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Structure of the O-polysaccharide of *Idiomarina zobellii* KMM 231^T containing two unusual amino sugars with the free amino group, 4-amino-4,6-dideoxy-D-glucose and 2-amino-2-deoxy-L-guluronic acid ☆

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Abstract—Mild acid degradation of the lipopolysaccharide of the bacterium *Idiomarina zobellii*, type strain KMM 231^T, with aq 2% HOAc at 100 °C, yielded an oligosaccharide, which represents one repeating unit of the O-polysaccharide. A polysaccharide was obtained by mild base degradation of the lipopolysaccharide. The following structure of the O-polysaccharide was elucidated by ^{1}H and ^{13}C NMR spectroscopy of the oligosaccharide and base-degraded lipopolysaccharide, including COSY, TOCSY, ROESY, ^{1}H, ^{13}C HSQC, HSQC-TOCSY and HMBC experiments:

→ 3)-α-D-Qui*p*4N-(1 → 4)-α-D-Glc*p*A-(1 → 6)-α-D-Glc*p*NAc-(1 → 4)-α-L-Gul*p*NA-(1 → 3)-β-D-Fuc*p*NAc-(1 →

The O-polysaccharide is distinguished by the presence of two unusual amino sugars, 4-amino-4,6-dideoxy-D-glucose (D-Qui4N) and 2-amino-2-deoxy-L-guluronic acid (L-GulNA), both having the free amino group. The unexpectedly high acid lability of the glycosidic linkage of 2-acetamido-2,6-dideoxy-D-galactose (D-FucNAc) could be associated with the presence of a free amino group adjacent to the site of attachment of FucNAc to Qui4N.

Keywords: *Idiomarina zobellii*; Marine bacteria; Lipopolysaccharide; Bacterial polysaccharide structure; 2-Amino-2-deoxy-L-guluronic acid; 4-Amino-4,6-dideoxy-D-glucose

1. Introduction

Bacterial strain KMM 231^T was isolated from a seawater sample taken at a depth of 4000 m from the northwestern Pacific Ocean (latitude 8°20 N, longitude 133°0 W) in July 1985. This deep-sea strain was found to be Gram-negative, halotrophic, psychrotolerant, heterotrophic and strictly aerobic. On the basis of polyphasic evidence, it was proposed that strain KMM 231 be classified in the new genus, *Idiomarina* gen. nov., as a representative of *Idiomarina zobellii* sp. nov.[1] The genus is related to the genera *Alteromonas*, *Pseudoalteromonas* and *Colwellia* within the family *Alteromonadaceae* in the subclass γ-*Proteobacteria*.[2] Structures of a number of surface polysaccharides of the marine bacteria of the

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genera *Alteromonas* and *Pseudoalteromonas* have been established.[3] Now we report on the structure of the O-polysaccharide from the lipopolysaccharide (LPS) of the new marine bacterium *I. zobellii* type strain KMM 231^T.

2. Results and discussion

Mild acid degradation of the LPS did not result in the expected high-molecular-mass polysaccharide. Rather, a single oligosaccharide (**1**), corresponding to one repeating unit of the O-polysaccharide (see below), was recovered. Therefore, the LPS was subjected to mild base degradation (O-deacylation), and a high-molecular-mass product was isolated by GPC on TSK HW-50 and examined by NMR spectroscopy.

The ^{13}C NMR spectrum of the base-degraded LPS (Fig. 1) showed, inter alia, signals for five anomeric carbons at δ 96.8–103.5, four nitrogen-bearing carbons of amino sugars at δ 51.1–60.0, two *C*H$_3$–C groups (C-6 of 6-deoxy sugars) at δ 16.8 and 18.9, two COOH groups (C-6 of uronic acids) at δ 174.2 and 175.4 and two *N*-acetyl groups at δ 23.3, 23.8 (both CH$_3$), 176.0 and 176.6 (both CO). Six signals were observed in a low-field region of the ^{1}H NMR spectrum at δ 4.5–5.4, which included five signals for anomeric protons and one signal for H-5 of a uronic acid (see below). Four signals for methyl groups were present in a high-field region of the spectrum at δ 1.28, 1.30 (both H-6 of 6-deoxy sugars), 2.09 and 2.10 (both *N*-acetyl groups).

Two-dimensional NMR spectroscopy of the base-degraded LPS resulted in very complex spectra, with overlapping resonances in the anomeric region making it difficult to commence detailed examination with no clear reporter groups for some residues present. Thus, oligosaccharide **1** was isolated by GPC on TSK HW-50 for examination by NMR spectroscopy.

The ^{13}C NMR spectrum of oligosaccharide **1** showed, inter alia, six signals in the anomeric region at δ 92.3–102.2, six signals for nitrogen-bearing carbons of amino sugars at δ 50.0–62.1, two signals for *C*H$_3$–C groups (C-6 of 6-deoxy sugars) at δ 16.8 and 18.4, three *N*-acetyl groups (CH$_3$ at δ 23.3–23.7) and CO groups (COOH and NAc) at δ 175.5–176.6. The attached-proton test (APT) revealed only one signal for a C–*C*H$_2$–C group at δ 67.0 and thus confirmed that the signal at δ 62.1 belongs to a C–*C*HN–C group. There were no non-anomeric sugar-ring signals in a lower field than δ 82, demonstrating that all constituent sugars are in the pyranoid form.[4] The ^{1}H NMR spectrum of **1** showed eight signals of differing intensity in a low-field region of δ 4.60–5.40, two signals for the *N*-acetyl groups at δ 2.08 and 2.11 (both s) and three signals for methyl groups (H-6 of 6-deoxy sugars) at δ 1.27, 1.24 and 1.19 (all d, $J_{5,6}$ 6–6.5 Hz).

The ^{1}H NMR spectrum of oligosaccharide **1** was assigned using 2D ^{1}H,^{1}H COSY and TOCSY experiments (Table 1). The general configurations of the monosaccharide were determined by a set of 1D ROE experiments in difference mode with excitation of each anomeric proton, which enabled the observation of the shape and the splitting of signals in the individual sugar residues and the measurement of coupling constant values for sugar ring protons. The positions of the amino groups in the amino sugars were confirmed by correlations between protons at the nitrogen-bearing carbons

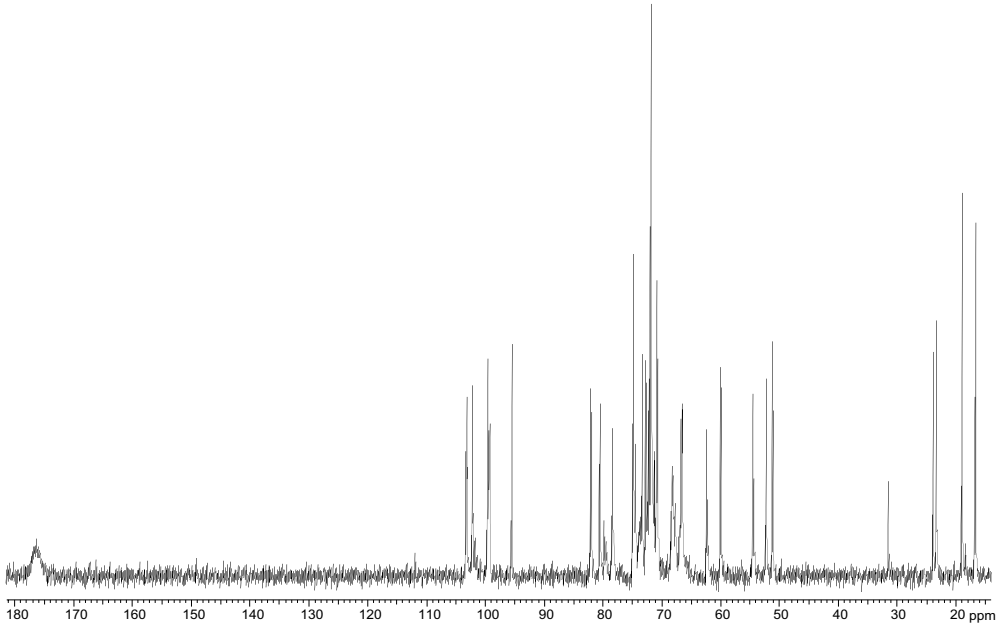


Figure 1. 100 MHz ^{13}C NMR spectrum of the base-treated LPS at 30 °C.

Table 1. 500 MHz ^{1}H NMR data (δ, ppm; J, Hz)

Sugar residue		H-1	H-2	H-3	H-4	H-5	H-6a, 6b
Oligosaccharide **1**							
α-D-Qui*p*4N-(1→	(**A**)	5.40 $J_{1,2}$ 3.65	3.58 $J_{2,3}$ ~9	3.66 $J_{3,4}$ ~9	2.88 $J_{4,5}$ 9.7	3.76 $J_{5,6}$ 6.1	1.27
→4)-α-D-Glc*p*A-(1→	(**B**)	5.00 $J_{1,2}$ ~4	3.64 $J_{2,3}$ ~10	3.99 $J_{3,4}$ ~10	3.77 $J_{4,5}$ ~10	4.10	
→6)-α-D-Glc*p*NAc-(1→	(**C**)	5.06 $J_{1,2}$ ~4	3.98 $J_{2,3}$ ~9	3.92 $J_{3,4}$ ~9	3.61 $J_{4,5}$ ~9	3.74	3.72, 4.03
→4)-α-L-Gul*p*NA-(1→	(**D***)	5.26 $J_{1,2}$ 3.6	3.60 $J_{2,3}$ ~3.2	4.16 $J_{3,4}$ ~3.5	4.22 $J_{4,5}$ <2	4.69	
→4)-α-L-Gul*p*NA-(1→	(**D**)	5.18 $J_{1,2}$ 3.7	3.60	4.19	4.22	4.69	
→3)-α-D-Fuc*p*NAc	(**E***)	5.21 $J_{1,2}$ 3.5	4.26	3.95	3.95	4.22 $J_{5,6}$ 6.5	1.19
→3)-β-D-Fuc*p*NAc	(**E**)	4.67 $J_{1,2}$ 9.1	3.95 $J_{2,3}$ ~10	3.76 $J_{3,4}$ ~3	3.87 $J_{4,5}$<2	3.78 $J_{5,6}$ 6.4	1.24
Base-degraded LPS							
→3)-α-D-Qui*p*4N-(1→	(**A**)	5.35	3.71	3.67	2.89	3.68	1.30
→4)-α-D-Glc*p*A-(1→	(**B**)	5.05	3.67	4.01	3.80	4.27	
→6)-α-D-Glc*p*NAc-(1→	(**C**)	5.07	3.99	3.83	3.66	3.79	3.74, 4.03
→4)-α-L-Gul*p*NA-(1→	(**D**)	5.24	3.64	4.15	4.24	4.98	
→3)-β-D-Fuc*p*NAc-(1→	(**E**)	4.61	4.03	3.79	4.28	3.78	1.28

Sugar residues in **1** with α-D-Fuc*p*NAc at the reducing end are indicated by asterisk. Chemical shifts for NAc are δ 2.08 (**C**) and 2.11 (**E** and **E***) in the oligosaccharide **1**; δ 2.09 (**C**) and 2.10 (**E**) in the base-degraded LPS.

and the corresponding carbons at δ 50.0–62.1 in the ^{1}H,^{13}C HSQC spectrum. Based on the coupling constant values (Table 1) and using published data,[5,6] it was inferred that oligosaccharide **1** consists of one residue each of α-Glc*p*N, α-Glc*p*A, 4-amino-4,6-dideoxy-α-glucopyranose (α-Qui*p*4N), 2-amino-2-deoxy-α-gulopyranuronic acid (α-Gul*p*NA) and 2-amino-2,6-dideoxygalactopyranose (Fuc*p*N). The latter monosaccharide occupied the reducing end of **1** and correspondingly occurred in an anomeric equilibrium of the α- and β-forms. The monosaccharides were designated as **A**–**E** according to their sequence in the oligosaccharide (see below).

Since the ^{1}H NMR spectrum showed the presence of only two *N*-acetyl groups, only two of four amino sugars are N-substituted, whereas the other two have a free amino group. Chemical shifts for H-2 of residues of α-Glc*p*N and α-Fuc*p*N were similar to those in the corresponding N-acetylated monosaccharides,[7,8] whereas those for H-4 of α-Qui*p*4N and H-2 of α-Gul*p*NA were different (compare δ 2.88 and 3.60 in **1** vs δ 3.58[9] and 4.25[10] in the corresponding N-acylated sugars). These data demonstrated the presence of the free amino group in Qui4N and GulNA.

A 2D H-detected ^{1}H,^{13}C HSQC experiment (Fig. 2) was applied for the assignment of the ^{13}C NMR spectrum of oligosaccharide **1** (Table 2). Significant downfield displacements (by >5 ppm) of the signals for C-4 of GlcA and GulNA, C-6 of GlcNAc and C-3 of FucNAc, when compared with their positions in the corresponding nonsubstituted monosaccharides, indicated the linkage position in each sugar residue.

The 2D ROESY spectrum of oligosaccharide **1** revealed the following inter-residue correlations between the anomeric protons and protons at the linkage carbons: **A** H-1,**B** H-4 at δ 5.40/3.77; **B** H-1,**C** H-6a and H-6b at δ 5.00/4.03 and 3.72; **C** H-1,**D*** and **D** H-4 at δ 5.06/4.22; **D*** H-1,**E*** H-3 at δ 5.26/3.95 and **D** H-1,**E** H-3 at δ 5.18/3.76. **C** H-1 gave also intense cross-peaks with **D*** and **D** H-3 at δ 5.06/4.16 and 4.19, which could be expected for the equatorial proton H-3 in GulNA.[11] These data were confirmed by a ^{1}H,^{13}C HMBC experiment, which showed the following inter-residue correlation peaks: **A** H-1,**B** C-4 at δ 5.40/78.4; **B** H-1,**C** C-6 at δ 5.00/67.0; **C** H-1,**D*** and **D** C-4 at δ 5.06/74.9. Therefore, oligosaccharide **1** has the following structure:

α-Qui*p*4N-(1→4)-α-Glc*p*A-(1→6)-α-Glc*p*NAc-(1→4)-α-Gul*p*NA-(1→3)-FucNAc

A **B** **C** **D**,**D*** **E**,**E***

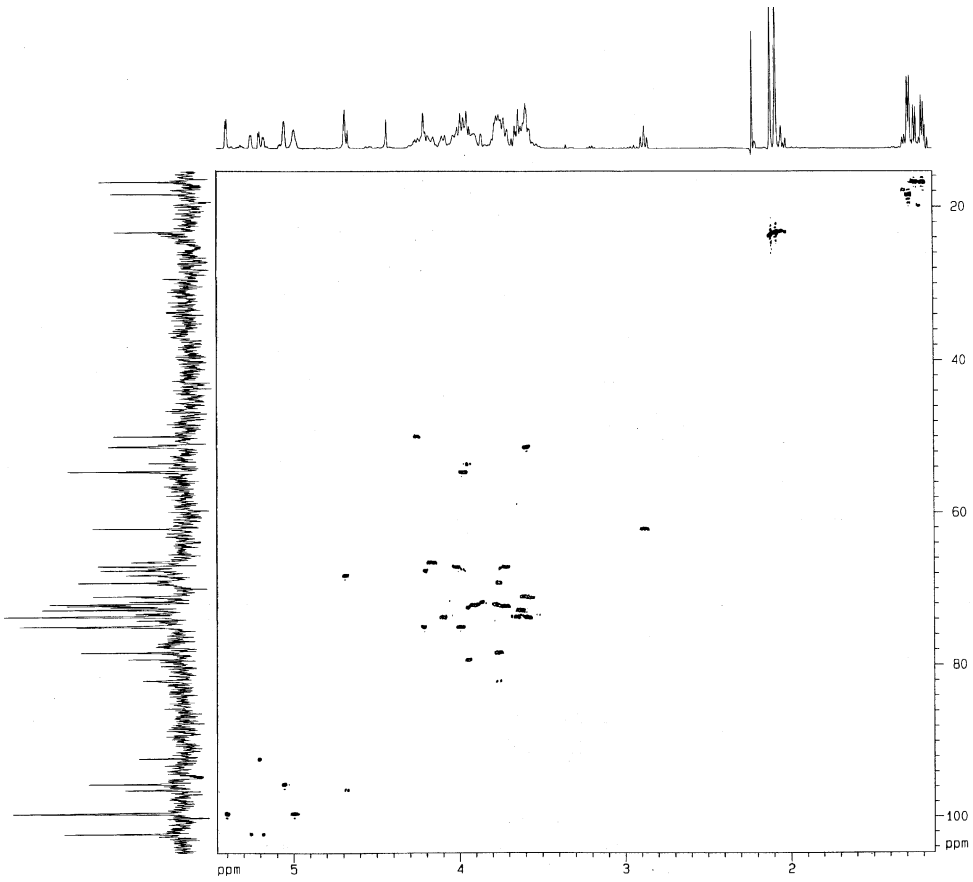


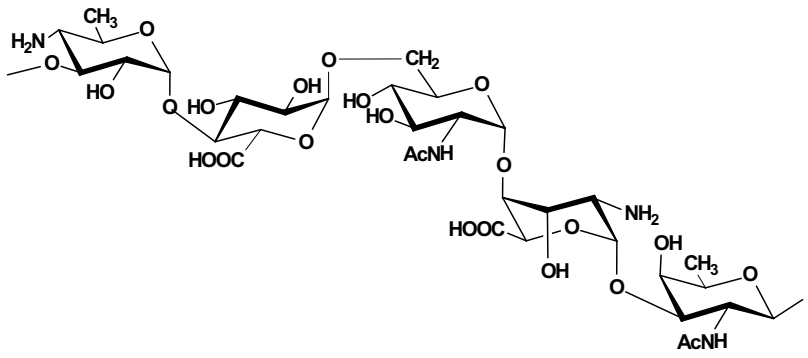
Figure 2. 500 MHz 2D $^{1}H,^{13}C$ HSQC spectrum of the oligosaccharide **1** at 55 °C. ^{1}H and ^{13}C NMR spectra are displayed along the horizontal and vertical axes, respectively.

Table 2. 125 MHz ^{13}C NMR data (δ, ppm)

Sugar residue		C-1	C-2	C-3	C-4	C-5	C-6
Oligosaccharide **1**							
α-D-Qui*p*4N-(1→	(**A**)	99.5	73.6	73.6	62.1	69.2	18.4
→4)-α-D-Glc*p*A-(1→	(**B**)	99.5	72.7	74.9 (+0.9)	78.4	73.6	175.5
→6)-α-D-Glc*p*NAc-(1→	(**C**)	96.6 (+4.5)	54.6	72.1	71.0	72.3	67.0
→4)-α-L-Gul*p*NA-(1→	(**D***)	102.2	51.4	66.5	74.9	68.2	176.0
→4)-α-L-Gul*p*NA-(1→	(**D**)	102.2	51.3	66.5	74.9	68.2	176.0
→3)-α-D-Fuc*p*NAc	(**E***)	92.3	50.0	79.3	72.4 (0.0)	67.6	16.8
→3)-β-D-Fuc*p*NAc	(**E**)	96.4	53.5	82.0	71.7 (−0.3)	72.05	16.8
Base-degraded LPS							
→3)-α-D-Qui*p*4N-(1→	(**A**)	100.2	72.0	80.4	60.0	70.7	18.9
→4)-α-D-Glc*p*A-(1→	(**B**)	99.5	72.5	74.4	78.4	72.0	175.4
→6)-α-D-Glc*p*NAc-(1→	(**C**)	96.8	54.5	71.9	71.1	72.3	67.3
→4)-α-L-Gul*p*NA-(1→	(**D**)	102.2	51.1	66.9	75.9	67.5	174.2
→3)-β-D-Fuc*p*NAc-(1→	(**E**)	103.5	52.2	82.8	72.4	71.9	16.8

Glycosylation effects used for determination of the relative absolute configurations of the monosaccharides are given in parentheses. Sugar residues in oligosaccharide **1** with α-D-Fuc*p*NAc at the reducing end are indicated by asterisk. Chemical shifts for NAc (CH_3/CO) are δ 23.3/176.6 (**C**), 23.5/176.3 (**E**) and 23.7/175.7 (**E***) in the oligosaccharide **1**; δ 23.3/176.6 (**C**) and 23.8/176.0 (**E**) in the base-degraded LPS.

Comparison of the ^{13}C NMR spectra of oligosaccharide **1** and the base-degraded LPS, together with analysis of the $^{1}H,^{13}C$ HSQC spectrum of the latter, showed that FucNAc is β-linked in the O-polysaccharide. Full assignment of the ^{1}H and ^{13}C spectra was also completed with the help of the previously assigned spectra of oligosaccharide **1** (Tables 1 and 2). A lower chemical shift for C-3 of Qui4N in the ^{13}C NMR spectrum of the base-degraded LPS when compared with that in the oligosaccharide **1** (δ 80.4 vs 73.6) indicated substitution of this sugar at position 3 and, hence, the O-polysaccharide is linear. The unexpectedly high



→3)-α-D-Qui*p*4N-(1→4)-α-D-Glc*p*A-(1→6)-α-D-Glc*p*NAc-(1→4)-α-L-Gul*p*NA-(1→3)-α-D-Fuc*p*NAc-(1→

A B C D E

Figure 3. Structure of the O-polysaccharide of *I. zobellii* KMM 231^T. FucNAc stands for 2-acetamido-2,6-dideoxygalactose, Qui4N for 4-amino-4,6-dideoxyglucose, and GulNA for 2-amino-2-deoxyguluronic acid.

lability of the glycosidic linkage of β-Fuc*p*NAc towards mild acid hydrolysis is evidently associated with the presence of a free amino group adjacent to the site of the attachment of FucNAc to Qui4N (Fig. 3).

The absolute configurations of GlcNAc and GlcA were determined to be D using GLC analysis of glycosides with chiral alcohols. In order to ascertain the relative absolute configurations of the other constituent monosaccharides, glycosylation effects[11,12] in the ^{13}C NMR spectrum of oligosaccharide **1** (Table 2) were examined. A small positive β-effect of 0.9 ppm for C-3 of D-Glc*p*A **B** showed that Qui*p*4N **A** and D-Glc*p*A **B** in an α(1→4)-linked disaccharide have the same absolute configurations (a higher by the absolute value negative β-effect would be expected if the absolute configurations were different). Hence, Qui4N is D. A relatively small α-effect of 4.5 ppm for C-1 of D-Glc*p*NAc **C** indicated different absolute configurations of the monosaccharides in the α-Glc*p*NAc-(1→4)-Gul*p*NA disaccharide, that is the L configuration of GulNA **D** (a larger value of ~8 ppm would show the same absolute configurations). The data for units **D** and **E** drew similar conclusions and showed that FucNAc is D. Indeed, a β-effect of 0.0 and −0.3 ppm for C-4 of α-Fuc*p*NAc and β-Fuc*p*NAc, respectively, indicated that the relative absolute configurations of Fuc*p*NAc **E** and Gul*p*NA **D** in an α1→3-linked disaccharide are opposite since a large absolute value negative β-effect of about −4 ppm would be expected for C-4 in the event of both sugars having the same absolute configuration.

On the basis of these data, it was concluded that the pentasaccharide repeating unit of the O-polysaccharide of *I. zobellii* KMM 231^T has the structure shown in Figure 3. The O-polysaccharide contains a rare sugar, 2-amino-2-deoxy-L-guluronic acid, which has been previously found in nature only in a few bacterial glycopolymers, including the acidic capsular polysaccharides of *Vibrio parahaemolyticus* K15[13] and the marine bacteria *Pseudoalteromonas nigrifaciens* KMM 158[14] and KMM 161,[15] as well as in the cell wall of *Halococcus* sp. strain 24.[16] Another rare component of the O-polysaccharide, 4-amino-4,6-dideoxy-D-glucose, has not been discovered previously with the free amino group but rather carrying various *N*-acyl substituents, including formyl,[17] acetyl,[18,19] *N*-acetylglycyl,[20,21] *N*-[(*R*)-3-hydroxybutyryl]-L-alanyl[9] and other groups.

3. Experimental

3.1. Bacterial growth and isolation of LPS

I. zobellii strain KMM 231^T was grown in the modified Youschimizu–Kimura medium.[22] The LPS was isolated in a yield of 6.5% from dry bacterial cells by phenol–water extraction[23] followed by removal of nucleic acid by precipitation with cold aq 50% CCl_3CO_2H.

3.2. Degradations of LPS

Mild acid degradation of the LPS (40 mg) with aq 2% HOAc (100 °C, 40 min) resulted in oligosaccharide **1** (30 mg), which was isolated by sequential GPC on columns of TSK HW-50 and TSK HW-40 in aq 0.3% HOAc.

Mild base degradation of the LPS (74 mg) was performed with 12.5% aq NH_4OH at 37 °C for 16 h, and the base-degraded LPS (50 mg) was isolated by GPC on TSK HW-40 (S) in H_2O.

3.3. Sugar analysis

Oligosaccharide **1** and the D-GlcA standard were subjected to methanolysis with 1 M HCl in MeOH (80 °C, 16 h) followed by 2-butanolysis [100 μL (*S*)-2-butanol, 15 μL CF_3CO_2H, 85 °C, 16 h] and acetylation with a 1:1

pyridine–Ac_2O mixture (100 °C, 2 h). The products were analysed by GLC on a Hewlett–Packard model 5890 Series II instrument equipped with a 30 m capillary column of SPB-5 (Supelco) using a temperature gradient of 150 °C (3 min) to 320 °C at 5 °C min^{-1}. The absolute configuration of GlcNAc was determined to be D by GLC of the (*S*)-2-octyl glycoside as described.[24,25]

3.4. NMR spectroscopy

Samples were deuterium exchanged by freeze drying three times from D_2O and then examined in solutions of 99.97% D_2O, using internal acetone as reference (δ_H 2.225, δ_C 31.45). NMR spectra were recorded at 55 °C (oligosaccharide **1**) or 30 °C (base-degraded LPS) at pH 2 on a JEOL Lambda spectrometer at 400 MHz equipped with a DEC AXP 300 computer workstation or a Bruker DRX-500 spectrometer and processed using IRIX5.3 or XWINNMR 2.6 software on an SGI workstation. The mixing time for the ROESY was 100 ms and the duration of the MLEV17 spin-lock for the TOCSY was 80 ms. Additional 2D NMR parameters were essentially the same as previously described.[26]

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