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Elucidation of the Structure of the Lipopolysaccharide Core and the Linkage between the Core and the O-Antigen in *Pseudomonas aeruginosa* Immunotype 5 Using Strong Alkaline Degradation of the Lipopolysaccharide

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Abstract—The products of the strong alkaline degradation of the lipopolysaccharide (LPS) of *Pseudomonas aeruginosa* immunotype 5 were separated by anion-exchange HPLC and studied by electrospray ionization mass spectrometry and NMR spectroscopy. It was found that two major products have the same inner core region and lipid A carbohydrate backbone but different outer core regions. The difference is in the position of a rhamnose residue, which is substituted with either an additional glucose residue or a disaccharide remainder of the degraded O-polysaccharide. The site and the configuration of the linkage between the O-polysaccharide and the core were determined and, together with published data, the structure of the so-called biological repeating unit of the O-antigen was defined. The glycosidic linkage of the 2-acetamido-2,6-dideoxy-D-glucose (N-acetyl-D-quinovosamine) residue is β when it links the O-polysaccharide to the core and α when it connects the interior repeating units of the O-polysaccharide to each other.

Key words: *Pseudomonas aeruginosa*, lipopolysaccharide, core oligosaccharide, repeating unit, O-antigen

Pseudomonas aeruginosa is an opportunistic human pathogen that causes severe infections in hosts with weakened defense mechanisms, often as a result of thermal burns, surgical operations, or another predisposing disease such as cystic fibrosis and cancer [1, 2]. The lipopolysaccharide (LPS) is the major surface antigen of *P. aeruginosa*, which plays an important role in interaction of the bacterium with its host. It is composed of lipid A, a core oligosaccharide, and an O-chain polysaccharide (O-antigen) built up of oligosaccharide repeating units. Lipid A and core are structurally conserved parts of the LPS, whereas the O-polysaccharide is highly variable in composition and structure. According to the O-antigen structures, strains of *P. aeruginosa* are classified in more than 20 O-serogroups [3-7]. The O-antigens that are heteropolysaccharides are synthesized by polymerization of an oligosaccharide, the so-called biological repeating unit, that is preassembled on an undecaprenyl phosphate carrier [8-10]. The structures of the O-polysaccharides of all serologically distinguishable smooth (S)-type *P. aeruginosa* strains have been determined [6, 11] but the biological repeating unit was defined only in serogroups O5 [12], O6 (immunotype 1) [13] and O11 [14]. Structures of the LPS core [13, 15-17] and lipid A [18, 19] have also been investigated in *P. aeruginosa* strains belonging to various O-serogroups.

Abbreviations: 6dHex) 6-deoxyhexose (rhamnose); 6dHexN) 6-deoxyhexosamine (quinovosamine); ESI MS) electrospray ionization mass spectrometry; Hep) heptose (L-glycero-D-manno-heptose); Hex) hexose (glucose); HexN) hexosamine (glucosamine or galactosamine); GalNAcA) 2-acetamido-2-deoxygalacturonic acid; ΔHexNA) 2-amino-2-deoxy-D-threo-hex-4-enuronic acid; Kdo) 3-deoxy-D-manno-oct-2-ulosonic acid; LPS) lipopolysaccharide; OS) oligosaccharide; QuiN) 2-amino-2,6-dideoxyglucose (quinovosamine); Und-*P*) undecaprenyl phosphate.

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The O-polysaccharide of *P. aeruginosa* immunotype 5 (serogroup O10) was studied earlier, and the following structure of the repeating unit was established [20-22]:

→3)-D-Qui*p*NAc-(α1→3)-L-Rha*p*2Ac-

-(α1→4)-L-Gal*p*NAcA-(α1→

where Rha2Ac, QuiNAc, and GalNAcA stand for 2-O-acetylrhamnose, 2-acetamido-2,6-dideoxyglucose (N-acetylquinovosamine), and 2-acetamido-2-deoxygalacturonic acid, respectively. The structure shown can either be identical to the biological repeating unit or differ from it in a cyclic permutation of the constituent monosaccharides.

In this paper, we present new structural data on the LPS of *P. aeruginosa* immunotype 5, including elucidation of the structures of the core and the biological repeating unit of the O-antigen as well as of the region between the O-polysaccharide and the core.

MATERIALS AND METHODS

Pseudomonas aeruginosa immunotype 5, strain 170034 was from the Hungarian National Collection of Medical Bacteria (National Institute of Hygiene, Budapest). It belongs to serogroup O10 of the International Antigenic Typing System (IATS) and is characterized by an O-antigen factor O10a,10b according to the classification scheme of Lanyi and Bergan [6, 21]. Cells were grown in Roux flasks with solid agar medium on Hottinger broth at 37°C for 18 h, then washed with physiological saline, separated by centrifugation, washed with acetone, and dried.

The LPS was isolated from dry bacterial cells by extraction with aqueous 45% phenol (30 min) at 65-68°C [23]. Cells were removed by centrifugation (4000*g*, 1 h), the supernatant was dialyzed against distilled water, and nucleic acids were precipitated by acidification with concentrated CCl_3CO_2H to pH 2.5 and removed by centrifugation (5000*g*, 1 h), the supernatant was dialyzed against distilled water and lyophilized.

The LPS (200 mg) was O-deacylated with anhydrous hydrazine (4 ml) for 1 h at 37°C and then 16 h at 20°C, hydrazine was flushed out in a stream of air at 30-33°C, and the residue washed with cold acetone and dried in vacuum. For further degradation the O-deacylated LPS was dissolved in 4 M NaOH (8 ml), oxygen was removed by flushing with nitrogen for 1 h with stirring, the solution was heated at 100°C for 16 h, after cooling acidified with concentrated HCl to pH 5.5, extracted twice with dichloromethane, and the aqueous solution desalted by gel chromatography on a column (60 × 2.5 cm) of Sephadex G-50. The isolated oligosaccharide mixture (15.8% of the LPS weight) was fractionated by anion-exchange HPLC on a semi-preparative CarboPac PA1 column (250 × 9 mm) using a linear gradient of AcONa (0.45→0.65 M) in 0.1 M NaOH at flow rate 1 ml/min for 90 min. Two-milliliter fractions were collected and analyzed by anion-exchange HPLC using a Dionex system (USA) with pulsed amperometric detection on an analytical CarboPac PA1 column (250 × 4.6 mm) using the same eluent at 1 ml/min for 30 min. After desalting on a column (40 × 2.6 cm) of Sephadex G-50, two major oligosaccharides (OS), $OS1_{NaOH}$ and $OS2_{NaOH}$, having retention times 15.3 and 19.6 min in analytical HPLC, were isolated in yields of 4.3 and 4.6% of the weight of the initial oligosaccharide mixture, respectively.

Electrospray ionization mass spectrometry (ESI MS) of oligosaccharides was performed in the negative ion mode using a Fourier transform ion cyclotron resonance (FT-ICR) mass analyzer (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) mixture of 2-propanol, water, and triethylamine at a concentration of ~20 ng/μl and sprayed with a flow rate of 2 μl/min.

The NMR spectra were obtained on a Bruker DRX-500 spectrometer (Germany) at 30°C in 99.96% D_2O. Prior to the measurements, the samples were lyophilized twice from D_2O. Chemical shifts are referenced to internal acetone (δ_H 2.225, δ_C 31.45) or external aqueous 85% H_3PO_4 (δ_P 0.0). Bruker software XWINNMR 2.1 was used to acquire and process the data. A mixing time of 200 or 100 μsec was used in 2D TOCSY and ROESY experiments, respectively.

RESULTS AND DISCUSSION

For elucidation of the carbohydrate backbone structure, the LPS was O-deacylated by mild hydrazinolysis and then N-deacylated by strong alkaline hydrolysis [24]. The latter was accompanied by depolymerization of the O-polysaccharide by β-elimination in 4-substituted L-GalNAcA residues, which were converted into residues of 2-amino-2-deoxy-D-threo-hex-4-enuronic acid (ΔHexNA) (Fig. 1). Two major oligosaccharides ($OS1_{NaOH}$ and $OS2_{NaOH}$) were isolated by anion-exchange HPLC (Fig. 2), and their structures that are shown in Fig. 3 were determined by ESI MS and NMR spectroscopy as follows.

The ESI mass spectra of $OS1_{NaOH}$ and $OS2_{NaOH}$ showed multiple-charged pseudomolecular ions derived from compounds with molecular masses 2519.64 and 2659.65 daltons, respectively. They correspond to the phosphorylated core-lipid A carbohydrate backbone $6dHexHex_4HexN_3Hep_2Kdo_2P_5$ ($OS1_{NaOH}$) and to an oligosaccharide, which contains one glucose residue less but bears a disaccharide remainder of the degraded O-

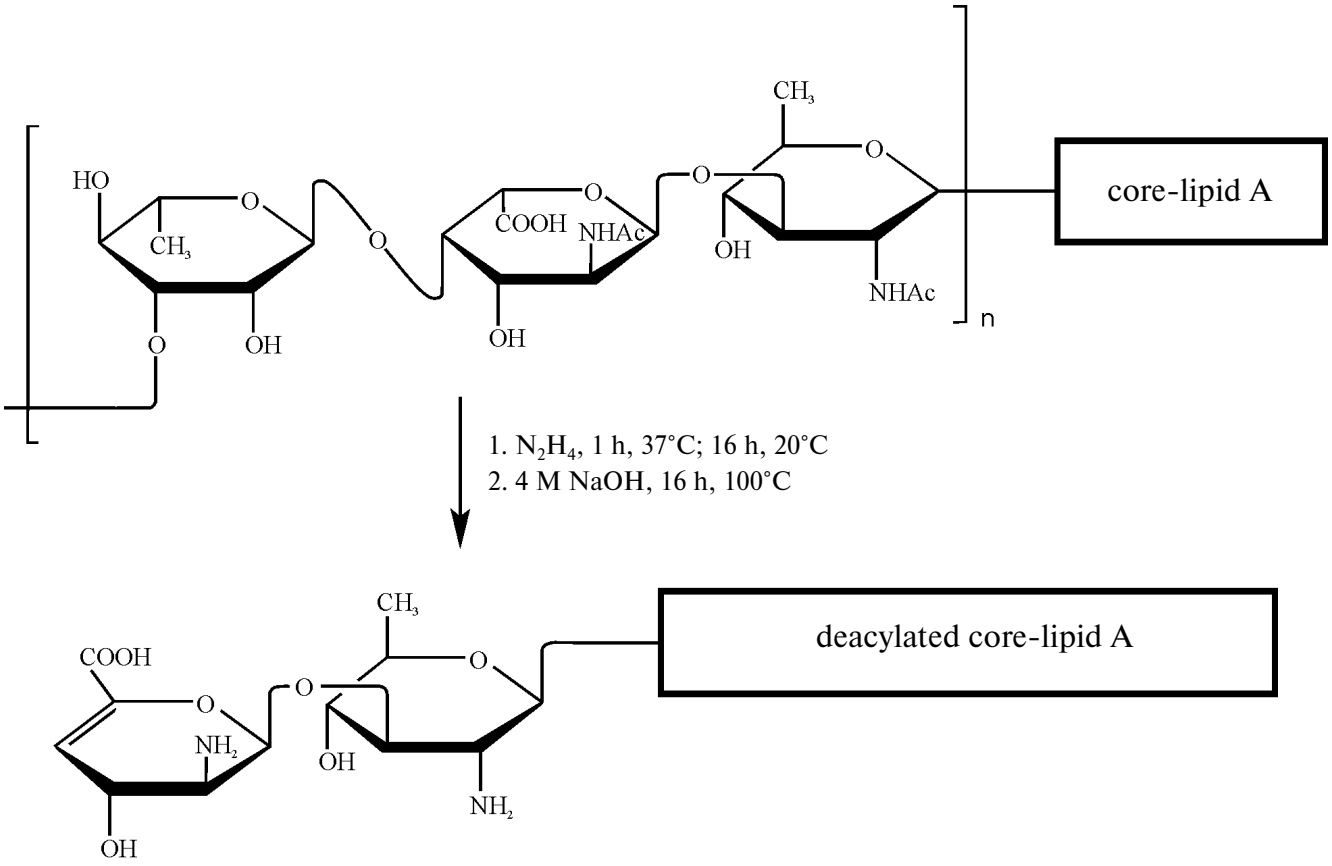


Fig. 1. Alkaline degradation of the LPS of *P. aeruginosa* immunotype 5. The glycosidic linkage of QuiN is α between the O-polysaccharide repeating units and β between the O-polysaccharide and the core.

polysaccharide and has the following composition: $\Delta HexNA6dHexN6dHexHex_3HexN_3Hep_2Kdo_2P_5$ ($OS2_{NaOH}$) (the calculated molecular masses 2519.57 and 2659.62 daltons, respectively).

The ^{1}H-NMR spectrum of $OS1_{NaOH}$ (Fig. 4) contained signals for 10 anomeric protons at δ 4.62-5.75, two methylene groups (axial and equatorial protons H3 of two Kdo) at δ 1.83-2.24, and one methyl group (H6 of Rha) at δ 1.32 (3H). The ^{13}C-NMR spectrum of $OS1_{NaOH}$ showed signals for anomeric carbons at δ 92.7-105.6, three nitrogen-bearing carbons (C2 of $GlcN^I$, $GlcN^{II}$, and GalN) at δ 51.7-56.6, a methyl group (C6 of Rha) at δ 18.3, and two methylene groups (C3 of Kdo) at δ 35.4 and 36.3. The ^{31}P-NMR spectrum of $OS1_{NaOH}$ contained signals for five phosphate groups at δ –0.9, 0.7, 0.9, and 2.1 (2P).

The ^{1}H-NMR spectrum of $OS2_{NaOH}$ (Fig. 4) contained signals for 11 anomeric protons at δ 4.67-5.75, two methylene groups (axial and equatorial protons H3 of Kdo) at δ 1.83-2.26, two methyl groups (H6 of Rha and QuiN) at δ 1.27 and 1.37, and a proton at a double bond (H4 of ΔHexNA) at δ 5.95. The ^{13}C-NMR spectrum of $OS2_{NaOH}$ showed signals for anomeric carbons at δ 92.9-105.7, five nitrogen-bearing carbons (C2 of $GlcN^I$, $GlcN^{II}$, GalN, QuiN, and ΔHexNA) at δ 51.9-56.8, methyl groups (C6 of Rha and QuiN) at δ 17.7 and 18.2, two methylene groups (C3 of Kdo) at δ 35.5 and 36.3, and C4 of ΔHexNA at δ 107.6. The ^{31}P-NMR spectrum of $OS2_{NaOH}$ contained signals for five phosphate groups at δ –0.2, 0.7, 0.9, and 2.1 (2P).

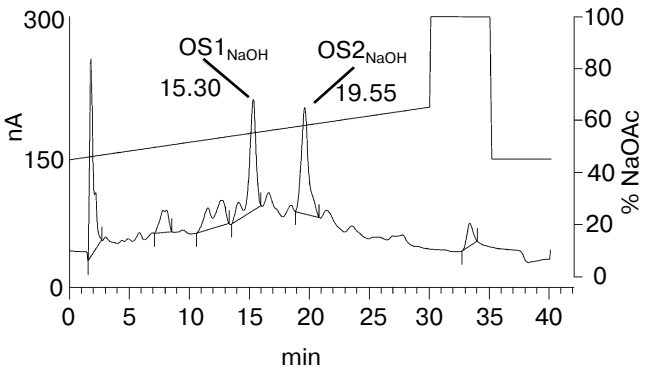
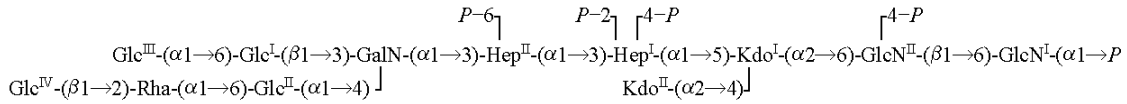


Fig. 2. Anion-exchange HPLC of the alkaline degradation products from the LPS of *P. aeruginosa* immunotype 5.

$OS1_{NaOH}$



$OS2_{NaOH}$

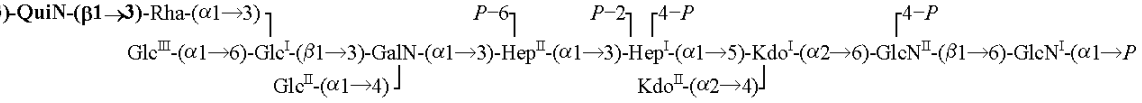


Fig. 3. Structures of the major alkaline degradation products from the LPS of *P. aeruginosa* immunotype 5 ($OS1_{NaOH}$ and $OS2_{NaOH}$). Kdo stands for 3-deoxy-D-manno-oct-2-ulosonic acid, Hep for L-glycero-D-manno-heptose, ΔHexNA for 2-amino-2-deoxy-D-threo-hex-4-enuronic acid, QuiN for 2-amino-2,6-dideoxyglucose; all sugars are in the pyranose form and have the D configuration, except for Rha, which has the L configuration. In $OS2_{NaOH}$, the remainder of the degraded O-polysaccharide is shown in bold type.

The ^{1}H- and ^{13}C-NMR spectra of $OS1_{NaOH}$ and $OS2_{NaOH}$ (Tables 1 and 2) were assigned using 2D shift-correlated NMR spectroscopy, including 2D ^{1}H,^{1}H COSY, TOCSY, ROESY, and H-detected ^{1}H,^{13}C HMQC experiments. The sugar spin systems were assigned based on the coupling constant values and those for amino sugars identified by correlation of the protons at the nitrogen-bearing carbons to the corresponding carbons. The configurations of the glycosidic linkages of the sugar residues with the gluco and galacto configurations (Glc, GlcN, GalN, QuiN, and ΔHexNA) were defined based on the $J_{1,2}$ coupling constant values and those of Rha, Hep^I, Hep^{II}, Kdo^I, and Kdo^{II} based on the typical ^{1}H-NMR chemical shifts (compare data shown at Table 1 with published data [13, 15]).

Linkage and sequence analyses of $OS1_{NaOH}$ and $OS2_{NaOH}$ were performed using 2D ROESY experiments as described [13, 15, 25], and the structures of the phos-

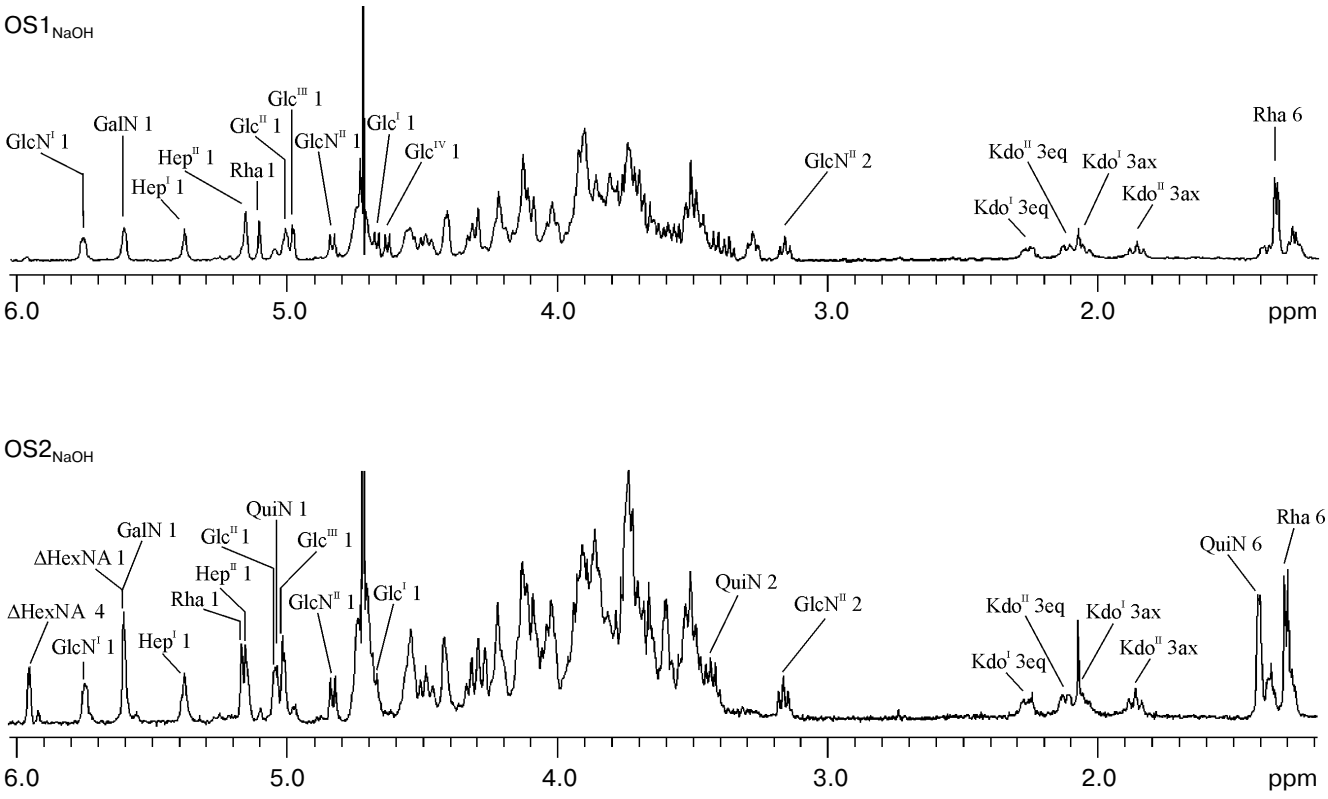


Fig. 4. ^{1}H-NMR spectra of $OS1_{NaOH}$ and $OS2_{NaOH}$. Arabic numerals refer to protons in sugar residues.

Table 1. ^{1}H-NMR data (δ, ppm)

Sugar residue	H1 H3a	H2 H3e	H3 H4	H4 H5	H5 H6	H6(6a) H7	H6b(7a) H8a	H7b H8b
				$OS1_{NaOH}$				
→6)-α-D-GlcN^{I}-1-*P*	5.75	3.48	3.93	3.65	4.12	3.79	4.29	
→6)-β-D-GlcN^{II}4*P*-(1→	4.83	3.14	3.91	3.86	3.77	3.48	3.73	
→4,5)-α-Kdo^{I}-(2→	2.04	2.24	4.10	4.29	3.72	3.86	3.60	3.90
α-Kdo^{II}-(2→	1.83	2.09	4.12	4.08	3.72	4.11	3.70	3.85
→3)-α-Hep^{I}2,4*P*-(1→	5.37	4.53	4.19	4.55	4.26	4.01	3.81	4.02
→3)-α-Hep^{II}6*P*-(1→	5.15	4.40	4.23	4.10	4.01	4.50	3.75	3.82
→3,4)-α-D-GalN-(1→	5.60	3.83	4.48	4.39	4.21	3.89		
→6)-β-D-Glc^{I}-(1→	4.66	3.26	3.54	3.26	3.70	3.80	3.91	
→6)-α-D-Glc^{II}-(1→	5.00	3.50	3.73	3.64	4.19	3.78	3.87	
α-D-Glc^{III}-(1→	4.97	3.56	3.69	3.45	3.67	3.78	3.87	
β-D-Glc^{IV}-(1→	4.62	3.35	3.50	3.39	3.47	3.70	3.91	
→2)-α-L-Rha-(1→	5.10	4.12	3.91	3.49	3.75	1.32		
				$OS2_{NaOH}$				
→6)-α-D-GlcN^{I}-1-*P*	5.75	3.47	3.93	3.65	4.13	4.31	3.80	
→6)-β-D-GlcN^{II}4*P*-(1→	4.83	3.14	3.89	3.85	3.77	3.72	3.47	
→4,5)-α-Kdo^{I}-(2→	2.03	2.26	4.11	4.29	3.73	3.88	3.61	3.92
α-Kdo^{II}-(2→	1.83	2.10	4.13	4.08	3.73	4.11	3.71	3.95
→3)-α-Hep^{I}2,4*P*-(1→	5.38	4.54	4.20	4.51	4.33	4.02	3.82	4.02
→3)-α-Hep^{II}6*P*-(1→	5.15	4.41	4.22	4.11	4.02	4.55	3.74	
→3,4)-α-D-GalN-(1→	5.60	3.86	4.48	4.42	4.21	3.90		
→3,6)-β-D-Glc^{I}-(1→	4.67	3.46	3.66	3.52	3.68	3.85	3.90	
α-D-Glc^{II}-(1→	5.04	3.50	3.75	3.52	4.06	3.85		
α-D-Glc^{III}-(1→	5.01	3.57	3.70	3.42	3.69	3.88	3.73	
→3)-α-L-Rha-(1→	5.17	4.26	4.03	3.65	4.07	1.27		
→3)-β-D-QuiN-(1→	5.02	3.43	4.13	3.59	3.59	1.37		
β-ΔHexNA-(1→	5.60	3.72	4.54	5.95				

phorylated carbohydrate core backbone were determined for both oligosaccharides (Fig. 3). It was found also that, in addition to the core, $OS2_{NaOH}$ includes a remainder of the degraded first repeating unit of the O-polysaccharide, which was shown to be a β-ΔHexNA-(1→3)-β-D-QuiN disaccharide attached to position 3 of the terminal Rha residue of the core.

The positions of the phosphate groups were determined using 2D ^{1}H,^{31}P HMQC experiments. The ^{1}H,^{31}P HMQC spectra of $OS1_{NaOH}$ and $OS2_{NaOH}$, which were almost identical, showed correlations for the phosphorus signals with the protons at phosphate-bearing carbons as shown in Fig. 5 for $OS1_{NaOH}$.

These data together established the structures of $OS1_{NaOH}$ and $OS2_{NaOH}$ from the LPS of *P. aeruginosa* immunotype 5 (serogroup O10), which are shown in Fig. 3. It should be mentioned that, in addition to the monosaccharides and phosphate groups, the LPS core of *P. aeruginosa* contains non-sugar components that are eliminated during strong alkaline degradation, such as a 7-O-carbamoyl group at Hep^{II} [26], an N-alanyl [27] group (less often N-acetyl group [16]) at GalN, ethanolamine diphosphate [28], and sometimes O-acetyl groups [13, 15].

$OS1_{NaOH}$ represents an unsubstituted phosphorylated core-lipid A carbohydrate backbone. It is similar to the corresponding compound isolated from the LPS of *P. aeruginosa* PAO1 (serogroup O5) [17] and differs from those of *P. aeruginosa* immunotype 1 [13] and strain A28 [17] (both serogroup O6) and a rough, cystic fibrosis isolate *P. aeruginosa* 2192 [15] in the presence of an additional, fourth glu-

Table 2. ^{13}C-NMR data (δ, ppm)

Sugar residue	C1	C2	C3	C4	C5	C6	C7	C8
				$OS1_{NaOH}$				
→6)-α-D-GlcN^{I}-1-*P*	92.7	55.3	70.3	70.2	73.7	70.5		
→6)-β-D-GlcN^{II}4*P*-(1→	100.1	56.6	72.7	75.5	74.9	63.5		
→4,5)-α-Kdo^{I}-(2→	[a]	[a]	35.4	72.3	69.1	73.3	70.1	64.8
α-Kdo^{II}-(2→	[a]	[a]	36.3	66.6	67.7	73.3	70.1	64.2
→3)-α-Hep^{I}2,4*P*-(1→	98.2	75.5	75.1	71.6	73.1	71.9	64.3	
→3)-α-Hep^{II}6*P*-(1→	103.1	70.2	78.7	66.6	72.7	73.9	62.5	
→3,4)-α-D-GalN-(1→	98.1	51.7	77.6	76.7	73.4	61.0		
→6)-β-D-Glc^{I}-(1→	105.6	74.5	76.8	71.6	75.8	68.7		
→6)-α-D-Glc^{II}-(1→	100.5	72.9	73.8	69.9	71.2	67.7		
α-D-Glc^{III}-(1→	99.4	72.4	74.3	70.3	73.1	61.8		
β-D-Glc^{IV}-(1→	105.3	74.5	76.7	70.5	76.8	61.8		
→2)-α-L-Rha-(1→	101.0	81.1	71.1	73.5	69.9	18.3		
				$OS2_{NaOH}$				
→6)-α-D-GlcN^{I}-1-*P*	92.9	55.6	70.6	70.7	74.0	70.8		
→6)-β-D-GlcN^{II}4*P*-(1→	100.4	56.8	72.9	75.7	75.0	63.7		
→4,5)-α-Kdo^{I}-(2→	[a]	[a]	35.5	72.5	69.4	73.3	70.4	65.0
α-Kdo^{II}-(2→	[a]	[a]	36.3	66.9	68.0	73.3	70.4	64.3
→3)-α-Hep^{I}2,4*P*-(1→	98.5	75.7	75.2	71.9	73.3	72.2	64.5	
→3)-α-Hep^{II}6*P*-(1→	103.2	70.6	79.2	66.9	72.8	74.2	62.6	
→3,4)-α-D-GalN-(1→	98.4	51.9	77.8	76.9	73.8	61.2		
→3,6)-β-D-Glc^{I}-(1→	105.7	74.9	81.2	69.6	75.9	67.8		
α-D-Glc^{II}-(1→	100.7	73.2	74.0	70.4	72.7	61.4		
α-D-Glc^{III}-(1→	99.4	72.6	74.4	70.9	73.6	62.1		
→3)-α-L-Rha-(1→	101.8	74.1	80.9	72.5	70.1	17.7		
→3)-β-D-QuiN-(1→	101.1	56.3	81.4	73.6	73.6	18.2		
β-ΔHexNA-(1→	95.3	54.0	63.7	107.6	[a]	[a]		

[a] Signal not found.

cose residue that is attached to the rhamnose residue. Whether this glucose residue is present or not, the core that has the structure of $OS1_{NaOH}$ and is called glycoform 1 core [13, 15] is not substituted with the O-polysaccharide.

$OS2_{NaOH}$ differs from $OS1_{NaOH}$ in the position of the rhamnose residue, the absence of the fourth glucose residue, and the presence of the remainder of the degraded O-polysaccharide. No compound with the same core called glycoform 2 core but without the O-polysaccharide remainder was detected. Hence, all molecules of the glycoform 2 core are substituted with the O-polysaccharide. This finding is in consistence with the earlier observations for *P. aeruginosa* serogroups O5 and O6 [12, 13].

As mentioned above, the structure of the O-polysaccharide of *P. aeruginosa* immunotype 5 LPS has been elucidated [20-22]. However, it could not be ascertained from those data what are the first and last monosaccharides in the repeating unit (i.e., what is the structure of the biological repeating unit), nor which monosaccharide is linked to the LPS core. From the structure of $OS2_{NaOH}$, it follows that the O-polysaccharide is attached to the core by the QuiNAc residue and the biological repeating unit has the following structure:

→3)-α-L-Rha*p*2Ac-(1→4)-α-L-Gal*p*NAcA-

-(1→3)-α-D-Qui*p*NAc-(1→

Elucidation of the structure of $OS2_{NaOH}$ enabled determination of not only the biological repeating unit

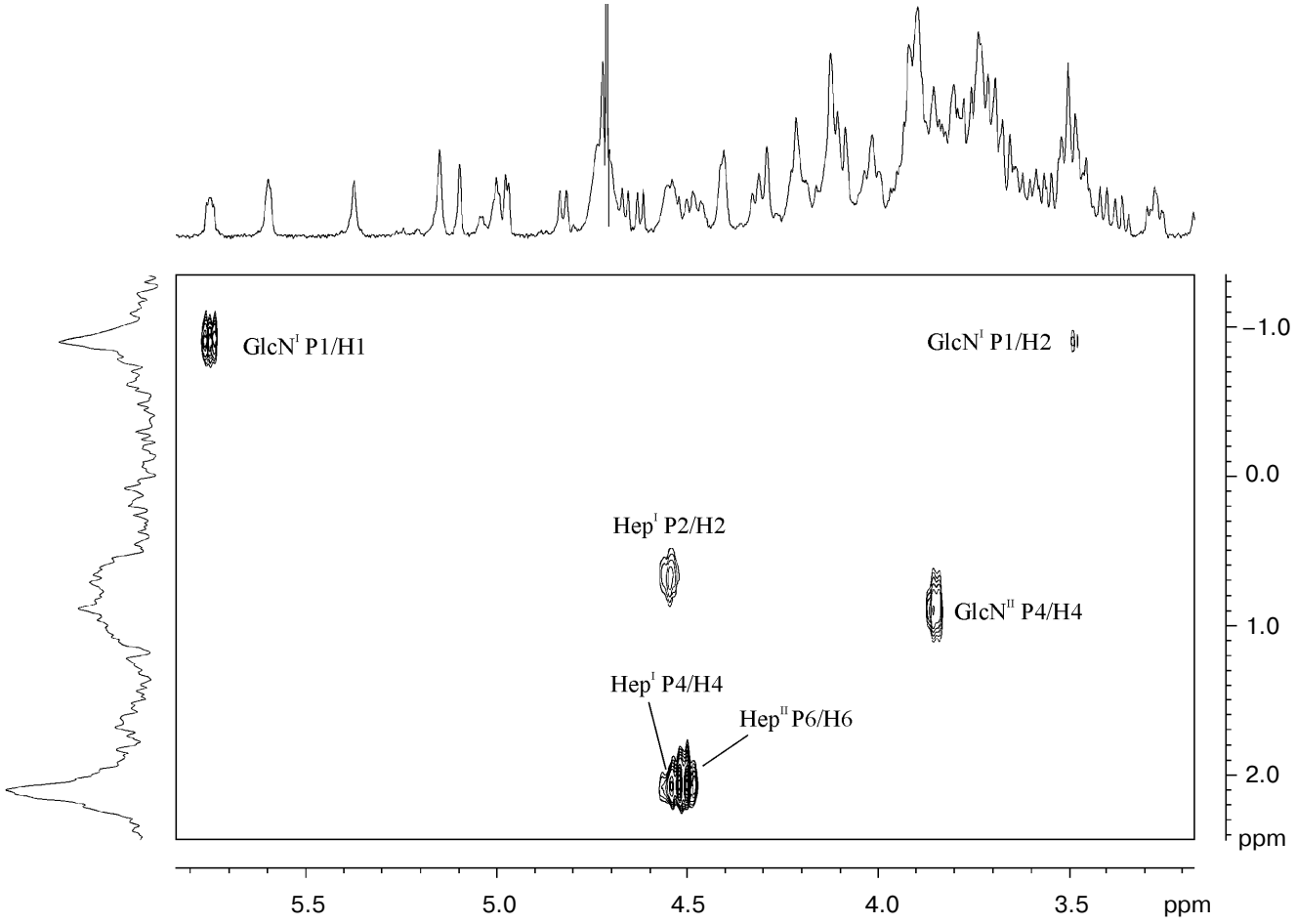


Fig. 5. 2D $^{1}H,^{31}P$ HMQC spectrum of $OS1_{NaOH}$. The corresponding parts of the 1D ^{1}H-NMR spectrum and the 1D ^{31}P-NMR spectrum are displayed along the horizontal and vertical axes, respectively.

but also of the mode and the site of the linkage between the O-polysaccharide and the core. It was found that the QuiNAc residue, which occupies the reducing end of the biological repeating unit, has the β configuration when it links the O-polysaccharide to the core but the α configuration when connects the interior repeating units of the O-polysaccharide to each other. This finding is in accordance with the biosynthesis pathway of O-polysaccharides, which involves multiple enzymes that mediate the formation of the QuiNAc glycosidic linkages, including O-antigen polymerase for polymerization of the repeating unit and ligase for attachment of the O-polysaccharide to the core [9].

All *P. aeruginosa* O-polysaccharides whose biological repeating unit structure is elucidated to date include either D-FucNAc (serogroups O5 [12] and O11 [14]) or D-QuiNAc (serogroups O6 [13] and O10 (this work)), and namely glycosidic linkages of these 6-deoxyamino sugars attach the biological repeating units to each other and the O-polysaccharide to the core. The structural data are consistent with biosynthesis of the O-polysaccharides in these serogroups, which is initiated by transfer of D-QuiNAc-1-*P* or D-FucNAc-1-*P* from UDP-D-QuiNAc or UDP-D-FucNAc to Und-*P* catalyzed by the corresponding enzymes called transferases WbpL [9]. Interestingly, these transferases show significant homology and each possesses substrate specificity for both sugar nucleotides [9]. The presence of D-QuiNAc or/and D-FucNAc in the O-polysaccharides of *P. aeruginosa* strains from most other serogroups [6] enables the suggestion that the initiation of the O-polysaccharide biosynthesis proceeds in these strains similarly.

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