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Note

Structure of the O-antigen of *Providencia stuartii* O20, a new polysaccharide containing 5,7-diacetamido-3,5,7,9-tetradeoxy-L-*glycero*-D-*galacto*-non-2-ulosonic acid

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Dedicated to the memory of Professor Nikolay K. Kochetkov

Abstract—The O-polysaccharide chain of the lipopolysaccharide (LPS) of *Providencia stuartii* O20 was found to contain D-glucuronic acid, *N*-acetyl-D-glucosamine, and a rarely occurring higher sugar 5,7-diacetamido-3,5,7,9-tetradeoxy-L-*glycero*-D-*galacto*-non-2-ulosonic acid (di-*N*-acetyl-8-epilegionaminic acid, 8eLeg5Ac7Ac). Degradation of the LPS with dilute acetic acid caused depolymerization of the polysaccharide chain by the ketosidic linkage to give a tetrasaccharide corresponding to the repeating unit of the polysaccharide. Based on sugar and methylation analyses of the tetrasaccharide and O-deacylated LPS as well as ESIMS, ^{1}H and ^{13}C NMR spectroscopy data, the structure of the O-polysaccharide of *P. stuartii* O20 was established.

Keywords: *Providencia stuartii*; O-Antigen; Lipopolysaccharide; Polysaccharide structure; 8-Epilegionaminic acid; 5,7-Diacetamido-3,5,7,9-tetradeoxynon-2-ulosonic acid

Bacteria of the genus *Providencia* are facultative pathogens, which are able to invade intestinal mucosa and other cell types and may cause intestinal infections. Particularly, *P. stuartii* is a well-recognized pathogen that causes urinary tract infections in patients with chronic indwelling urinary catheters.[1] The O-antigen-based serological classification scheme of *P. alcalifaciens*, *P. rustigianii*, and *P. stuartii* includes 63 O-serogroups.[2,3] At present, more than 25 *Providencia* O-polysaccharide structures have been established. In this paper, we report on a new structure of an acidic O-polysaccharide from the lipopolysaccharide (LPS) of *P. stuartii* serogroup O20.

Mild acid degradation of the LPS cleaved the polysaccharide chain to give an oligosaccharide (OS), thus indicating the presence of an acid–labile linkage. Also, the LPS was treated with aqueous ammonia to cleave *O*-acyl groups.

Sugar analyses of the OS and O-deacylated LPS by GLC of the acetylated alditols and anion-exchange chromatography using a sugar analyzer showed the presence of 2-amino-2-deoxyglucose (GlcN) and glucuronic acid (GlcA), respectively. The D configuration of both monosaccharides was determined by GLC of the acetylated (*S*)-2-octyl glycosides.

GLC–MS of the partially methylated alditol acetates derived from the methylated LPS by acid hydrolysis revealed the presence of a 3-substituted GlcN. When the methylated LPS was carboxyl-reduced prior to hydrolysis, in addition to the amino sugar, 2,3-di-*O*-methylglucose was identified, which was evidently derived from a 4-substituted GlcA. A similar methylation analysis of the OS, including carboxyl-reduction, revealed the

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presence of 2,3,4-tri-*O*-methylglucose and 2,3-di-*O*-methylglucose, which were derived from terminal and 4-substituted GlcA residues, respectively.

The ^{13}C NMR spectrum of the OS (Fig. 1a) showed four signals for the anomeric carbons at δ 94.1–104.3, that at δ 98.0 belong to a quaternary carbon atom (data of an attached-proton-test experiment[4]). The spectrum also contained signals for three nitrogen-bearing carbons of amino sugars at δ 53.3 and 54.4 (2C), 14 non-anomeric oxygen-bearing sugar-ring carbons at δ 68.9–82.9, one CH_2OH group at δ 61.9, one CH_3–CH, and one CH–CH_2–CH group at δ 15.2 and 41.3, respectively, three CH_3CON groups at δ 23.3–23.6, and six CO groups (CH_3CON and CO_2H) at δ 175.1–177.8.

The ^{1}H NMR spectrum of the OS contained, inter alia, signals for three anomeric protons at δ 4.98 (d, $J_{1,2}$ 3.4 Hz), 4.50 and 4.42 (both d, $J_{1,2}$ 7.9 Hz), one CH_3–CH group at δ 1.15 (d, J 6.0 Hz), one CH–CH_2–CH group at δ 1.82 (J 4.5 and 13.5 Hz) and 2.20 (J 10.5 and 13.5 Hz), other sugar signals in the region δ 3.3–4.1, and three CH_3CON groups at δ 1.99–2.01 (all s).

The ^{1}H NMR spectrum of the OS was assigned using 2D ^{1}H,^{1}H COSY, and TOCSY experiments (Table 1) and then the ^{13}C NMR spectrum was assigned using a 2D ^{1}H,^{13}C gHSQC experiment (Table 2). Based on $J_{H,H}$ coupling constants and H-2,C-2 correlations, two residues of β-Glc*p*A (**A**, **B**) and one residue of α-Glc*p*NAc (**C**) were identified. The remaining signals in the spectra (Tables 1 and 2) belonged evidently to a 5,7-diacetamido-3,5,7,9-tetradeoxynon-2-ulosonic acid residue (**D**). This conclusion was confirmed by the negative ion electrospray ionization mass spectrum of the OS, which showed an intense peak for a $[M-H]^-$ pseudomolecular ion of a compound with the molecular mass 889.28 Da, that is the calculated monoisotopic molecular mass.

Relatively large $J_{4,5}$ and $J_{5,6}$ coupling constants of 10.4–10.5 Hz in unit **D** (Table 1) confirmed the axial orientation of the pyranose-ring protons H-4, H-5, and H-6, that is the *xylo* configuration of the C-4–C-6 fragment. A small $J_{6,7}$ coupling constant of 2 Hz is typical of 5,7-diamino-3,5,7,9-tetradeoxynon-2-ulosonic acids with the equatorial NH-5 group and shows the trans-like relationship for H-6 and H-7.[5] Altogether, this pattern is characteristic of the D-*glycero*-D-*galacto* or L-*glycero*-D-*galacto* isomers (legionaminic acid, Leg; or 8-epilegionaminic acid, 8eLeg, respectively).[5] A 2D ROESY experiment with the OS revealed a strong H-6,H-9 correlation in unit **D** at δ 4.06/1.15, which is characteristic of 8eLeg but not Leg.[5] The ^{1}H NMR chemical sifts and $J_{H,H}$ coupling constants of unit **D** in the OS were similar to those of 8eLeg in a trisaccharide

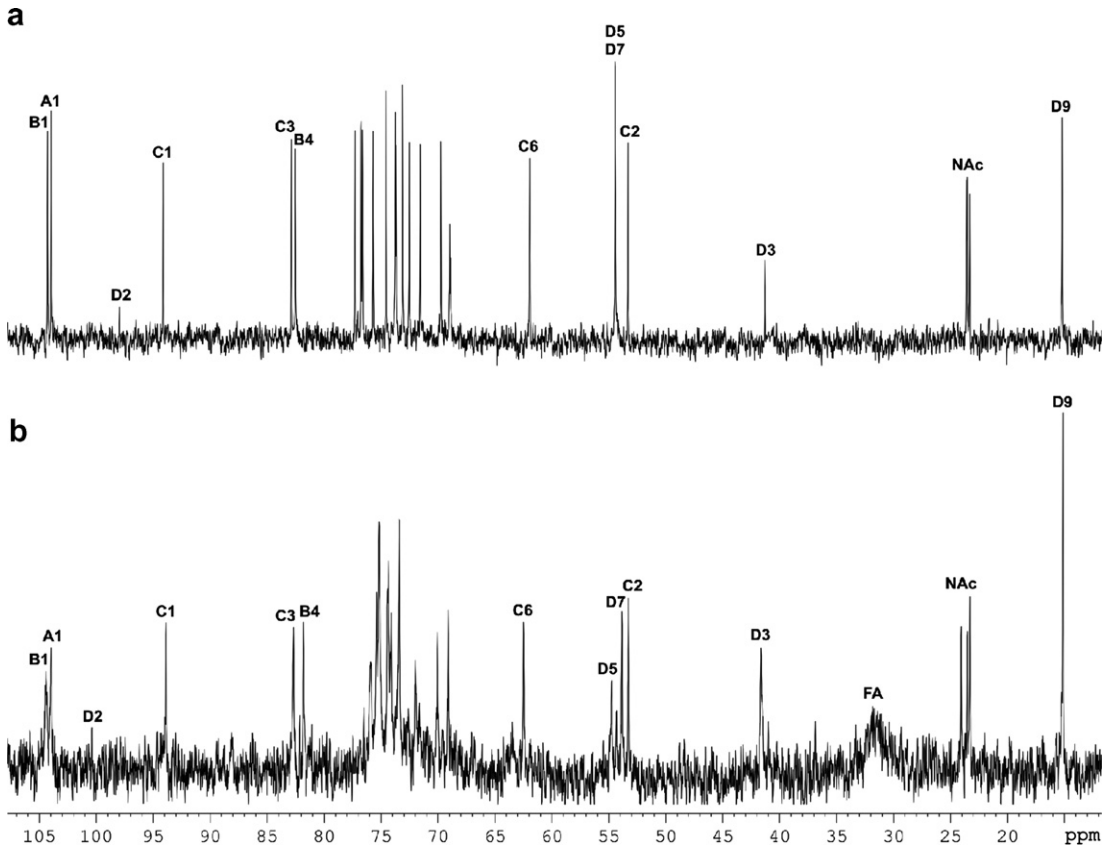

Figure 1. ^{13}C NMR spectra of the OS (a) and the O-deacylated LPS (b) from *P. stuartii* O20. CO resonances are not shown. Arabic numerals refer to carbons in sugar residues denoted as shown in Tables 1 and 2. NAc, *N*-acetyl groups; FA, fatty acyl groups.

Table 1. ^{1}H NMR data of the OS and O-deacylated LPS from *P. stuartii* O20 (δ, ppm; $J_{H,H}$, Hz)

Sugar residue		H-1	H-2	H-3	H-4	H-5	H-6a	H-6b	
OS									
β-D-Glc*p*A-(1→	**A**	4.50	3.36	3.53	3.52	3.76			
→4)-β-D-Glc*p*A-(1→	**B**	4.42	3.38	3.66	3.70	3.95			
→3)-α-D-Glc*p*NAc-(1→	**C**	4.98	4.09[a]	3.62	3.57	3.60	3.77	3.85	
		H-3eq	H-3ax	H-4	H-5	H-6	H-7	H-8	H-9
		$J_{3eq,3ax}$	$J_{3ax,4}$	$J_{3eq,4}$	$J_{4,5}$	$J_{5,6}$	$J_{6,7}$	$J_{7,8}$	$J_{8,9}$
→8)-β-8eLeg*p*5Ac7Ac	**D**	1.82 (1.86)	2.20 (2.29)	3.91 (3.97)	3.73[b] (3.79)	4.06 (4.14)	3.95[c] (4.00)	3.84 (3.83)	1.15 (1.13)
		13.5	10.5 (11.5)	4.5 (4.9)	10.4 (10.1)	10.4 (10.3)	2.0 (2.2)	9.4 (9.5)	6.0 (6.1)
		H-1	H-2	H-3	H-4	H-5	H-6a	H-6b	
O-Deacylated LPS									
→4)-β-D-Glc*p*A-(1→	**A**	4.48	3.32	3.53	4.02	3.96			
→4)-β-D-Glc*p*A-(1→	**B**	4.42	3.35	3.67	3.73	4.12			
→3)-α-D-Glc*p*NAc-(1→	**C**	4.98	4.06[d]	3.57	3.50	3.68	3.67	3.92	
		H-3eq	H-3ax	H-4	H-5	H-6	H-7	H-8	H-9
→8)-α-8eLeg*p*5Ac7Ac-(2→	**D**	1.66	2.67	3.61	3.90[e]	3.88	3.58[f]	3.86	1.21

Data of an oligosaccharide with an α-D-Glc*p*NAc-(1→8)-β-8eLeg*p*5Acyl7Ac fragment at the reducing end from *Salmonella arizonae* O61[6] are given in parentheses.

Additional chemical shifts for CH_3CON are δ [a]2.01; [b]2.00; [c]1.99; [d]1.94; [e]1.95; [f]2.05.

Table 2. ^{13}C NMR data of the OS and O-deacylated LPS from *P. stuartii* O20 (δ, ppm)

Sugar residue		C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9
OS										
β-D-Glc*p*A-(1→	**A**	103.9	74.6	76.6	73.1	77.3	177.0			
→4)-β-D-Glc*p*A-(1→	**B**	104.3	73.7	75.7	82.5	76.2	176.1			
→3)-α-D-Glc*p*NAc-(1→	**C**	94.1	53.3[a]	82.9	69.7	73.6	61.9			
→8)-β-8eLeg*p*5Ac7Ac	**D**	177.8	98.0	41.3	68.9	54.4[b]	71.5	54.4[c]	72.5	15.2
O-Deacylated LPS										
→ 4)-β-D-Glc*p*A-(1→	**A**	104.0	74.3	75.4	74.3	75.9	174.5			
→4)-β-D-Glc*p*A-(1→	**B**	104.4	73.4	75.2	81.8	75.2	173.0			
→3)-α-D-Glc*p*NAc-(1→	**C**	93.9	53.3[d]	82.7	70.0	73.4	62.5			
→8)-α-8eLeg*p*5Ac7Ac-(2→	**D**	n.d.	100.0	41.6	69.1	54.7[e]	74.1	53.9[f]	72.0	15.1

Additional chemical shifts for CH_3CON are δ [a]23.3 (CH_3) and 175.9 (CO); [b]23.5 and 175.1; [c]23.6 and 175.3; [d]23.3 and 175.3; [e]23.5 and 176.1; [f]24.1 and 175.4.

from *Salmonella arizonae* O61 containing an α-D-Glc*p*NAc-(1→8)-8eLeg fragment at the reducing end[6] (Table 1). The absolute configuration at C-8 and, hence, the entire configuration of residue **D** was inferred based on glycosylation effects in the ^{13}C NMR spectrum (see below).

The monosaccharide sequence and positions of the linkages in the OS were established using 2D ROESY and ^{1}H,^{13}C HMBC experiments. The ROESY spectrum showed interresidue cross-peaks between the following anomeric protons and protons at the linkage carbons: **A** H-1,**B** H-4 at δ 4.50/3.70; **B** H-1,**C** H-3 at δ 4.42/3.62; and **C** H-1,**D** H-8 and H-9 at δ 4.98/3.84 and 4.98/1.15, respectively. The following interresidue correlations were revealed by the HMBC experiment: **A** H-1,**B** C-4 at δ 4.50/82.5; **B** H-4,**A** C-1 at δ 3.70/103.9; **B** H-1,**C** C-3 at δ 4.42/82.9; **C** H-3,**B** C-1 at δ 3.62/104.3; and **C** H-1,**D** C-8 at δ 4.98/72.5. Downfield displacements of the signals for **B** C-4 and **C** C-3 to 82.5 and 82.9, compared with their positions in the corresponding non-substituted monosaccharides,[7] confirmed the modes of glycosylation of these monosaccharides. In accordance with the terminal position of unit **A**, the chemical shifts for C-2–C-4 of unit **A** were similar to those in β-Glc*p*A.[7]

In order to establish the absolute configuration of the nonulosonic acid, a similarity of its C-7–C-9 fragment to the C-2–C-4 fragment of threonine and allothreonine was employed. According to the glycosylation effects on ^{13}C NMR chemical shifts, glycosides of the model amino acids can be subdivided into two groups.[8] One group for α-D-glycosides of D-threonine, and L-allothreonine having the L configuration at C-3 is characterized by a relatively small α-effect (1.4–3.4 ppm) on C-1 of the glycon and a relatively large (by the absolute value) β-effect (−4 to −5 ppm) on C-4 (CH_3 group) of the aglycon. In the other group for α-D-glycosides of L-threonine, and D-allothreonine having the D configuration at C-3,

the α-effect on C-1 of the glycon is relatively large (5.6 to +7.6 ppm), whereas the β-effect on C-4 of the aglycon is small (−0.4 to −1.2 ppm). The glycosylation effects in the OS are +2.0 ppm for C-1 of unit **C** (α-D-Glc*p*NAc) and −4.6 ppm for C-9 of unit **D**, determined by comparison with the data for the corresponding monosaccharides,[5,7] which showed that the nonulosonic acid is homomorphic to the amino acids from the first group. Therefore, the configuration at C-8 of the nonulosonic acid is L that finally confirms the 5,7-diacetamido-3,5,7,9-tetradeoxy-L-*glycero*-D-*galacto*-non-2-ulosonic acid structure (8eLeg5Ac7Ac).

Based on the data obtained, it was concluded that the OS has the following structure:

β-D-Glc*p*A-(1→4)-β-D-Glc*p*A-(1→3)-α-D-Glc*p*NAc-(1→8)-8eLeg5Ac7Ac

A **B** **C** **D**

The ^{1}H and ^{13}C NMR (Fig. 1b) spectra of the O-deacylated LPS differ from the spectra of the OS in a significant broadening of the signals for the carbohydrate moiety and the presence of signals for *N*-acyl groups in the lipid moiety. The spectra of the O-deacylated LPS were assigned using 2D NMR experiments as described above for the OS (Tables 1 and 2), and the structure of the repeating unit was fully confirmed by ROESY and $^{1}H,^{13}C$ HMBC (Fig. 2) spectra.

Most ^{13}C NMR chemical shifts were similar in the spectra of the OS and O-deacylated LPS, except for small distinctions for units **A** and **D**. In unit **A** of the O-deacylated LPS, the signal for C-4 shifted by 0.8 ppm downfield and those for C-3 and C-5 by 1.2–1.4 ppm upfield, thus indicating substitution of unit **A** at position 4 with a keto sugar, that is with 8eLeg. The 2→4-linkage between units **D** and **A** was confirmed by an **A** H-4,**D** C-2 correlation peak at δ 4.02/100.0 in the HMBC spectrum of the O-deacylated LPS (Fig. 2). Hence, the O-polysaccharide of the LPS is linear.

A downfield displacement of the signal for C-6 of unit **D** from δ 71.5 in the OS to δ 74.1 in the O-deacylated LPS reflects a change in the orientation of the carboxyl group from equatorial in the former to axial in the latter compound. The anomeric configuration of the nonulosonic acid was confirmed by a difference of 1.01 ppm between the H-3eq and H-3ax chemical shifts in unit **D** of the O-deacylated LPS, which is characteristic of the axial orientation of the carboxyl group,[9] corresponding to the α configuration of 8eLeg.[5] Remarkably, as opposite to the O-polysaccharide studied in this work, all O-polysaccharides containing derivatives of α-8eLeg studied earlier[6,9,10] were stable under the conditions of mild acid degradation of the LPS.

The data obtained showed that the O-polysaccharide of *P. stuartii* O20 has the following structure:

Most likely,[11] the structure shown represents the biological repeating unit of the O-polysaccharide with D-GlcNAc as the first monosaccharide, whose transfer to an undecaprenol carrier initiates biosynthesis of the O-polysaccharide.

1. Experimental

1.1. Bacterial strain and isolation of the lipopolysaccharide

P. stuartii O20:H4, strain 247, obtained from the Hungarian National Collection of Medical Bacteria (National Institute of Hygiene, Budapest) was cultivated under aerobic conditions in tryptic soy broth supplemented with 0.6% yeast extract. The bacterial mass was harvested at the end of the logarithmic growth phase, centrifuged, washed with distilled water, and lyophilized. The LPS was isolated in a yield of 6.8% of dry bacterial cells weight by phenol–water extraction[12] followed by dialysis of the extract without layer separation and centrifugation to remove insoluble contaminations. The crude LPS preparation was purified by treatment of the aqueous solution with cold aq 50% CCl_3CO_2H; after centrifugation the aqueous layer was dialyzed and freeze-dried.

1.2. Degradations of the lipopolysaccharide

A LPS sample (150 mg) was heated with 2% AcOH for 2.5 h at 100 °C, the carbohydrate-containing supernatant was fractionated by GPC on a column (60 × 2.5 cm) of Sephadex G-50 (S) in 0.05 M pyridinium acetate buffer, pH 4.5, and the oligosaccharide fraction was fractionated by GPC on a column (80 × 1.5 cm) of TSK HW-40 in 1% AcOH to give the OS in a yield of 30% of the LPS weight.

For O-deacylation, a LPS sample (130 mg) was heated with aq 12% ammonia at 37 °C for 16 h and the supernatant was fractionated by GPC on Sephadex G-50 (S) as described above to give the O-deacylated LPS (60%).

1.3. Monosaccharide analysis

For sugar analysis, OS and LPS samples (0.5 mg each) were hydrolyzed with 2 M CF_3CO_2H for 2 h at 120 °C. Alditol acetates were prepared by reduction with an excess of $NaBH_4$ (20 °C, 2 h) followed by acetylation

→8)-α-8eLeg*p*5Ac7Ac-(2→4)-β-D-Glc*p*A-(1→4)-β-D-Glc*p*A-(1→3)-α-D-Glc*p*NAc-(1→

D **A** **B** **C**

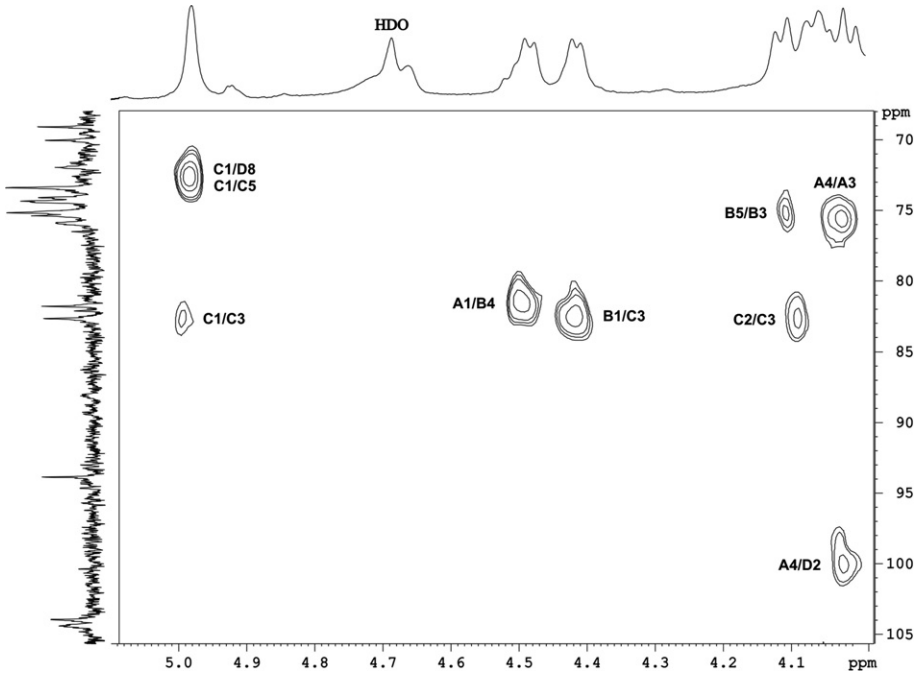

Figure 2. Part of a $^{1}H,^{13}C$ HMBC spectrum of the O-deacylated LPS from *P. stuartii* O20. The corresponding parts of ^{1}H and ^{13}C NMR spectra are displayed along the horizontal and vertical axes, respectively. Arabic numerals before slash refer to protons and after slash to carbons in sugar residues denoted by letters as shown in Tables 1 and 2.

(0.2 mL Ac_2O, 0.2 mL pyridine, 100 °C, 1 h) and analyzed by GLC on a Hewlett-Packard 5890 Series II instrument equipped with an HP-1 fused silica column (0.25 mm × 30 m), using a temperature program from 170 to 180 °C at 1 °C min^{-1}, and then from 180 to 230 °C at 7 °C min^{-1}. Uronic acids were analyzed using a Biotronik LC-2000 sugar analyzer as described.[13]

For determination of the absolute configuration of GlcN and GlcA,[14] an OS sample (0.5 mg) was either hydrolyzed with 2 M CF_3CO_2H (120 °C, 2 h) and then N-acetylated (400 μL $NaHCO_3$, 60 μL Ac_2O, 0 °C, 1 h) or subjected to methanolysis (1 mL MeOH, 0.1 mL AcCl, 16 h, 80 °C), respectively. The products were heated with (*S*)-2-octanol (100 μL) in the presence of CF_3CO_2H (15 μL) at 120 °C for 16 h, acetylated and analyzed by GLC as above.

1.4. Methylation analysis

Prior to methylation, a sample of the O-deacylated LPS was treated with an Amberlite IR-120 (H^+-form) resin to remove cations and lyophilized. Methylation of the O-deacylated LPS and OS was performed by the Hakomori procedure,[15] the products were recovered using a Sep-Pak cartridge and divided into two parts, one of which was reduced with $LiBH_4$ in aq 70% 2-propanol (20 °C, 2 h). Partially methylated monosaccharides were obtained by hydrolysis with 2 M CF_3CO_2H for 2 h at 120 °C, converted into the alditol acetates and analyzed by GLC–MS on a TermoQuest Finnigan model Trace series GC 2000 instrument equipped with an EC-1 column (0.32 mm × 30 m) using a temperature gradient from 150 °C (2 min) to 250 °C at 10 °C min^{-1}.

1.5. Electrospray ionization MS

Ion cyclotron resonance Fourier transform ESIMS was performed using an ApexII instrument (Bruker Daltonics, USA) equipped with a 7 T actively screened magnet and an Apollo ion source. An OS sample was dissolved in 30:30:0.01 2-propanol–water–Et_3N at concentration ~20 ng μL^{-1} and sprayed at a flow rate of 2 μL min^{-1}. Capillary entrance voltage was set to 3.8 kV and drying gas temperature to 150 °C.

1.6. NMR spectroscopy

Prior to measurements, samples were deuterium-exchanged by freeze-drying twice from D_2O. ^{1}H and ^{13}C NMR spectra were recorded with a Bruker DRX-500 spectrometer at 30 °C in 99.96% D_2O using internal TSP (δ_H 0) and acetone (δ_C 31.45) as references. 2D NMR experiments were performed using standard Bruker software. TOCSY spectra were acquired using a MLEV17 spin-lock duration of 200 ms. A mixing time of 300 ms was used in ROESY experiments. HMBC experiments were optimized for the coupling constant $J_{H,C}$ 8 Hz.

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