D-Glc1*P*, D-GlcNAc, L-Fuc, 6-deoxy-D-talose (D-6dTal), 4-acetamido-4,6-dideoxy-D-glucose (D-Qui4NAc), and an *O*-acetyl group. A partially degraded OPS was obtained by hydrolysis of LPS with 0.25 M sodium acetate in aqueous 0.5% acetic acid. Fractionation of LPS on Sephadex G-200 in DOC buffer allowed isolation of long-chain LPS species which, together with OPS, were studied by methylation analysis, chemical degradations (*O*-deacetylation, dephosphorylation with 48% hydrofluoric acid, Smith degradation), and $^{1}H$ and $^{13}C$ NMR spectroscopy, including 2D COSY, TOCSY, NOESY, and H-detected $^{1}H,^{13}C$ heteronuclear single-quantum coherence (HSQC) experiments. The following structure of the repeating unit of OPS was established:




3)-α-D-Glc*p*NAc-(1→3)-β-D-Qui*p*4NAc-(1→3)-α-D-6dTal*p*-(1→3)-α-L-Fuc*p*-(1→6)-α-D-Glc*p*-1-*P*-(O→

## INTRODUCTION

*Hafnia alvei* is an enterobacterial opportunistic pathogen which is found in many incidents of nosocomial infections, including wounds and enteric, urinary, and respiratory tract disorders.[2-4] Based on the lipopolysaccharide (LPS, O-antigen) specificity, *H. alvei* strains could be divided into 39 O-serotypes.[5] To create the chemical basis for the classification, structures of more than 20 different O-specific polysaccharides (OPSs) of *H. alvei* LPSs have been elucidated (refs. 6-10 and refs. cited therein). OPSs are typically acidic, hexuronic acids being common components. Some OPSs include unusual neutral and acidic O-antigen components, such as 3-amino-3,6-dideoxyhexoses, 4-amino-4,6-dideoxyhexoses, sialic acid, D-allothreonine, phosphates (glycerol, arabinitol, ethanolamine, or glycosyl phosphate), 3-hydroxybutyryl and formyl groups.

Now we report on the structure of OPS of *H. alvei* strain 23, a new O-antigen containing an oligosaccharide-phosphate repeating unit.



## RESULTS AND DISCUSSION

LPS was isolated from dry bacterial cells by the phenol-water procedure.[11] In SDS-PAGE (Fig. 1b) LPS displayed a ladder-like migrating pattern typical of S-form LPS with OPS with variable chain length. R-form LPS containing no OPS, but core and lipid A moieties, was present as well. LPS was fractionated by gel-permeation chromatography on Sephadex G-200 in DOC buffer (Fig. 1a). As a result, LPS species with long-chain OPS (fraction I), short-chain OPS (fractions II and III) and no OPS (fraction IV) were obtained (Fig. 1b).

Degradation of LPS with aqueous 1% acetic acid (100 °C, 1 h) resulted in complete depolymerisation of OPS. Therefore, milder degradation conditions were used, namely, 0.1 M sodium acetate buffer pH 4.2 or 0.25 M sodium acetate in aqueous 0.5% acetic acid (100 °C, 3-4 h). $^{13}C$ NMR spectroscopic analysis (see below) showed that the least destruction of OPS occurred with the latter reagent. The degraded material was fractionated on Sephadex G-50 to give OPS, three oligosaccharide fractions, and low-molecular-mass products (3-deoxyoctulosonic acid and salts).

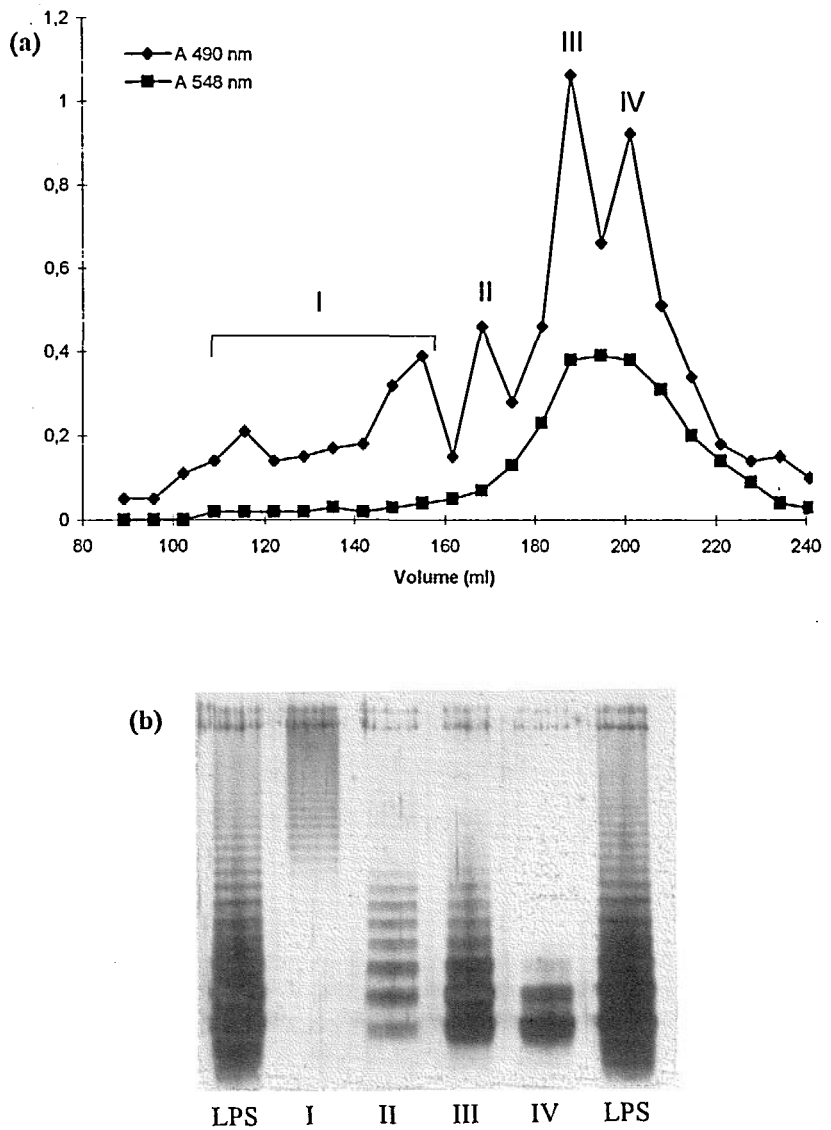



**Figure 1.** Fractionation of LPS of *H. alvei* 23. **(a)** Gel-permeation chromatography on Sephadex G-200 in sodium deoxycholate buffer. Fractions were analysed for 3-deoxyoctulosonic acid (-■-) and, after dialysis, for total sugar (-♦-); **(b)** SDS-PAGE of non-fractionated LPS and LPS fractions I-IV from Sephadex G-200. 1-2 μg of each LPS was applied. Gel was silver stained.

Chemical analyses showed that OPS and two higher-molecular mass oligosaccharide fractions had the same composition and, hence, differed in the chain length only. Fraction I LPS had the same composition as well, and, therefore, no acid-labile component was split off during degradation of LPS.

Sugar analysis using GLC-MS of alditol acetates obtained after hydrolysis of OPS with 2 M $CF_3CO_2H$ (120 °C, 2 h) revealed glucose, fucose and 6-deoxytalose (6dTal) as the main components, together with smaller amounts of 4-amino-4,6-dideoxyglucose (Qui4N) and GlcN (Table 1). When hydrolysis was performed with 10 M HCl (80 °C, 30 min), the content of Qui4N increased, but that of 6dTal dropped sharply, thus indicating lower stability of 6dTal compared to Fuc (Table 1).

The D configuration of Glc and GlcN was established enzymatically using D-glucose oxidase and hexokinase, respectively. Qui4N was identified by comparison with the authentic sample from the O-specific polysaccharide of *H. alvei* 1205[12] using GLC-MS and paper chromatography. The D configuration of Qui4N and the L configuration of Fuc were established using $^{13}C$ NMR chemical shift data of OPS (see below).



6dTal was isolated from the hydrolysate by preparative paper chromatography and identified using GLC-MS and authentic samples from the O-antigen of *Yokonella* 2476[13] and 6-deoxy-D-talan from *Burkholderia plantarii,*[14] and by comparison of the $^1H$ NMR data with published data.[15] The D configuration of 6dTal was determined by the specific optical rotation value.

Using colorimetric methods, in addition to the monosaccharides (glucose, 19%, and 6-deoxyhexoses, 36.5%), phosphate (0.84 μmol/mg) and *O*-acetyl groups (0.88 μmol/mg) were detected. Thus, the lability of OPS towards mild acid hydrolysis may be accounted for by the presence of a glycosyl-phosphate group in the main chain of the polysaccharide, as has been recently reported for some other O-antigens.[8,10,16]

The $^{13}C$ NMR spectrum of OPS contained two series of signals (Fig. 2). The major series belonged to a polysaccharide with a pentasaccharide repeating unit (there were five signals for anomeric carbons at δ 96.6, 98.5, 100.3, 102.8, and 104.2), which includes three 6-deoxy sugars (Fuc, 6dTal, and Qui4NAc, the signals for $CH_3$-C groups at δ 16.4, 16.7, and 17.6), two *N*-acetylated amino sugars [GlcNAc and Qui4NAc, the signals for carbons bearing nitrogen at δ 54.2 and 57.6, and two *N*-acetyl groups at δ 23.3 and 23.6 ($CH_3$)], and one *O*-acetyl group (the signal for $CH_3$ at δ 21.7).

**Table 1.** Sugar Analysis Data[a]

| Sugar | $t_R$ | GLC detector response | | | |
|---|---|---|---|---|---|
| | | OPS | | OS | |
| | | $CF_3CO_2H$ | HCl | $CF_3CO_2H$ | HCl |
| 6dTal | 0.68 | 0.55 | 0.06 | 0.52 | 0.03 |
| Fuc | 0.69 | 0.67 | 0.61 | 0.69 | 0.62 |
| Qui4N | 0.90 | 0.24 | 0.80 | 0.33 | 0.86 |
| Glc | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| GlcN | 1.17 | 0.10 | 0.15 | 0.74 | 1.07 |

a. $t_R$, retention time in GLC for the corresponding alditol acetate related to hexa-*O*-acetylglucitol. Hydrolysis of OPS and OS was performed with 2 M $CF_3CO_2H$ (120 °C, 2 h) or 10 M HCl (80 °C, 30 min).



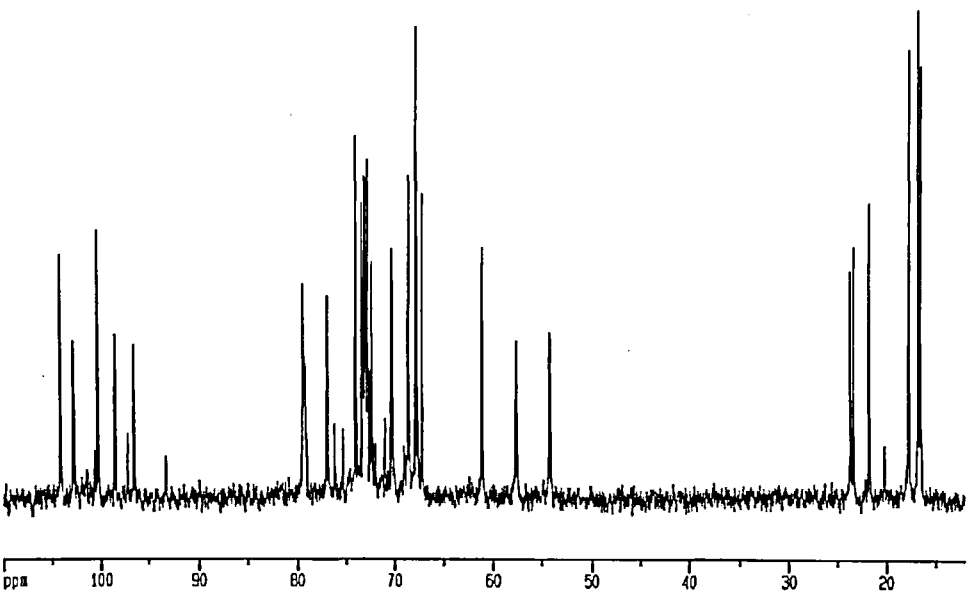



**Figure 2.** $^{13}C$ NMR spectrum (125 MHz) of the O-specific polysaccharide of *Hafnia alvei* 23

The minor series of signals in the $^{13}C$ NMR spectrum of OPS included, *inter alia*, signals for C-1 of a reducing sugar residue at δ 93.3 and 97.2 (α- and β-forms, respectively). Hence, this series belonged to a terminal oligosaccharide repeating unit resulting from the selective cleavage of OPS during mild acid degradation. Based on the ratio of the intensities of the signals in the minor and major series, the content of the terminal repeating unit was estimated on the average as about one fourth of the total repeating units.

Accordingly, the $^{1}H$ NMR spectrum of OPS contained, *inter alia*, six major signals in a low-field region at δ 4.59-5.53 (five anomeric protons and a proton at a carbon carrying an acetoxy group) and two minor signals at δ 5.23 and 4.65 (for H-1 of α- and β-forms of the reducing monosaccharide, respectively). In a high-field region, there were signals for $CH_3$-C groups of Fuc, 6dTal, and Qui4NAc at δ 1.13, 1.14, and 1.21, two *N*-acetyl groups of GlcNAc and Qui4NAc at δ 1.96 and 2.06, and one *O*-acetyl group at δ 2.20.

In accordance with these data, the $^{31}P$ NMR spectrum of OPS contained two signals at δ -0.57 and 5.32 in the ratio ~3:1, which could be assigned to a phosphodiester group in the non-degraded OPS repeating units and a phosphomonoester group in the terminal non-reducing oligosaccharide unit resulting from the cleavage of the glycosyl-phosphate linkage, respectively.

Therefore, OPS has a pentasaccharide repeating unit containing one residue each of D-Glc, L-Fuc, D-6dTal, D-GlcNAc and D-Qui4NAc, as well as one phosphate group and one *O*-acetyl group. A lower-than-expected release of GlcN by acid hydrolysis in sugar analysis of OPS may be accounted for by its phosphorylation. When dephosphorylation of OPS with 48% HF was performed prior to hydrolysis, the content of GlcN in the hydrolysate of the resultant oligosaccharide (OS) was comparable to those of other monosaccharides (Table 1). Depolymerisation of OPS during dephosphorylation confirmed the suggestion that the phosphate group is located in the main chain.

Methylation analysis of OPS (Table 2) revealed partially methylated monosaccharides derived from 6-substituted Glc and 3-substituted Fuc, 6dTal, and Qui4NAc. In addition, a methylated derivative from terminal GlcNAc was detected, which evidently resulted from dephosphorylation of this residue during methylation. As

**Table 2.** Methylation Analysis Data[a]

| Methylated sugar | $t_R$ | GLC detector response | | | |
|---|---|---|---|---|---|
| | | OPS | | OS | |
| | | $CF_3CO_2H$ | HCl | $CF_3CO_2H$ | HCl |
| 2,4-$Me_2$6dTal | 0.86 | 0.85 | <0.05 | 1.35 | <0.05 |
| 2,4-$Me_2$Fuc | 0.96 | 0.90 | 1.48 | 0.92 | 0.37 |
| 2,3,4-$Me_3$Glc | 1.29 | 1.00 | 1.00 | 1.08 | 0.50 |
| 2-MeQui4NMeAc | 1.62 | 0.19 | 1.47 | 0.52 | 0.72 |
| 3,4,6-$Me_3$GlcNMeAc | 1.67 | 0.18 | 0.67 | 1.00 | 1.00 |

a. $t_R$, retention time in GLC for the corresponding alditol acetate related to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol (2,3,4,6-$Me_4$Glc). Hydrolysis of methylated OPS and OS was performed with 2 M $CF_3CO_2H$ (120 °C, 2 h) or 10 M HCl (80 °C, 30 min).



in sugar analysis, hydrolysis of the methylated OPS with 10 M HCl (80 °C, 30 min), instead of 2 M $CF_3CO_2H$ (120 °C, 2 h), gave better yields of methylated amino sugars but destroyed methylated 6dTal.

Methylation analysis of OS revealed a stoichiometric amount of terminal GlcNAc (Table 2). When OS was borodeuteride-reduced prior to methylation, 1,2,3,4,5-penta-*O*-methylglucitol-1-d was detected instead of 2,3,4-tri-*O*-methylglucose. Hence, the reducing end of OS is occupied by Glc which was derived from a glucosyl-phosphate group.

Smith degradation of OS resulted in destruction of GlcNAc and Glc and appearance of terminal Qui4NAc (methylation analysis data). A repeated Smith degradation destroyed Qui4NAc to give an oligosaccharide with 6dTal at the non-reducing end. In OPS, GlcNAc was stable towards periodate oxidation and, thus, is phosphorylated at position 3 or 4.

These data suggested that OPS is linear and that the repeating units are connected by a glycosyl-phosphate linkage between the residues of Glc and GlcNAc. They also revealed the glycosylation pattern and the sequence of the monosaccharides in the repeating unit of OPS.

The major series in the $^{1}H$ and $^{13}C$ NMR spectra of OPS were completely assigned using 2D COSY, TOCSY, and H-detected $^{1}H,^{13}C$ HSQC experiments (Tables 3 and 4).

Relatively small $J_{1,2}$ coupling constant values (<4 Hz) for the H-1 signals demonstrated that Fuc, and GlcNAc residues are α-linked, whereas a relatively large $J_{1,2}$ value of 7.5 Hz showed that Qui4NAc is β-linked. The H-1 signal for Glc was broadened and not well resolved due to coupling to phosphorus. The α configuration of Glc and 6dTal followed from a relatively low-field position of the signals for H-1 at δ 5.53 and 5.17, respectively. These two and three other anomeric configurations were confirmed by a NOESY experiment which revealed interresidue H-1,H-2 correlations for the four α-linked sugar residues and H-1,H-3 and H-1,H-5 correlations for β-Qui4NAc.

The signal for H-3 of GlcNAc was shifted downfield (to δ 4.36, as compared with its position in the spectrum of nonsubstituted α-GlcNAc at δ 3.75[17]) and split additionally ($J$ ~9 Hz) due to coupling to phosphorus. Therefore, GlcNAc is phosphorylated at position 3. The phosphorylation pattern of OPS was confirmed by an H-detected $^{1}H,^{31}P$ HMQC experiment which showed two cross-peaks for the major phosphate group with Glc H-1 and GlcNAc H-3 at δ -0.57/5.53 and -0.57/4.36, respectively.

A downfield displacement to δ 5.35 of the signal for H-4 of 6dTal, as compared with its position at δ 3.76 in nonsubstituted α-6dTal,[15] resulted from a deshielding effect of the *O*-acetyl group and, thus, showed that the *O*-acetyl group is located at position 4 of 6dTal.

$^{13}C$ chemical shift data (Table 4) provided support for the substitution pattern of OPS. Thus, the signals for C-3 of Fuc, 6dTal and Qui4NAc, as well as for C-6 of Glc were markedly shifted downfield to δ 79.4, 74.0, 79.3, and 67.8 due to α-effects of glycosylation (compare reported chemical shifts[15,17,18] of the corresponding signals in the nonsubstituted monosaccharides at δ 70.30, 66.3, 74.8, and 61.84, respectively). In accordance with phosphorylation at position 3, the signal for C-3 of GlcNAc shifted downfield from δ 71.74[17] to 76.8.

**Table 3.** $^{1}H$ NMR Chemical Shifts[a] of the O-specific Polysaccharide of *H. alvei* 23

| Proton | Residue | | | | |
|---|---|---|---|---|---|
| | αGlcNAc | βQui4NAc | α6dTal4Ac | αFuc | Glc1*P* |
| H-1 | 5.10 | 4.59 | 5.15 | 4.94 | 5.53 |
| H-2 | 3.98 | 3.46 | 4.15 | 3.90 | 3.62 |
| H-3 | 4.36 | 3.68 | 4.33 | 3.99 | 3.76 |
| H-4 | 3.72 | 3.72 | 5.35 | 3.87 | 3.64 |
| H-5 | 4.16 | 3.50 | 4.30 | 4.14 | 3.98 |
| H-6(a) | 3.84 | 1.14 | 1.13 | 1.21 | 3.94 |
| H-6b | 3.78 | | | | 3.84 |
| Ac | 2.06 | 1.96 | 2.20 | | |

a. In ppm (using internal acetone at δ 2.225) in $D_2O$ at 47 °C.

**Table 4.** $^{13}C$ NMR Chemical Shifts[a] of the O-specific Polysaccharide of *H. alvei* 23

| Carbon | Residue | | | | |
|---|---|---|---|---|---|
| | αGlcNAc | βQui4NAc | α6dTal4Ac | αFucα | Glc1*P* |
| C-1 | 98.5 | 102.8 | 104.2 | 100.3 | 96.6 |
| C-2 | 54.2 | 73.3 | 67.7 | 68.5 | 72.7 |
| C-3 | 76.8 | 79.3 | 74.0 | 79.4 | 74.0 |
| C-4 | 70.2 | 57.6 | 73.1 | 73.0 | 70.2 |
| C-5 | 72.7 | 72.3 | 67.2 | 67.8 | 73.1 |
| C-6 | 61.0 | 17.6 | 16.7 | 16.4 | 67.8 |
| Ac | | | | | |
| $CH_3$ | 23.6 | 23.3 | 21.7 | | |
| CO | 175.4 | 175.0 | 175.0 | | |

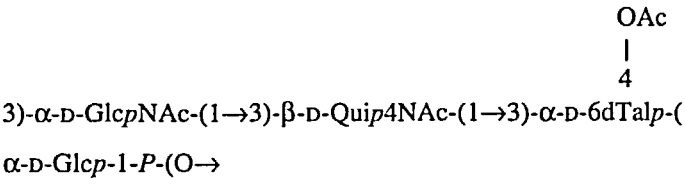
a. In ppm (using internal acetone at δ 31.45) in $D_2O$ at 47 °C.

Analysis of β-effects of glycosylation in the $^{13}C$ NMR spectrum of OPS allowed determination of the absolute configurations of Fuc and Qui4NAc. Thus, the β-effect on C-4 of Fuc was insignificant (+0.2 ppm) that was indicative of different absolute configurations of the constituent monosaccharides in the disaccharide fragment α-D-6dTal*p*-(1→3)-α-Fuc*p*, i.e. of the L configuration of Fuc (an effect of about -4 ppm would be expected for D-Fuc).[19] The β-effect on C-2 of 6dTal was relatively large by the absolute value (-3.5 ppm) and, hence, the glycosylating sugar Qui4NAc in the disaccharide fragment β-Qui*p*4NAc-(1→3)-α-D-6dTal*p* has the same D configuration as the glycosylated sugar 6dTal (an effect less than 1 ppm would be expected in the case of different absolute configurations of the monosaccharides).[20]

The NOESY spectrum of OPS revealed the following interresidue correlations between transglycosidic protons: GlcNAc H-1,Qui4NAc H-3 at δ 5.10/3.68, Qui4NAc H-1,6dTal H-3 at δ 4.59/4.33, 6dTal H-1,Fuc H-3 at δ 5.15/3.99, and Fuc H-1,Glc H-6a,6b at δ 4.94/3.94 and 4.94/3.84. In addition to the cross-peak with the transglycosidic proton, Qui4NAc H-1 gave a cross-peak with 6dTal H-2. According to published data for 3-substituted Man,[21] such correlation is typical of the same absolute configuration of the β1→3-linked monosaccharides and, thus, confirmed again the D configuration of Qui4NAc. As expected, no interresidue cross-peak was observed for Glc which is attached to a phosphate group. The NOESY data were consistent with the linkage and sequence patterns of OPS demonstrated by chemical methods.



On the basis of the data obtained, it was concluded that the O-specific polysaccharide of *H. alvei* 23 has a pentasaccharide-phosphate repeating unit with the following structure:




Although polysaccharides of this structural type have been previously reported in lipopolysaccharides of *Hafnia*[8,10] and *Proteus*,[15] they are rather uncommon for gram-

negative bacteria. Interestingly, unlike in other enterobacteria, most O-specific polysaccharides in the two genera mentioned above are acidic and many of them are phosphorylated, including teichoic acid-like polysaccharides.[22] Another peculiar feature of the polysaccharide studied is the presence of L-fucose and 6-deoxy-D-talose, which are new components of *H. alvei* lipopolysaccharides.

## EXPERIMENTAL

**Bacterial Strain, Preparation of LPS and OPS.** *Hafnia alvei* strain 23 was from the collection of the Institute of Immunology and Experimental Therapy (Wroclaw, Poland). Bacteria were cultivated in liquid medium with aeration at 37 °C for 24 h, then harvested and freeze-dried.[23]



LPS was isolated from dried bacterial mass by phenol-water extraction[11] and purified on Sepharose 2B as described earlier.[24] The yield of LPS was 2.5% of dry bacterial mass. Fractionation of LPS was performed according to Rivera et al.[25] on a column (2.2 × 64 cm) of Sephadex G-200 equilibrated with 0.01 M Tris-HCl buffer pH 8.0, containing 0.2 M NaCl, 0.001 M EDTA, 0.02% $NaN_3$ and 0.25% sodium deoxycholate. Elution was monitored by the phenol-sulfuric acid reaction. Four fractions were obtained and analysed by SDS-PAGE as described earlier[26,27] and for 2-keto-3-deoxyoctonic acid.[28]

OPS was obtained by mild acid degradation of LPS with 0.1 M sodium acetate buffer pH 4.2 or 0.25 M NaOAc in aqueous 0.5% HOAc at 100 °C for 3-4 h followed by gel-permeation chromatography of the carbohydrate portion on a column (2 × 100 cm) of Sephadex G-50 in 0.05 M pyridinium acetate buffer pH 4.5.

**Chemical Analyses.** For sugar analysis, hydrolysis was performed with 2 M $CF_3CO_2H$ for 2 h at 120 °C or 10 M HCl for 30 min at 80 °C and followed by evaporation in vacuum, reduction with $NaBH_4$, acetylation with $Ac_2O$ in pyridine, and analysis of the resulting alditol acetates by GLC-MS using a Hewlett-Packard 5971A system with an HP-1 glass capillary column (0.2 mm × 12 m) and temperature program of 8°/min from 150 to 270 °C.

Determination of *O*-acetyl groups, phosphate, and absolute configurations of sugars using enzymatic methods was performed essentially as described earlier.[29] For

hexokinase reaction, OS was hydrolysed with 4 M HCl (100 °C, 18 h) and acid was removed in vacuum over solid KOH.

6-Deoxy-D-talose (2.2 mg) was isolated by preparative paper chromatography on Whatman 1 paper in a system of butanol/pyridine/water (6:4:3, v/v) after hydrolysis of OPS (20 mg) with 2 M $CF_3CO_2H$ (2 mL, 120 °C, 2 h). It was identified by the specific optical rotation value, $[\alpha]_D^{20}$ +19.1 ° (*c* 0.1, water), measured using a Jasco DIP 300 polarimeter; compare published data,[30] $[\alpha]_D$ +21.0° (water).

Methylation was performed according to the Gunnarson procedure,[31] methylated products were purified by extraction with chloroform/water (1:1, v/v), then hydrolysed with 2 M $CF_3CO_2H$ (120 °C, 2 h) or 10 M HCl ( 80 °C, 30 min), reduced with $NaBD_4$, acetylated, and analysed as above.

**Dephosphorylation and Smith Degradation.** Fraction I LPS or OPS was dephosphorylated with aqueous 48% HF for 48 h at 4 °C, HF was removed in the stream of nitrogen, and OS was isolated by gel-permeation chromatography on a column (1.6 × 100 cm) of Bio-Gel P4 in a yield of 62% of the OPS weight.

OS (8 mg) was oxidised with 0.1 M $NaIO_4$ (0.8 mL, 4 °C, 48 h, in dark), treated with ethylene glycol (0.03 mL), and reduced with $NaBH_4$ (30 mg). After acidification with aqueous 50% HOAc and desalting by gel filtration on a column (1.5 × 25 cm) of Sephadex G-10, the product (6.3 mg) was hydrolysed with aqueous 2% HOAc (0.7 mL, 100 °C, 2 h), a portion was methylated, and the rest (4.3 mg) was repeatedly degraded as above.

**NMR Spectroscopy.** $^1H$ and $^{13}C$ NMR spectra were recorded with a Bruker DRX-500 spectrometer for solutions in $D_2O$ at 47 °C. Chemical shifts are reported with internal acetone ($\delta_H$ 2.225, $\delta_C$ 31.45) as reference. A $^{31}P$ NMR spectrum was recorded at 30 °C using external aqueous 85% phosphoric acid ($\delta_P$ 0) as reference. Two-dimensional experiments were performed using standard Bruker software. Mixing times of 100 and 250 ms were used in TOCSY and NOESY experiments, respectively.

## ACKNOWLEDGMENTS

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