Characterization of the carbohydrate backbone of *Vibrio parahaemolyticus* O6 lipopolysaccharides

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Abstract

Structural characterization studies have been carried out on the carbohydrate backbone of *Vibrio parahaemolyticus* serotype O6 lipopolysaccharides (LPS). The carbohydrate backbone isolated from O6 LPS by sequential derivatization, i.e., dephosphorylation, *O*-deacylation, pyridylamination, *N*-deacylation and *N*-acetylation, is a nonasaccharide consisting of 3 mol of D-glucosamine (GlcN) (of which one is pyridylaminated), 2 mol of L-*glycero*-D-*manno*-heptose (Hep), and 1 mol each of D-galactose (Gal), D-glucose (Glc), D-glucuronic acid (GlcA) and 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo). Structural analyses by NMR spectroscopy and fast-atom bombardment mass spectrometry (FABMS) demonstrated that the carbohydrate backbone is β-Gal*p*-(1→2)-α-Hep*p*-(1→3)-α-Hep*p*-(1→5)-α-Kdo*p*-(2→6)-β-Glc*p*NAc-(1→6)-GlcNAc-PA, in which the 3-substituted α-Hep*p* is further substituted by β-Glc*p*NAc-(1→4)-β-Glc*p* at position 4 and by β-Glc*p*A at position 2. In native O6 LPS, an additional 1 mol of D-galacturonic acid, which is liberated by dephosphorylation in hydrofluoric acid, is present at an unknown position. Our previous report described that of 13 O-serotype LPS of *V. parahaemolyticus*, the only LPS from which Kdo was detected was from O6 LPS after mild acid hydrolysis. In the present study, we demonstrate that only 1 mol of Kdo is present at the lipid A proximal position, which is common component among the LPS in all serotypes of the bacterium, and that no additional Kdo is present in the carbohydrate backbone of O6 LPS. Enzyme-linked immunosorbent assay (ELISA) and ELISA inhibition analysis using antisera against O6 and *Salmonella enterica* Minnesota R595 and LPS of both strains further revealed that Kdo is not involved as an antigenic determinant of O6 LPS.

Key words: lipopolysaccharides, *Vibrio parahaemolyticus* O6, polysaccharide, structural analysis

Introduction

A halophilic marine bacterium, *Vibrio parahaemolyticus,* is a causative agent of a form of food poisoning mainly associated with seafood. This bacterium is serologically divided into 13 O-serotypes based on differences in serological properties of the cell surface somatic antigen (O-antigen), i.e., the lipopolysaccharides (LPS) (1, 2). All O-serotypes of *V. parahaemolyticus* produce LPS belonging to the type of lipooligosaccharides (LOS) that lack a high-molecular-weight O-specific polysaccharide chain. Instead, these LOS have a short carbohydrate chain corresponding to a core oligosaccharide typical of gram-negative bacterial LPS bound to the lipid A moiety (3). The LPS of *V. parahaemolyticus* exhibit their own antigenic specificities, although their molecular construction is chemically similar to that of rough-type (R-type) LPS. Therefore, in the case of *V. parahaemolyticus* LPS, the structure of the short carbohydrate chain is responsible for their serological specificities which determine the O-serotype of the parental bacterial cells (4, 5).

An earlier report (6, 7) described that *V. parahaemolyticus* O6, O7 and O12 LPS are positive in the periodate-2-thiobaribituric acid (TBA) reaction for 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) after mild acid hydrolysis. However, Kdo is determined only in hydrolysate of O6 LPS; the TBA reaction-positive substance detected in LPS from O7 and O12 LPS was later identified as 3-deoxy-D-*threo*-hex-2-ulosonic acid (8). Another report described that a Kdo phosphorylated at position 4, which might be present at the lipid A proximal position binding the distal part of the carbohydrate portion of LPS, is present in LPS of all O-serotypes of *V. parahaemolyticus* (9). As well known, the Kdo inter-linked between polysaccharide and lipid A moieties is not liberated by mild acid hydrolysis because of the presence of glycosidic linkage of the polysaccharide portion to the Kdo. These earlier investigations suggest that O6 LPS might have an inner core region (Kdo region) with a unique chemical structure; i.e. branched Kdo binding to the carbohydrate residue via acid labile ketosidic linkage, that differs from that of the other serotypes of LPS. These findings suggested that the Kdo detected in O6 LPS might be

immune-dominant constituent of O6 LPS. In the present study, we characterized the carbohydrate backbone of O6 LPS as part of a larger investigation to clarify the chemical structures of the carbohydrate backbones from LPS of all 13 O-serotypes of the vibrios. In addition, we aimed to clarify whether an additional Kdo is a constituent of the carbohydrate backbone of O6 LPS.

Materials and Methods

Bacterial strains and LPS

The strains of *V. parahaemolyticus* used were O6:K18 (V86-129), O6:K18 (Pilot), O6:K46 (AQ4618) and O6:KUT; these strains were grown in nutrient broth supplemented with 3% NaCl at 37˚C for 12 hr with aeration. *Salmonella enterica* Minnesota R595 was cultivated in nutrient broth at 37˚C for 16 hr with aeration. The bacterial cells were killed by exposure to high heat, harvested by centrifugation, then were washed successively with water, ethanol, acetone and diethylether, and dried. LPS of *V. parahaemolyticus* were extracted by the hot phenol/water extraction technique (10) and, after sequential digestion with DNase, RNase and proteinase K, the LPS were recovered and washed with distilled water by repeated ultracentrifugation. LPS of *S. enterica* Minnesota R595 were extracted from dried cells with phenol/chloroform/petroleum ether (2:5:8) (11) and purified as described previously (12).

Preparation of chemically modified LPS and the carbohydrate backbone of O6 LPS

The carbohydrate portion (PS) of LPS was prepared by hydrolysis of O6:K18 (V86-129) LPS in 5% acetic acid at 100˚C for 2.5 hr, followed by gel chromatography using a column (2.5 x 100 cm) of Sephadex G-25 superfine (GE Healthcare UK Ltd., Buckinghamshire, England) and pyridine/acetic acid/water (8:5:2000) as the eluting solvent. The dephosphorylated carbohydrate portion (HF-PS) of LPS was isolated from dephosphorylated (50% hydrofluoric acid, 4˚C for 48 hr) O6 LPS by hydrolysis in 5% acetic acid at 100˚C for 2.5 hr, and HF-PS was separated by G-25 gel-chromatography as described above. Deacylated LPS (dAc-LPS) were prepared according to a published method (13) by treatment of O6 LPS with anhydrous hydrazine at 37˚C for 1 hr (*O*-deacylation), followed by heating in 4 M KOH at 100˚C for 16 hr (*N*-deacylation) and then *N*-acetylation. The dAc-LPS were fractionated by G-25 gel chromatography as above.

Deacylated, dephosphorylated, pyridylaminated and *N*-acetylated carbohydrate backbone

(PSPA) was prepared as follows: LPS (1550 mg) of O6:K18 (V86-129) were *O*-deacylated and dephosphorylated as described above. Pyridylamination of the *O*-deacylated and dephosphorylated LPS (dAcP-LPS) was carried out basically according to the method of Kondo and co-workers (14); dAcP-LPS (665 mg) was pyridylaminated in 6.8 ml of a reagent [1.38 g of 2-aminopyridine (Wako Pure Chemicals, Osaka, Japan) dissolved in 1.0 ml of glacial acetic acid (Wako Pure Chemicals)] at 90˚C for 60 min. After cooling to room temperature, 6.8 ml of reducing reagent [4.0 g of dimethylamine-borane (Wako Pure Chemicals) dissolved in 1.6 ml of glacial acetic acid and 1.0 ml of distilled water)] was added to the solution and heated at 85˚C for 35 min. The reaction mixture was chilled in an ice bath and the pH was adjusted to pH 10 by adding 28% NH_4OH dropwise; after dialysis against distilled water, the reaction mixture was freeze-dried (yield: 664 mg). The product was *N*-deacylated in 26 ml of 4 M KOH at 100˚C for 16 hr. The solution was chilled and acidified by adding 4 M or 1 M HCl in an ice bath, and the released fatty acids were extracted with $CHCl_3$. The *O*-deacylated, dephosphorylated, pyridylaminated and *N*-deacylated carbohydrate backbone of LPS recovered by G-25 gel-chromatography as described above was *N*-acetylated (14) and fractionated by G-25 (water, 1.5 x 50 cm) gel-chromatography to yield PSPA (231 mg). The PSPA was further purified by high-performance liquid chromatography (HPLC) (HITACHI L-6000 system) (HITACHI, Tokyo, Japan) using a CAPCELL PACK (Shiseido, Tokyo, Japan) (1 x 25 cm) column and 0→ 5% gradient (45 min) of methanol in 20 mM $NH_4H_2PO_4$ as the eluting solvent; the eluent was monitored by UV (HITACHI L-400 UV-VS-Detector). The two major products were individually collected, desalted by G-25 gel-chromatography (water, 1.5 x 50 cm), and freeze-dried (PSPA-A: 46 mg; PSPA-B: 37 mg).

Analytical methods

Neutral and amino sugars were analyzed by GC and GC-MS as previously described. Uronic acid, Kdo and phosphorous were estimated by a colorimetric assay (15). The absolute

configuration of each component sugar was determined by GC-MS of their peracetylated (*S*)-(+)- and (*R*)-(-)-2-butylglycosides (16). Methylation analysis of HF-PS was performed according to the method of Hakomori (17). The permethylated HF-PS was hydrolyzed in 2 M TFA, reduced with $NaBH_4$, peracetylated and carboxy-methylated with diazomethane, and analyzed by GC and GC-MS.

GC and GC-MS spectrometry

GC was carried out using a GC-14A gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a fused silica capillary column coated with DB210 (J&W Scientific, Folsom, CA) or HR52 (Chromato Packing Center, Kyoto, Japan) with a temperature program of 180˚C (3 min) raised to 240˚C at 5˚C/min for neutral and amino sugar analysis (DB210), and150˚C (3 min) to 320˚C at 5˚C/min for partially acetylated alditol acetates (HR52). GC-MS was performed on a JMS-700 (JEOL, Tokyo, Japan) instrument using the same column as used in GC.

FABMS spectrometry

FAB mass spectra were recorded in negative-ion mode on a JMS-700 (JEOL) instrument. Samples were dissolved in 0.1% TFA (30 mg/ml) and CH_3CN was added to give a final concentration of 25% (v/v). Glycerol was used as a matrix.

NMR spectroscopy

NMR spectra were recorded on a JEOL A-500 spectrometer using standard JEOL software. The lyophilized samples were dissolved in D_2O. Measurements (25˚C) were made at 500.0 MHz and 125.7 MHz for ^{1}H and ^{13}C atoms, respectively, and chemical shifts were referenced to the methyl resonances of internal acetone at 2.225 ppm for ^{1}H and 30.07 ppm for ^{13}C. The assignments of spectra were made with the help of COSY, TOCSY, NOESY, C/H-HMQC and C/H-HMBC experiments.

Serological methods

V. parahaemolyticus O6-specific rabbit antiserum (non-diluted diagnostic antiserum that specifically reacts with O6 heat-killed cells) was obtained from Denka-Seiken Co., Ltd. (Tokyo, Japan), and serum against *S. enterica* Minnesota R595 was prepared by immunizing rabbits with heat-killed whole cells (18). ELISA and ELISA inhibition tests were performed as described previously (19). In ELISA experiments, *V. parahaemolyticus* O6 V86-129 and *S. enterica* Minnesota R595 LPS were used as coated antigens (500 ng/well). Maxi-sorp U-bottom microplates (Nalge Nunc International, Roskilde, Denmark) coated with antigen were washed with 150 mM NaCl containing 15 mM Na_2HPO_4 (pH 7.2, NaCl/Pi) and blocked with NaCl/Pi containing 2.5% casein (casein-NaCl/Pi). After removal of the blocking solution, serial two-fold dilutions of homologous and heterologous antiserum (diluted 10-fold with casein-NaCl/Pi) were added and incubated at 37˚C for 1 hr. After washing with NaCl/Pi, a casein-NaCl/Pi solution of peroxidase-conjugated goat anti-rabbit IgG H+L chains (Southern Biotechnology Associates, Birmingham, AL) was added and the plate was incubated at 37˚C for 1 hr. After washing with NaCl/Pi following by 100 mM sodium citrate buffer (pH 4.5), 2,2'-azino-bis(3-ethylbenzthiazolinesulfonic acid) diammonium salt (Sigma Aldrich, St. Louis, MO) and H_2O_2 were added to each well and the optical density of the reaction with peroxidase was read at 405 nm. The end point titers were taken at $A_{405} > 0.4$ as the reciprocal of the highest dilution of antiserum. For the inhibition tests the concentration of inhibitor LPS causing 50% inhibition of the reaction using diluted anti-serum to give A_{405} 1.0 in ELISA was expressed as the 50% inhibition dose.

Results

Chemical analysis of O6 LPS and chemically modified LPS

Table 1 shows the sugar composition of LPS from four *V. parahaemolyticus* O6 strains determined in this study, as well as compositions given in an earlier report (4). The component sugars D-galactose (Gal), D-glucose (Glc), L-*glycero*-D-*manno*-hepotose (Hep), uronic acid [D-glucuronic (GlcA) and D-galacturonic acid (GalA)], D-glucosamine (GlcN) and Kdo were detected in all O6 LPS, although their relative concentrations differed depending on the strain from which the LPS were derived.

Methylation analysis (Table 2) of HF-PS revealed the presence of the following pyranosidic sugar residues: terminal Gal, GlcN and GlcA, 4-substituted Glc, 2-substituted Hep, and 2,3,4-substituted Hep. No partially methylated derivative of GalA was detected since these compounds would have been removed during dephosphorylation of LPS in hydrofluoric acid.

FABMS of dAc-LPS and PSPA

FABMS of O6 dAc-LPS (negative-ion mode) (Fig. 1) showed an ion peak at *m/z* 2050.4, corresponding to the calculated molecular weight (2051 Da) of phosphorylated nonasaccharide consisting of 3 mol of *N*-acetyl-D-glucosamine (GlcNAc), 2 mol of Hep, 1 mol each of Gal, Glc, GlcA and Kdo, and four phosphate groups. The ion peaks at *m/z* 1970.5, 2130.4 and 2215.4 were assigned to the nonasaccharide carrying three phosphate groups, five phosphate groups, and one ethanolamine in addition to five phosphate groups, respectively. No ion peak suggesting the presence of an additional Kdo in dAc-LPS was detected. Since *O*-deacylation of LPS with anhydrous hydrazine and KOH does not release a ketosidically binding Kdo residue, we assumed that, in O6 LPS, only one Kdo molecule is present in the carbohydrate backbone.

In the FAB mass spectrum (Fig. 2) of PSPA before purification by HPLC, two major ion peaks, at *m/z* 1605.6 and 1809.6, were detected. These peaks correspond to the calculated molecular weights of PSPA-A (pyridylaminated octasaccharide, 1606 Da) and PSPA-B

(pyridylaminated nonasaccharide, 1809 Da). FABMS of purified PSPA-A and PAS-B demonstrated that the ion peaks at *m/z* 1605.6 and 1809.6 in the respective mass spectra (data not shown) were the only major ion peaks in this mass range. Since FABMS of dAc-LPS demonstrated that 3 mol of GlcNAc were involved in the carbohydrate backbone of O6 LPS, PSPA-A must be a byproduct of PSPA-B generated by the loss of 1 mol GlcNAc.

NMR spectroscopy of PSPA

^{1}H spectra of purified PSPA, PSPA-A and PSPA-B are shown in Fig. 3, and the corresponding ^{13}C-NMR spectra are shown in Fig. 4. The signals observed in the spectra were assigned with the help of COSY, TOCSY, C/H HMQC and C/H HMBC experiments, and are summarized in Table 3 and 4. The ^{1}H-NMR and COSY spectra of PSPA-A confirmed the presence of 7 ring-systems. The ring systems gave rise to two anomeric proton signals at δ 5.127 and 5.172. The rings were assigned a *manno*-configuration on the basis of the small J_{H1-H2} and J_{H2-H3} values (≈ 1.0 Hz) and large J_{H3-H4} (10.3 Hz). Moreover the C-1 signals (δ 99.27 and 101.99) showed large J_{C1-H1} values (171 and 172 Hz, respectively) in proton coupled ^{13}C-NMR and C/H HMBC (Fig. 5) experiments, indicating that the two ring systems are attributed to α-Hep residues (referred to here as Hep-I and Hep-II, in the order of their chemical shift values in the ^{1}H-NMR spectrum). The three ring systems with anomeric protons at δ 4.423, 4.451 and 4.574, were assigned a *gluco*-configuration, as these anomeric protons showed large J_{H1-H2} values (7.7-8.4 Hz). These data, together with the chemical shifts of carbon signals attributed to three residues observed in the ^{13}C-NMR spectra, lead to these ring systems being assigned to β-GlcA, β-GlcNAc and β-Glc. The ring protons included an anomeric proton (δ 4.230) that showed large J_{H1-H2}, J_{H2-H3} (8.0-8.8 Hz) and small J_{H3-H4} and J_{H4-H5} (< 1.7 Hz) values, indicating that the ring system is in a β-Gal configuration. The remaining ring system had no anomeric proton; this ring consisted of 8 carbons, and according to the C/H HMQC and C/H HMBC experiments, carried a carboxyl group at position 1 and a methylene group at position 3. These

data strongly suggest that this ring system is attributed to a Kdo residue. The triplet and doublet-doublet signals assigned to H-3ax and H-3eq of Kdo (δ 1.882 and 2092, respectively) are characteristic of a pyranose ring-form having an α-D-configuration (20, 21, 22). In the high-field portion of the ^{1}H and ^{13}C-NMR spectra of PSPA-A, 4 proton signals (δ 6.913-7.922) and 5 carbon signals (δ 113.87-154.02) were observed. The chemical shift values and *J* coupling of the signals clearly demonstrated that these signals arise from a pyridylamino (PA) group. A C/H HMBC experiment clearly showed cross-peaks between C-2 of PA (δ 153.90) and methylene protons at position 1 of a 2-amino-hexitol residue. The data suggest that the GlcN residue, which is localized at the reducing terminus of the LPS, is labeled with a PA group (GlcNAc-PA). These data showed that the PSPA-A prepared from the LPS of *V. parahaemolyticus* O6 is an octasaccharide consisting of 2 mol each of Hep and GlcNAc, and 1 mol each of Glc, GlcA, Gal and Kdo. In addition, one of the GlcNAc residues is labeled with a PA group at a reducing terminus of the polysaccharide.

To confirm the sequence of octasaccharide PSPA-A, C/H HMBC and NOESY experiments were performed. In the C/H HMBC spectrum (Fig. 5), inter-residue long-range couplings were observed between C-1 of Hep-I and H-5 of Kdo, C-1 of Hep-II and H-3 of Hep-I, C-1 of Glc and H-4 of Hep-I, C-1 of GlcNAc and H-4 of Glc, C-1 of Gal and H-2 of Hep-II, and C-2 of Kdo and H-6b of GlcNAc-PA. Inter-residue NOE observed in the NOESY spectrum (Fig. 6 and Table 5) strongly supported the sequence of PSPA-A suggested by the C/H HMBC experiment.

On the other hand, the ^{1}H-NMR (Table 3) and COSY spectrum of PSPA-B clearly demonstrated the presence of one additional ring system compared with PSPA-A. The ring system characteristic of PSPA-B had an anomeric proton with a large $J_{H1\text{-}H2}$ (8.0 Hz) value and was assigned a *gluco* configuration. In ^{13}C-NMR (Table 4) and C/H HMQC spectra of PSPA-B, the chemical shift of C-2 was observed at δ 55.98, indicating that the signals arise from a second β-GlcNAc (GlcNAc-II). Most of the long-range couplings observed in PSPA-A were also present in the C/H HMBC spectrum of PSPA-B, with the exception of that between C-2 of Kdo

and H-6b of GlcNAc-PA. In the C/H HMBC of PSPA-B, inter-residue long-range couplings were observed between C-1 of GlcNAc-II and H-6a/b of GlcNAc-PA, and C-2 of Kdo and H-6b of GlcNAc-II. In the NOESY spectrum of PSPA-B (Fig. 6 and Table 5), inter-residue NOE between H-1 of GlcNAc-II and H-6a/b were observed in addition to that of PSPA-B. These results show that PSPA-B is a nonasaccharide consisting of 3 mol of GlcNAc, 2 mol of Hep, and 1 mol each of Glc, GlcA, Gal and Kdo; in addition, one of the GlcNAc residues is labeled with a PA group at a reducing terminus of the polysaccharide (Fig. 7). Thus the carbohydrate backbone of *V. parahaemolyticus* O6 LPS is absent from the second Kdo which linked via ketosidic linkage giving positive TBA reaction after mild acid hydrolysis of the LPS. As mentioned above, the FABMS spectrum of dAc-LPS demonstrated that there are 3 mol of GlcNAc in the carbohydrate backbone of O6 LPS. The NMR data clarified that the nonasaccharide obtained as PSPA-B is the carbohydrate backbone of *V. parahaemolyticus* O6 LPS, and that PSPA-A is a byproduct of PSPA-B generated by the loss of 1 mol of GlcNAc. In the previous studies, the structural elucidations of carbohydrate backbone of the LPS were performed on *V. parahaemolyticus* O2 (15) and KX-V212 (O-untypeable strain barring a common O-antigen with O2) (23) strains. In both cases, reducing terminal GlcN of GlcN disaccharide of the lipid A backbone was partially released in dephosphorylation (50% hydrofluoric acid, 4˚C for 48 hr) process, although the reason why the β-(1→6)-linkage in lipid A backbone was cleaved under the mild acidic condition is remaining unclear.

ELISA and ELISA inhibition analysis

The contribution of Kdo to the serological specificity of O6 LPS was examined by ELISA and ELISA-inhibition tests, using LPS from O6 and *S. enterica* Minnesota R595 and antiserum against O-antigen of both strains. The results showed that anti-O6 and anti-R595 antiserum reacted with homologous O6 and R595 LPS at high dilutions (400 and 240, respectively), whereas marked cross-reactivity of these two antisera with heterologous LPS was not observed.

ELISA-inhibition analysis was carried out using O6 LPS and chemically modified O6 LPS as inhibitors against a reaction system containing O6 LPS / anti-O6 antiserum. If an additional ketosidically binding Kdo was present in the carbohydrate portion of O6 LPS, it would be present in the dAc-LPS but not in dAcP-LPS or PS. The 50% inhibition values of the chemically modified O6 LPS were very high (> 110 μg/ml) compared with that of native O6 LPS (3.8 μg/ml). *S. enterica* Minnesota R595 strain is an Re-mutant, that produces LPS composed of α-Kdo-(2→4)-α-Kdo-(2→6)-lipid A, in which terminal Kdo residue plays an important role as an immuno-dominant epitope (24). These results therefore demonstrate that Kdo is not involved as an immunological epitope that determines the serological specificity of O6 LPS.

Discussion

Of 13 O-serotype LPS of *V. parahaemolytius*, O6 LPS was the only LPS in which Kdo was detected by the TBA reaction and high-voltage paper electrophoresis (6). In the present study, it was demonstrated that one mole of Kdo is present in the carbohydrate backbone of O6 LPS connecting the lipid A moiety and carbohydrate part of the LPS; however, no evidence for the presence of an additional Kdo was obtained. *V. parahaemolyticus* O6 produces K-antigen (K18, K46 or KUT), depending on the strain. These K-antigens (or extracellular polysaccharides) prepared from a culture of O6 strain contain Kdo (unpublished data); therefore, the detection of Kdo in O6 LPS might be due to contamination with K-antigens or extracellular polysaccharides that could not be removed during purification of O6 LPS. Our conclusion is that O6 LPS do not contain an additional, ketosidically binding Kdo.

All O-serotypes of *V. parahaemolyticus* produce LPS composed of lipid A and low-molecular-weight carbohydrate chains (3). The structures of these carbohydrate chains contribute to determining their serological specificities (4, 5). Out of 13 O-serotype LPS of *V. parahaemolyticus*, the structures of the carbohydrate chains have been elucidated for only two O-serotypes: O2 is a nonasaccharide (15), and O12 is a decasaccharide (25). Native O6 and O12 LPS contain GalA, and O12 contains an additional 3-deoxy-D-*threo*-hex-2-ulosonic acid with an acid labile linkage (7, 8). In the present study, the structure of the carbohydrate backbone of O6 LPS was characterized to be a nonasaccharide (Fig. 8). Acid-labile GalA was also present in the native O6 LPS. The structures of the carbohydrate backbones of O6 and O12 LPS share the same partial structure

β-Gal*p*-(1→2)-α-Hep*p*-(1→3)-α-Hep*p*-(1→5)-α-Kdo*p*-(2→6)-β-Glc*p*NAc-(1→6)-Glc*p*NAc

carrying β-Glc*p* and β-Glc*p*A at the 4 and 2 positions, respectively, of the α-Hep residue proximal to α-Kdo. The carbohydrate backbone of O6 LPS carries β- Glc*p*NAc at position 4 of the β- Glc*p* residue, in contrast to

β-3-acetamido-3,6-dideoxy-D-glucopyranose-(1→3)-β-Gal*p*NAc disaccharide in the carbohydrate backbone of O12 LPS. Since a marked antigenic cross-reactivity is not apparent between O6 and O12 LPS, these structural variations might play a role as a dominant epitope(s) responsible for determining the serological specificities of O-serotypes O6 and O12.

Most of LPS from strains belonging to *Enterobacteriaceae* and *Vibrionaceae* with exception of *V. parahaemolyticus* (26, 27) carry 3 mol or more Hep in the core region of their LPS. In contrast, *V. parahaemolyticus* O6 and O12 LPS share in common partial structure α-Hep*p*-(1→3)-α-Hep*p*-(1→5)-α-Kdo*p* which consists of 2 mol of Hep. The similar structure has been reported for LPS from bacteria belonging to *Neisseria gonorrhoeae* (28, 29), *N. meningitidis* (30, 31), *Pseudomonas aeruginosa* (32) and *P. syringae* (33) to date. It is further interesting that bacterial species in genus *Neisseria* produce LOS type low molecular weight LPS. Also, among these bacteria, heterogeneity of the sugar components of the carbohydrate moiety of the LPS was found, reflecting the serological specificity of these bacteria (29, 34).

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Figure legends

Fig.1 FAB mass spectrum (negative mode) of *O*- and *N*-deacylated LPS (dAc-LPS) from *V. parahaemolyticus* O6.

The dominant ion peak at *m/z* 2050.4 was attributed to a nonasaccharide carrying 4 mol of phosphate (P) groups. The ion peaks at *m/*z 1970.5, 2130.4 and 2215.4 were respectively assigned to nonasaccharide carrying three phosphate groups, five phosphate groups, and one *N*-acetylated ethanolamine (EtNAc) in addition to five phosphate groups.

Fig.2 FAB mass spectrum (negative mode) of the carbohydrate backbone (PSPA) from *V. parahaemolyticus* O6.

Two major ion peaks at *m/z* 1605.6 and 1809.6 were detected, and corresponded to the calculated molecular weights of PSPA-B (pyridylaminated nonasaccharide, 1809 Da) and of PSPA-A (pyridylaminated octasaccharide, 1606 Da). PSPA-A lacked 1 mol of GlcNAc residue found in PSPA-B.

Fig. 3 ^{1}H-NMR spectra (500.0 MHz) of the carbohydrate backbone, PSPA-A (a) and PSPA -B(b), prepared from *V. parahaemolyticus* O6 lipopolysaccharides.

Fig. 4 ^{13}C-NMR spectra (125.7 MHz) of the carbohydrate backbone, PSPA-A (a) and PSPA -B(b), prepared from *V. parahaemolyticus* O6 lipopolysaccharides.

Fig. 5 Part of the C/H HMBC spectra of the carbohydrate backbone, PSPA-A (a) and PSPA -B(b), prepared from *V. parahaemolyticus* O6 lipopolysaccharides.

Fig. 6 Part of the NOESY spectra of the carbohydrate backbone, PSPA-A(a) and

PSPA-B(b), prepared from *V. parahaemolyticus* O6 lipopolysaccharides.

Fig. 7 Structure of the carbohydrate backbone (PSPA-B) of *V. parahaemolyticus* O6 lipopolysaccharides.
PSPA-A is a octasaccharide derived from PSPA-B by lack of GlcNAc-II residue.

Fig. 8 Proposed structure of the carbohydrate backbone of *V. parahaemolyticus* O6 lipopolysaccharides.

Table 1 Sugar composition of lipopolysaccharides (LPS) isolated from *V. parahaemolyticus* serotype O6 possessing different K-antigens.

Component sugars	V89-129 (O6:K18)	AQ4618 (O6:K46)	O6:KUT†	Pilot (O6:K18)	Pilot‡ (O6:K18)
D-Galactose	49 (0.1)	88 (0.7)	92 (0.5)	30 (0.2)	22 (0.1)
D-Glucose	469 (2.5)	281 (2.1)	212 (1.1)	410 (1.9)	305 (1.3)
L-*Glycero*-D-*manno*-heptose	197 (1.0)	225 (1.7)	224 (1.1)	400 (1.9)	376 (1.7)
Uronic acid (D-Glucuronic acid and D-Galacturonic acid)	323 (1.7)	384 (2.8)	492 (2.5)	350 (1.7)	196 (0.9)
D-Glucosamine	371 (2.0)	263 (2.0)	389 (2.0)	420 (2.0)	453 (2.0)
3-Deoxy-D-*manno*-octo-2-ulosonic acid	23 (0.1)	193 (1.4)	176 (0.9)	110 (0.5)	160 (0.7)
Phosphate	1219 (6.5)	1204 (9.0)	1191 (6.0)	1360 (6.5)	nd§

Values are expressed as nmol/mg of LPS. Values in parentheses are molar ratios (D-Glucosamine: 2.0).

† KUT: K-antigen untypeable

‡ Data are quoted from previous report (4).

§ nd: not determined

Table 2 Partially methylated alditol acetates detected by methylation analysis of the dephosphorylated carbohydrate portion of *V. parahaemolyticus* O6 lipopolysaccharides.

Partially methylated alditol acetates	Position of substitution	Molar ratio†
1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-galactitol	-	1.2
methyl-(1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl)-gulonate	-	1.0
1,5-di-*O*-acetyl-(2-methylacetamido)-3,4,6-tri-*O*-methyl-2-deoxy-glucitol	-	0.8
1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-glucitol	4	0.8
1,2,5-tri-*O*-acetyl-3,4,6,7-tetra-*O*-methyl-L-*glycero*-D-*manno*- heptitol	2	0.8
1,2,3,4,5-penta-*O*-acetyl-6,7-di-*O*-methy-L-*glycero*-D-*manno*-heptitol	2, 3, 4	1.3

† molar ratios: methyl-(1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl)-gulonate =1.0

Table 3 ^{1}H-NMR data for the carbohydrate backbone (PSPA-A and PSPA-B) of *V. parahaemolyticus* O6 lipopolysaccharides.

Sugar residues	Proton atom (ppm)							
	1 a/b	2	3ax/eq	4	5	6 a/b	7 a/b	8 a/b
PSPA-A								
Hep-I	5.172	4.153	4.185	4.143	4.105	3.686	3.718	
							3.750	
Hep-II	5.127	4.217	3.895	3.742	3.754	4.068	3.521	
							3.694	
Glc	4.574	3.271	3.638	3.324	3.503	3.674		
						3.807		
GlcNAc	4.451	3.726	3.549	3.424	3.477	3.714		
						3.887		
GlcA	4.423	3.396	3.501	3.585	3.738			
Gal	4.230	3.529	3.626	3.887	3.537	3.730		
						3.819		
GlcNAc-PA	3.493	4.254	3.948	3.597	3.819	3.489		
	3.650					3.505		
Kdo			1.882	4.161	4.105	3.714	3.754	3.678
			2.092					3.920
PA			7.821	6.913	7.922	7.057		
PSPA-B								
Hep-I	5.171	4.143	4.157	4.202	4.111	3.679	3.708	
							3.733	
Hep-II	5.117	4.223	3.899	3.717	3.787	4.086	3.521	

Table 3 continued

							3.700	
Glc	4.573	3.272	3.642	3.314	3.513	3.675		
						3.800		
GlcNAc-I	4.510	3.725	3.550	3.430	3.422	3.717		
						3.887		
GlcNAc-II	4.493	3.708	3.509	3.451	3.517	3.585		
						3.604		
GlcA	4.406	3.461	3.496	3.579	3.671			
Gal	4.223	3.534	3.625	3.887	3.534	3.737		
						3.825		
GlcNAc-PA	3.501	4.186	3.978	3.567	3.795	3.704		
	3.650					4.020		
Kdo			1.882	4.157	4.120	3.721	3.762	3.654
			2.113					3.962
PA			7.831	6.917	7.931	7.074		

N-Ac signals: 1.963 and 2.051 for PSPA-A; 1.951, 2.011, and 2.054 for PSPA-B

Abbreviations: Hep; L-*glycero*-D-*manno*-heptose, Glc; D-glucose, GlcNAc; *N*-acetyl-D-glucosamine, GlcA; D-glucuronic acid, Gal; D-galactose, GlcNAc-PA; pyridylamino labeled *N*-acetyl-D-glucosamine, Kdo; 3-deoxy-D-*manno*-oct-2-ulosonic acid.

Table 4 ^{13}C-NMR data for the carbohydrate backbone (PSPA-A and PSPA-B) of *V. parahaemolyticus* O6 lipopolysaccharides.

Sugar residues	Carbon atom (ppm)							
	1	2	3	4	5	6	7	8
PSPA-A								
Hep-I	99.27	78.79	75.10	74.02	72.35	72.19	63.87	
Hep-II	101.99	78.67	70.92	68.04	72.54	68.90	64.24	
Glc	103.07	74.27	78.03	80.99	76.64	62.19		
GlcNAc	102.29	56.51	74.31	70.80	77.18	61.64		
GlcA	102.79	73.20	76.07	72.59	77.79	175.88		
Gal	104.05	71.36	72.81	69.40	76.64	62.02		
GlcNAc-PA	43.80	51.82	69.71	72.81	69.40	65.22		
Kdo	175.55	100.65	35.64	66.57	74.76	72.19	70.36	64.09
PA		153.90	136.09	113.87	154.02	113.87		
PSPA-B								
Hep-I	98.92	78.22	74.57	73.85	72.10	71.89	63.49	
Hep-II	101.58	78.22	70.50	67.70	71.94	68.38	63.83	
Glc	102.62	73.82	74.38	80.56	76.21	61.80		
GlcNAc-I	101.83	56.05	74.63	70.32	76.74	61.19		
GlcNAc-II	101.16	55.98	74.73	70.68	74.50	62.42		
GlcA	102.31	72.73	75.73	72.20	77.97	176.24		
Gal	103.58	70.89	72.73	68.95	76.21	61.57		
GlcNAc-PA	43.36	51.61	68.91	72.20	70.21	71.45		
Kdo	174.94	100.12	35.20	66.18	74.38	71.89	70.13	63.99
PA		153.57	135.84	113.44	144.54	113.44		

N-Ac signals: 175.53 and 22.78, 175.55 and 23.05 for PSPA-A; 175.02 and 22.35,

(Table 4 continued)

175.09 and 22.70, 175.19 and 22.59 for PSPA-B

Abbreviations: Hep; L-*glycero*-D-*manno*-heptose, Glc; D-glucose, GlcNAc; *N*-acetyl-D-glucosamine, GlcA; D-glucuronic acid, Gal; D-galactose, GlcNAc-PA; pyridylamino labeled *N*-acetyl-D-glucosamine, Kdo; 3-deoxy-D-*manno*-oct-2-ulosonic acid.

Table 5 Inter-residue NOE effects observed in the NOESY spectra of the carbohydrate backbone (PSPA-A and PSPA-B) of *V. parahaemolyticus* O6 lipopolysaccharides.

Sugar residues	Atom No.	NOE effect observed with	
		PSPA-A	PSPA-B
Hep-I	H-1	Kdo H-5	Kdo H-5
		GlcA H-1	GlcA H-1
Hep-II	H-1	Hep-I H-3	Hep-I H-3
		Gal H-1	Gal H-1
Glc	H-1	Hep-I H-4 and H-2	Hep-I H-4
GlcNAc-I	H-1	Glc H-4	Glc H-4
GlcNAc-II	H-1		GlcNAc-PA H-6a/b
GlcA	H-1	Hep-I H-2	Hep-I H-2
Gal	H-1	Hep-II H-2 and H-3	Hep-II H-2 and H-3

Abbreviations: Hep; L-*glycero*-D-*manno*-heptose, Glc; D-glucose, GlcNAc; *N*-acetyl-D-glucosamine, GlcA; D-glucuronic acid, Gal; D-galactose GlcNAc-PA; pyridylamino labeled *N*-acetyl-D-glucosamine,.

List of Abbreviations

deacylated LPS; dAcP-LPS, *O*-deacylated and dephosphorylated LPS; ELISA, enzyme-linked immunosorbent assay; EtNAc, *N*-acetylated ethanolamine; FABMS, fast-atom bombardment mass spectrometry; Gal, D-galactose; GalA, D-galacturonic acid; Glc, D-glucose; GlcA, D-glucuronic acid; GlcN, D-glucosamine; GlcNAc, *N*-acetyl-D-glucosamine; GlcNAc-PA, pyridylamino labeled *N*-acetyl-D-glucosamine; Hep, L-*glycero*-D-*manno*-heptose; HF-PS, dephosphorylated carbohydrate portion; HPLC, high-performance liquid chromatography; Kdo, 3-deoxy-D-*manno*-oct-2-ulosonic acid; LOS, lipooligosaccharides; LPS, lipopolysaccharides; P, phosphate; PS, carbohydrate potion; PA, pyridylamino; PSPA, deacylated, dephosphorylated, pyridylaminated and *N*-acetylated carbohydrate backbone; R-type, rough-type; TBA, periodate-2-thiobarbituric acid.

Fig.1

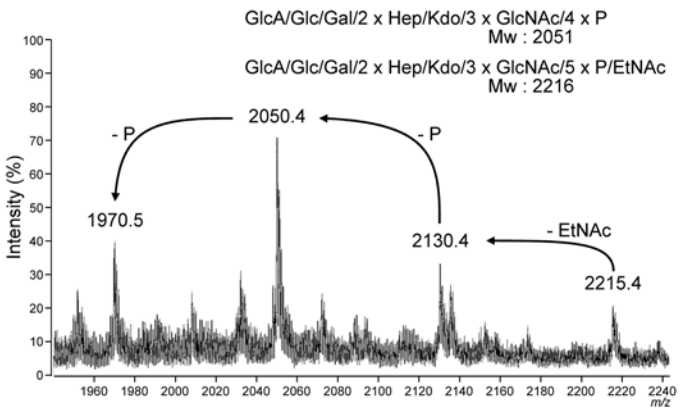

GlcA/Glc/Gal/2 x Hep/Kdo/3 x GlcNAc/4 x P
Mw : 2051
GlcA/Glc/Gal/2 x Hep/Kdo/3 x GlcNAc/5 x P/EtNAc
Mw : 2216
2050.4
- P
- P
1970.5
2130.4
- EtNAc
2215.4
Intensity (%)
m/z

Fig.2

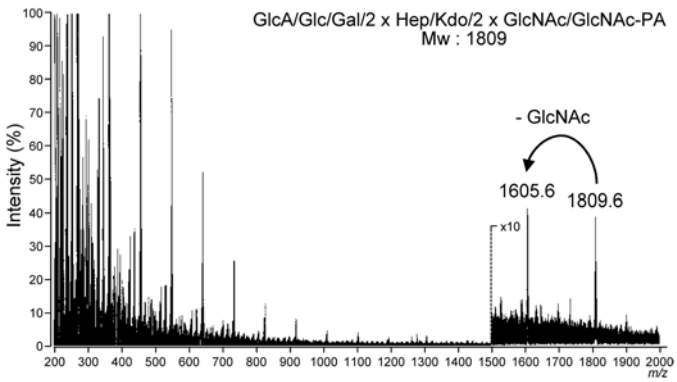

GlcA/Glc/Gal/2 x Hep/Kdo/2 x GlcNAc/GlcNAc-PA
Mw : 1809
- GlcNAc
1605.6
1809.6
x10
Intensity (%)
m/z

Fig.3

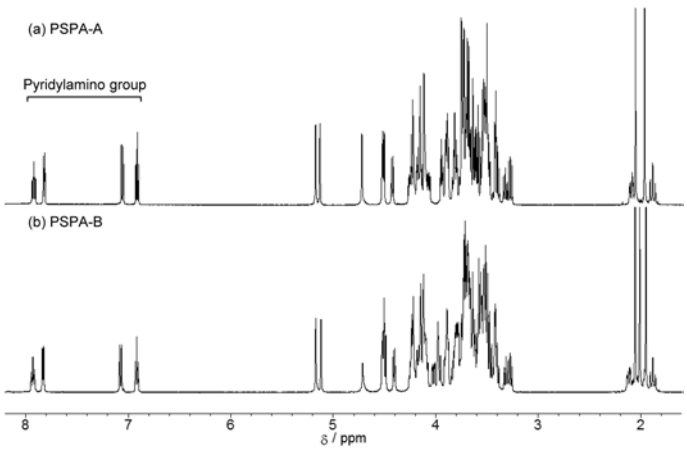


Fig.4

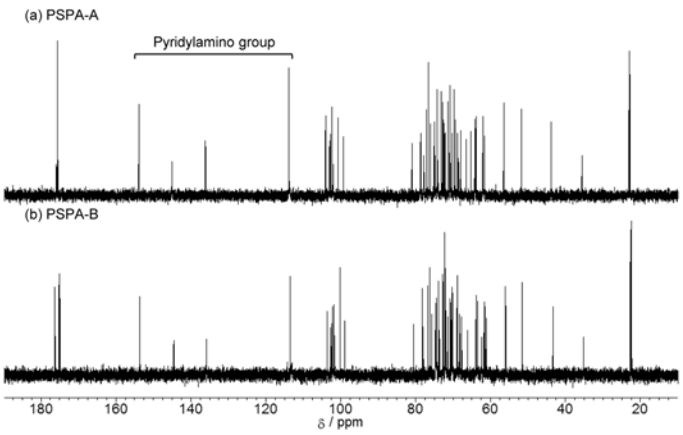

(a) PSPA-A
Pyridylamino group
(b) PSPA-B
180
160
140
120
100
80
60
40
20
δ / ppm

Fig.5

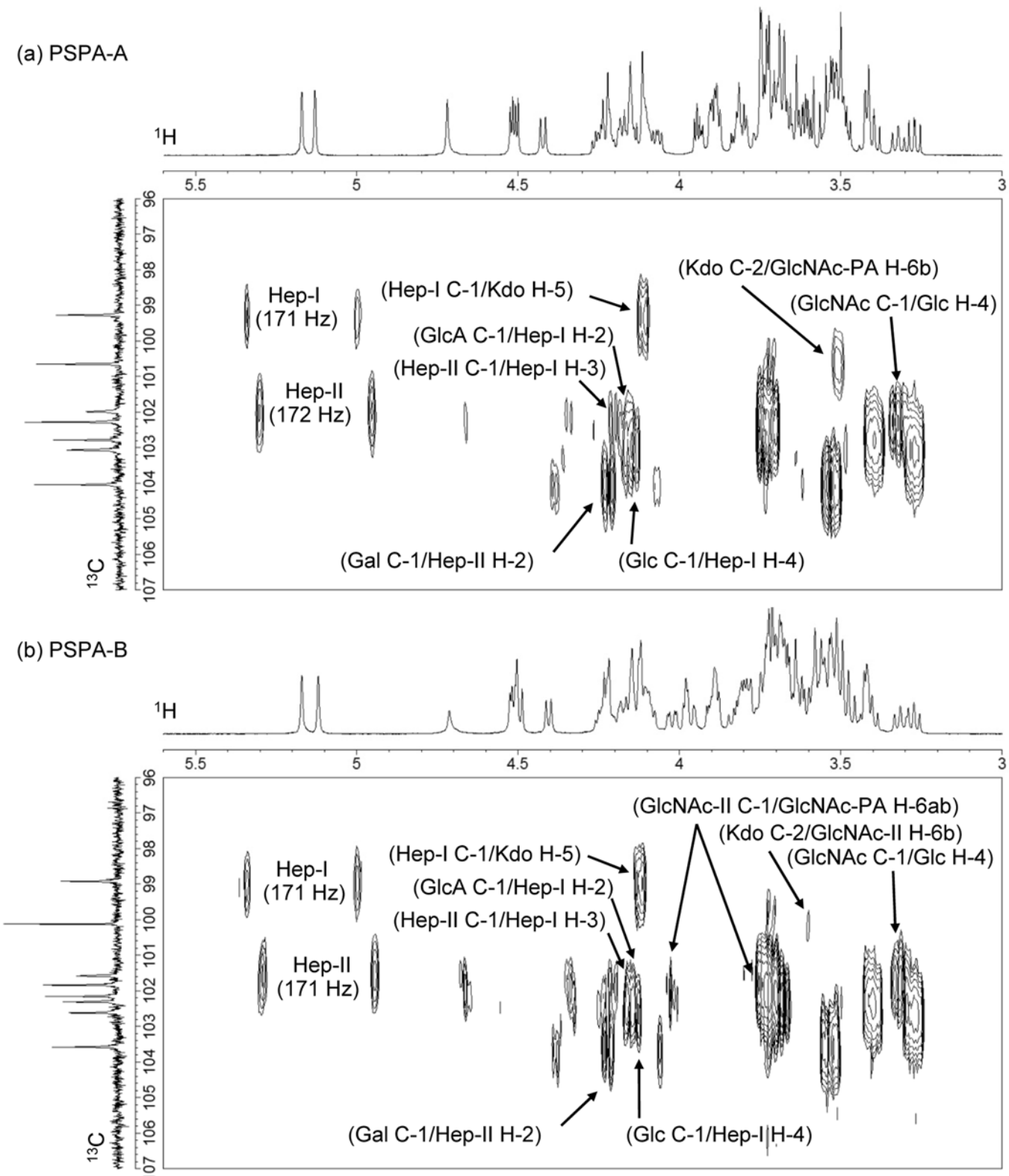
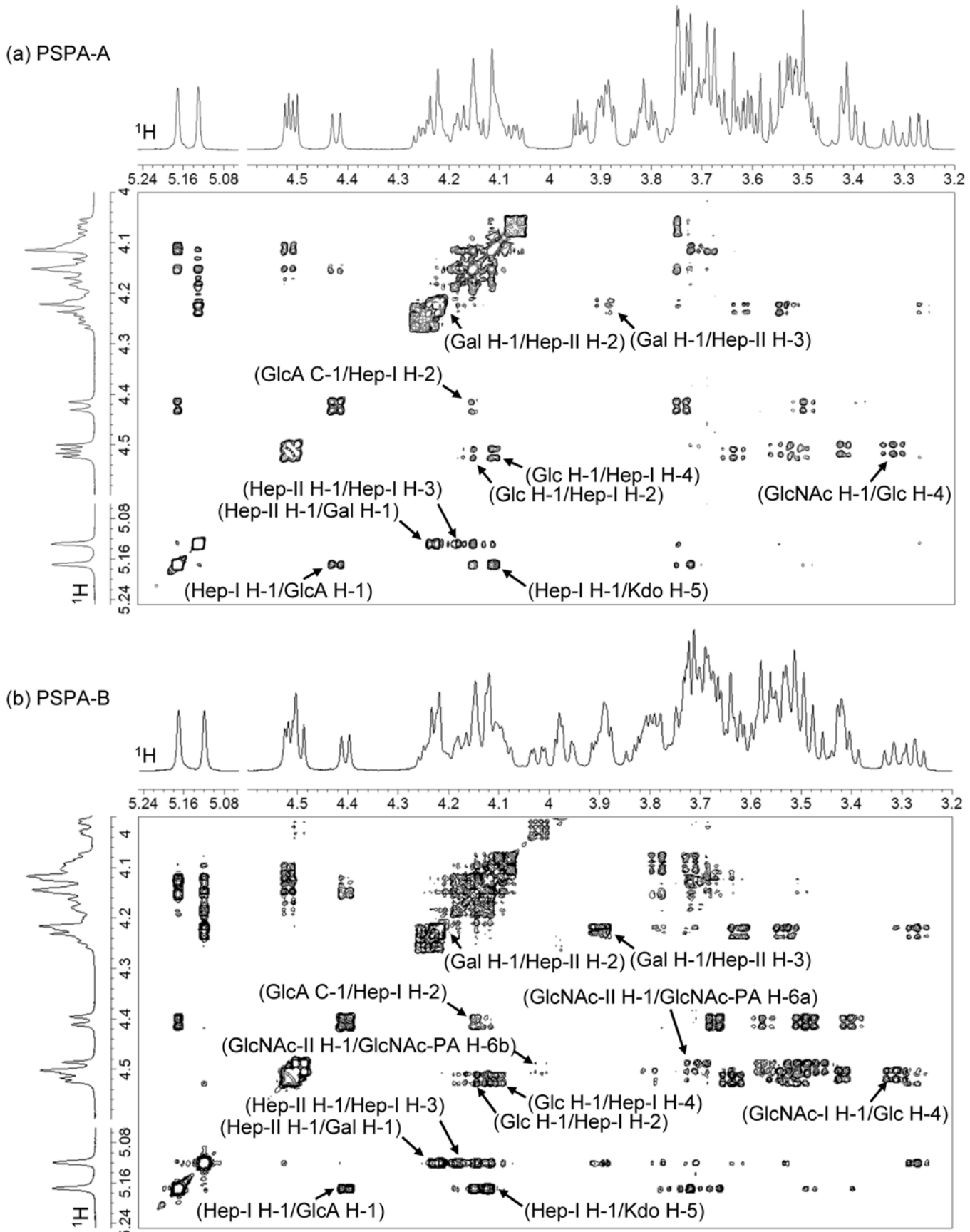

(a) PSPA-A
^{1}H
5.5
5
4.5
4
3.5
3
^{13}C
96
97
98
99
100
101
102
103
104
105
106
107
Hep-I
(171 Hz)
Hep-II
(172 Hz)
(Hep-I C-1/Kdo H-5)
(GlcA C-1/Hep-I H-2)
(Hep-II C-1/Hep-I H-3)
(Kdo C-2/GlcNAc-PA H-6b)
(GlcNAc C-1/Glc H-4)
(Gal C-1/Hep-II H-2)
(Glc C-1/Hep-I H-4)
(b) PSPA-B
^{1}H
5.5
5
4.5
4
3.5
3
^{13}C
96
97
98
99
100
101
102
103
104
105
106
107
Hep-I
(171 Hz)
Hep-II
(171 Hz)
(Hep-I C-1/Kdo H-5)
(GlcA C-1/Hep-I H-2)
(Hep-II C-1/Hep-I H-3)
(GlcNAc-II C-1/GlcNAc-PA H-6ab)
(Kdo C-2/GlcNAc-II H-6b)
(GlcNAc C-1/Glc H-4)
(Gal C-1/Hep-II H-2)
(Glc C-1/Hep-I H-4)

Fig.6


(a) PSPA-A
^{1}H
(Gal H-1/Hep-II H-2)
(Gal H-1/Hep-II H-3)
(GlcA C-1/Hep-I H-2)
(Glc H-1/Hep-I H-4)
(Glc H-1/Hep-I H-2)
(Hep-II H-1/Hep-I H-3)
(Hep-II H-1/Gal H-1)
(GlcNAc H-1/Glc H-4)
(Hep-I H-1/GlcA H-1)
(Hep-I H-1/Kdo H-5)
(b) PSPA-B
^{1}H
(Gal H-1/Hep-II H-2)
(Gal H-1/Hep-II H-3)
(GlcA C-1/Hep-I H-2)
(GlcNAc-II H-1/GlcNAc-PA H-6a)
(GlcNAc-II H-1/GlcNAc-PA H-6b)
(Hep-II H-1/Hep-I H-3)
(Glc H-1/Hep-I H-4)
(Glc H-1/Hep-I H-2)
(GlcNAc-I H-1/Glc H-4)
(Hep-II H-1/Gal H-1)
(Hep-I H-1/GlcA H-1)
(Hep-I H-1/Kdo H-5)