Two Kdo-heptose regions identified in *Hafnia alvei* 32 lipopolysaccharide: the complete core structure and serological screening of different *Hafnia* O-serotypes

Running Title: Kdo-heptose core regions in *Hafnia alvei* lipopolysaccharides

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The abbreviations used are: COSY, correlated spectroscopy; DEPT, distortionless enhancement by polarization transfer; ESI, electrospray ionisation; FAB, fast atom bombardment; GC, gas chromatography; L-α-D-Hep, α-L-glycero-D-manno-heptose; HMBC, heteronuclear multiple bond correlation; HMQC, heteronuclear multiple quantum coherence; HSQC, heteronuclear single quantum coherence; Kdo, 3-deoxy-D-manno-oct-2ulosonic acid; LPS, lipopolysaccharide; MALDI-TOF, matrix-assisted laser-desorption/ionization time-of-flight; MS, mass spectrometry; NOESY, nuclear Overhauser effect spectroscopy; PBS, phosphate buffered saline; ROESY, rotating frame nuclear Overhauser effect spectroscopy; TOCSY, total correlation spectroscopy.
*Hafnia alvei*, a Gram-negative bacterium, is an opportunistic pathogen associated with mixed hospital infections, bacteremia, septicemia, and respiratory diseases. Various Kdo (3-deoxy-D-manno-oct-2-ulosonic acid)-containing fragments, different from already known structures of core oligosaccharides, were previously found among fractions obtained by mild acid hydrolysis of some *H. alvei* lipopolysaccharides. However, the position of these segments in LPS structure was not known. Analysis of de-N,O-acylated LPS by NMR spectroscopy and mass spectrometry allowed for determination of the location of Kdo-containing trisaccharide in the structure of *H. alvei* PCM 32 LPS. It was established that the trisaccharide \[L-\alpha-D-Hep-(1\rightarrow4)-[\alpha-D-Galp6OAc-(1\rightarrow7)]-\alpha-Kdo-(2\rightarrow)\] is an integral part of the outer core oligosaccharide of *H. alvei* 32 LPS. The ketosidic linkage of very labile nature between trisaccharide’s \(\rightarrow4,7\)-\(\alpha\)-Kdo and \(\rightarrow2\)-Glc in the core oligosaccharide was identified. Screening for this Kdo-containing trisaccharide was performed on the group of 37 O-serotypes of *H. alvei* LPSs using monospecific antibodies recognizing this structure. It was established that this trisaccharide is a characteristic component of the outer core oligosaccharides of *H. alvei* 2, 32, 600, 1192, 1206, and 1211 LPSs. The weaker cross-reactions with LPSs of strains 974, 1188, 1198, 1204, and 1214 suggest the presence of similar structures in these LPSs as well. Thus we have identified new example of endotoxins among those elucidated so far. This type of core oligosaccharide deviates from the classical scheme by the presence of the structural Kdo-containing motif in the outer core region.
Lipopolysaccharide (LPS, endotoxin) is the main surface antigen and important virulence factor of most of the Gram-negative bacteria pathogenic for humans and animals (46). LPS contributes greatly to the structural integrity of bacteria and constitutes a "pathogen-associated molecular pattern" for host infection (46). As one of the most potent natural activators of the innate immune system, LPS is recognized by different classes of receptors present on macrophages, monocytes, B and T cells, neutrophiles, endothelial cells, and epithelial cells (46). Endotoxins stimulate these cells to produce multiple inflammatory mediators responsible for the immunotoxicity (e.g., TNFα, IL-1, IL-6, IL-8, INFγ/α, NO, PAF, and endorphins). Interaction of LPS with CD14/TLR4 (Toll-like receptor)/MD-2 receptor complex constitutes a major mechanism responsible for innate immune response to Gram-negative infection (1, 46). Large amount of LPS released into the bloodstream triggers the excessive inflammatory response of innate immune system leading to sepsis and septic shock (6). High levels of inflammatory mediators have profound effects on the cardiovascular system, kidneys, lungs, liver, central nervous system, and coagulation system. Following their action, renal failure, myocardial dysfunction, acute respiratory distress syndrome (ARDS), hepatic failure, and disseminated intravascular coagulation can occur, which may result in death (6). Despite intense research on etiology and treatment of sepsis its severe form still carries high mortality rate (6, 46).

*Hafnia alvei* has been reported as opportunistic human pathogen. This Gram-negative bacterium and its LPS are among the identified causative agents of bacteremia and septicemia in humans and animals (19). Annually up to 42 cases of *H. alvei* bacteremia were reported in the United Kingdom for years 2001 – 2003. Most of them were monomicrobial infections and in ~ 33% of the cases *H. alvei* was isolated not only from blood, but also from hepatic abscesses, pancreatic pseudocyst fluid, sputum, feces, and central venous catheter (19). Besides bacteremia and sepsis, which seem to be the most common syndromes reported, *H. alvei* is also associated with respiratory diseases and mixed hospital infections in humans. Since gastrointestinal and respiratory tracts represent very common habitats for *hafniae*, most cases of *H. alvei* bacteremia are usually of these origins. *H. alvei* sepsis is also a serious clinical problem
in animal production industry, as infections of *H. alvei* can be severe, in the case of commercial laying hens, pullets and rainbow trout yielding septicemia (19).

Our knowledge concerning the pathogenicity of *H. alvei* is limited. LPS is the major virulence factor in cases of *H. alvei* septicemia and bacteriemia (19). Studies concerning other virulence factors of *H. alvei* reported only on the iron scavenging mechanism, mannose-sensitive/mannose-resistant hemagglutinins, and plasmids encoding bacteriocins and alveicins (19).

Most of the elucidated structures of *H. alvei* LPS are smooth type molecules built up of O-specific polysaccharide (PS), core oligosaccharide (OS) and lipid A. The O-antigens of *H. alvei* are subdivided into 40 O-serotypes (2, 28, 42). The structures of the O-specific polysaccharides from 30 serologically different *H. alvei* strains were elucidated (15, 24, 26, 28, 42). So far four types of core OS were identified for *H. alvei* LPSs (9, 17, 27, 30, 43). The most common core oligosaccharide, isolated by mild acidic hydrolysis from LPSs of smooth *H. alvei* strains, is a hexasaccharide composed of two D-Glc, three L,D-Hep, and one 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo). Two L,D-Hep residues are substituted by phosphoethanolamine (PEtn) and phosphoryl groups (P) (9, 17, 25). In LPSs isolated from *H. alvei* PCM 1185 and 1204, core oligosaccharides are terminated with D-Galp instead of D-Glcp (27). LPSs of *H. alvei* containing non-typical core oligosaccharides, identical with these found in LPS of *Escherichia coli* R4 (strain 23 and 1222) and *Salmonella enterica* Ra (strain 39), were also identified (43).

The chemical structures of *H. alvei* O-specific chains and core oligosaccharides were elucidated using fractions obtained by mild acid hydrolysis of LPS. The procedure was optimized for the delipidation of LPS, exploiting the susceptibility of a ketosidic linkage between the inner-core and lipid A to acid. However, other acid-labile linkages within the LPS could also be affected, leading to a partial degradation of the isolated molecules.

The presence of Kdo-containing OSs among fractions obtained by mild acid hydrolysis of LPSs, other than previously identified core OSs, makes the structural analysis of entire *H. alvei* LPSs difficult. Two
types of trisaccharides were previously identified: (i) L-\(\alpha\)-D-Hep\(-(1\rightarrow4)-[\alpha\-D-Gal\p-(1\rightarrow7)]\)-\(\alpha\-Kdo\) for 94 strains 2, 1211, 32, 1192 (16, 23), (ii) and \(\alpha\-D-Gal\p-(1\rightarrow2)-L\-\(\alpha\)-D-Hep\-(1\rightarrow4)-\(\alpha\)-Kdo\) for strains 1188 and 1196 (22). These Kdo-containing motifs were never located in any of the LPS segments. Thus it is of interest to complete the structure of \(H. alvei\) LPSs with the location of such acid-labile motifs in the structure of LPSs isolated from these bacteria.

We now report on structural studies of de-N,O-acylated lipopolysaccharide of \(H. alvei\) 32 containing carbohydrate backbone of lipid A, core OS, an additional trisaccharide in the outer region of the core OS, and all of the acid labile substituents. Additionally, data obtained previously for trisaccharide isolated from \(H. alvei\) 32 LPS (16) was complemented with detailed \(^1\)H and \(^{13}\)C NMR analyses and the assignment of all proton and carbon signals. The screening for presence of these acid-labile trisaccharides identical with those found in the 32 LPS was performed on 37 different O-serotypes of \(H. alvei\) LPSs with the antibodies against the conjugate of de-N,O-acylated \(H. alvei\) 32 endotoxin fragment with BSA (bovine serum albumin), specific for the isolated trisaccharide.

**MATERIALS AND METHODS**

**Bacteria.** \(H. alvei\) strains PCM 1, 1M, 2, 17, 23, 31, 32, 37, 38, 39, 481, 600, 744, 974, 981, 1188, 1190, 1191, 1192, 1195, 1198, 1200, 1203, 1204, 1205, 1206, 1207, 1209, 1210, 1211, 1212, 1213, 1214, 1215, 1221, 1224, 4221 were obtained from the Polish Collection of Microorganisms (PCM) at the Institute of Immunology and Experimental Therapy (Wroclaw, Poland). The bacteria were grown in Davis medium, killed with 0.5% phenol and centrifuged using a CEPA flow laboratory centrifuge (39).

**Preparation of lipopolysaccharides and core oligosaccharides.** LPSs were extracted from bacterial cells by the hot phenol/water method (53) and purified by ultracentrifugation as previously described (39). The yields of LPS preparations were 2 - 3.5%. Poly-, and oligosaccharides were isolated
by mild acidic hydrolysis (1.5% acetic acid) at 100 °C, for 2 h, fractionated and purified as previously described (16, 37).

**De-N,O-acylation of LPS.** De-O-acylation of LPS 32 was achieved by mild hydrazinolysis (14) modified as previously described (36). Briefly, LPS (200 mg) was dissolved in anhydrous hydrazine (5 ml) and the reaction was carried out at 37 °C for 30 min. The mixture was cooled and added to cold acetone (−20 °C) to terminate the reaction by conversion of hydrazine to acetone hydrazone. The precipitate of the de-O-acylated LPS was collected by centrifugation (4000 × g, −20 °C, 30 min), dissolved in water and freeze-dried. The de-O-acylated LPS was dissolved in aqueous 4 M KOH (10 ml) and hydrolyzed in a sealed tube under nitrogen (120 °C, 16 h). The reaction mixture was neutralized with HClO₄ at 0 °C. Most of the insoluble KClO₄ sediment was removed by centrifugation and further desalted using a Bio-Gel P-2 column equilibrated with 0.05 M pyridine/acetic acid buffer at pH 7.0. Fraction of the highest molecular weight was further fractionated by gel permeation chromatography, performed on a column (1.6 cm × 100 cm) of Bio-Gel P-10 equilibrated with 0.05 M pyridine/acetic acid buffer at pH 7.0. Fractions were collected, freeze-dried and checked by 1D, 2D NMR spectroscopy. Fraction showing NMR resonances of the structure reporter groups typical for the intact Kdo-containing core regions (OS₃₂) was rechromatographed and chosen for further structural analysis.

**Analytical procedures.** Methylation was performed on oligosaccharide according to the method of Hakomori (13). Alditol acetates and partially methylated alditol acetates were analysed by GC-MS with a Hewlett-Packard 5972 system using the HP-1 fused-silica capillary column (0.2 mm × 12.5 m) and a temperature program 150 → 270 °C at 8 °C min⁻¹. The absolute configurations of monosaccharides were determined as described by Gerwig et al. using (R)-2-butanol for the formation of 2-butyl glycosides (10, 11). Prior to the absolute configuration analysis dephosphorylation of residues substituted by P or PPEtn was performed as previously described (34). Briefly, oligosaccharides (2 mg) were treated with aqueous 48% HF (1 ml, 72 h, 4°C), then concentrated to dryness by evaporation. The trimethylsilylated butyl glycosides were identified by comparison with the authentic samples produced from carbohydrate
standards (Sigma, St. Louis, MO, USA) and (R/S)-2-butanol (Fluka, Buchs, Switzerland) on GC-MS.

This analysis was carried out with a Hewlett-Packard 5971A system using an HP-1 fused-silica capillary column (0.2 mm × 12 m) and a temperature program, 100 → 270 °C at 8 °C/min.

**Mass spectrometry.** Matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry was carried out on a Kratos Kompact-SEQ instrument as described previously (34). Trihydroxyacetophenone (25 mg/ml, acetonitrile:water, 1:1, v/v) was used as matrix for the analysis of OSs. ESI-MS analysis of the de-N,O-acylated *H. alvei* 32 LPS (OS₃₂) was performed on micrOTOF-Q spectrometer (Bruker Daltonics, Bremen, Germany) in the negative-ion mode. The sample was dissolved in acetonitrile:water solution (1:1, v/v; 0.2 mg/ml) and analysed by direct infusion at a rate of 3 µl/min. Spectra were scanned in the range of m/z 120-2000. The ion source temperature was 180 °C, the flow rate was 4 l/min and the pressure of nitrogen was 0.4 bar. External calibration in the negative-ion mode was applied using the Tunemix™(neg) mixture (Bruker Daltonics, Germany) in quadratic regression mode, in m/z range of 113 - 2234 Da.

**NMR spectroscopy.** All NMR spectra were obtained on Bruker DRX 400 and DRX 600 spectrometers. NMR spectra of the isolated poly- and oligosaccharides were obtained for ²H₂O solutions at 30 °C using acetone (δH 2.225, δC 31.05) as internal reference. In ³¹P NMR spectroscopy experiments, phosphorus resonances were referenced to external 85% phosphoric acid (δ 0 ppm) in a separate experiment. Oligosaccharides were repeatedly exchanged with ²H₂O (99.95%) with intermediate lyophilisation. The data were acquired and processed using standard Bruker software. The processed spectra were assigned with the help of SPARKY (12). The signals were assigned by two-dimensional experiments (COSY, clean-TOCSY, NOESY, ROESY, HMBC, HSQC-DEPT and HSQC with and without carbon decoupling). In the clean-TOCSY experiments the mixing times used were 30, 60 and 100 ms. The delay time in HMBC was 60 ms, and the mixing time for NOESY was 200 ms.

**Conjugation of OS₃₂ with BSA.** Bovine serum albumin (BSA, 10 mg) was activated with glutardialdehyde under nitrogen (1%, carbonate/bicarbonate buffer pH 9.0, 4 h, 21 °C). Excess of
glutardialdehyde was removed by dialysis of the mixture against carbonate/bicarbonate buffer (50 mM, pH 9.0). The activated BSA was mixed with the dodecasaccharide OS$_{32}$ (5 mg) and the conjugation was carried out for 16 h at 21°C, followed by treatment with NaBH$_4$ (10 mg/ml, 2 h) and dialysed extensively against phosphate buffered saline (PBS, pH 7.4) (5, 21).

**Immunization procedures.** Rabbits were housed at the animal facility of the Institute of Immunology and Experimental Therapy (Wroclaw, Poland). Rabbits were immunized with 50 µg of the OS$_{32}$-BSA conjugate, suspended in the complete Freund’s adjuvant and polyclonal antibodies against the conjugate were obtained by the procedures previously described (21, 32). All experiments were carried out according to the procedures approved by the Local Ethical Commission.

**Isolation of trisaccharide-specific antibodies from polyclonal serum.** Trisaccharide-specific antibodies were prepared by absorbing crude antiserum obtained by immunization of rabbits with OS$_{32}$-BSA conjugate with *H. alvei* 1207 bacterial cells, whose LPS is devoid of trisaccharide (C. Lugowski, T. Niedziela, W. Jachymek, and J. Lukasiewicz, unpublished data). Killed, freeze-dried bacteria (2 g) were suspended in PBS for 24 h. Bacteria were centrifuged (1000 × g at 4°C for 30 min) and rinsed three times with 30 ml of PBS. The serum (10 ml, 1:3 diluted in PBS) was added to bacteria suspended in PBS. Mixture was incubated on a rocking platform, 16 h at 22°C and centrifuged. Supernatant was added to the fresh suspension of *H. alvei* 1207 bacteria, incubated (16 h, at 22°C) and centrifuged. Supernatant was filtered (0.2-µm pores), collected in sterile vials and stored at −70°C.

**ELISA and ELISA inhibition test.** Enzyme-linked immunosorbent assay (ELISA), using LPS as solid-phase antigen, was performed by a modification (35) of the method described by Voller *et al.* (52). In the inhibition studies the trisaccharide-specific serum (100 µl) at concentration twice as high as that giving $A_{405}$ in the range 0.5–0.8 was mixed with serial dilutions of the trisaccharide isolated from *H. alvei* 32 (100 µl) and incubated for 1 h at 37°C. The mixture (100 µl) was then transferred into the wells of a microtiter plate coated with *H. alvei* 32 LPS and the reaction was carried with shaking (15 min, 22°C, pH
192 7.3). Washing of the wells, reaction with second antibody conjugated with alkaline phosphatase and 193 colour development were performed as described for ELISA.

194 **SDS-PAGE.** The LPS was analyzed by SDS-PAGE according to the method of Laemmli (29) 195 with modifications previously described (15). LPS bands were visualised by the silver staining method 196 (45).

197 **Immunoblotting.** Immunoblotting was performed on the SDS-PAGE separated LPS fractions as 198 previously described (31). A goat anti-rabbit IgG conjugated with alkaline phosphatase (Bio-Rad) was 199 used as the second antibody and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium was 200 applied as a detection system.

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**RESULTS**

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205 Isolation and initial analysis of the de-N,O-acylated LPS and the Kdo-containing 206 trisaccharide. The mixture of the de-N,O-acylated LPSs of *H. alvei* 32 was fractionated by gel filtration 207 (Supplemental Material, Fig. S1A, B). All isolated fractions were checked by 1D and 2D $^1$H, $^{13}$C NMR 208 spectroscopy. Fraction showing characteristic NMR resonances of three pairs of signals originating from 209 deoxy protons of Kdo, and two signals of nitrogen-bearing carbons from aminosugars of lipid A (Fig. 1, 209 2) was selected for structural analysis of the intact Kdo-containing core regions (OS$_{32}$, 11 mg).

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The trisaccharide component of the core oligosaccharide of *H. alvei* 32 LPS was isolated as previously 211 described (16). Briefly, poly- and oligosaccharides were released by mild acidic hydrolysis of *H. alvei* 32 212 LPS, fractionated by gel filtration on Bio-Gel P-10 (Supplemental Material, Fig. S1C), followed by Bio- 213 Gel P-2 chromatography (Supplemental Material, Fig. S1D). Isolated poly- and oligosaccharides were 214 examined by MALDI-TOF mass spectrometry (data not shown) and compared with results published 215 previously for O-specific chain of *H. alvei* 32 and core OS of *H. alvei* 32 and 1192 (16, 17, 33). The 216 trisaccharide-containing fraction was identified by $^1$H and $^{13}$C NMR and selected for further investigation.
Structural analysis of the purified de-N,O-acylated LPS isolated from *H. alvei* 32. Monosaccharide and absolute configuration analyses of the dephosphorylated OS_{32} revealed the presence of D-GlcN, L-glycero-D-manno-Hep, D-Glc, D-Gal. Methylation analysis of native OS_{32} showed the presence of terminal D-Glcp, 3-substituted D-Glcp, and terminal L,D-Hepp residues identified previously for core OSs obtained by mild acid hydrolysis of *H. alvei* 32 LPS (17). The analysis revealed also additional terminal D-Galp and 2-substituted D-Glcp. These terminal D-Galp and 2-substituted D-Glcp have not been identified before in core OS of *H. alvei* 32 obtained by mild acid hydrolysis of this LPS.

The oligosaccharide OS_{32} was investigated by NMR spectroscopy. Two-dimensional NMR experiments (COSY, TOCSY, HSQC-TOCSY, NOESY, HSQC-DEPT) were recorded and analyzed. All NMR analyses were performed at pH ~ 8.5, using 1.9 mm capillary tubes.

Nine major anomeric proton and carbon resonances, two signals of nitrogen-bearing carbons, and in addition three Kdo spin systems were identified in spectra of OS_{32} (Fig. 1, 3A; Table 1, 2). Residues are denoted by capital letters as shown in the structure of the isolated dodecasaccharide (Fig. 1, inset structure). These letters refer to the corresponding residues through the entire text, tables and figures.

Residue A with the H-1/C-1 signals at δ 5.48/94.0 ppm, J_{H-1,H-2} ~ 3.4 Hz, was recognized as the 6-substituted α-D-GlcpN1P. The low chemical shift of the C-2 signal (δ 56.4), the high chemical shift of the C-6 signal (δ 70.3) and the large vicinal coupling constants between H-2 and H-3, H-3 and H-4, H-4 and H-5 (J_{H-2,H-3}, J_{H-3,H-4} and J_{H-4,H-5} ~ 10 Hz) were observed. ^1^H,^3^P-correlation experiment showed the connectivity between the phosphate monoester peak at δP 1.8 ppm and H-1 (δ 5.48) and H-2 (δ 3.00) of residue A (3J_{H-1,P} ~ 8 Hz).

Residue B with the H-1/C-1 signals at δ 4.68/103.1 ppm, J_{H-1,H-2} 8.6 Hz, was assigned as the 6-substituted β-D-GlcpN, on the basis of the typical chemical shifts of the H-1, C-1 and C-2 (δ 57.5) signals and the large J-values for the vicinal couplings between all ring protons. The small down-field shift of the C-6 signal (δ 62.7) indicated the substitution by a Kdo residue, which in general induces only a weak α-
effect on C-6 of residue B (4, 38). The residues A and B constitute the disaccharide backbone →6)-β-D-
GlcN-(1→6)-α-D-GlcN1P of the lipid A as was further supported by HMBC data.

Residues C, D and K were identified as different Kdo molecules. Chemical shifts values of H-3ax and H-3eq protons in the 1.7-2.38 ppm range, together with the chemical shift values of H-5 and C-2 indicated the α-pyranosidic configuration of all Kdo residues (3, 47).

Residue C was identified as the 4,5-disubstituted pyranose form of 3-deoxy-α-D-manno-oct-2-ulosonic acid (α-Kdop) on the basis of characteristic deoxy proton signals at δ 1.90 ppm (H-3ax) and δ 2.21 ppm (H-3eq), and high chemical shifts of the C-4 (δ 72.3) and C-5 (δ 69.6) signals.

Residue D was identified as terminal α-Kdop. Characteristic deoxy proton signals were found at δ 1.76 ppm (H-3ax) and δ 2.14 ppm (H-3eq). Chemical shift values of the assigned resonances were in agreement with previously published data for terminal α-Kdop (44, 47, 49).

Residue K was identified as the 4,7-disubstituted α-Kdop on the basis of characteristic deoxy proton signals at δ 1.88 ppm (H-3ax) and δ 2.30 ppm (H-3eq), and high chemical shifts of the C-4 (δ 72.3) and C-7 (δ 78.9) signals.

Residue E with the H-1/C-1 signals at δ 5.22/100.0 ppm, $J_{H-1,C-1} \sim 172$ Hz was recognized as 3-substituted L-glycero-α-D-manno-Hepp4P from the $^1$H and $^{31}$C chemical shifts, small vicinal couplings between H-1 and H-2, H-2 and H-3, and the relatively large chemical shift of the C-3 signal (δ 78.2).

$^1$H,$^{31}$P-HMQC experiment revealed the connectivity between the phosphate monoester peak at δp 2.8 ppm and H-4 (δ 4.32) of residue E, indicating that the →3)-L-α-D-Hepp residue was substituted at O-4 with a phosphate group.

Residue F with the H-1/C-1 signals at δ 5.16/103.8 ppm, $J_{H-1,H-2} < 2$ Hz, $J_{H-1,C-1} \sim 176$ Hz, was recognized as the 3,7-disubstituted L-glycero-α-D-manno-Hepp4P from the $^1$H and $^{31}$C chemical shifts, small J-values for the vicinal couplings between H-1 and H2, H-2 and H-3, and the high chemical shifts of the C-3 (δ 79.4) and C-7 (δ 70.1) signals. $^1$H,$^{31}$P-HMQC experiment revealed the connectivity between
the phosphate monoester signal at δP 2.8 ppm and H-4 (δ 4.34) of residue F, indicating that the \(\rightarrow 3,7\)-L-α-d-Hepp residue was substituted at O-4 with a phosphate group.

Residue G with the H-1/C-1 signals at δ 4.89/101.7 ppm, \(J_{H-1,H-2} < 2\) Hz, \(J_{H-1,C-1} \sim 170\) Hz and residue L with the H-1/C-1 signals at δ 5.08/98.7 ppm, \(J_{H-1,H-2} < 2\) Hz, \(J_{H-1,C-1} \sim 173\) Hz were recognized as terminal L-glycero-α-d-manno-Hepp due to the small vicinal couplings between H-1 and H-2, and H-3.

Residue H with the H-1/C-1 signals at δ 5.23/102.4 ppm, \(J_{H-1,H-2} 2.7\) Hz, was assigned as 3-substituted α-d-Glc\(\text{p}\) based on the large chemical shift of the C-3 signal (δ 80.3) and the large vicinal couplings among H-2, H-3, H-4 and H-5.

Residue J with the H-1/C-1 signals at δ 5.46/96.8 ppm, \(J_{H-1,H-2} 2.6\) Hz, was assigned as the 2-substituted α-d-Glc\(\text{p}\) residue based on the relatively high chemical shift of the C-2 signal (δ 74.0) and the large vicinal couplings between H-2 and H-3, H-3 and H-4, H-4 and H-5.

Residue M with the H-1/C-1 signals at δ 5.20/101.8 ppm, \(J_{H-1,H-2} 3.8\) Hz, was assigned as terminal α-d-Gal\(\text{p}\) residues due to the large coupling constant between H-2 and H-3 and the small vicinal couplings between H-4 and H-5.

All assigned resonances were in agreement with previously published data (16, 17, 20, 44, 47, 49).

Each disaccharide element in the OS\(_{32}\) was identified by HMBC (Fig. 2) and NOESY (Fig. 3) experiments showing inter-residue connectivities between adjacent sugar residues and providing the sequence of monomers in the dodecasaccharide (Fig. 1, inset structure).

Interresidual NOEs were identified between H-1 of L and H-4 of K, H-1 of M and H-7 of K, H-1 of J and H-3 of H, H-1 of H and H-3 of F, H-1 of G and H-7a,b of F, H-1 of F and H-3 of E, H-1 of E and H-5 of C, and H-1 of B and H-6a of A. Additionally, the inter-residue NOE signals between H-3ax (δ 1.90) and H-3eq (δ 2.21) of residue C and H-6 of residue D (δ 3.65) (Fig. 3) supported the presence of disaccharide element in the inner core region: α-Kdop-(2→4)-α-Kdop-(2→3). The HMBC spectra showed cross-peaks between the anomeric proton and the carbon at the linkage position and between the
anomeric carbon and the proton at the linkage position (Table 2), which confirmed the sequence of sugar residues in the dodecasaccharide.

Residues K, L, and M constitute the trisaccharide previously found among fractions released by the mild acid hydrolysis of *H. alvei* LPS 32 (16). HMBC correlation between C-2 of residue K and H-2 of residue J confirmed the presence of acid-labile α-(2→2) ketosidic linkage between the α-Kdo (K) of the trisaccharide and the α-D-Glc (J).

Two additional signals were present in all NMR spectra (Fig. 1, 2, 3 and Table 1, 2). Residue j (H-1/C-1 signals at δ 5.40/99.9 ppm, J_H-1,H-2 ~ 2.7 Hz, J_H-1,C-1 ~ 171 Hz) was recognized as terminal α-D-Glc. Residue j represented a nonsubstituted variant of residue J (→2)-D-Glc and formed a non-reducing end in oligosaccharide devoid of the trisaccharide. Residue h (H-1/C-1 signals at δ 5.28/101.8 ppm, J_H-1,H-2 < 2 Hz) was identified as a variant of residue H (3-substituted α-D-Glcp ) effected by the lack of the trisaccharide.

This minor structural heterogeneity of OS32 was also examined with the ESI-MS analysis (Fig. 4, Table 3). ESI-MS spectra of OS32 showed several multiply charged deprotonated ions [M-2H]^{2-} and [M-3H]^{3-}. Main ions corresponded to the structures of dodecasaccharide (OS32), which differed only by phosphate group substitution and the presence of terminal Kdo residue in the inner core OS (Table 3). The ions at m/z 999.18, m/z 959.18, m/z 665.8, and m/z 639.11 corresponded to the OS32 devoid of the trisaccharide (Table 3). Additionally the [M-H_{2}O-H]^{1-} ion m/z 573.14 corresponding to the trisaccharide was also identified.

**Intact structure of the Kdo-containing trisaccharide.** Procedure used for the isolation of the intact Kdo-containing core region of *H. alvei* 32 LPS - OS32 entails anhydrous hydrazine and aqueous 4 M KOH treatment and always leads to the loss of the alkali-labile substituents (*i.e.* O-acetyl/acyl, N-acetyl/acyl, PEtn). Structure of the trisaccharide isolated from *H. alvei* 32 was elucidated previously only by GC-MS, FAB-MS and 1D ^1^H and ^13^C NMR spectroscopy (16), but the presence of O- and N-substituents susceptible to hydrazine and KOH treatment was not reported. Moreover, both papers
presented incomplete NMR assignments of spin systems of the Kdo-containing trisaccharides. Since none of the alkali-labile components were retained in the OS$_{32}$ we have also analyzed the trisaccharide isolated by mild acid hydrolysis of LPS 32 and completed $^1$H, $^{13}$C NMR spectroscopy analysis (Fig. 5, Table 4, 5) with emphasis on the presence of the labile constituents.

Residue K* was identified as a 4,7-disubstituted $\alpha$-Kdo on the basis of characteristic deoxy proton signals at $\delta$ 1.91 (H-3ax) and $\delta$ 2.11 ppm (H-3eq), the large chemical shift values of the C-4 ($\delta$ 71.9) and C-7 signals ($\delta$ 78.6).

Residue L* with the H-1/C-1 signals at $\delta$ 5.08/97.9 ppm, $J_{H1,H2} < 2$ Hz, $J_{H1,C1} \sim 172$ Hz was recognized as terminal L-glycero-$\alpha$-d-manno-Hepp due to the small vicinal couplings between H-1, H-2 and H-3 and the similarity of the chemical shifts with those of the terminal L-$\alpha$-d-Hepp.

Residue M* with the H-1/C-1 signals at $\delta$ 5.19/101.0 ppm, $J_{H1,H2} = 2.6$ Hz, $J_{H1,C1} \sim 172$ Hz, was assigned as terminal $\alpha$-d-Galp6OAc due to the large couplings between H-2 and H-3 and the small vicinal coupling constants between H-3, H-4 and H-5. The H-6a and H-6b of this residue resonated at $\delta$ 4.25 and $\delta$ 4.31 ppm. This observed downfield shift is consistent with the substitution of the O-6 with an acetyl group. It was further supported by the HMBC connectivities observed between H-6a,b of residue M* ($\delta_H$ 4.25, 4.31 ppm), the acetyl carbonyl carbon ($\delta_{CO}$ 174.5 ppm) and the acetyl methyl protons ($\delta_H$ 2.13 ppm).

HMBC experiment exhibited inter-residue connectivities between adjacent monosaccharides and thus provided the sequence: [L-$\alpha$-d-Hepp-(1$\rightarrow$4)-[$\alpha$-d-Galp6OAc-(1$\rightarrow$7)]-$\alpha$-Kdo-(2$\rightarrow$)] (Table 5).

**Trisaccharide-specific antibodies.** In immunoblotting tests antisera obtained against the OS$_{32}$-BSA conjugate reacted strongly with fast migrating fractions of homologous H. alvei 32 LPS as well as LPSs of H. alvei PCM 1192 and 1207, containing core oligosaccharide-lipid A molecules not substituted with O-specific chain (Supplemental Material, Fig. S2A). This observation was in agreement with structural data obtained previously for the examined LPSs (15-18).
A fraction of immunoglobulins specific for the trisaccharide, \( \text{L-} \alpha\text{-D}-\text{Hepp-(1} \rightarrow 4)[\alpha\text{-D}-\text{Galp-(1} \rightarrow 7)]\alpha\text{-Kdo-(2} \rightarrow \text{)} \) was obtained by absorption of anti OS\(_{32}\)-BSA serum with \( H. \text{alvei} \) 1207 bacterial cells. \( H. \text{alvei} \) strain 1207 was chosen for absorption of antibodies specific for the core oligosaccharide identical with that found for strain 32, but devoid of the trisaccharide (C. Lugowski, T. Niedziela, W. Jachymek, and J. Lukasiewicz, unpublished data).

The reactivity of the absorbed serum was assessed in ELISA and immunoblotting with \( H. \text{alvei} \) 32, 1192 and 1207 LPSs (Supplemental Material, Fig. S2C, B). Contrary to non-absorbed serum, strong reactions were observed only for LPSs of strains 32 and 1192. The antibodies did not react with LPS of strain 1207. Specificity of the absorbed serum was examined by ELISA inhibition test with the use of the trisaccharide of \( H. \text{alvei} \) 32 as the inhibitor. The trisaccharide showed 50% inhibition of the reaction of the absorbed serum with \( H. \text{alvei} \) 32 LPS at concentration 8 \( \mu \text{M} \) and 90% at concentration 98 \( \mu \text{M} \). Similar inhibitory activity of the trisaccharide was observed also for reaction with \( H. \text{alvei} \) PCM 1192 LPS (9.8 \( \mu \text{M} \) and 120 \( \mu \text{M} \) - 50% and 90% inhibitory concentration, respectively). This suggested that the absorption yielded antibodies recognizing only epitopes of the trisaccharide of \( H. \text{alvei} \) 32 LPS.

**Screening of \( H. \text{alvei} \) LPSs for the presence of Kdo-containing trisaccharide.** Specific anti-trisaccharide antibodies were used to scan all available LPSs of \( H. \text{alvei} \) comprising 37 different O-serotypes for the presence of epitopes similar to those found in the \( H. \text{alvei} \) 32. LPSs separated by SDS-PAGE (Fig. 6A) were transferred from the gel onto the nitrocellulose membrane and subjected to an immunoblotting test (Fig. 6B). Most of the LPSs represented S forms and showed a high molecular mass pattern of bands characteristic for smooth strains (Fig. 6A). Strong reactions were observed with fast-migrating fractions of six \( H. \text{alvei} \) strains, i.e. 2, 32, 600, 1192, 1206, and 1211, suggesting the presence of a common trisaccharide epitope in an outer core region of these strains. Cross-reactions that were observed for \( H. \text{alvei} \) 2, 32, 1192, and 1211 LPSs are in agreement with previously published results of structural analysis of trisaccharides isolated form these LPSs (16, 23). All of the observed reactions of anti-conjugate serum were detected mainly for fast migrating LPS fractions (lipid A substituted with core.
Weaker reactions detected for *H. alvei* 974, 1188, 1198, 1204, and 1214 suggest the presence of epitopes with structure similar to the trisaccharide.

**DISCUSSION**

Structures of LPSs isolated from *H. alvei* have been widely studied over the past 20 years. A number of O-specific polysaccharides and four types of core OSs were identified for *H. alvei* LPSs to date (9, 15, 17, 24-28, 30, 42, 43). Studies of these LPS focused on structural analysis of isolated and chemically modified fragments, *i.e.* poly- and oligosaccharides obtained by mild acid treatment – a procedure typically used for the delipidation of LPS. However, this method leads to hydrolysis of all ketosidic linkages in the LPS molecules. Therefore such strategy has to be further supplemented by other methods of structural analysis, providing insight into structural details of the isolated LPS molecules. The standard delipidation procedure, allows only determination of structures of discrete regions: O-specific repeats, the core OS and Kdo-containing trisaccharides (16, 18). The key part of this study was the investigation of an additional Kdo-containing fragment and its location in *H. alvei* 32 LPS segments. These fragments were often found among poly- and oligosaccharides obtained by mild acid hydrolysis of *H. alvei* LPSs (16, 22, 23, 40), but their origin was not explained. Thus, the results presented herein and the methods used have pieced together the data on regions of *H. alvei* 32 LPS.

We present here the complete structure of the dodecasaccharide OS_{32} obtained by de-N,O-acylation of *H. alvei* 32 LPS, with all acid-labile ketosidic linkages preserved. The dodecasaccharide OS_{32} represented the complete structure of the core oligosaccharide containing →4,5)-α-Kdop residue in the inner core region, substituted by terminal α-Kdop and the trisaccharide 1-α-D-Hepp-(1→4)-[α-D-Galp6OAc-(1→7)]-α-Kdop-(2→ as integral part of the outer core OS.

The acid-labile α-(2→2) linkage between α-Kdop (K) of the trisaccharide and α-D-Glcp (J) of the outer core OS (Fig. 1, inset structure) was identified using NMR analyses and the trisaccharide was
located in the outer core region of *H. alvei* 32 LPS. The presence of →2)-GlcΔ among the components of the OS$_{32}$ was further supported by the methylation analysis and suggested that this residue was substituted by the trisaccharide. The 2-substituted α-D-GlcΔ (residue J) had never been identified among constituents of *H. alvei* core OSs obtained by mild acid hydrolysis (16, 33). Due to the heterogeneity of OS$_{32}$ revealed by NMR and ESI-MS analyses and corresponding to the presence or the lack of the trisaccharide, two forms of α-D-GlcΔ were observed: →2)-α-D-GlcΔ (residue J) and terminal α-D-GlcΔ (j). The presence of the terminal α-D-GlcΔ could be explained by the even higher susceptibility of the ketosidic linkage between →4,7)-α-Kdop and →2)-α-D-GlcΔ to acidic conditions than the bond between →4,5)-α-Kdop and lipid A. The inter-residue connectivities observed in NOESY experiment identified linkage between two other α-Kdop residues (residue C and residue D) and thus allowed for an unambiguous localization of the α-Kdop-(2→4)-α-Kdop-(2→) disaccharide segment in the inner core region. Furthermore the assigned resonances of the remaining OS$_{32}$ residues [→3)-α-GlcΔ, →3,7)-L-α-D-HepΔ, →3)-L-α-D-HepΔ, terminal L-α-D-HepΔ] were similar to those described previously for core OSs (17, 20, 33, 44, 47, 49).

We isolated and analyzed the de-N,O-acylated population of *H. alvei* 32 LPS molecules nonsubstituted with O-specific polysaccharide (rough type population). Further studies will be required to characterize the position of substitution of the core OS by O-specific PS. Mild acid hydrolysis of some LPSs isolated from other species of Gram-negative bacteria usually gives fractions, which contain core OSs substituted by one or a few repeating units. Structural analysis of these fractions provides information on the biological repeating unit and the linkage between the core OS and the O-specific chain. Such fractions, containing previously identified hexasaccharide core OS substituted directly with one O-repeat have never been isolated among poly- and oligosaccharides obtained by mild acid hydrolysis of 32 LPS (C. Lugowski, T. Niedziela, W. Jachymek, and J. Lukasiewicz, unpublished data). The lack of such fraction could be explained by the presence of Kdo-containing fragments between O-specific PS and core OS in *H. alvei* LPSs.
Ravenscroft and coworkers (40) in their studies on *H. alvei* 2 LPS identified an oligosaccharide that was built of the fragment of the O-repeat linked to C-7 of Kdo in the disaccharide: $\rightarrow$7-[$\text{L-}\alpha\text{-D-}$

$\text{Hepp-}(1\rightarrow8)]$-\(\alpha\)-Kdop. The authors suggested that this Kdo-containing element could constitute an alternative or a predominant way the O-specific polysaccharide was linked to the core OS, but this linkage in *H. alvei* 2 LPS was not proven (40). Katzenellenbogen *et al.* isolated fractions from *H. alvei* LPSs 1185, 1188, 1189, 1196, 1199, 1204, 1205, 1211, 1216, and 1546, built of sugar residues characteristic both for core OS (Hepp) and for O-repeats as shown by sugar and methylation analysis (25). Analysis of constituents and their molar ratios in these fractions and fractions of unsubstituted core oligosaccharides revealed biological repeating units of ten studied strains, but also indicated that neither the outer core hexoses nor terminal heptose residue were substituted with the O-repeat of these LPSs (25).

In immunoblotting analysis, identical or similar elements have been found in seven *H. alvei* LPSs, among them three LPSs (strains 1188, 1204, 1211) examined by Katzenellenbogen *et al.* (25). Another previously identified type of trisaccharide, Galp-$(1\rightarrow2)$-\(\text{L-}\alpha\text{-D-Hepp-}(1\rightarrow4)$-\(\alpha\)-Kdop (22), sharing part of the epitope of trisaccharide of LPSs 32 could explain weaker cross-reactivity of antibodies specific for the trisaccharide of *H. alvei* 32 with LPS of strain 1188. All these data suggest that Kdo-containing trisaccharide is an interlinking segment for the O-specific chain in these LPSs.

Hypothesis formulated by Ravenscroft *et al.* for *H. alvei* LPS (40) holds for *Klebsiella pneumoniae* LPSs, for which similar Kdo-containing oligosaccharide fragments were identified as an interlinking segment between the O-specific chain and the core OS (49). The presence of the third Kdo residue in the core part of the LPS was detected previously for *K. pneumoniae* O3, and the disaccharide Hepp-$(1\rightarrow4)$-\(\alpha\)-Kdop-$(2\rightarrow$ was identified in the outer region of the core OS (50). Further studies showed that this region represents the conserved feature of *K. pneumoniae* LPS. Moreover, Vinogradov and coworkers identified this acid labile element as the ligation site for the O-antigens in the LPSs of *K. pneumoniae* (49). Position C-5 of the $(\rightarrow4)-\(\alpha\)$-Kd0 is a common attachment point of the O-specific polysaccharides to the core OSs in *Klebsiella* LPSs (49). In the case of *R. etli* CE358, CE359, and CE166
Kdo residue is either an attachment point for O-specific polysaccharide (7) or constitutes the outer core region of LPS (8).

Similar feature could also be characteristic for *H. alvei* LPSs. Different Kdo-containing motifs might represent the way the O-specific polysaccharide is linked to the core OS, as core hexasaccharide substituted directly with one or a few repeating units has not been identified among fractions obtained by mild acid hydrolysis of majority of *H. alvei* LPSs. In our serological studies we have found that 6 out of 37 LPSs of *H. alvei* have outer core regions \[\text{L-}\alpha-\text{D}-\text{Hep}(1\rightarrow4)-[\text{a}-\text{D}-\text{Galp}(1\rightarrow7)]-\alpha-\text{Kdop}(2\rightarrow)\] identical with the structure described herein. Six other strains could have similar structures. Different types of outer core Kdo-containing oligosaccharides identified previously (16, 22, 23), with a Kdo residue as a common feature responsible for high-sensitivity to acid hydrolysis suggest, that other types of linkage between the core OS and the O-specific polysaccharide in *H. alvei* LPSs, could not be excluded.

Lipopolysaccharides of *H. alvei* are yet another example of enterobacterial endotoxins deviating from the classical scheme of LPS by the presence of an additional Kdo residue in the outer core region. This finding demonstrates how important it is to use the complementary instrumental techniques and chemical analyses in LPS structure elucidation to avoid the loss of significant structural information. Interesting cases of acid-labile interlinking LPS segments among *K. pneumoniae*, *R. etli*, *S. marcescens* (51) and *H. alvei* could prompt researchers to look for similar motifs in structural analyses of other lipopolysaccharides.

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**FIGURE LEGENDS**

**Figure 1.** $^1$H-NMR spectrum of the dodecasaccharides OS$_{32}$ (inset structure) isolated from de-N,O-acylated *H. alvei* 32 LPS. The $^1$H-NMR spectrum was obtained for $^2$H$_2$O solutions (pH ~ 8.5) at 600 MHz and 30 °C. Residual water was removed by processing. The capital letters in the anomeric regions of the $^1$H-NMR spectra refer to carbohydrate residues as shown in the structure. The Arabic numerals refer to protons in the respective residue. Residues h and j are constituents of oligosaccharide devoid of the trisaccharide (framed with a dashed line) and represent variants of residue H and J (Table 2). M indicates substitution of the terminal $\alpha$-d-Galp residue (M) at O-6 by an O-acetyl group identified for the trisaccharide isolated by mild acid hydrolysis of *H. alvei* LPSs 32.

**Figure 2.** Selected parts of HSQC-DEPT and HMBC spectra of the OS$_{32}$ isolated from de-N,O-acylated *H. alvei* 32 LPS. The spectra were obtained for $^2$H$_2$O solutions (pH ~ 8.5) at 600 MHz and 30 °C. The capital letters refer to carbohydrate residues as shown on the structure in Fig. 1, and the numbers refer to protons and carbons in the respective residue. Residues h and j are constituents of oligosaccharides devoid of the trisaccharide and represents form of residue H and J.

**Figure 3.** The parts of NOESY spectra of the OS$_{32}$ isolated from de-N,O-acylated *H. alvei* 32 LPS. (A) Region of deoxy protons of $\alpha$-Kdo residues. (B) Region of anomeric protons. The cross-peaks are labeled as explained in the legend to Fig. 1.

**Figure 4.** Negative ion ESI-Q-TOF mass spectrum of the dodecasaccharide OS$_{32}$. The spectra were obtained for acetonitrile:water solutions (0.2 mg/ml). Number of charges is indicated above m/z values. The inset structures show the structural heterogeneity of fraction OS$_{32}$. Detailed interpretation of ions is described in Table 1.

**Figure 5.** Parts of the 600 MHz HSQC-DEPT spectrum of the trisaccharide isolated by mild acid hydrolysis of *H. alvei* 32 LPS. The inset spectrum contains the deoxy resonances of Kdo residue. The capital letters refer to carbohydrate residues as shown on the inset structure, and the numbers refer to
protons and carbons in the respective residue. Spin systems assigned with small letters belong to constituents of the trisaccharide: \( L-\alpha-D-\text{Hepp-(1\(\rightarrow\)4)-(}\alpha-D-\text{Galp6OAc-(1\(\rightarrow\)7)\}\alpha-Kdop \).}

**Figure 6.** Reactivity of affinity-purified antibodies specific for the trisaccharide \( L-\alpha-D-\text{Hepp-(1\(\rightarrow\)4)-(}\alpha-D-\text{Galp-(1\(\rightarrow\)7)\}\alpha-Kdop-(2\(\rightarrow\) with \( H. alvei \) LPSs. The affinity purified trisaccharide-specific antibodies against \( OS_{32}-\text{BSA conjugate were used for immunoblotting (B). LPSs were analyzed by SDS-PAGE (5 \( \mu \)g/lane) using a 15\% polyacrylamide-bisacrylamide separating gel and visualized by silver staining (A). LOS of \( H. alvei \) 1M was isolated from the rough strain and represented low-molecular weight form built of lipid A substituted with core oligosaccharide only (41). Spot intensities of the observed cross-reactions in immunoblotting were compared with this for reaction between \( H. alvei \) 32 LPS and absorbed serum (positive, strong reaction).
TABLE 1. $^1$H and $^{13}$C NMR chemical shifts of the dodecasaccharide isolated from de-N,O-acylated *H. alvei* 32 (OS$_3$2, pH ~ 8.5).$^a$

<table>
<thead>
<tr>
<th>Residue</th>
<th>H1/C1</th>
<th>H2/C-2</th>
<th>H3/C3</th>
<th>H4/C4</th>
<th>H5/C5</th>
<th>H6a, 6b/C6</th>
<th>H7a, 7b/C7</th>
<th>H8a, 8b/C8</th>
</tr>
</thead>
<tbody>
<tr>
<td>A →6)-α-D-GlcpN1P$^b$</td>
<td>5.48 / 94.0</td>
<td>3.00 / 56.4</td>
<td>3.74 / 73.2</td>
<td>3.45 / 71.3</td>
<td>4.08 / 73.3</td>
<td>3.81 / 4.19 / 70.3</td>
<td>(H3ax, H3eq)</td>
<td></td>
</tr>
<tr>
<td>B →6)-β-D-GlcpN-(1→</td>
<td>4.68 / 103.1</td>
<td>2.86 / 57.5</td>
<td>3.46 / 75.6</td>
<td>3.47 / 71.2</td>
<td>3.57 / 75.8</td>
<td>3.56 / 62.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C →4,5)-α-Kdo-(2→</td>
<td>nd</td>
<td>- / 101.0</td>
<td>(1.90, 2.21)/35.6</td>
<td>4.14 / 72.3</td>
<td>4.21 / 66.6</td>
<td>3.62 / 73.1</td>
<td>nd</td>
<td>3.56, 3.91 / 65.2$^c$</td>
</tr>
<tr>
<td>D α-Kdo-(2→</td>
<td>nd</td>
<td>- / 103.3</td>
<td>(1.76, 2.14)/36.6</td>
<td>4.16 / 67.1</td>
<td>4.05 / 63.1</td>
<td>3.65 / 73.3</td>
<td>nd</td>
<td>$^i$, 3.94 / 64.5$^c$</td>
</tr>
<tr>
<td>E →3)-L-glycero-α-D-manno-Hepp4P-(1→$^d$</td>
<td>5.22 / 100.0</td>
<td>4.01 / 72.5</td>
<td>4.03 / 78.2</td>
<td>4.32 / 70.4</td>
<td>4.26 / 70.6</td>
<td>4.03 / 70.4</td>
<td>$^i$ / 64.3</td>
<td></td>
</tr>
<tr>
<td>F →3,7)-L-glycero-α-D-manno-Hepp4P-(1→$^e$</td>
<td>5.16 / 103.8</td>
<td>4.43 / 71.3</td>
<td>4.05 / 79.4</td>
<td>4.34 / 69.2</td>
<td>3.74 / 65.4</td>
<td>4.27 / 68.9</td>
<td>3.65, 3.68 / 70.1</td>
<td></td>
</tr>
<tr>
<td>G L-glycero-α-D-manno-Hepp-(1→</td>
<td>4.89 / 101.7</td>
<td>3.95 / 71.5</td>
<td>3.85 / 72.2$^f$</td>
<td>3.84 / 67.6</td>
<td>3.64 / 67.0</td>
<td>nd</td>
<td>$^i$ / 64.5</td>
<td></td>
</tr>
<tr>
<td>H →3)-α-D-Glc-(1→</td>
<td>5.23 / 102.4</td>
<td>3.54 / 71.9</td>
<td>4.01 / 80.3</td>
<td>3.68 / 72.3</td>
<td>3.81 / 65.5</td>
<td>3.73, 3.89$^d$ / 62.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>h →3)-α-D-Glc-(1→$^h$</td>
<td>5.28 / 101.8</td>
<td>3.54 / 71.9</td>
<td>3.99 / 80.4</td>
<td>3.57 / 72.0</td>
<td>3.90 / 65.5</td>
<td>3.69, 3.91$^d$ / 61.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>J →2)-α-D-Glc-(1→</td>
<td>5.46 / 96.8</td>
<td>3.70 / 74.0</td>
<td>3.80 / 73.1</td>
<td>3.27 / 72.6</td>
<td>4.18 / 72.2</td>
<td>3.57, 3.93 / 62.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>j α-D-Glc-(1→$^i$</td>
<td>5.40 / 99.9</td>
<td>3.53 / 73.3</td>
<td>3.75 / 73.3</td>
<td>3.34 / 71.6</td>
<td>4.09 / 73.0</td>
<td>3.68, 3.91$^d$ / 62.2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>K →4,7)-α-Kdo-(2→</td>
<td>nd</td>
<td>- / 100.0</td>
<td>(1.88, 2.30)/34.5</td>
<td>4.20 / 72.3</td>
<td>4.33 / 64.0</td>
<td>4.39 / 71.2</td>
<td>3.94 / 78.9</td>
<td>3.73, 4.00 / 62.8</td>
</tr>
<tr>
<td>L L-glycero-α-D-manno-Hepp-(1→</td>
<td>5.08 / 98.7</td>
<td>3.94 / 71.6</td>
<td>3.85 / 71.1$^d$</td>
<td>3.82 / 67.6</td>
<td>3.65 / 73.2</td>
<td>nd</td>
<td>nd</td>
<td>nd / 64.5</td>
</tr>
<tr>
<td>M α-D-Galp-(1→</td>
<td>5.20 / 101.8</td>
<td>3.82 / 69.6</td>
<td>3.88 / 70.7</td>
<td>3.99 / 70.7</td>
<td>4.15 / 70.3$^j$</td>
<td>3.73$^j$ / 64.5$^j$</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Spectra were obtained for $^2$H$_2$O solutions at 30 °C. Acetone ($\delta_H$ 2.225, $\delta_C$ 31.05) was used as internal reference. *nd*, not determined.

$^b$ Residue substituted with a glycosidically linked phosphate group at O-1. The connectivity between the phosphate monoester peak at $\delta_P$ 1.8 ppm and H-1 and H-2 of residue A was deduced from an $^1$H, $^{31}$P HMQC experiment.

$^c$ Other values for $\delta_P$ 1.8 ppm.

$^d$ Other values for $\delta_P$ 1.8 ppm.

$^e$ Other values for $\delta_P$ 1.8 ppm.

$^f$ Other values for $\delta_P$ 1.8 ppm.

$^g$ Other values for $\delta_P$ 1.8 ppm.

$^h$ Other values for $\delta_P$ 1.8 ppm.

$^i$ Other values for $\delta_P$ 1.8 ppm.

$^j$ Other values for $\delta_P$ 1.8 ppm.
c) Tentative assignment in agreement with the reference (48).

d) Residue substituted with a phosphate group at O-4. The connectivity between the phosphate monoester peak at δ_2.8 ppm and H-4 of residue E was deduced from an \(^1\)H,\(^{31}\)P-HMQC.

e) Residue is substituted with a phosphate group at O-4. The connectivity between the phosphate monoester peak at δ_2.8 ppm and H-4 of residue F was deduced from an \(^1\)H,\(^{31}\)P-HMQC.

f) Not resolved.

g) Assignments of resonances could be interchanged.

h) Residue h represents a form of residue H in the outer core OS devoid of the trisaccharide fragment.

i) Residue j represents an unsubstituted form of residue J, terminal α-D-Glcp in the core OS devoid of the trisaccharide.

j) Tentative assignment.
TABLE 2. Selected inter-residue NOE and $^3J_{H,C}$ connectivities from the anomeric atoms of the dodecasaccharide OS$_{32}$ (pH ~ 8.5) isolated from de-N,O-acylated *H. alvei* 32 LPS.$^a$

<table>
<thead>
<tr>
<th>Residue</th>
<th>Atom Connectivities to Inter-residue atom/residue</th>
<th>$\delta_H / \delta_C$ (ppm)</th>
<th>$\delta_C$</th>
<th>$\delta_H$</th>
</tr>
</thead>
<tbody>
<tr>
<td>B $\to$6)-β-D-GlcpN-(1→</td>
<td></td>
<td>4.68</td>
<td>70.3</td>
<td>3.81, 4.19</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>C6, H-6a,b of A</td>
</tr>
<tr>
<td>C $\to$4,5)-α-Kdop-(2→</td>
<td>(H-3ax) 1.90</td>
<td>-</td>
<td>3.65*</td>
<td>H-6 of D</td>
</tr>
<tr>
<td></td>
<td>(H-3eq) 2.21</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E $\to$3)-L-glycero-α-D-manno-Hepp4P-(1→</td>
<td>5.22</td>
<td>69.7</td>
<td>4.21*</td>
<td>C-5, H-5 of C</td>
</tr>
<tr>
<td></td>
<td>100.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F $\to$3,7)-L-glycero-α-D-manno-Hepp4P-(1→</td>
<td>5.16</td>
<td>78.2</td>
<td>4.03</td>
<td>C-3, H-3 of E</td>
</tr>
<tr>
<td></td>
<td>103.8</td>
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<td></td>
<td></td>
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<tr>
<td>G L-glycero-α-D-manno-Hepp-(1→</td>
<td>4.89</td>
<td>70.1</td>
<td>3.65*, 3.69*</td>
<td>C-7, H-7a,b of F</td>
</tr>
<tr>
<td></td>
<td>101.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H $\to$3)-α-D-GlcP-(1→</td>
<td>5.23</td>
<td>-</td>
<td>4.07*</td>
<td>H-3 of F</td>
</tr>
<tr>
<td></td>
<td>102.4</td>
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<tr>
<td>h $\to$3)-α-D-GlcP-(1→</td>
<td>5.28</td>
<td>-</td>
<td>4.07</td>
<td>H-3 of F</td>
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<td>J $\to$2)-α-D-GlcP-(1→</td>
<td>5.46</td>
<td>-</td>
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<td>H-3 of H</td>
</tr>
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<tr>
<td>---</td>
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<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>j</td>
<td>α-D-Glcp-((1\rightarrow)^c)</td>
<td>5.40</td>
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<td>3.99</td>
</tr>
<tr>
<td>K</td>
<td>→⁴,⁷-α-Kdo-((2\rightarrow))</td>
<td>(C-2) 100.0</td>
<td>-</td>
<td>3.70</td>
</tr>
<tr>
<td>L</td>
<td>L-glycero-α-D-manno-Hepp-((1\rightarrow))</td>
<td>5.08</td>
<td>72.3</td>
<td>4.21*</td>
</tr>
<tr>
<td>M</td>
<td>α-D-Galp-((1\rightarrow))</td>
<td>5.20</td>
<td>79.0</td>
<td>3.94</td>
</tr>
</tbody>
</table>

676 a) The values marked with asterisks represent NOE connectivities only.

677 b) Residue h represents a form of residue H in the outer core OS devoid of the trisaccharide fragment.

678 c) Residue j represents an unsubstituted form of residue J, terminal α-D-Glc in the core OS devoid of the trisaccharide.
| Oligosaccharides                                      | Calculated mass (M) | Calculated mass (M) | Observed ion (m/z) | Interpreted ion (M – H₂O – H)⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻˓  | Interpreted ion (M – 3H)⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻˓ | Interpreted ion (M – 2H)⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻˓ | Interpreted ion (M – 2H)⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻˓ |
| GlcN₂•Kdo₂•Hep₁•Glc₂•P₃  •  Kdo•Hep•Gal ⁹ | 2494.63 | 1246.28 | [M – 2H]⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻˓ | 1246.31 |
| GlcN₂•Kdo₂•Hep₁•Glc₂•P₄  •  Kdo•Hep•Gal ⁹ | 2574.60 | 857.15  | [M – 3H]⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻˓ | 857.19 |
| GlcN₂•Kdo₁•Hep₁•Glc₂•P₂  •  Kdo•Hep•Gal ⁹ | 2194.61 | 1096.23 | [M – 2H]⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻нием | 1096.30 |
| Kdo₁•Hep₁•Glc₂•P₂  •  Kdo•Hep•Gal ⁹ | 1854.46 | 617.12  | [M – H₂O – H]⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻-Semitism | 617.14 |
| GlcN₂•Kdo₂•Hep₁•Glc₂•P₄ | 2000.43 | 999.18  | [M – 2H]⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻NegativeButton | 999.21 |
| GlcN₂•Kdo₂•Hep₁•Glc₂•P₃ | 1920.46 | 959.18  | [M – 2H]⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻˓ | 959.22 |
| Kdo•Hep•Gal ⁹ | 592.19 | 573.14  | [M – H₂O – H]⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻˓ | 573.16 |

⁹ Kdo•Hep•Gal – the trisaccharide.
TABLE 4. $^1$H and $^{13}$C NMR chemical shifts of the trisaccharide isolated from *H. alvei* 32 LPS. Spectra were obtained for $^2$H$_2$O solutions at 30 °C. Acetone ($\delta_1$ 2.225, $\delta_2$ 31.05) was used as internal reference. Residues are denoted by capital letters with asterisk to distinguish them from residues of the trisaccharide present in the dodecasaccharide.  

<table>
<thead>
<tr>
<th>Residue</th>
<th>Chemical shift [ppm]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H1/C1</td>
</tr>
<tr>
<td>K* $\rightarrow$4,7)-$\alpha$-Kdop</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td>nd</td>
</tr>
<tr>
<td>L* $\rightarrow$L-glycero-$\alpha$-D-manno-Hepp-(1$\rightarrow$5.08 / 97.9 (5.00 / 99.8) b</td>
<td>3.97 / 70.7</td>
</tr>
<tr>
<td>M* $\rightarrow$$\alpha$-D-Galp6OAc-(1$\rightarrow$5.19 / 101.0 (5.12 / 100.5) b</td>
<td>3.95 / 68.8</td>
</tr>
</tbody>
</table>

---

a) Since Kdo (residue K*) represented reducing end of the trisaccharide, resonances of the furanose isomer 4,7-disubstituted-$\alpha$-Kdof along with the effected spin systems belonging to constituents of L-$\alpha$-D-Hepp-(1$\rightarrow$4)-[$\alpha$-D-Galp6OAc-(1$\rightarrow$7)]-$\alpha$-Kdof were also identified in the NMR spectra of the trisaccharide.

b) Chemical shift values of the residue substituting $\rightarrow$4,7)-$\alpha$-Kdof. All other resonances were not effected.

c) Long-range HMBC couplings between H-6 of M ($\delta_1$ 4.25, 4.31 ppm), the acetyl carbonyl carbon ($\delta_2$ 174.5 ppm) and the acetyl methyl protons ($\delta_1$ 2.13 ppm) supported the position of the O-acetyl group.
<table>
<thead>
<tr>
<th>Residue</th>
<th>Atom</th>
<th>Connectivities to</th>
<th>Inter-residue atom/residue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H1 / C1 (ppm)</td>
<td>δC</td>
<td>δH</td>
</tr>
<tr>
<td>M*</td>
<td>5.19 / 101.0</td>
<td>78.6</td>
<td>3.91</td>
</tr>
<tr>
<td>α-D-Galp6OAc-(1→</td>
<td>5.12 / 100.5</td>
<td>80.5</td>
<td>3.68</td>
</tr>
<tr>
<td>L*</td>
<td>5.08 / 97.9</td>
<td>71.7</td>
<td>4.12</td>
</tr>
<tr>
<td>L-glycero-α-D-manno-Hepp-(1→</td>
<td>5.00 / 99.8</td>
<td>78.9</td>
<td>4.49</td>
</tr>
</tbody>
</table>

a) Chemical shifts of anomeric atoms and $^3J_{\text{H,C}}$ connectivities characteristic for constituents of the trisaccharide L-α-D-Hepp-(1→4)-[α-D-Galp6OAc-(1→7)]-α-Kdo, containing the furanose form of Kdo.
L-α-D-Hepp

α-D-GlcPS- (1→3)-α-D-GlcP- (1→3)-L-α-D-Hepp4
R4 (1→3)-L-α-D-Hepp4
R3 (1→5)-α-Kdop- (2→6)-β-D-GlcP-NR2- (1→6)-α-D-GlcP-N1P

R1 = α-Kdop
R2 = H
R3, R4 = P
(m/z 1246.28, m/z 830.5)

R1 = H
R2 = H
R3 or R4 = P
(m/z 1096.23, m/z 730.5)

R1 = α-Kdop
R2 = P
R3, R4 = P
(m/z 999.18, m/z 665.80)