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Structural heterogeneity of the sialic-acid-containing oligosaccharides from the lipopolysaccharide of *Hafnia alvei* strain 2 as detected by FABMS studies☆

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Abstract

The structure of four oligosaccharide fractions from the *Hafnia alvei* strain 2 lipopolysaccharide (LPS) have been assigned by FABMS. This approach corroborates data previously established by NMR spectroscopy for the major oligosaccharides in these fractions [A. Gamian, E. Romanowska, U. Dabrowski, J. Dabrowski, *Biochemistry* 30 (1991) 5032–5038; E. Katzenellenbogen, A. Gamian, E. Romanowska, U. Dabrowski, J. Dabrowski, *Biochem. Biophys. Res. Commun.* 194 (1993) 1058–1064; N. Ravenscroft, A. Gamian, E. Romanowska, *Eur. J. Biochem.* 227 (1995) 889–896]. In addition, the MS/MS with B/E linked scan technique allowed the detection of an additional oligosaccharide with the structure:

α-D-Glc*p*
1
↓
6
α-D-Glc*p*-(1→4)-β-D-Gal*p*-(1→6)-β-D-Glc*p*-(1→3)-β-D-Gal*p*-(1→3)-β-D-Gal*p*NAc-(1→4)-Neu5Ac

lacking the branched O-6 linked glucopyranose residue at the 3-linked Gal unit, which indicates a structural heterogeneity for the major oligosaccharide fraction.

Keywords: Lipopolysaccharide; Core oligosaccharide; FABMS; Sialic acid; *Hafnia alvei*

1. Introduction

The polysaccharide moiety of bacterial endotoxic lipopolysaccharide (LPS) is built up of a core oligosaccharide and oligosaccharide O-specific repeating units which, in some bacterial strains, contain sialic acid [1]. Usually, the

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occurrence of sialic acid is associated with an increased pathogenicity of bacteria. In the LPS of *Hafnia alvei* strain 2 (PCM 2386), where an O-specific polysaccharide containing sialic acid was found, the repeating unit is a branched octasaccharide [2]. In the native LPS, this repeating unit is terminated at the nonreducing end by sialic acid [3]. A core oligosaccharide and a trisaccharide fragment, where 3-deoxy-octulosonic acid (Kdo) is disubstituted with a Gal residue and a heptose residue, were then described in this LPS [4,5]. In the same LPS, a unique hexasaccharide [6] was further found, which has Kdo at the reducing end. The structure of this oligosaccharide bears a similarity to the fragment of the O-specific repeating unit which is attached to Kdo. The structure of the oligosaccharides was previously deduced in part from NMR experiments. In order to firmly assess the structural parameters for such complex oligosaccharide structure, it is worth combining NMR and mass spectroscopy methodologies. In the case of *Escherichia coli* O24 and O56 O-specific polysaccharides, both MS [7] and NMR [8] approaches proved essential in the correct structure assignment. In the present report, four major oligosaccharide fractions isolated from the *H. alvei* strain 2 LPS were subjected to FABMS investigation. This complementary approach allowed detection of additional oligosaccharides which could not be seen by NMR spectroscopy, and reflect a structural heterogeneity of the oligosaccharide components. The possible location of the oligosaccharides in such a complex LPS is discussed.

2. Experimental

Bacterial strain, isolation of the lipopolysaccharide and oligosaccharides.—*H. alvei* strain 2 (PCM 2386) was obtained from the collection of the Institute of Immunology and Experimental Therapy, Wrocław, Poland. Isolation of the LPS with phenol–water and purification on a Sepharose 2B column were performed as described [2,9]. The LPS (200 mg) was treated with 1% AcOH (20 mL) for 25 min at 100 °C and the carbohydrate containing supernatant was fractionated into six oligosaccharide fractions as shown before [2]. Fractions 2-S1, 2-S2, 3 and 4 were the subject of the present investigation.

HPLC purification.—Gel permeation chromatography of oligosaccharides was performed on a Waters apparatus equipped with an M600E pump system and a M996 photodiode array detector, both controlled by a Millennium 2010 software. A Knauer differential refractometric detector of type 188 was also used. A TSK G2500 PWXL column (7.5 × 250 mm, Supelco) was run in the isocratic mode with 1% AcOH in water at 1 mL/min.

Oligosaccharide derivatization.—Peracetylation of the oligosaccharides was performed under acidic conditions as described [10], with 2:1 trifluoroacetic anhydride–AcOH. For deuteroacetylation, AcOH was replaced with ^{2}H-labelled glacial AcOH. Peracetylation was also carried out with 1:1 pyridine–Ac_2O or ^{2}H-labelled Ac_2O for 24 h at room temperature. After evaporation of the reagents, the procedure was repeated. Methylation was carried out according to Hakomori [11] and the product was cleaned up using a Sep Pak C18 cartridge with elution by aq MeCN [10]. The permethylated oligosaccharide present in the 1:1 MeCN–water fraction was then evaporated with a stream of N_2. When the methylation was performed according to Ciucanu and Kerek [12], the product was extracted from the water phase with $CHCl_3$.

FABMS.—A Fisons-VG type ZAB2-SEQ double focusing mass spectrometer, working at 8 kV accelerating voltage, was used. The LSIMS ion source was equipped with a Cs ion gun, giving a beam of 2 μA/35 keV in positive and negative modes. Thioglycerol was used as the liquid matrix in positive mode, and triethanolamine in negative mode. For derivatized compounds solubilized in MeOH, *m*-nitrobenzyl alcohol was the matrix in positive mode. Aqueous 0.1 M NaI (1 μL) was added for cationization. The B/E linked scans were driven by informatic command after calibration with cesium iodide, setting the pressure of the argon gas used for the collision induced dissociation experiments (CID) so that the height of the considered peak was decreased by 30% of its original value.

3. Results

The carbohydrate material obtained from the mild acidic treatment (1% acetic acid, 100 °C, 25 min) of the LPS of *H. alvei* strain 2 was fractionated, according to the described protocol [2], into six oligosaccharide fractions 1, 1a, 2-S1, 2-S2, 3 and 4 [2,5,6]. Fraction 1 contained a dimer of the O-specific unit, whereas Fraction 1a was a fragment of the repeating unit and the core region. The current analyses were performed on an oligosaccharide Fraction 2-S1, a monomeric branched octasaccharide repeating unit containing Neu5Ac at the reducing end, Fraction 2-S2, the core oligosaccharide, and Fractions 3 and 4 which are Kdo containing hexa- and tri-saccharide, respectively [4–6]. The purity of the fractions was checked by HPLC analysis on a gel permeation column.

Fraction 2-S1.—The FAB^+MS of the cationized native material showed mainly two high mass peaks at m/z 1507 and 1345, in a ratio of about 7:3, which are the cationized quasimolecular ions for two molecules (M_1 and M_2). The FAB^-MS showed one high mass ion at m/z 1483. From these values, the molecular mass for each molecule, 1484 and 1322 respectively, was inferred.

The distinct cationized quasimolecular ions in the FAB^+MS of the permethylated Fraction 2-S1, scanned in *m*-nitrobenzyl alcohol in the presence of sodium iodide, confirmed the presence of a mixture of two main components and a minor one (Table 1). The B/E linked scans [13–16] on these ions, with collision induced dissociation with argon, gave specific spectra for each molecule. For the main molecule M_1, the spectrum in Fig. 1(a) was in agreement with the branched octasaccharide structure previously assigned by NMR spectroscopy techniques [2]. The following three types of fragmentation were in support of the structure: (i) cationized X* ions, formed by classical remote fragmentation [13] at m/z 1709: X^*_{5a}, X^*_{2b}, X^*_{3b}; m/z 1505: X^*_{4a}, and m/z 1301: X^*_{3a}; m/z 893: X^*_{2a}. These ions indicate two substitutions of a Gal unit by hexose residues and also two substitutions of a GalNAc residue by hexose residues. The lack of ions at m/z 1097 and 689 was in agreement with branching of the main chain with two glucose residues; (ii) ions C*, from remote fragmentation of the molecule cationized at the non reducing end at m/z 1524: C^*_5; m/z 1075: C^*_4; m/z 667 and 635: C^*_3 and C^*_3–MeOH, respectively; (iii) double radical elimination. These ions, formed by a high energy process, correspond to remote fragmentation at the non reducing end of the molecule, with the charge at the reducing end. Two kinds of such ions were observed, corresponding to the elimination of: (a) a methoxyl radical and vicinal C-3 or C-4 substituents, respectively ($^\bullet$OR). They are labelled K^*_i where *i* denotes the number of sugar residues in the main chain a from the reducing end, with (*) indicating cationized ions, and b for the side chains, i.e. m/z 1837: K*, $[MNa]^+ - 2MeO^\bullet$; m/z 1633: K^*_{5a}, $[MNa]^+ - (MeO^\bullet + HexO^\bullet)$; m/z 1225: K^*_{3a}, $[MNa]^+ - (MeO^\bullet + Hex \rightarrow Hex \rightarrow HexO^\bullet)$; m/z 817: K^*_{2a}, $[MNa]^+ - (MeO^\bullet + Hex \rightarrow Hex \rightarrow Hex \rightarrow (Hex\text{-}6) \rightarrow HexO^\bullet)$: and (b) a methoxyl radical and the substituent at C-5 of the hexose moiety ($^\bullet CH_2OR$), labelled L^*_i, i.e. m/z 1823: L*, $[MNa]^+ - (MeO^\bullet + MeOCH_2^\bullet)$; m/z 1619: L^*_{2b}, L^*_{3b}, $[MNa]^+ - (MeO^\bullet + HexOCH_2^\bullet)$; m/z 1415: L^*_{4a}, $[MNa]^+ - (MeO^\bullet + Hex \rightarrow HexOCH_2^\bullet)$. These L^*_i ions allow differentiation of the C-6 substitution in the glycopyranose residues. Thus, the linkage between hex-2 and hex-3 was proved as (1→6). Double elimination of C-3- or C-4-substituent, along with a vicinal methoxyl radical are not differentiated, as this is usually the case with source spectra [17], i.e., when comparing fragments m/z 1633 (C-4 elimination) and m/z 1225 (C-3 elimination).

The Y_1 ion at m/z 376 is a noncationized fragment which could be assigned to a proto-

Table 1
Molecular composition of Fraction 2-S1, as deduced from the FAB^+MS of the permethylated material

Component	m/z $[MNa]^+$	MW	Abundance (%)	No. of methyl groups
M_1	1899	1876	74	25
M_2	1695	1672	23	22
M_3	1538	1515	3	19

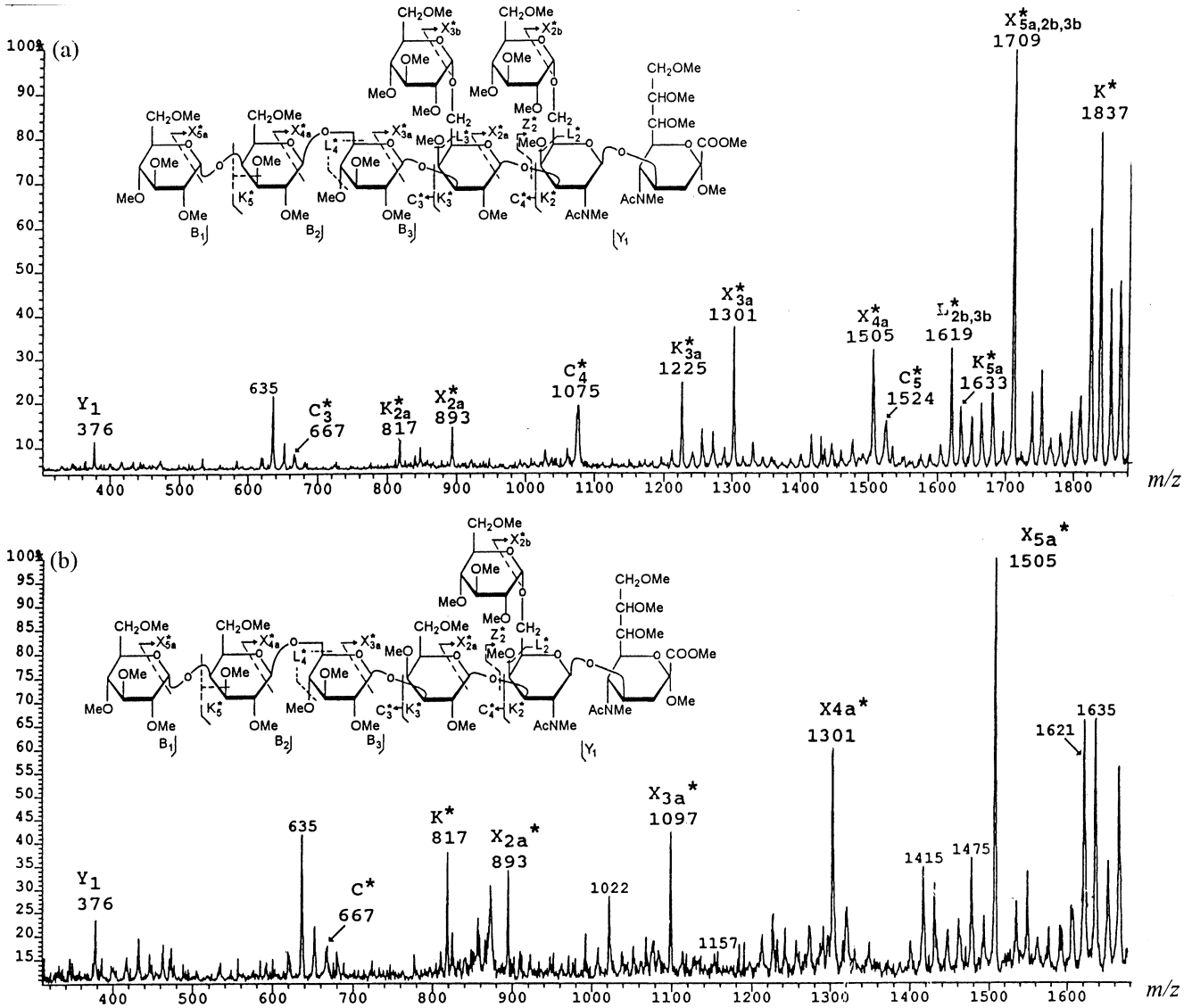


Fig. 1. CID–B/E mass spectra of $[M+Na]^+$ ions in permethylated Fraction 2-S1: (a) from ion at m/z 1899 in the main component M_1; (b) from ion at m/z 1695 in the main secondary component M_2. In all cases, * indicates cationized ions with sodium.

nated, dehydrated Neu5Ac residue, since it was not shifted when sodium was replaced by potassium as cationizing agent. An abundant ion for Y_1–MeOH at m/z 344 was furthermore seen in the source spectrum.

Analysis of the B/E CID linked scan spectrum for M_2 in Fraction 2-S1 (MW 1672; $[MNa]^+$: m/z 1695, Fig. 1(b)) showed that one lateral glucose unit was missing at the fourth hexose residue from the nonreducing end. The corresponding fragments X_i^* were found at m/z 1505: X^*_{5a}; m/z 1301: X^*_{4a}; m/z 1097: X^*_{3a} and m/z 893: X^*_{2a}.

Fraction 2-S2.—FABMS analysis of the native phosphorylated sample in positive and negative modes indicated a molecular weight of 1421 for the main component (m/z 1422 in FAB^+ and m/z 1420 in FAB^-) consistent with a composition: 2 hexoses, 3 heptoses, Kdo, phosphoric acid and a pyrophosphorylethanolamine residue. A sample, dephosphorylated by HF treatment [4], and then peracetylated, was analysed by FAB^+MS. The spectrum showed the presence of two different oligosaccharides (Fig. 2). One corresponded to the dephosphorylated hexasaccharide with two hexoses, three heptoses and one Kdo residue. Its structure was assigned from

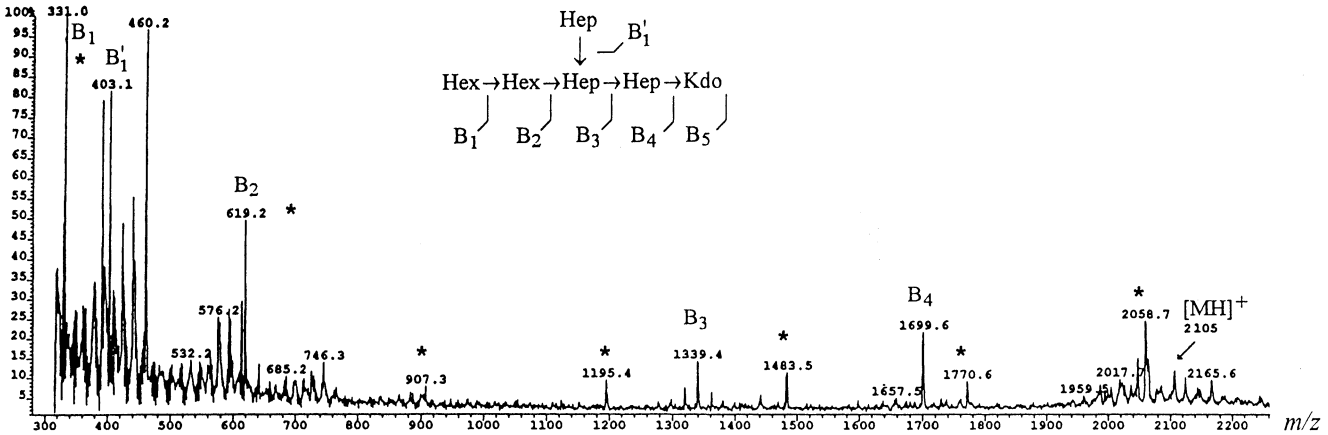


Fig. 2. Positive FABMS of peracetylated dephosphorylated Fraction 2-S2.

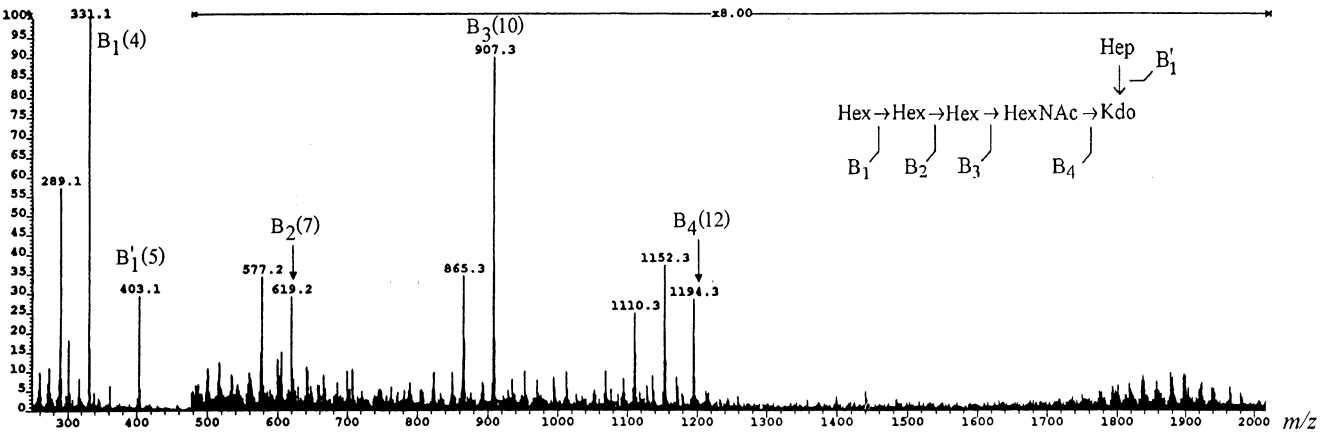


Fig. 3. Positive FABMS of peracetylated Fraction 3 with, in brackets, the number of acetyl groups.

ions at m/z 2105 $[MH]^+$, 1699 (B_4), 1339 (B_3), 619 (B_2), 331 (B_1 for hexose), 403 (B'_1 for heptose). The other corresponded to a linear heptasaccharide with 6 hexoses and one 2-acetamido-2-deoxy-hexose {m/z 2058 $[MH-AcOH]^+$, m/z 1770 (B_6), m/z 1483 (B_5), m/z 1195 (B_4), m/z 907 (B_3), m/z 619 (B_2), m/z 331 (B_1)}. This oligosaccharide was separated from the core oligosaccharide, taking into account a different solubility in chloroform after peracetylation of Fraction 2-S2. No further investigation was carried out on this compound.

Fraction 3.—The molecular weight for this Kdo-containing hexasaccharide is 1119, as deduced from the comparative FAB^+ and FAB^- spectra where $[MH]^+$ and $[MH]^-$ were found at m/z 1120 and 1118, respectively, in agreement with the composition Kdo, Hep, HexNAc, Hex_3. Similarly, as for Fraction 2-S2, the molecular ion region in the FAB^+MS of peracetylated Fraction 3 was found to be complicated and not usable. The rest of the spectrum showed, however, distinct B_i acetylated ions (Fig. 3) indicating the presence of: (i) two terminal sugar units, a hexose (m/z 331, B_1 type ion with 4 acetyl groups), and a heptose (m/z 403, B'_1 type ion with five acetyl groups); and (ii) a linear tetrasaccharide (set of B_i fragments at m/z 331, four acetyl groups, m/z 619, seven acetyl groups, m/z 907, ten acetyl groups; and m/z 1194, 12 acetyl groups). The FAB^+MS of permethylated Fraction 3 showed a major cationized pseudomolecular ion at m/z 1450 $[MNa]^+$. The B/E linked scan spectrum (Fig. 4) contained fragments of the same kind as for Fraction 2-S1: (i) m/z 1260: X^*_{4a}; m/z 1056: X^*_{3a}; m/z 852: X^*_{2a}; m/z 607: X^*_{1a}; m/z 1216: X^*_{1b}. The X^*_i ions ($i = 1-4$) indicated furthermore a structure of linear oligosaccharide linked to Kdo. A heptose substituent of Kdo is consistent with ions X^*_{1a} and X^*_{1b}; (ii) ions C*: C^*_3 at m/z 667 and C^*_2 at m/z 463, with m/z 431 (simultaneous

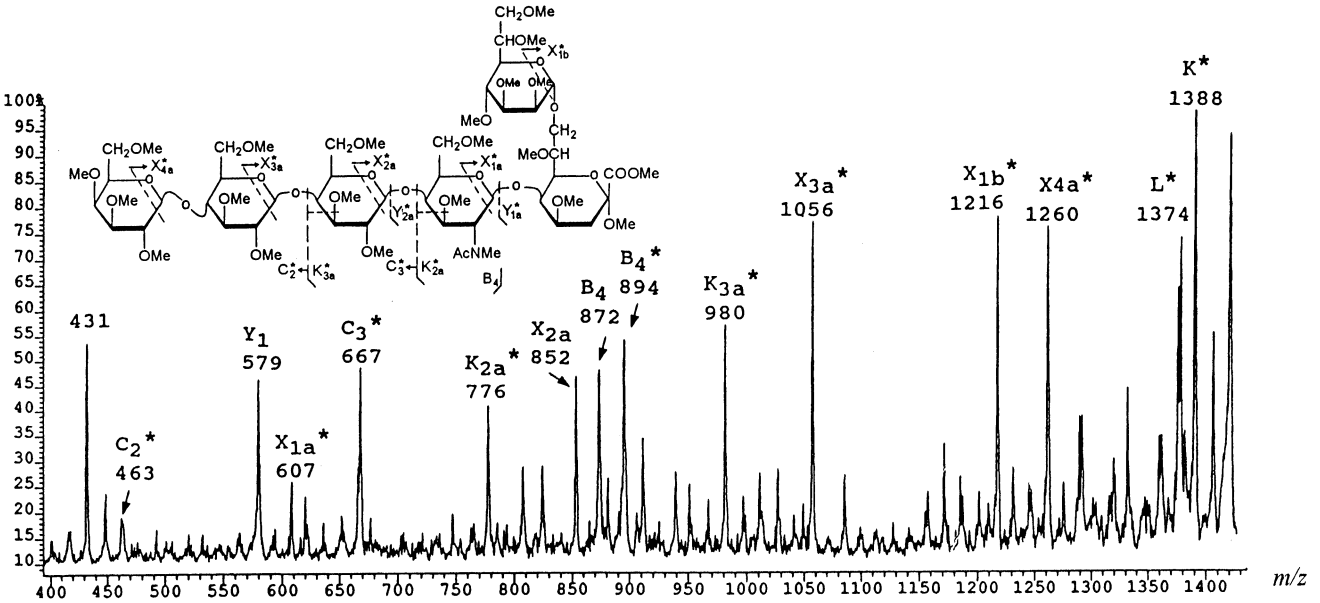


Fig. 4. CID–B/E mass spectrum of the cationized molecular ion $[M + Na]^+$ at m/z 1450 of permethylated Fraction 3.

elimination of MeOH) and; (iii) double radical eliminations with K ions at m/z 1388: K*, $[MNa]^+ - (2MeO^\bullet)$; m/z 980: K^*_{3a}, $[MNa]^+ - (MeO^\bullet + Hex \rightarrow HexO^\bullet)$; m/z 776: K^*_{2a}, $[MNa]^+ - (MeO^\bullet + Hex \rightarrow Hex \rightarrow HexO^\bullet)$; and L ions at m/z 1374: L*, $[MNa]^+ - (MeO^\bullet + MeOCH_2^\bullet)$. This fragmentation agreed with the presence of a $(1 \rightarrow 3)$ or $(1 \rightarrow 4)$ linkage between glycose residues 2,3 and 3,4, when numbered from the nonreducing end. For the linkage between residues 1 and 2, two ions of low intensity at m/z 1184 and m/z 1170 did not allow to determine whether the linkage position was $(1 \rightarrow 6)$ or $(1 \rightarrow 3$ or $1 \rightarrow 4)$, although ion at m/z 1170 was twice as high as that of m/z 1184.

The Y ions furthermore confirmed the position of the heptose constituent as a substituent of the Kdo residue with ions at m/z 824: Y^*_{2a}; and m/z 579: Y^*_{1a}. The presence of ions B^*_4 and B_4 (m/z 894 and m/z 972) appeared to be related with the collision conditions.

Fraction 4.—A molecular weight of 634 was inferred for this component from the FAB^+ and FAB^- quasimolecular ions at m/z 635 and 633, respectively, which is compatible with a trisaccharide molecule containing Kdo, a hexose, a heptose residue and one acetyl group. The FAB^+MS (Fig. 5) of peracetylated Fraction 4 showed two main fragments, B_1 (m/z 331) and B'_1 (m/z 403). Fragment B_1 was displaced at m/z 340 by perdeuteroacetylation, in agreement with the presence of one native acetyl group in an hexose moiety. Fragment B'_1, which incorporates five acetyl groups, agreed with a heptose residue linked to Kdo. The molecular region appeared to be cationized with cesium since an $[MCs]^+$ quasimolecular ion was seen at m/z 1229, together with fragments at m/z 487 (ketene loss), at m/z 1169 (acetic acid loss), and m/z 1125 (carbon dioxide loss). This indicate that the molecule is composed of Kdo substituted by a monoacetylated hexose and an heptose.

4. Discussion

The *H. alvei* strain 2 (PCM 2386) LPS comprises a long-chain polysaccharide structure representing a defined O-specific serotype [2]. This LPS does not release an O-specific polysaccharide on mild acid hydrolysis, but instead gives a set of oligosaccharides due to the presence of an intrachain sialic acid constituent. In the present results, a heterogeneity involving branched Glc residues in the O-specific unit was detected by use of the MS/MS with B/E linked scan CID technique. Fraction 2-S1 contains two molecules, in an approximate molar proportion 7:3. A sequence of sugars as previously inferred from NMR data

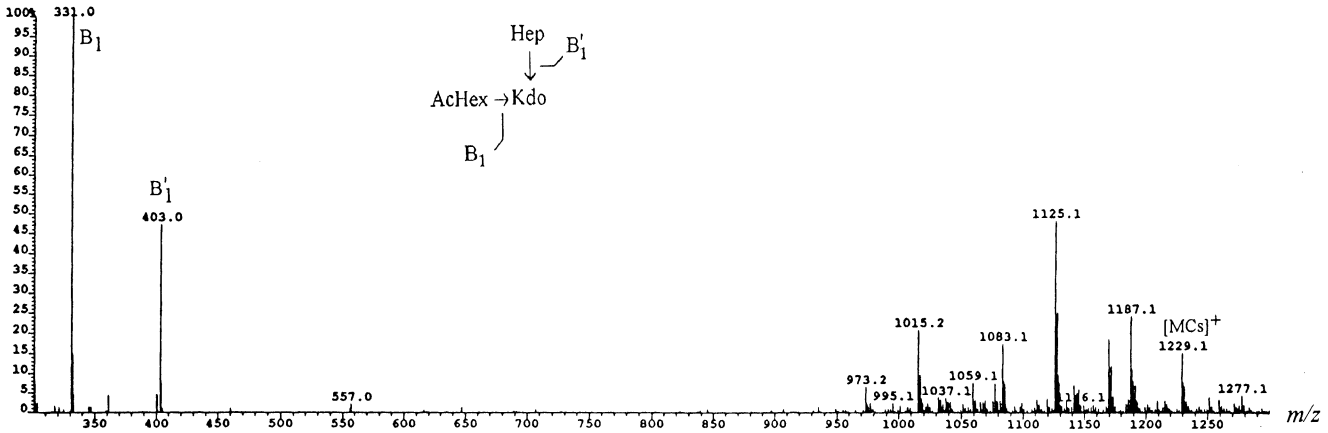


Fig. 5. Positive FABMS of peracetylated Fraction 4.

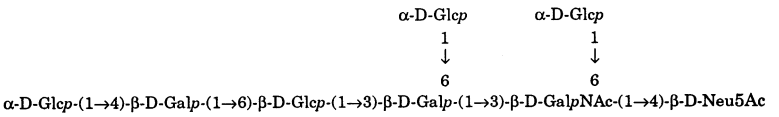
[2] for the major component, representing the O-specific repeating unit, was confirmed (Scheme 1(a)). The minor component, to which the structure in Scheme 1(b) was assigned, is probably an incomplete subunit since it is devoid of the terminal Glc which, in the complete O-specific oligosaccharide is present at C-6 of the three-linked galactose residue. It was shown previously that this Glc residue is a site of sialylation, bridging O-specific units in the dimer [2]. Thus, the question arises if the addition of this glucose unit is essential for chain lengthening. Further analysis of the third component detected, although present in trace amounts, should give valuable information concerning the order of the biosynthesis. It corresponds probably to a molecule lacking sialic acid.

The FABMS approach furthermore provided valuable information on the mass fragmentation of this type of oligosaccharides. Fragments arising from double radical elimination, specific for (1→6) linkages, were consistent with methylation analysis and NMR spectroscopy studies. This approach is thus a complementary technique for the determination of linkage position. The molecular region of peracetylated Fraction 2-S1 included a group of peaks corresponding respectively to the cationized ion, incomplete acetylation of the Neu5Ac residue and fragmentation with elimination of acetic acid and carbon dioxide. The origin of this complexity was confirmed by comparison with the spectrum of a peracetylated neuraminic acid (not shown).

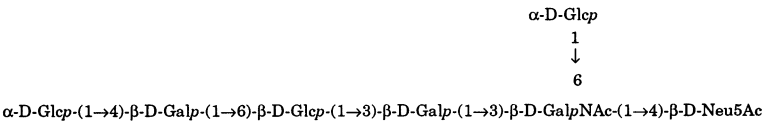
Fraction 2-S2 contains the core oligosaccharide [2,4] as the major component (Scheme 1(c)). The FABMS corroborate the composition of this core oligosaccharide with pyrophosphorylethanolamine [4]. Fraction 2-S2 contains furthermore a linear oligosaccharide detected by FABMS, which comprises five hexose constituents and a hexosamine branched with an additional hexose residue. The hexasaccharide Fraction 3 (Scheme 1(d)) shows a similarity to Fraction 2-S1, taking into account fragments arising from double radical elimination. A tetrasaccharide which substitutes a Kdo residue is also found in the O-specific unit where it is linked to sialic acid [6]. The FABMS results confirm the structure of the trisaccharide Fraction 4, as proposed previously based on NMR data [4] (Scheme 1(e)). This oligosaccharide may arise from a nonreducing terminal disubstituted Kdo residue.

The mode of linkage of the O-chain with the core oligosaccharide in the LPS of the genus *Hafnia* was suggested only for *H. alvei* strain 2. From the LPS of this strain, we have previously characterized Fraction 1a, in which a pentasaccharide segment of the O-specific unit is linked to the subterminal glucose of the core [2]. However, this may not be the major mode of attachment of the O-chain to the core region, because this fraction constitutes only 1.7% of the whole carbohydrate material isolated from the LPS. Thus, the tetrasaccharide fragment of the O-specific unit linked to Kdo in Fraction 3 (Scheme 1(d)), comprising 20% of the LPS carbohydrate material, may repre-

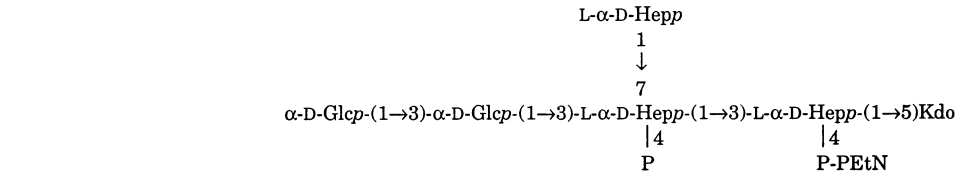
a) Fraction 2-S1, oligosaccharide M1



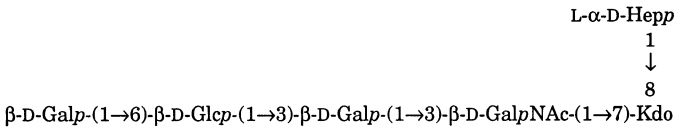
b) Fraction 2-S1, oligosaccharide M2



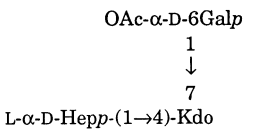
c) Fraction 2-S2



d) Fraction 3



e) Fraction 4



Scheme 1. Oligosaccharides components from the *Hafnia alvei* strain 2 LPS.

sent an alternative way of linking the O-chain to the core in *H. alvei* 2 LPS. The question arises on the arrangement of this hexasaccharide in the LPS: whether it is linked to lipid A directly, forming a second LPS, or whether this oligosaccharide is a side chain linked to the terminal Kdo in the same LPS molecule. In turn, this oligosaccharide may be linked via its Kdo to the terminal Glc of the core. Such a structure was found in some LPSs of *Rhizobium*, where the Kdo residue present at the reducing end of the O-specific chain was found linked to a hexosidic outer region of the core [18]. Since NeuAc bears some structural similarity with Kdo [19], a competition for the substrate may occur during the biosynthesis step between polymerase and enzymes synthesizing the branched octasaccharide unit [20].

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