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Carbohydrate Research 338 (2003) 1801–1806
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Note

Structure of the biological repeating unit of the O-antigen of *Pseudomonas aeruginosa* immunotype 4 containing both 2-acetamido-2,6-dideoxy-D-glucose and 2-acetamido-2,6-dideoxy-D-galactose

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Received 28 April 2003; accepted 5 June 2003

Abstract

A phosphorylated core-lipid A backbone oligosaccharide that carries a disaccharide remainder of the first O-antigen repeating unit was derived by strong alkaline degradation following mild hydrazinolysis of the lipopolysaccharide of *Pseudomonas aeruginosa* immunotype 4 (serogroup O-1). The structure of the oligosaccharide was determined using ESI MS and NMR spectroscopy and it was demonstrated that 2-acetamido-2,6-dideoxy-D-glucose is the first monosaccharide of the O-polysaccharide that is linked to the LPS core. These data define the structure of the biological repeating unit of the O-antigen.
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Keywords: Lipopolysaccharide; O-Polysaccharide; O-Antigen; Repeating unit; *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is an opportunistic human pathogen causing significant infections in immunocompromised individuals and cystic fibrosis patients. Lipopolysaccharide (LPS) is one of the virulence factors and the major surface antigen of this bacterium. Based on the immunospecificity of the O-polysaccharide chains of the LPS (O-antigens), strains of *P. aeruginosa* are divided into more than 20 O-serotypes.[1–5] The chemical structure of the O-polysaccharide has been established in all serotypes.[5,6] However, until recently, the structure of the actual biological repeating unit remained unknown. This represents the properly ordered oligosaccharide, which is assembled on a lipid carrier and then, after having been polymerized into the O-polysaccharide or as such, transferred to the LPS core. To know the structure of the biological repeating unit is essential for elucidation of the O-antigen biosynthesis pathway and understanding on the molecular level the immunospecificity of the bacteria, which is substantially defined by a saccharide that occupies the non-reducing end of the O-polysaccharide.

Biosynthesis of the O-polysaccharides of *P. aeruginosa*[7] proceeds by typical O-antigen polymerase (Wzy)-dependent pathway, which is initiated by a galactosylphosphate transferase (WbpL)-mediated transfer of the first monosaccharide of the repeating unit to undecaprenyl phosphate according to the following scheme: UDP-Sug + Und-*P* = Und-*P*-*P*-Sug + UMP. It was proposed,[7] and later confirmed chemically,[8,9] that in *P. aeruginosa* O-5 and O-6 this sugar is 2-acetamido-2,6-dideoxy-D-galactose (D-FucNAc) or 2-acetamido-2,6-dideoxy-D-glucose (D-QuiNAc), respectively. Recently, a 2-acetamido-2,6-dideoxy-D-hexose (D-FucNAc or D-

Abbreviations: FucN, 2-amino-2,6-dideoxy-D-galactose; GlcN3NA, 2,3-diamino-2,3-dideoxyglucuronic acid; Hep, L-*glycero*-D-*manno*-heptose; HPAEC, high-performance anion-exchange chromatography; Kdo, 3-deoxy-D-*manno*-oct-2-ulosonic acid; LPS, lipopolysaccharide; QuiN, 2-amino-2,6-dideoxy-D-glucose; ΔHexN3NA, 2,3-diamino-2,3-dideoxy-L-*threo*-hex-4-enuronic acid.

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doi:10.1016/S0008-6215(03)00262-3

QuiNAc) has been demonstrated to be the first monosaccharide of the O-polysaccharide also in the LPS of serogroups O-11[10] and O-10,[11] respectively. This feature could be common for most other *P. aeruginosa* serogroups, in whose O-polysaccharides D-QuiNAc and D-FucNAc are abundant.[5,6] In this work, we further confirmed this suggestion and showed that when D-FucNAc and D-QuiNAc are present simultaneously, the latter is attached to the core and, thus, is the first monosaccharide of the biological repeating unit.

A peculiar feature of the O-polysaccharide of *P. aeruginosa* immunotype 4 (serogroup O-1) is the presence of 2,3-diacetamido-2,3-dideoxy-D-glucuronic acid (D-GlcNAc3NAcA), together with D-GalNAc, D-QuiNAc and D-FucNAc. The structure of the chemical tetrasaccharide repeating unit has been established by studies of the polysaccharide, which was released by mild acid degradation of the LPS.[5,12] For elucidation of the structure of the biological repeating unit, the LPS was deacylated by strong alkaline treatment following O-deacylation by mild hydrazinolysis.[13] The resulting products were fractionated by high-performance anion-exchange chromatography (HPAEC) to give two major fractions, I and II (Fig. 1).

Fraction II contained two isomeric core-lipid A backbone oligosaccharide pentakisphosphates (**1** and **2**) in the ratio ~7:1, which differ in the site of attachment of the terminal rhamnose residue in the outer core region and are called core glycoforms 1 and 2, respectively.[8,9,11,14] Both **1** and **2** in the ratio ~2.5:1 were obtained earlier from the LPS of a rough cystic fibrosis isolate, *P. aeruginosa* 2192, and characterized as *N*-acetyl derivatives.[14] The major compound **1** is identical to the core glycoform 1 oligosaccharide derived from the LPS of *P. aeruginosa* immunotype 1 (serogroup O-6)[9] and O-12.[15] The glycoform 2 oligosaccharide in all smooth *P. aeruginosa* strains studied previously is linked to an O-polysaccharide by a base-stable bond and, therefore, was not released on strong alkaline treatment.[8,9,11,15]

Fraction I contained two other oligosaccharide pentakisphosphates (**3a,b**) in similar amounts, which represent the core-lipid A backbone bearing a D-FucN→D-QuiN disaccharide remainder of the first O-antigen repeating unit (Fig. 2). **3b** differs from **3a** in the presence of an *N*-acetyl group at D-QuiN and, hence, resulted from incomplete N-deacylation during strong alkaline treatment of the LPS. As expected, **3** has the glycoform 2 outer core region (see above). Therefore, in the strain studied the majority of the glycoform 2 core is substituted with the O-polysaccharide giving rise to compounds **3**, whereas an unsubstituted minority afforded compound **2**.

The structures of **1**–**3** were determined using ESI FT-MS and NMR spectroscopy. The ESI mass spectra showed molecular masses of 2357.52 Da for **1** and **2**, 2647.66 Da for **3a** and 2689.68 Da for **3b**. The ^{1}H NMR spectra of **1** and **3** (Table 1, Fig. 3) were assigned using 2D ^{1}H, ^{1}H COSY and TOCSY experiments as described previously.[9,11,14] The ^{1}H NMR chemical shift, ^{1}H,^{13}C and ^{1}H,^{31}P HMQC and NOE correlation patterns of the core-lipid A backbone moiety in **1** and **3** were practically identical to those in the corresponding compounds isolated previously from the LPS of *P. aeruginosa* immunotypes 1 (O-6).[9] The structure and the attachment site of the D-FucN→D-QuiN disaccharide fragment in **3** was established by a ROESY experiment, which showed FucN H-1,QuiN H-3 and QuiN H-1,Rha H-3 correlations at δ 5.68/3.83 and 4.91/4.01 in **3a** or δ 5.49/3.70 and 4.73/3.86 in **3b**, respectively. The α configuration of the FucN linkage and the β configuration of the QuiN linkage were determined by $J_{1,2}$ values of 3.5 and 7.5 Hz, respectively. Compound **3b** was distinguished from **3a** by a significantly lower-field position of the H-2 signal of QuiN (3.86 vs. 3.19).

On strong alkaline treatment, 4-substituted hexuronic acids and 2-acylamino-2-deoxyhexuronic acids undergo β-elimination and are converted into the corresponding hex-4-enuronic acids. As a result, the O-polysaccharides of *P. aeruginosa* immunotypes 1 (O-6)[9] and 5 (O-10)[11] that contain 2-amino-2-deoxygalacturonic acid derivatives, were depolymerised during strong alkaline treatment to give the core-lipid A backbone oligosaccharides bearing a remainder of the first repeating unit that is terminated with a 2-amino-2-deoxyhex-4-enuronic acid residue. Formation of **3** shows that the O-polysaccharide of *P. aeruginosa* immunotype 4 (O-1) was depolymerized too but afforded no expected product **4**, probably owing to a deeper degradation of GlcNAc3NAcA. An attempt to isolate **4** by a decrease of the reaction time from 16 to 4 h was unsuccessful and resulted only in an increase of the content of **3b** in the isolated **3a,b** mixture.

The structure of **3** in combination with that of the chemical repeating unit[12] defined the following full structure of the O-antigen of *P. aeruginosa* immunotype 4 (serogroup O-1):

α-D-Gal*p*NAc-(1→4)-β-D-Glc*p*NAc3NAcA-(1→3)-α-D-Fuc*p*NAc-(1→3)-α-D-Qui*p*NAc-(1-
[→4)-α-D-Gal*p*NAc-(1→4)-β-D-Glc*p*NAc3NAcA-(1→3)-α-D-Fuc*p*NAc-(1→3)-α-D-Qui*p*NAc-(1-]$_n$
→4)-α-D-Gal*p*NAc-(1→4)-β-D-Glc*p*NAc3NAcA-(1→3)-α-D-Fuc*p*NAc-(1→3)-β-D-Qui*p*NAc-(1→

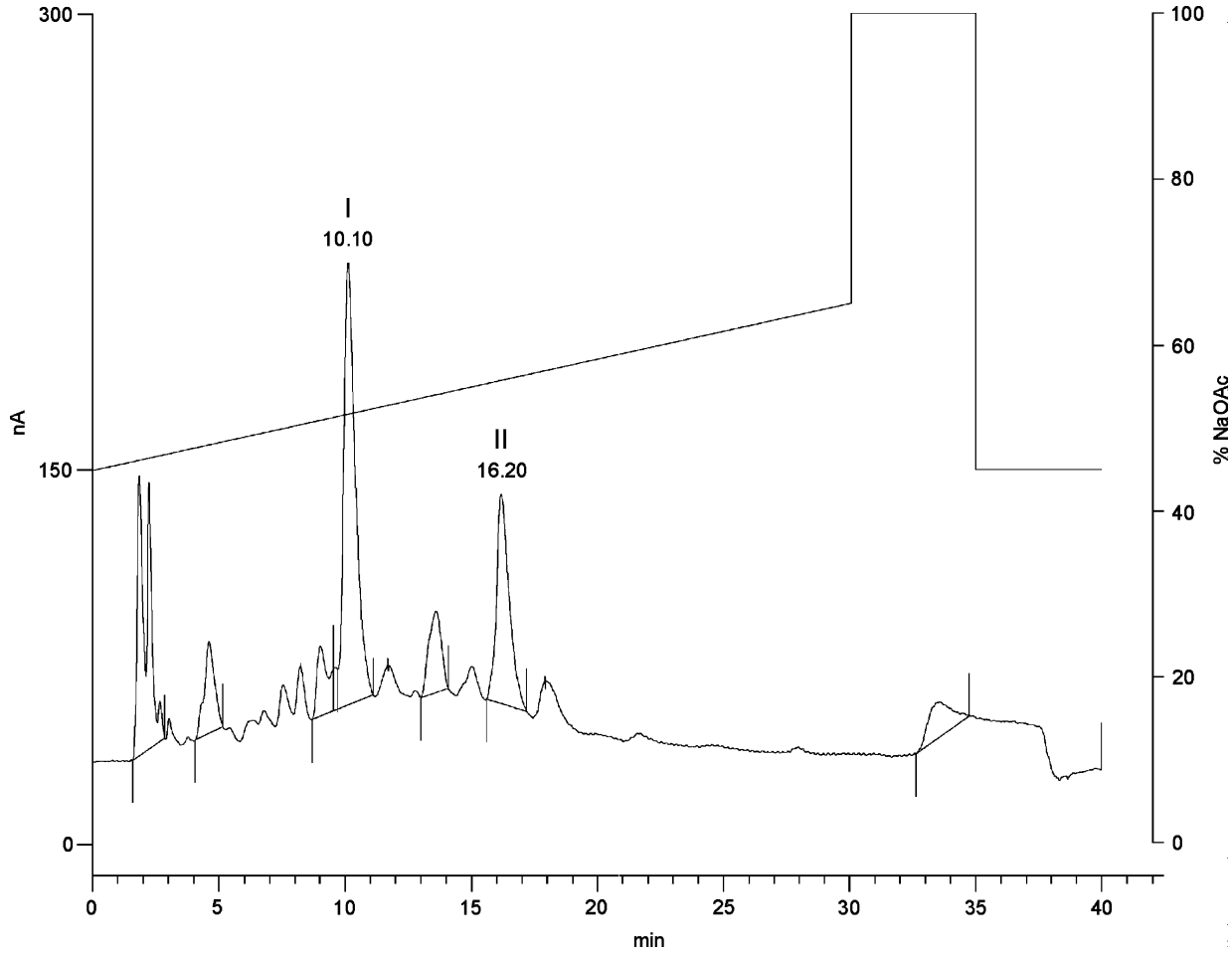

Fig. 1. HPAEC of the alkaline degradation products from the LPS of *P. aeruginosa* immunotype 4. Fractions I and II correspond to the core-lipid A backbone oligosaccharide bearing a D-FucN → D-QuiN disaccharide remainder of the first O-antigen repeating unit and the unsubstituted core-lipid A backbone oligosaccharide, respectively.

In contrast to the three other constituent monosaccharides, the anomeric configuration of QuiNAc in the first and interior O-polysaccharide repeating units is different. This finding is in accordance with the involvement of different enzymes in the processes with formation of the two types of the QuiNAc linkage, namely, O-antigen polymerase (Wzy) in connection of the repeating units to each other and ligase (WaaL) in transfer of the O-polysaccharide to the core.[7]

According to our preliminary data, D-QuiNAc is also the first monosaccharide in the biological repeating unit of the O-polysaccharide of *P. aeruginosa* O-9, which contains D-QuiNAc, D-FucNAc and a di-*N*-acyl derivative of a nonulosonic (pseudaminic) acid.[5,16] Therefore, when present, D-QuiNAc is the monosaccharide whose transfer to Und-*P* initiates biosynthesis of the O-antigen whether or not D-FucNAc is present. In *P. aeruginosa* O-9, as well as in the other *P. aeruginosa* strains studied (Refs. 8, 9, 11, 15 and this work), the 2-acetamido-2,6-dideoxy-D-hexose is attached to the core by the β-linkage.

1. Experimental

1.1. Bacterial strain, growth and isolation of the lipopolysaccharide

P. aeruginosa immunotype 4, strain 170014, was from the Hungarian National Collection of Medical Bacteria (National Institute of Hygiene, Budapest). It belongs to serogroup O-1 of the international antigenic typing system (IATS). Cells were grown in Roux flasks with solid agar medium on Hottinger broth at 37 °C for 18 h, then washed in physiological saline, separated by centrifugation, washed with acetone and dried. LPS was isolated from dry bacterial cells by extraction with aq 45% phenol for 30 min at 65–68 °C.[17] Cells were removed by centrifugation. The supernatant was dialyzed against running water, nucleic acids were precipitated by acidification with aq 50% CCl_3CO_2H to pH 2.5 and removed by centrifugation. The supernatant was dialyzed against distilled water and lyophilized.

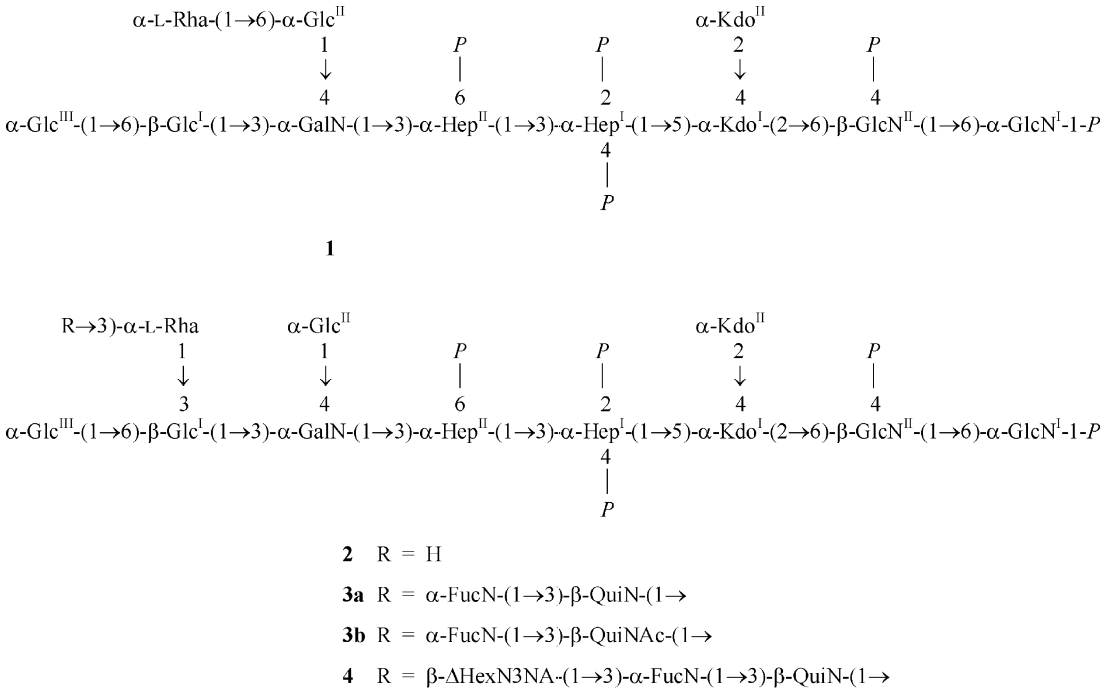

Fig. 2. Structures of the alkaline degradation products from the LPS of *P. aeruginosa* immunotype 4. Abbreviations: ΔHexN3NA, 2,3-diamino-2,3-dideoxy-L-*threo*-hex-4-enuronic acid; FucN, 2-amino-2,6-dideoxygalactose; Hep, L-*glycero*-D-*manno*-heptose; Kdo, 3-deoxy-D-*manno*-oct-2-ulosonic acid; QuiN, 2-amino-2,6-dideoxyglucose. All monosaccharides are in the pyranose form and have the D configuration unless stated otherwise.

1.2. Alkaline degradation of the lipopolysaccharide

LPS (200 mg) was treated with anhyd hydrazine (4 mL) for 1 h at 37 °C, diluted with water, dialyzed against distilled water and lyophilized. The product was dissolved in 4 M NaOH (8 mL), flushed with nitrogen for 1 h with stirring, heated at 100 °C for 16 h, cooled, acidified with conc HCl to pH 5.5, extracted twice with

Table 1
^{1}H NMR data of **3** (δ, ppm)

Sugar residue	H-1 H-3ax	H-2 H-3eq	H-3 H-4	H-4 H-5	H-5 H-6	H-6(6a) H-7	H-6b(7a) H-8a	H-7b H-8b
→6)-α-D-Glc*p*N^{I}-1-*P*	5.68	3.45	3.93	3.64	4.16	3.80	4.32	
→6)-β-D-Glc*p*N^{II}-(1→	4.88	3.09	3.87	3.78	3.78	3.46	3.72	
→4,5)-α-Kdo*p*^{I}-(2→	2.01	2.23	4.11	4.30	3.68 [a]	3.87	3.63	3.92
α-Kdo*p*^{II}-(2→	1.82	2.08	4.16	4.09	3.74 [a]	4.13	3.81	4.03
→3)-α-Hep*p*^{I}2,4*P*-(1→	5.38	4.56	4.21	4.50	4.35	4.01	3.72	3.96
→3)-α-Hep*p*^{II}6*P*-(1→	5.17	4.43	4.25	4.15	4.03	4.53	3.75	3.81
→3,4)-α-D-Gal*p*N-(1→	5.62	3.85	4.48	4.42	4.23	3.89	3.91	
→3,6)-β-D-Glc*p*^{I}-(1→	4.68	3.49	3.68	3.54	3.69	3.84	3.92	
α-D-Glc*p*^{II}-(1→	5.05	3.51	3.77	3.52	4.08	3.85	3.85	
α-D-Glc*p*^{III}-(1→	5.02	3.59	3.73	3.42	3.74	3.74	3.88	
→3)-α-L-Rha*p*-(1→	5.17	4.28	4.01	3.66	4.07	1.25		
	(5.15)	(4.24)	(3.86)	(3.51)	(4.02)	(1.22)		
→3)-β-D-Qui*p*N-(1→	4.91	3.19	3.83	3.49	3.58	1.35		
	(4.73)	(3.86)	(3.70)	(3.43)	(3.54)	(1.33)		
α-D-Fuc*p*N-(1→	5.68	3.54	4.18	3.89	4.22	1.25		
	(5.49)	(3.47)	(4.01)	(3.83)	(3.97)	(1.22)		

When different, data for **3b** are given in parentheses. The signal for the NAc group is at δ 2.04. For abbreviations see legend to Fig. 2.

[a] Tentative assignment.

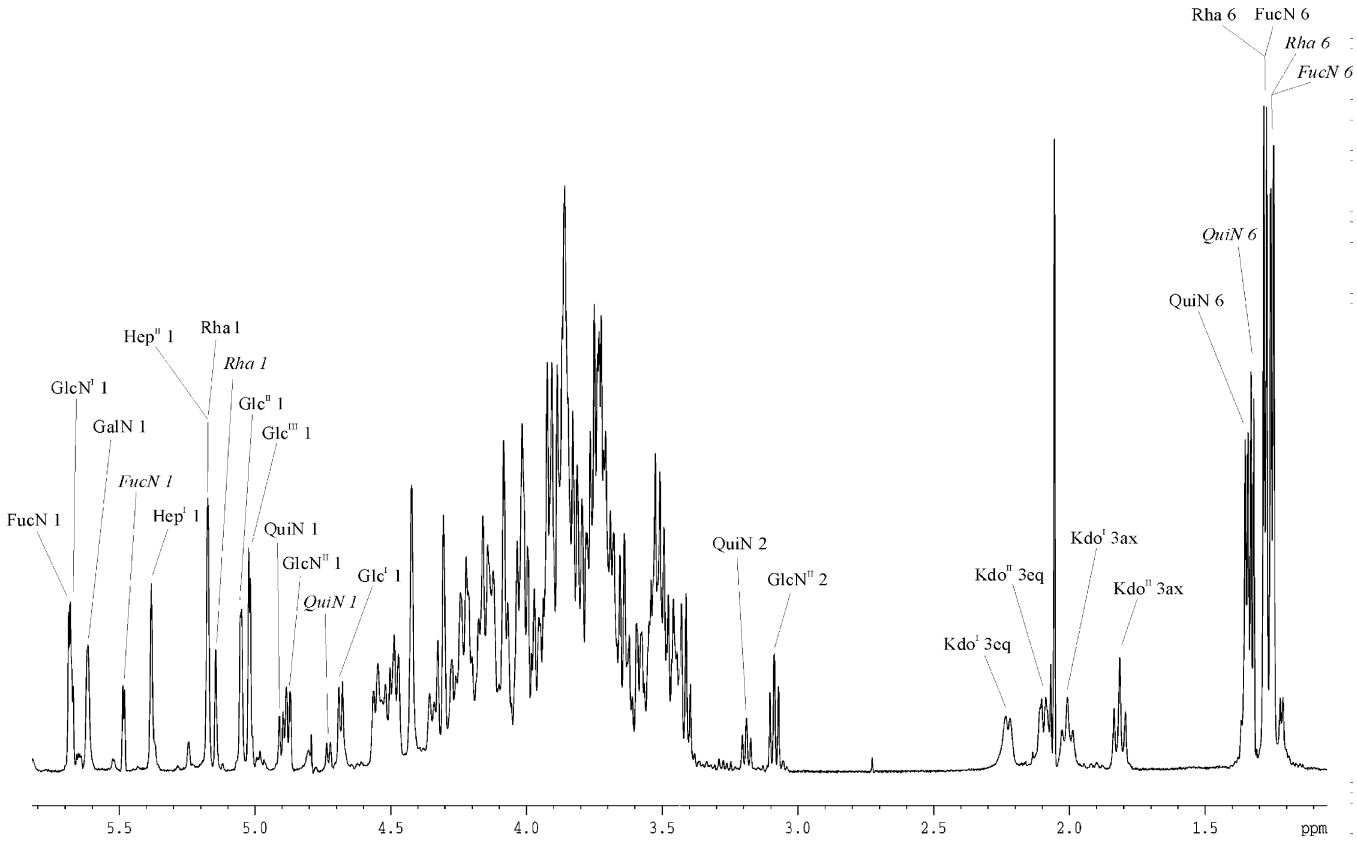

Fig. 3. ^{1}H NMR spectrum of a mixture of compounds **3a,b**. Arabic numerals refer to protons in sugar residues. Designations for Rha, QuiN, and FucN in **3a** and **3b** are not italicized and italicized, respectively.

CH_2Cl_2, the aq layer was desalted by GPC on a column (40 × 2.6 cm) of Sephadex G-50 (S) in 0.1 M NH_4HCO_3 buffer (7.91 g NH_4HCO_3 and 10 mg NaN_3 in 1 L water) at 30 mL h^{-1} monitored with a Knauer differential refractometer. The yield of oligosaccharides was 11.4% of the LPS weight. In an unsuccessful attempt to avoid or reduce degradation of GlcN3NA, treatment of the LPS (200 mg) with 4 M NaOH was performed for a shorter time (4 h) to give oligosaccharides in a yield 8.5% of the LPS weight.

The combined oligosaccharides from both experiments (39 mg) were fractionated by HPAEC on a semi-preparative CarboPac PA1 column (Dionex, USA; 250 × 9 mm) using a linear gradient of 0.40 → 0.60 M NaOAc in 0.1 M NaOH at flow rate 1 mL min^{-1} for 90 min. 2 mL fractions were collected and analyzed by HPAEC with pulsed amperometric detection (Dionex, USA) on an analytical CarboPac PA1 column (250 × 4.6 mm) using a linear gradient of 0.45 → 0.65 M NaOAc in 0.1 M NaOH at 1 mL min^{-1} for 30 min. After desalting on a column (40 × 2.6 cm) of Sephadex G-50, two major oligosaccharides, I and II, having retention times 10.1 and 16.2 min in analytical HPAEC (Fig. 1), were isolated in yields of 21.4 and 10.8% on the initial oligosaccharide mixture weight.

1.3. NMR spectroscopy

NMR spectra were obtained on a DRX-600 spectrometer (Germany) in 99.96% D_2O at pD 7 and 30 °C using internal acetone (δ_H 2.225, δ_C 31.45) or external aq 85% H_3PO_4 (δ_P 0.0) as reference. Prior to the measurements, the samples were lyophilized twice from D_2O. Bruker software XWINNMR 2.6 was used to acquire and process the data. Mixing times of 100 and 225 ms were used in TOCSY and ROESY experiments, respectively.

1.4. Mass spectrometry

ESI FT-MS was performed in the negative ion mode using a Fourier transform ion cyclotron resonance mass analyser (ApexII, Bruker Daltonics, USA) equipped with a 7 T actively shielded magnet and an Apollo electrospray ion source. Samples were dissolved in a 30:30:0.01 (v/v/v) mixture of 2-propanol, water and

Et_3N at a concentration of ~ 20 ng μL^{-1} and sprayed with a flow rate of 2 $\mu L\ min^{-1}$.

Acknowledgements

This work was supported by grants from the Deutsche Forschungsgemeinschaft (U.Z., SFB 470 project B4; U.Z. and Y.A.K., 436 RUS 113/314; B.L., LI-448-1), the Russian Foundation for Basic Research (Y.A.K., 02-04-04005), INTAS (O.B., YS 2001/2-6) and NIH (G.B.P., AI22535).

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