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Structure of the O-Specific Polysaccharide from *Shewanella japonica* KMM 3601 Containing 5,7-Diacetamido-3,5,7,9-tetradeoxy-D-*glycero*-D-*talo*-non-2-ulosonic Acid

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Abstract—Structure of the O-specific polysaccharide chain of the lipopolysaccharide (LPS) of *Shewanella japonica* KMM 3601 was elucidated. The initial and O-deacylated LPS as well as a trisaccharide representing the O-deacetylated repeating unit of the O-specific polysaccharide were studied by sugar analysis along with ^{1}H and ^{13}C NMR spectroscopy. The polysaccharide was found to contain a rare higher sugar, 5,7-diacetamido-3,5,7,9-tetradeoxy-D-*glycero*-D-*talo*-non-2-ulosonic acid (a derivative of 4-epilegionaminic acid, 4eLeg). The following structure of the trisaccharide repeating unit was established:

→4)-α-4eLeg*p*5Ac7Ac-(2→4)-β-D-Glc*p*A3Ac-(1→3)-β-D-Gal*p*NAc-(1→.

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Key words: *Shewanella japonica*, O-specific polysaccharide, lipopolysaccharide, marine bacteria, NMR spectroscopy, 5,7-diacetamido-3,5,7,9-tetradeoxy-D-*glycero*-D-*talo*-non-2-ulosonic acid

The genus *Shewanella* consists currently of 54 species of Gram-negative facultatively anaerobic Proteobacteria (see Taxonomy Browser at http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=22) and is associated mainly with aquatic habitats. The bacteria of this genus have received a significant amount of attention due to their important roles in cometabolic bioremediation of halogenated organic pollutants [1], destructive oxidation of crude petroleum [2], and dissimilatory reduction of magnesium and iron oxides [3]. Phylogenetic relationships, the taxonomy of these proteobacteria, and an improved approach for identification of newly isolated wild strains have been considered elsewhere [4].

Structures of surface polysaccharides of a number of *Shewanella* species containing unusual acidic N-acylamino sugars and various non-sugar substituents have been determined by us [5-8] or others [9-11] and reviewed recently [12, 13]. A highly unusual C-branched 9-carbon sugar named shewanellose was identified as a component of a hydrophobic antigenic polysaccharide from *Shewanella putrefaciens* clinical strain A6 [7]. Earlier, we established the structure of the O-specific polysaccharide from *S. japonica* type strain KMM 3299^{T} containing rare 6-deoxyamino sugars [8]. Now, we report on a new structure of the O-polysaccharide from *S. japonica* strain KMM 3601 and identification, as its component, of a higher acidic diamino sugar.

Abbreviations: COSY, correlation spectroscopy; DLPS, O-deacetylated LPS; ESI-MS, electrospray ionization-mass spectrometry; GlcA, glucuronic acid; HSQC, heteronuclear single-quantum coherence spectroscopy; *J*, coupling constant; LPS, lipopolysaccharide; MS/MS, tandem mass spectrometry; OS, oligosaccharide; ROESY, rotating-frame nuclear Overhauser effect spectroscopy; TOCSY, total correlation spectroscopy.

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MATERIALS AND METHODS

Shewanella japonica strain KMM 3601 was isolated from a seawater sample from a depth of 0.5-1.5 m at the Pacific Institute of Bioorganic Chemistry of the Marine Experimental Station, Troitza Bay, Gulf of Peter the Great, Japan Sea. Bacteria were grown with shaking (160 rpm) on modified Yoshimizu–Kimura medium (36 h, 20°C) [14]. Wet bacterial cells from 20 liters of the cultural fluid were extracted with hot aqueous 45% phenol as described [15]. The aqueous layer was separated by centrifugation, dialyzed against distilled water, concentrated, and freeze-dried to yield lipopolysaccharide (LPS) (720 mg).

The LPS (65 mg) was treated with aqueous 12.5% ammonia at 20°C for 16 h, and the solution was desalted on a column (90 × 2.5 cm) of TSK HW-40 (S) (Merck, Germany) in water and freeze-dried to give O-deacetylated LPS (DLPS) (48 mg).

The DLPS (40 mg) was hydrolyzed with aqueous 2% HOAc (100°C, 3 h), and the precipitate was removed by centrifugation (13,000*g*, 10 min). The water-soluble portion was fractionated by gel chromatography on TSK HW-40 (S) as above to afford oligosaccharide (OS) (24 mg).

An OS sample was hydrolyzed with 2 M CF_3CO_2H (120°C, 2 h). The monosaccharides were reduced with 0.25 M $NaBH_4$ in aqueous 1 M ammonia (25°C, 1 h), acetylated with a 1 : 1 (v/v) mixture of pyridine and Ac_2O (120°C, 0.5 h), and analyzed by GLC. Methanolysis of the OS (1 mg) was carried out using 1 M HCl-MeOH (85°C, 16 h) followed by acetylation with Ac_2O in pyridine (120°C, 30 min). The absolute configurations of the monosaccharides were determined by GLC of acetylated (*S*)-(+)-2-butyl glycosides according to published methods [16, 17]. GLC was performed using a Hewlett-Packard 5890 Series II instrument equipped with an HP-5 fused silica column (0.25 mm × 30 m) using a temperature program of 170 to 180°C (1°C/min) followed by 180 to 230°C (7°C/min).

ESI-MS was performed on a Bruker Daltonics $MicroTOF_Q$ mass spectrometer in the positive mode using as solvent water–isopropanol (1 : 1) with 0.2% formic acid. The MS/MS experiment used nitrogen as collision gas.

Samples were deuterium-exchanged by freeze-drying from D_2O and then examined at 30°C in solutions of 99.97% D_2O, using internal sodium 3-trimethylsilyl-[2,2,3,3$^2H_4$]propanoate (δ_H 0) and external acetone (δ_C 31.45) as references. NMR spectra were recorded on a Bruker DRX-500 spectrometer (Germany). Two-dimensional NMR spectra were obtained using standard Bruker software, and the XWINNMR 2.6 program (Bruker) was used to acquire and process the NMR data. Mixing times of 200 and 100 msec were used in TOCSY and ROESY experiments, respectively.

RESULTS AND DISCUSSION

The LPS was isolated from *S. japonica* strain KMM 3601 by the phenol–water procedure [15]. On degradation with dilute acetic acid to split off lipid A from the carbohydrate moiety, the LPS afforded no polysaccharide owing to cleavage of the O-polysaccharide chain at an acid-labile glycosidic linkage of a higher aldulosonic acid (see below). Therefore, the LPS was treated with aqueous ammonia under mild conditions to give an O-deacylated high-molecular-weight product (DLPS). Mild acid degradation of the DLPS resulted in an O-deacetylated oligosaccharide (OS). The DLPS and OS were isolated by gel chromatography on TSK HW-40.

Sugar analysis by GLC of the alditol acetates after full acid hydrolysis of the OS revealed GalNAc only. Methanolysis of the OS followed by GLC of the acetylated methyl ester methyl glycosides showed the presence of glucuronic acid (GlcA). GLC analysis of the acetylated glycosides with (*S*)-2-butanol showed that GalNAc and GlcA have the D-configuration. Further studies by NMR spectroscopy revealed also a derivative of 5,7-diamino-3,5,7,9-tetradeoxy-D-*glycero*-D-*talo*-non-2-ulosonic acid (4-epilegionaminic acid, 4eLeg) as the third OS component.

The ^{13}C NMR spectrum of the OS (Fig. 1) showed signals for three anomeric carbons at 105.5, 99.1, and 97.3 ppm (according to attached-proton test, the latter signal belonging to the non-protonated carbon C2 of 4eLeg). There were also signals for three nitrogen-bearing carbons at 54.8, 52.7, and 49.2 ppm, a HO*C*H_2–C group (C6 of GalNAc) at δ 62.4, a *C*H_3–C group (C9 of 4eLeg) at 20.7 ppm, a C–*C*H_2–C group (C3 of 4eLeg) at 34.3 ppm, 10 oxygen-bearing non-anomeric sugar carbons in the region 66.9-81.5 ppm, three N-acetyl groups at 23.2, 23.4, and 23.8 ppm (all CH_3), and five carbonyl groups (CO of three N-acetyl groups, C1 of 4eLeg and C6 of GlcA) at 174.8-176.7 ppm. The ^1H NMR spectrum contained, *inter alia*, three doublet signals in a low-field region (two anomeric protons and H6 of 4eLeg) at 4.53-4.65 ppm, one signal for H9 of 4eLeg at 1.17 ppm (d, $J_{8,9}$ 6 Hz), and three singlet signals for N-acetyl groups at 1.97-2.05 ppm. These data indicate that the OS is a trisaccharide containing one residue each of D-GlcA, D-GalNAc, and 4eLeg5Ac7Ac.

The ^1H NMR spectrum of the OS was assigned using two-dimensional COSY and TOCSY experiments (table). Tracing connectivities in the spectra revealed three spin systems corresponding to Glc*p*A, Gal*p*NAc, and 4eLeg*p*5Ac7Ac (Fig. 2). Relatively large $^3J_{H1,H2}$ coupling constant values of ~8 Hz indicated that Glc*p*A and Gal*p*NAc are β-linked. The configuration of the C4–C8 centers of 4eLeg5Ac7Ac was inferred from a close similarity of the $^3J_{H,H}$ values (table) with those of the synthetic counterpart [18]. A relatively small difference between $H3_{eq}$ and $H3_{ax}$ chemical shifts (0.30 ppm) was character-

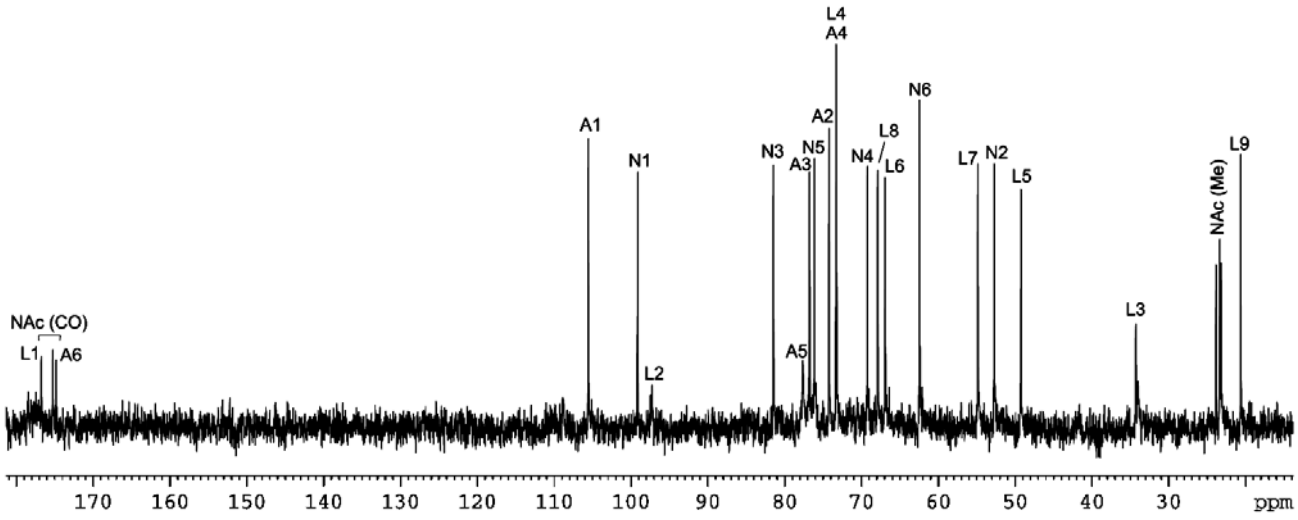

Fig. 1. ^{13}C-NMR spectrum of the OS. Numbers refer to carbons in sugar residues denoted as follows: N, GalN; A, GlcA; L, 4eLeg.

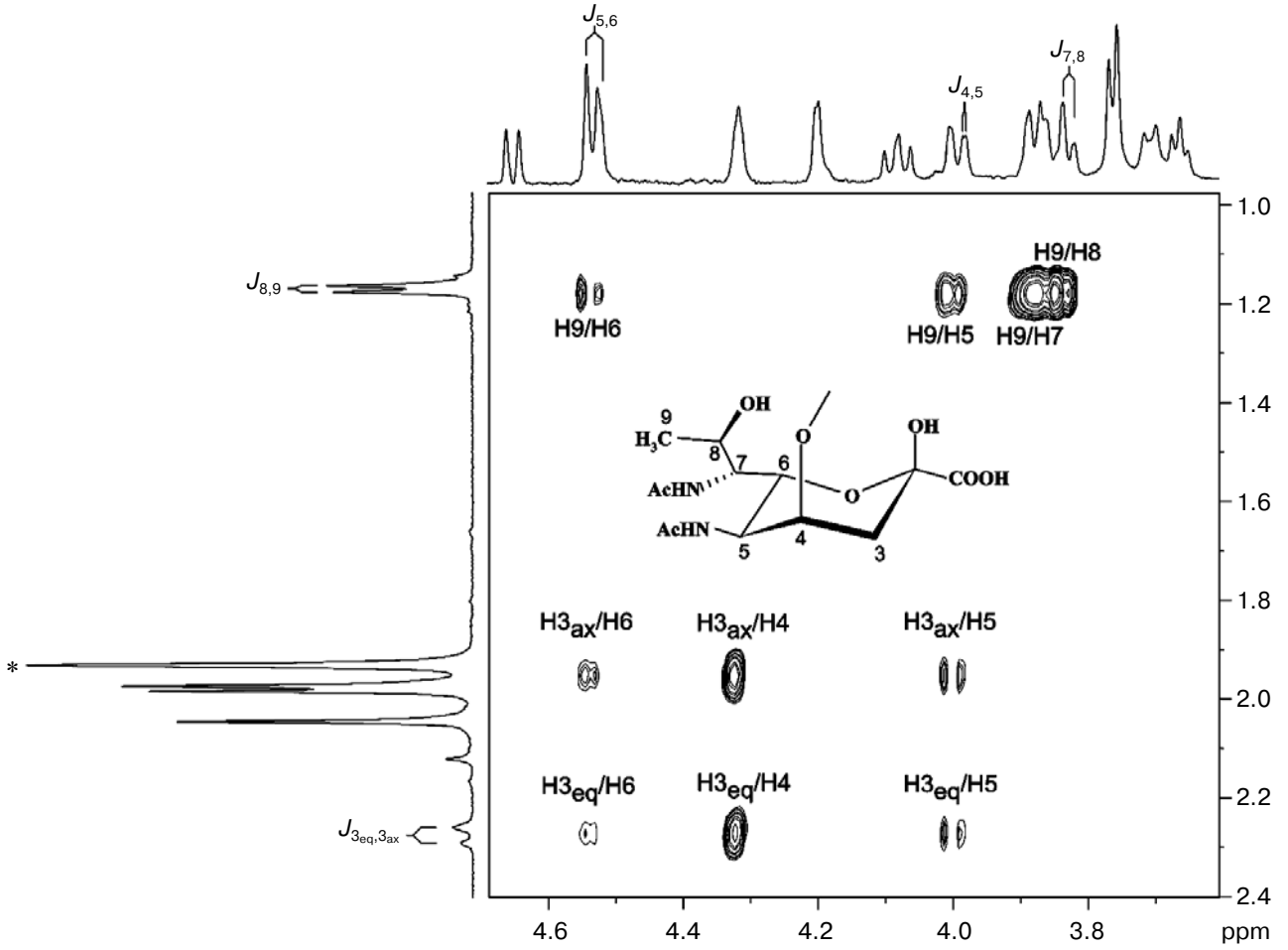

Fig. 2. Part of a two-dimensional ^{1}H,^{1}H TOCSY spectrum of the OS. The corresponding parts of the ^{1}H NMR spectrum are displayed along the axes. Arabic numerals refer to protons in cross-peaks in the spin system of 4eLeg; coupling constants are indicated for 4eLeg. The structure of 4eLeg5Ac7Ac is shown in the inset. The signal of contaminating sodium acetate is marked with the asterisk.

^{1}H and ^{13}C NMR data of the OS, DLPS, and LPS from *S. japonica* strain KMM 3601 (δ, ppm)

Sugar residue	H1 C1	H2 C2	H3 (3_{eq}; 3_{ax}) [$J_{3eq,3ax}$] C3	H4 [$J_{3eq,4}$] C4	H5 [$J_{4,5}$] C5	H6 (6a; 6b) [$J_{5,6}$] C6	H7 [$J_{6,7}$] C7	H8 [$J_{7,8}$] C8	H9 [$J_{8,9}$] C9
OS*									
β-Glc*p*A-(1→	4.53 105.5	3.35 74.2	3.51 76.8	3.50 73.3	3.72 77.6	 174.8			
→3)-β-Gal*p*NAc-(1→	4.65 99.1	4.08 52.7	3.87 81.5	4.20 69.2	3.66 76.1	3.76; 3.76 62.4			
→4)-β-4eLeg5Ac7Ac	 176.7	 97.3	2.27; 1.97 [15] 34.3	4.32 [~3] 73.3	3.99 [2.3] 49.2	4.53 [~10] 66.9	3.83 [<2] 54.8	3.87 [8.8] 67.9	1.17 [6] 20.7
DLPS**									
→4)-β-Glc*p*A-(1→	4.47 105.2	3.29 74.0	3.49 76.2	3.64 78.1	3.82 75.0	 174.6			
→3)-β-Gal*p*NAc-(1→	4.60 99.3	3.93 52.6	3.77 82.0	4.17 69.0	3.64 76.2	3.73; 3.73 62.3			
→4)-α-4eLeg*p*5Ac7Ac-(2→	 176.7	 97.4	2.86; 1.80 35.5	4.26 71.7	3.91 49.1	4.33 70.3	3.63 55.8	4.03 67.7	1.10 19.4
LPS***									
→4)-β-Glc*p*A3Ac-(1→	4.53 105.2	3.40 73.2	4.90 76.3	3.72 77.9	3.70 78.0	 174.8			
→3)-β-Gal*p*NAc-(1→	4.61 97.0	3.95 52.5	3.77 82.6	4.17 69.0	3.64 76.3	3.73; 3.73 62.4			
→4)-α-4eLeg*p*5Ac7Ac-(2→	 176.6	 97.7	2.85; 1.69 34.6	4.24 71.3	3.93 49.3	4.35 70.4	3.64 55.9	4.00 67.8	1.10 19.4

Note: Coupling constant values ($^3J_{H,H}$, Hz) are given in square brackets.

* Chemical shifts for the N-acetyl groups are: δ_H 1.97, 1.98, 2.05 and δ_C 23.2, 23.4, 23.8 (3 Me), and 174.8-176.7 (3 CO).

** Chemical shifts for the N-acetyl groups are: δ_H 1.90, 2.01, 2.02 and δ_C 23.3, 23.6, 24.2 (3 Me), and 174.8-176.7 (3 CO).

*** Chemical shifts for the N-acetyl groups are: δ_H 1.90, 2.01, 2.02 and δ_C 23.3, 23.7, 23.9 (3 Me), and 174.8-176.7 (3 CO); for the O-acetyl group δ_H 2.10 and δ_C 21.9.

istic of the β-configuration of the nonulosonic acid in the OS, which was confirmed by the C6 chemical shift of 66.9 ppm (compare with 66.5 and 70.3 ppm for β-4eLeg5Ac7Ac and α-4eLeg5Ac7Ac, respectively [18]).

Monosaccharide sequence in the OS was determined using a two-dimensional ROESY experiment, which showed inter-residue cross-peaks at δ 4.53/3.87 and 4.65/4.32 ppm assigned to GlcA H1/GalNAc H3 and GalNAc H1/4eLeg5Ac7Ac H4 correlations, respectively.

The ^{13}C NMR spectrum of the OS was assigned using a two-dimensional ^{1}H,^{13}C HSQC experiment (table). The ^{13}C NMR signals for C3 of GalNAc and C4 of 4eLeg5Ac7Ac were shifted downfield to 81.5 and 73.3 ppm, as compared with their positions in the spectra of the corresponding non-substituted monosaccharides at 72.4 and 67.1 ppm, respectively [18, 19]. These displacements were due to α-glycosylation effects and confirmed the positions of substitution of the monosaccharide residues. These data together demonstrated that the OS has the following structure:

β-D-Glc*p*A-(1→3)-β-D-Gal*p*NAc-(1→4)-β-4eLeg5Ac7Ac.

The absolute configuration of 4eLeg5Ac7Ac was determined by analysis of the glycosylation effects in the β-D-Gal*p*NAc-(1→4)-β-4eLeg5Ac7Ac disaccharide fragment of the OS using the known regularities [20]. The values of α-effects on C1 of β-D-Gal*p*NAc (+2.5 ppm) and C4 of 4eLeg5Ac7Ac (+6.2 ppm) as well as the value of a β-effect on C3 of 4eLeg5Ac7Ac (−3.3 ppm) demonstrated the D-*glycero*-D-*talo* configuration of the nonulosonic acid (in case of the opposite, L-*glycero*-L-*talo* configuration, both α-effects would be 7.5-9 ppm and the β-effect ~0 ppm).

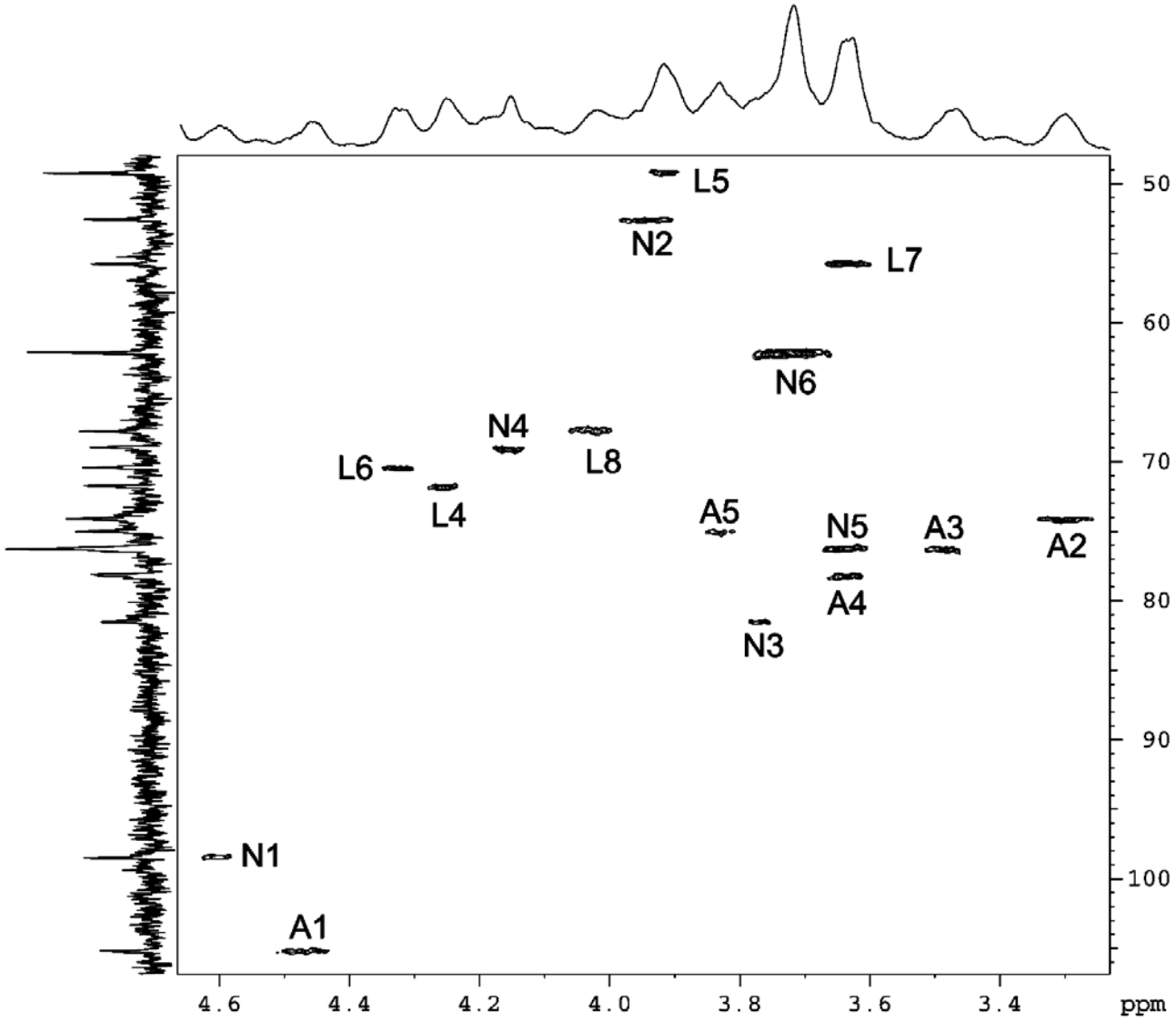

Fig. 3. Part of a two-dimensional $^{1}H,^{13}C$ HSQC spectrum of the DLPS. The corresponding parts of the ^{1}H and ^{13}C NMR spectra are displayed along the horizontal and vertical axes, respectively. Arabic numerals refer to cross-peaks in the sugar residues designated as in Fig. 1.

The positive mode electrospray ionization mass spectrum of the OS contained a peak for an $[M+Na]^+$ ion at *m/z* 736.2370 (calculated mass 736.2383 Da for $C_{27}H_{43}O_{19}N_3Na$), which was consistent with a trisaccharide composed of HexNAc, HexA, and 4eLeg5Ac7Ac residues. An MS/MS spectrum showed daughter ions at *m/z* 380.1193 for $C_{14}H_{22}O_{11}N$ from an A_1-type cleavage and *m/z* 560.2063 for $C_{21}H_{35}O_{13}N_3Na$ corresponding to a B-type fragment (calculated masses 380.1187 and 560.2062 Da, respectively). The ion at *m/z* 533.16 for a B-type fragment, which was not present, would have been the result from an oligosaccharide with a reversed HexA and HexNAc sequence. These data confirmed the HexA-HexNAc-4eLeg5Ac7Ac sequence in the OS.

The ^{1}H NMR spectra of the OS and DLPS were rather similar, differing only in a few details. A major distinction was observed for the chemical shifts of $H3_{eq}$ and $H3_{ax}$: 2.86 and 1.80 ppm ($\Delta\delta$ 1.06) in the DLPS *versus* 2.27 and 1.97 ppm ($\Delta\delta$ 0.30) in the OS. The large chemical shift difference in the DLPS is characteristic of α-4eLeg5Ac7Ac [18]. The ^{1}H and ^{13}C NMR spectra of the DLPS (table) were assigned as described above for the OS. The C6 chemical shift of 70.3 ppm confirmed the α-configuration of 4eLeg5Ac7Ac. A down-field shift of the C4 signal from 73.3 ppm in the OS to 78.1 ppm in the DLPS indicated substitution of GlcA at position 4 in the polysaccharide (Fig. 3).

The ^{13}C NMR spectrum of the intact LPS showed a signal at 21.9 ppm characteristic of an O-acetyl group. Accordingly, the ^{1}H NMR spectrum of the LPS contained a singlet at 2.10 ppm for OAc and a doublet of a doublet at 4.90 ppm ($^{3}J_{H,H}$ ~10 Hz) for a proton at an

OAc-bearing carbon. The latter was assigned using two-dimensional COSY and TOCSY experiments to H3 of GlcA, thus showing location of the O-acetyl group at position 3 of this sugar residue. The other ^{1}H NMR chemical shifts of the DLPS and LPS were similar (table).

Therefore, the repeating unit of the O-specific polysaccharide of *S. japonica* KMM 3601 has the following structure:

→4)-α-4eLeg*p*5Ac7Ac-(2→4)-β-D-Glc*p*A3Ac-(1→3)-β-D-Gal*p*NAc-(1→.

The polysaccharide contains a novel sugar, a 5,7-di-N-acetyl derivative of 4-epilegionaminic acid. Earlier, a homopolymer of a 5-*N*-acetimidoyl-7-N-acetyl derivative of 4eLeg has been found in the LPS of *Legionella pneumophila* non-1 serogroups [21].

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