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Structure of a highly acidic O-specific polysaccharide of lipopolysaccharide of *Pseudoalteromonas haloplanktis* KMM 223 (44-1) containing L-iduronic acid and D-QuiNHb4NHb[1]

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Abstract

An acidic O-specific polysaccharide was obtained by mild acid degradation of the lipopolysaccharide isolated by phenol–water extraction of *Pseudoalteromonas haloplanktis* strain KMM 223 (44-1). L-Iduronic acid (IdoA) was found to be a component of the polysaccharide and identified by NMR spectroscopy and after carboxyl-reduction followed by acid hydrolysis and acetylation, by GLC-MS as 2,3,4-tri-*O*-acetyl-1,6-anhydroidose. On the basis of ^{1}H and ^{13}C NMR spectroscopic studies, including 1D NOE, 2D NOESY, HSQC and HMBC experiments, the following structure of the branched pentasaccharide repeating unit of the polysaccharide was established:

$$\rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{A}^{\text{I}}\text{-}(1\rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{A}^{\text{II}}\text{-}(1\rightarrow 3)\text{-}\beta\text{-D-Qui}p\text{NHb4NHb}^{\text{II}}\text{-}(1\rightarrow 2)\text{-}\alpha\text{-L-Ido}p\text{A-}(\rightarrow$$

$$\begin{array}{c} 4 \\ \uparrow \\ 1 \\ \alpha\text{-D-Qui}p\text{NAc4NAc}^{\text{I}} \end{array}$$

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where QuiNAc4NAc and QuiNHb4NHb are 2,4-diacetamido-2,4,6-trideoxyglucose and 2,4,6-trideoxy-2,4-di[(*S*)-3-hydroxybutyramido]glucose, respectively. This is the first report of L-iduronic acid in a lipopolysaccharide and of D-QuiNHb4NHb in nature.

Keywords: *Pseudoalteromonas haloplanktis*; O-specific polysaccharide structure; L-Iduronic acid; 2,4-Diamino-2,4,6-trideoxy-D-glucose; (*S*)-3-Hydroxybutyric acid; NMR spectroscopy

1. Introduction

The bacterial genera *Alteromonas* and *Pseudoalteromonas* have originated from division of the former genus *Alteromonas* which now includes only one species, *Alteromonas macleodi*, while all other species fall in the genus *Pseudoalteromonas* [1]. These bacteria are obligatory marine procaryotes widespread among heterotrophic bacteria which inhabit marine water [2,3]. They are known to produce a number of biologically active compounds, such as antibiotics, toxins, enzymes, antitumor and antiviral agents [4,5]. Studies on the lipopolysaccharides of *Alteromonas* and *Pseudoalteromonas* have recently been initiated with data on the structure of the O-specific polysaccharides (O-antigens) being reported for a small number of strains [6–10]. Here we report on the structure of a new O-specific polysaccharide isolated from *P. haloplanktis* strain KMM 223 (44-1).

2. Results and discussion

Bacterial cells were extracted with aq 45% phenol, and the lipopolysaccharide was recovered from the aqueous phase and degraded with dilute acetic acid to give the O-specific polysaccharide.

Acid hydrolysis of the polysaccharide gave glucuronic acid and another hexuronic acid having the same retention time in anion-exchange chromatography as iduronic acid and altruronic acid, which could not be separated on the Dionex A×8–11 resin used. Methanolysis of the polysaccharide followed by carboxyl reduction, acid hydrolysis, acetylation and GLC-MS analysis resulted in the identification of 2,3,4-tri-*O*-acetyl-1,6-anhydroidose which was undistinguished from the reference compound obtained by the same procedure from heparin but different by retention time from 2,3,4-tri-*O*-acetyl-1,6-anhydroaltrose from the O-specific polysaccharide of *Proteus mirabilis* O10 [11]. Another monosaccharide component, 2,4-diamino-2,4,6-trideoxyglucose (QuiN4N), was not detected; it was identified in the course of NMR spectroscopic studies of the polysaccharide (see below).

The ^{13}C NMR spectrum of the polysaccharide (Fig. 1, Table 1) contained signals for five anomeric carbons at δ 95.1, 100.5, 103.3, 103.6, and 104.7, four carbons bearing nitrogen (C-2 and C-4 of two QuiN4N residues) at δ 55.2, 56.6, 57.6, and 57.9, two CH_3-C groups (C-6 of QuiN4N) at δ 18.0 and 18.5, three COOH groups (C-6 of GlcA and IdoA) at δ 172.3, 172.8, and 174.1, as well as two *N*-acetyl and two *N*-(3-hydroxybutyryl) (Hb) groups {CH_3 at δ 23.3–23.9, CO at δ 175.1–175.6, C-2 and C-3 of Hb at δ 46.3 (2 C) and 66.1–66.2, respectively; compare published data, e.g., ref. [12]}. The $^1J_{C,H}$ coupling constant values determined from the gated-decoupling spectrum of the polysaccharide, showed that all sugar residues are in the pyranoid form [13] and that three of them are β-linked ($^1J_{C,H}$ 161–162 Hz), while two others are α-linked ($^1J_{C,H}$ 171–172 Hz) [14].

The low-field region of the ^1H NMR spectrum of the polysaccharide (Fig. 2, Table 2) contained six signals at δ 4.445, 4.579, 4.644, 5.091, 5.109, and 5.181, five of which belonged to anomeric protons and the remaining one to H-5 of IdoA (see below). The high-field region of the spectrum contained signals for two *N*-acetyl groups (CH_3 at δ 2.034 and 2.136, both s), four more CH_3 groups (Hb H-4 and QuiN4N H-6) at δ 1.189–1.253 (all d), and two CH_2 groups (Hb H-2) at δ 2.358 and 2.440 (both d) (cf. published data [12,15]).

Therefore, the polysaccharide has a pentasaccharide repeating unit containing two residues of QuiN4N and three residues of hexuronic acids (two GlcA and one IdoA), two amino groups of the diamino sugar residues being acetylated and two others acylated by the 3-hydroxybutyryl group. The absence from the ^{13}C NMR spectrum of any signals for non-anomeric sugar carbons at a lower field than δ 81 demonstrated again the pyranoid form of all sugar residues [16].

GLC of acetylated (*R*)-2-butyl glycosides and trifluoroacetylated (*S*)-2-octyl esters showed that glucuronic acid has the D configuration and 3-hydroxybutyric acid the *S* configuration, respectively. The L configuration of IdoA and the D configuration of QuiN4N were determined by analysis of the glycosylation effects in the ^{13}C NMR spectrum of the polysaccharide (see below).

The ^{1}H NMR spectrum of the polysaccharide was assigned using 2D COSY and H,H-relayed COSY experiments (Table 2). The spin-systems for two GlcA and two QuiN4N residues were identified based on the $J_{2,3}$, $J_{3,4}$, and $J_{4,5}$ coupling constant values (Table 2) and, for QuiN4N, confirmed by correlations of the protons at carbons bearing nitrogen (H-2 and H-4) to the corresponding carbons, which were revealed in an HSQC experiment (Table 1). The $J_{1,2}$ coupling constant values demonstrated that one of the QuiN4N residues (QuiN4N^{I}) is α-linked ($J_{1,2}$ 3.5 Hz), while the second QuiN4N residue (QuiN4N^{II}) and both GlcA residues (GlcA^{I} and GlcA^{II}) are β-linked ($J_{1,2}$

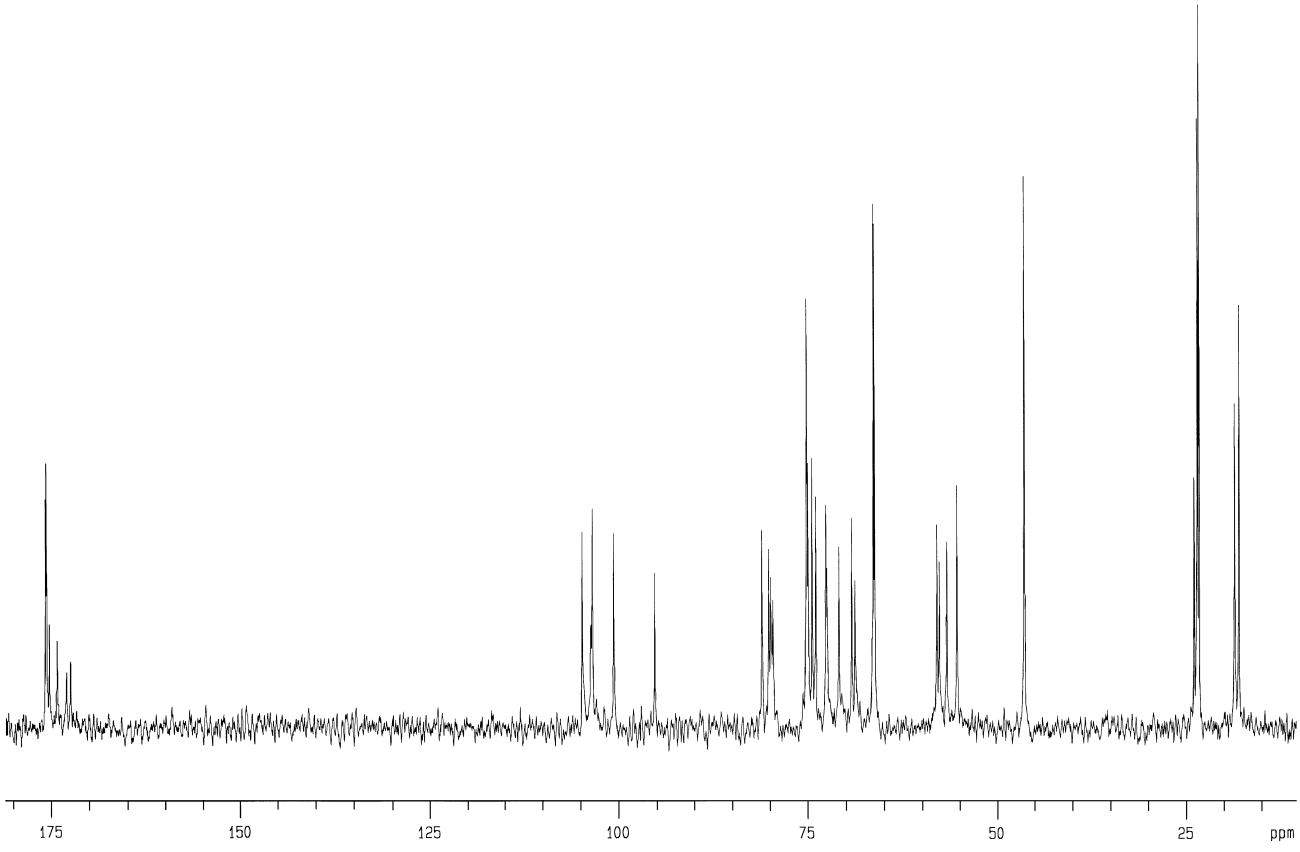


Fig. 1. ^{13}C NMR spectrum of the O-specific polysaccharide

Table 1
^{13}C NMR chemical shifts for the O-specific polysaccharide. QuiN4N, 2,4-diamino-2,4,6-trideoxy-D-glucose; Hb, (*S*)-3-hydroxybutyryl

Sugar residue	C-1	C-2	C-3	C-4	C-5	C-6	2-*N*-Ac or 2-*N*-Hb				4-*N*-Ac or 4-*N*-Hb			
							C-1	C-2	C-3	C-4	C-1	C-2	C-3	C-4
→4)-β-D-Glc*p*A^{I}-(1→	103.3	73.8	75.0	80.0	74.8	172.8								
→4)-β-D-Glc*p*A^{II}-(1→	104.7	73.8	75.0	80.9	75.0	172.3								
→3)-β-D-Qui*p*NHb4NHb^{II}-(1→	103.6	57.6	79.7	56.6	72.5	18.5	175.4	46.3	66.1	23.5	175.5	46.3	66.2	23.3
→ 2)-α-L-Ido*p*A-(1 →	100.5	79.5	66.4	72.3	68.6	174.1								
4 ↑														
α-D-Qui*p*NAc4NAc^{I}-(1 →	95.1	55.2	70.8	57.9	69.1	18.0	175.1	23.9			175.6	23.6		

7.5 Hz). The coupling constant values ($J_{2,3}$ 3, $J_{3,4}$ 3, and $J_{4,5}$ 1.5 Hz) for the fifth sugar residue were close to those ($J_{2,3}$ 3.3, $J_{3,4}$ 3.3, and $J_{4,5}$ 1.6 Hz) for β-L-Ido occurring in the conformation $^1C_4$ but differed dramatically from those for α-L-Ido in the $^4C_1$ conformation, the $J_{1,2}$ value being small (< 2 Hz) in both forms [17]. Taking into account that the repeating unit contains two α-linked

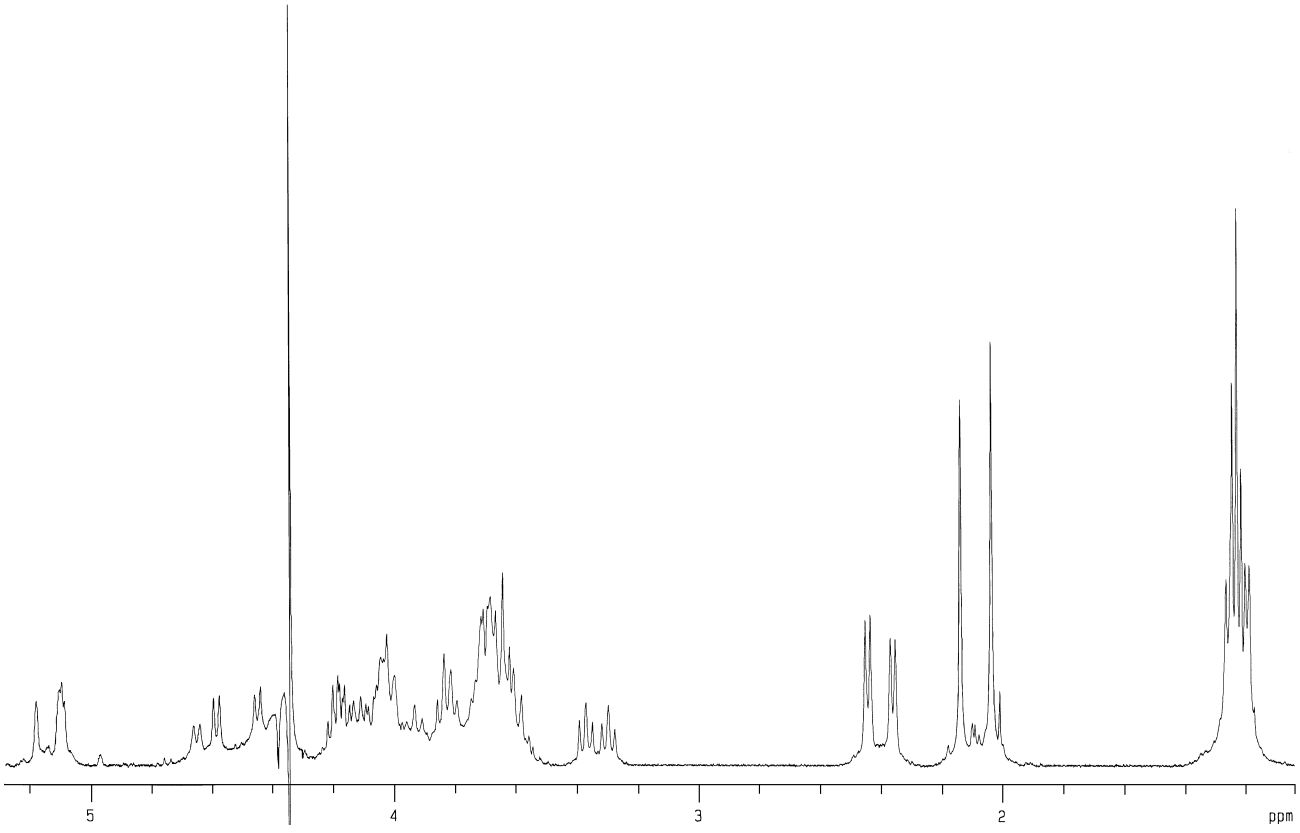


Fig. 2. ^{1}H NMR spectrum of the O-specific polysaccharide

Table 2
^{1}H NMR data for the O-specific polysaccharide. QuiN4N, 2,4-diamino-2,4,6-trideoxy-D-glucose; Hb, (*S*)-3-hydroxybutyryl

Sugar residue							2-*N*-Ac or 2-*N*-Hb			4-*N*-Ac or 4-*N*-Hb		
	H-1	H-2	H-3	H-4	H-5	H-6	H-2	H-3	H-4	H-2	H-3	H-4
→4)-β-D-Glc*p*A^{I}-(1→	4.579	3.367	3.369	3.808	4.110							
	$J_{1,2}$ 7.5	$J_{2,3}$ 9	$J_{3,4}$ 9	$J_{4,5}$ 9								
→4)-β-D-Glc*p*A^{II}-(1→	4.445	3.292	3.639	3.831	4.027							
	$J_{1,2}$ 7.5	$J_{2,3}$ 9	$J_{3,4}$ 9	$J_{4,5}$ 9								
→3)-β-D-Qui*p*NHb4NHb^{II}-(1→	4.644	3.684	3.936	3.636	3.713	1.253	2.358	4.169	1.216	2.440	4.188	1.233
	$J_{1,2}$ 7.5	$J_{2,3}$ 10	$J_{3,4}$ 10	$J_{4,5}$ 10	$J_{5,6}$ 6		$J_{2,3}$ 6	$J_{3,4}$ 6		$J_{2,3}$ 6	$J_{3,4}$ 6	
→ 2)-α-L-Ido*p*A-(1 →	5.181	3.690	4.039	4.002	5.109							
4 ↑	$J_{1,2}$ < 1.5	$J_{2,3}$ 3	$J_{3,4}$ 3	$J_{4,5}$ 1.5								
α-D-Qui*p*NAc4NAc^{I}-(1 →	5.091	4.081	3.576	3.710	3.770	1.189	2.136			2.034		
	$J_{1,2}$ 3.5	$J_{2,3}$ 10	$J_{3,4}$ 10	$J_{4,5}$ 10	$J_{5,6}$ 6							

sugars (see above), it was suggested that L-IdoA is α-linked and exists in the conformation $^1C_4$ with an equatorial carboxyl group and four axial oxygen substituents.

Sequence and linkage analyses of the polysaccharide were performed using both 1D NOE experiments with sequential, selective pre-irradiation of each of the anomeric protons and a 2D NOESY experiment. The following interresidue cross-peaks were observed in the 1D NOE experiments: GlcA^{I} H-1/GlcA^{II} H-4 at δ 4.579/3.831 (strong); GlcA^{II} H-1/QuiN4N^{II} H-3 at δ 4.445/3.936 (strong); QuiN4N^{II} H-1/IdoA H-2 at δ4.644/3.690 (strong); IdoA H-1/GlcA^{I} H-2 and H-4 at δ 5.181/3.367 (weak) and 5.181/3.808 (strong), respectively; QuiN4N^{I} H-1/IdoA H-3 and H-4 at δ 5.091/4.039 and 5.091/4.002 (both strong), respectively. In addition to the NOEs listed above, pre-irradiation of QuiN4N^{I} H-1 and QuiN4N^{II} H-1 caused weak NOEs on some neighbouring protons of IdoA, which were most likely due to spin-diffusion typical of polysaccharides. In accordance with the configurations of the anomeric centres, intraresidue NOEs were observed on H-2, H-3 and H-5 of the β-linked GlcA^{I}, GlcA^{II} and QuiN4N^{II} residues and on H-2 of the α-linked IdoA and QuiN4N^{I} residues.

Thus, the 1D NOE data revealed the glycosylation pattern and the full monosaccharide sequence in the main chain of →4)-GlcA^{I}-(1→4)-GlcA^{II}-(1→3)-QuiN4N^{II}-(1→2)-IdoA-(1→ and a side chain of QuiN4N^{I} attached to either O-3 or O-4 of IdoA at the branching point. A 2D NOESY experiment (Fig. 3) exhibited the expected cross-peaks between the transglycosidic protons within the main chain and a strong cross-peak between QuiN4N^{I} H-1 and IdoA H-4 at δ 5.091/4.002, thus confirming the structure of the main chain and demonstrating a disaccharide fragment QuiN4N^{I}-(1→4)-IdoA.

The ^{13}C NMR spectrum of the polysaccharide was assigned using 2D H-detected ^{1}H,^{13}C HSQC (Table 1). Analysis of the chemical shifts in the spectrum confirmed the positions of substitution of the monosaccharide residues (in particular, the substitution of IdoA at O-2 and O-4) and allowed

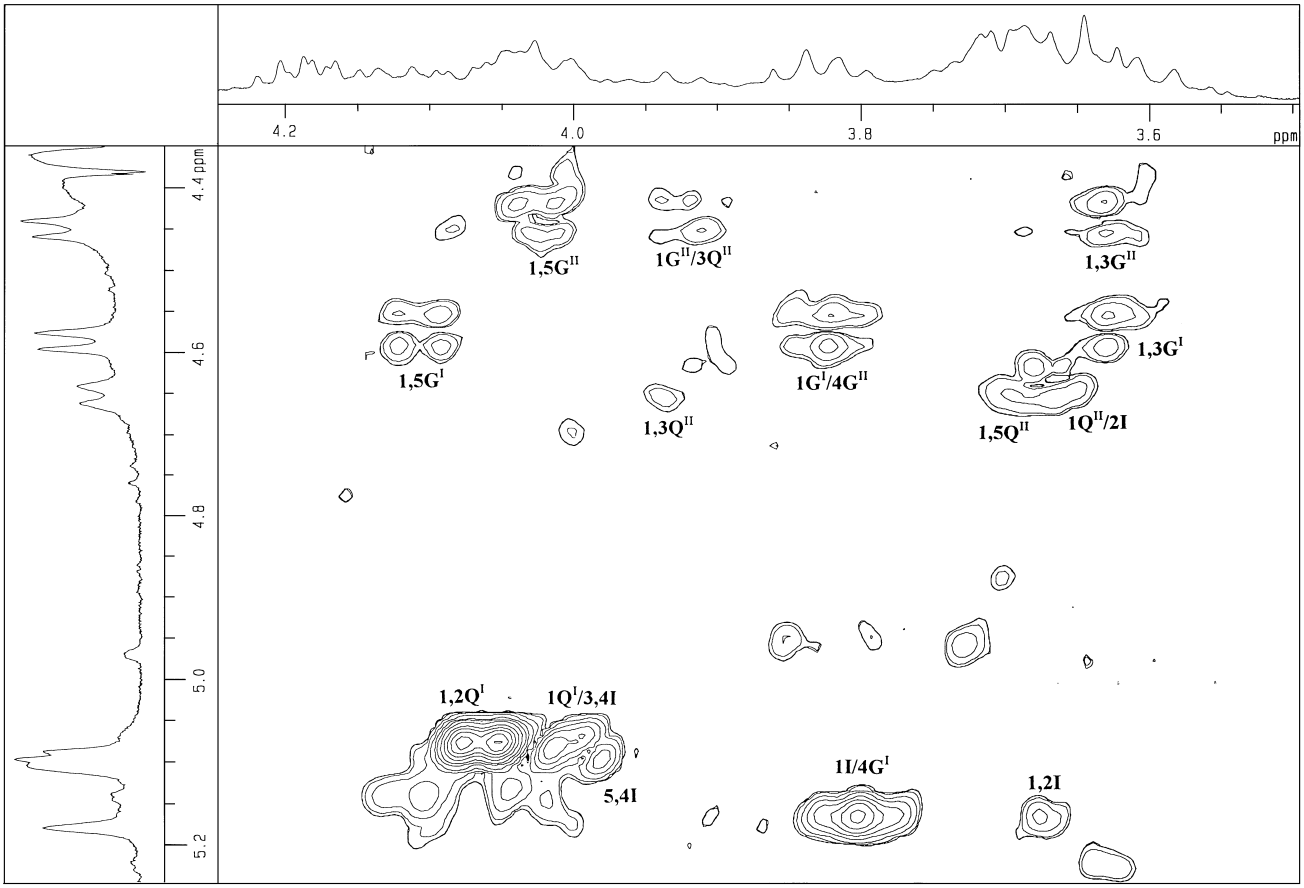
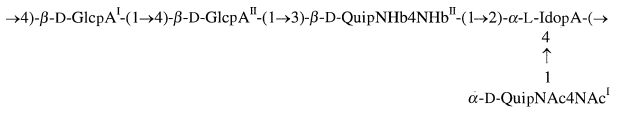


Fig. 3. 2D NOESY spectrum of the O-specific polysaccharide. The corresponding parts of the ^{1}H NMR spectrum are displayed along the axes

determination of the absolute configurations of IdoA and QuiN4N. Thus, small positive and large negative β-effects on C-2 and C-4 of QuiN4N^{II} (+0.1 and −1.3 Δ ppm, respectively) caused by its glycosylation at position 3 with β-D-GlcA^{II}, proved the same absolute configuration of the two monosaccharides, i.e. the D configuration of QuiN4N^{II} [18,19]. A relatively large (absolute value) negative β-effect of glycosylation (−1.8 Δ ppm) on C-3 of D-GlcA^{I} caused by its glycosylation at position 4 with α-IdoA demonstrated different absolute configurations of these sugar residues, i.e., the L configuration of IdoA (in the case of the same absolute configuration the β-effect would be close to 0) [18,19]. Finally, a small positive α-effect of glycosylation (~3 Δ ppm) on C-1 of α-QuiN4N^{I} indicated different absolute configurations of QuiN4N^{I} and L-IdoA substituted by this sugar residue at position 4. Hence, QuiN4N^{I} has the D configuration (an effect of 7–8 ppm would be observed in the case of the L configuration of QuiN4N^{I}; compare published data for 4-substituted L-guluronic acid having the same configurations at C-3 and C-4 and the same $^{1}C_4$ conformation as L-IdoA [19]).

A HMBC experiment confirmed the linkage and the sequence patterns of the polysaccharide (Table 3) and revealed the location of the acyl groups. The carbonyl signals at δ 175.1 and 175.6 were assigned to the *N*-acetyl groups based on their connectivities to the methyl proton signals (CH_3–CO) at δ 2.136 and 2.034, respectively. Correlation peaks were also displayed with H-2 and H-4 of QuiN4N^{I} at δ 4.081 and 3.710, respectively, indicating that QuiN4N^{I} is di-*N*-acetylated. Two additional carbonyl signals at δ 175.4 and 175.5 belonged, thus, to the *N*-[(*S*)-3-hydroxybutyryl] groups which are located at N-2 and N-4 of QuiN4N^{II}. This was confirmed by their correlations to Hb H-2 (CH_2), H-3 and H-4 (CH_3) (Table 2), and to H-2 and H-4 of QuiN4N^{II} at δ 3.684 and 3.636, respectively.

Therefore, on the basis of the data obtained, it was concluded that the O-specific polysaccharide of *P. haloplanktis* strain KMM 223 (44-1) has the following structure:



Remarkably, the polysaccharide is highly acidic and includes three negatively charged groups in the pentasaccharide repeating unit, while other known O-antigens, if acidic, usually contain not more than one negatively charged group per repeating unit [20]. One of the acidic monosaccharides present, L-iduronic acid, is a well-known component of mammalian glycosaminoglycans (dermatan sulfate, heparan sulfate and heparin) [21], but is uncommon in bacterial polysaccharides. To the best of our knowledge, this is the first time that L-iduronic acid is found in a lipopolysaccharide. Iduronic acid of unknown absolute configuration has been hitherto found in surface polysaccharides of only one bacterial species, *Clostridium perfringens* [22,23]. Various derivatives of another unusual monosaccharide, D-QuiN4N, including D-QuiNAc4NAc, D-QuiNHb4NAc and D-QuiNAc4NHb, have been recently reported as components of a

Table 3
Heteronuclear interresidue connectivities for transglycosidic atoms in the 2D HMBC spectrum of the O-specific polysaccharide. QuiN4N, 2,4-diamino-2,4,6-trideoxy-D-glucose; Hb, (*S*)-3-hydroxybutyryl; no interresidue cross-peak was observed for Qui*p*NAc4NAc^{I}

Sugar residue	Chemical shifts for correlating atoms				Connectivity to
	δ_{H-1}	δ_{C-1}	δ_{H}	δ_{C}	
→4)-β-D-Glc*p*A^{I}-(1→	4.579			80.9	Glc*p*A^{II} C-4
		103.3	3.831		Glc*p*A^{II} H-4
→4)-β-D-Glc*p*A^{II}-(1→	4.445			79.7	Qui*p*N4N^{I} C-3
		104.7	3.936		Qui*p*N4N^{I} H-3
→3)-β-D-Qui*p*NHb4NHb^{II}-(1→	4.644			79.5	Ido*p*A C-2
		103.6	3.690		Ido*p*A H-2
→ 2)-α-L-Ido*p*A-(1 →[a]	5.181			80.0	Glc*p*A^{I} C-4
4					
↑		100.5	3.808		Glc*p*A^{I} H-4

[a] In addition to the interresidue cross-peaks, a cross-peak for Ido*p*A H1/C5 was observed at δ 5.181/68.6.

few other bacterial polysaccharides [12,15,20,24]. However, we report here in the polysaccharide of *P. haloplanktis* the first occurrence in nature of the di(3-hydroxybutyryl) derivative, D-QuiNHb4NHb.

3. Experimental

Bacterial strain, growth, and isolation of the polysaccharide.—*P. haloplanktis* strain KMM 223 (44-1) was isolated in June 1985 from marine water in the north-west of the Pacific Ocean. The bacterium was grown in the modified Youschimizu–Kimura medium [25]. The lipopolysaccharide produced by the microorganism was isolated from dried bacterial cells by the Westphal procedure in a yield of 5% [26] and degraded with 1% HOAc for 2 h at 120 °C to give the O-specific polysaccharide isolated in a yield of 62% by GPC on a column (2.5×90 cm) of TSK 50(F) in water.

Chemical analyses.—Hydrolysis of the polysaccharide was performed with 2 M CF_3CO_2H at 120 °C for 2 h, and hexuronic acids were analysed using a Biotronik LC-2000 sugar analyser, a Dionex A×8–11 anion-exchange resin, and 0.02 M potassium phosphate buffer (pH 2.4) at 60 °C. Commercial glucuronic acid (Reanal, Hungary), iduronic acid from heparin (Spopha, Czechia) and altruronic acid from the O-specific polysaccharide of *P. mirabilis* O10 [11] were used as references.

Methanolysis of the polysaccharide was performed with 1 M HCl in MeOH at 80 °C for 16 h, products were carboxyl-reduced with $LiBH_4$ in aq 70% 2-propanol at 20 °C for 16 h, hydrolysed as above and, after conventional reduction with $NaBH_4$ and full acetylation with Ac_2O in pyridine, analysed by GLC on a Hewlett–Packard 5890 instrument equipped with a glass capillary column (13 m×0.25 mm) coated with DB-5 stationary phase using a temperature program of 160 °C (3 min) to 250 °C at 10 °C/min and by GLC-MS (EI) using the same chromatograph equipped with a Hewlett–Packard 5970 mass spectrometer.

The absolute configuration of glucuronic acid was determined by the published method [27] modified as described [10]. The absolute configuration of 3-hydroxybutyric acid was determined by the published method [28] modified [15] as follows: the polysaccharide hydrolysate was concentrated to dryness, the residue dried over P_2O_5 for 16 h at 20 °C, heated with (*R*)-2-octanol in the presence of conc. HOAc for 8 h at 120 °C, the mixture concentrated to dryness, the residue acylated with $(CF_3CO)_2O$ for 16 h at 4 °C, and analysed by GLC.

NMR spectroscopy.—Samples were deuterium-exchanged by freeze-drying three times from D_2O and then examined in solutions of 99.97% D_2O. Spectra were recorded at 70 °C on a Jeol 400 MHz spectrometer equipped with a DEC AXP 300 computer. A mixing time of 1 s was used in 1D NOE experiments. The parameters used for 2D experiments were as follows: COSY (256×512 data matrix; zero-filled to 512 data points in t_1; 16 scans per t_1 value; spectral width 1908.0 Hz; recycle delay 0.7317 s; unshifted sine-bell filtering in t_1 and t_2); NOESY (256×512 data matrix; zero-filled to 512 data points in t_1; 16 scans per t_1 value; spectral width 1912.0 Hz; mixing time 200 ms; shifted sine-squared filtering in t_1 and t_2); H,H-relayed COSY (256×512 data matrix; zero-filled to 512 data points in t_1; 8 scans per t_1 value; spectral width 1912.0 Hz; delay for coupling constants 9 Hz; recycle delay 1.0 s; unshifted sine-bell filtering in t_1 and t_2); HSQC (256×512 data matrix; zero-filled to 512 data points in t_1; 64 scans per t_1 value; spectral width in t_1 1749.2 Hz and in t_2 9643.2 Hz; delay for coupling constants 145 Hz; recycle delay 1.0 s; shifted sine-squared filtering in t_1 and t_2); HMBC (512×1024 data matrix; zero-filled to 1024 data points in t_1; 56 scans per t_1 value; spectral width in t_1 1873.0 Hz and in t_2 16977.9 Hz; delay for coupling constants 145 Hz; recycle delay 1.5 s; shifted sine-squared filtering in t_1 and t_2).

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