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Full Structure of the Lipopolysaccharide of *Pseudomonas aeruginosa* Immunotype 5

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Abstract—The lipopolysaccharide (LPS) of the opportunistic human pathogen *Pseudomonas aeruginosa* immunotype 5 was delipidated by mild acid hydrolysis, and the products were separated by high-performance anion-exchange chromatography and analyzed by ESI MS and NMR spectroscopy. LPS species of three types were found, including those with an unsubstituted core and the core substituted with one O-polysaccharide repeating unit or with an O-polysaccharide of a variable number of repeating units. The core region is highly phosphorylated, the major species containing two monophosphate groups and one ethanolamine diphosphate group. Based on these and published data on the O-polysaccharide structure, the full structure of the LPS of *P. aeruginosa* immunotype 5 was established.

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

Pseudomonas aeruginosa causes severe infections in hosts with weakened defense mechanisms. The lipopolysaccharide (LPS) plays an important role in interaction of the bacterium with its host. It consists of a lipid part (lipid A) and a core oligosaccharide with an O-polysaccharide chain (O-antigen) attached, which defines the immunospecificity of the bacterium. Based on the O-antigens, strains of *P. aeruginosa* are classified in more than 20 O-serogroups (reviews [1, 2]). In the Wzy-dependent pathway of LPS biosynthesis, the O-polysaccharide is synthesized by polymerization of the pre-assembled oligosaccharide, the so-called biological repeating unit [3]. The O-polysaccharide structures were established in all O-serogroups of *P. aeruginosa* [2, 4], and in a few of them, the structure of the biological repeating unit was defined [5-7]. The core structure was elucidated in type strains of several serogroups [6, 8, 9] and in a number of rough strains with an O-polysaccharide-lacking LPS [8-11].

Recently, we have determined the structure of the biological repeating unit of the O-polysaccharide and the core-lipid A carbohydrate backbone of *P. aeruginosa* immunotype 5 (serogroup O10) and established the mode of the linkage between the O-polysaccharide and the core [12]. It was found that the LPS core of one type is substituted with the O-polysaccharide or one repeating unit of the O-polysaccharide, and the core of the other type is not substituted. The substituted and unsubstituted cores differ in the position of a rhamnose residue and in the number of glucose residues. These data were obtained by analysis of the products that were derived from the LPS by strong alkaline degradation.

In this work, the LPS of *P. aeruginosa* immunotype 5 was studied by mild acid degradation, which enabled confirmation of the previous structural data and determination of the acylation and phosphorylation patterns of the core, including the location of the ethanolamine diphosphate group. As a result, the full LPS structure of *P. aeruginosa* immunotype 5 was established.

Abbreviations: 6dHex) 6-deoxyhexose (rhamnose); 6dHexN) 6-deoxyhexosamine; ESI MS) electrospray ionization mass spectrometry; Hep) L-*glycero*-D-*manno*-heptose; Hex) hexose; HexN) hexosamine; HMQC) heteronuclear multi-quantum coherence; GalNAcA) 2-acetamido-2-deoxygalacturonic acid; Kdo) 3-deoxy-D-*manno*-oct-2-ulosonic acid; LPS) lipopolysaccharide; OS) oligosaccharide; QuiNAc) 2-acetamido-2,6-dideoxyglucose (quinovosamine); Rha) rhamnose.

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MATERIALS AND METHODS

Pseudomonas aeruginosa immunotype 5, strain 170034, belongs to serogroup O10 of the International Antigenic Typing System (IATS) and serotype O10a,10b of the Lanyi–Bergan classification [1, 2]. Bacterial cells were grown on solid agar medium and the LPS was isolated from dry bacterial cells by extraction with aqueous 45% phenol [13] and purified using precipitation with aqueous 50% CCl_3CO_2H as described [12].

The LPS (160 mg) was dissolved in aqueous 1% HOAc and heated for 6 h at 100°C. The precipitate was removed by centrifugation (12,000*g*, 10 min), and the supernatant fractionated by gel chromatography 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 one liter of water) at 30 ml/h; elution was monitored with a Knauer differential refractometer (Germany). A polysaccharide and two oligosaccharides (OS-III and OS-IV) were isolated in yields of 12.3 and 9.4% of the LPS weight. OS-IV was fractionated by high-performance anion-exchange chromatography on a semi-preparative CarboPac PA1 column using a linear gradient of 0.02→0.52 M NaOAc at flow rate 1.5 ml/min for 140 min. Three-milliliter fractions were collected and analyzed on an analytical CarboPac PA1 column using the Dionex system (USA) and the same eluent at 1.5 ml/min for 30 min; before pulse amperometric detection the eluate was mixed (3 : 1) with 1.5 M NaOH. After desalting on Sephadex G-50 four major oligosaccharides, OS-IV-1 to OS-IV-4, having retention times 8.9, 10.7, 16.9, and 19.7 min on the analytical column, were isolated in yields of 6.4, 3.1, 8.9, and 7.8% of the OS-IV weight, respectively.

OS-III and OS-IV (40 μg each) were hydrolyzed with 4 M HCl (80 μl) at 100°C for 16 h. Amino components were analyzed as the phenyl isothiocyanate derivatives by high-performance chromatography on a Pico-Tag reversed-phase column (150 × 3.9 mm) using buffers for Pico-Tag amino acid analysis of protein hydrolyzates (Waters, Germany) at 42°C and a flow rate 1 ml/min for 10 min; monitoring was performed with a dual λ absorbance detector (Waters, Germany) at 254 nm.

ESI MS was performed in the negative ion mode using a Fourier transform ion cyclotron resonance 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 at a flow rate of 2 μl/min.

NMR spectra were obtained on a Bruker DRX-600 spectrometer (Germany) in 99.96% D_2O at pD 7 and 27°C using internal sodium 3-trimethylsilylpropanoate-d_4 (δ_H 0) or external aqueous 85% H_3PO_4 (δ_P 0) as references. 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 msec were used in two-dimensional TOCSY and ROESY experiments, respectively.

RESULTS AND DISCUSSION

Phosphorylated oligosaccharides have been previously isolated by strong alkaline degradation of the LPS of *P. aeruginosa* immunotype 5 and identified as the core-lipid A carbohydrate backbone substituted with a remainder of the first repeating unit of the O-antigen (OS-I) and the unsubstituted core-lipid A backbone (OS-II) (Fig. 1, products (a)) [12]. However, information about alkali-labile substituents, such as acyl and diphosphate groups, has been lost upon alkaline degradation of the LPS. Therefore, in this wok mild acid hydrolysis was used for delipidation of the LPS, which resulted in cleavage of the acid-labile glycosidic linkages of both residues of 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo^I and Kdo^{II}), including the linkage between the core and lipid A. Fractionation of the products by gel chromatography on Sephadex G-50 yielded a high-molecular-mass O-polysaccharide and two oligosaccharides that represented a core substituted with one O-antigen repeating unit (OS-III) and an unsubstituted core (OS-IV) (Fig. 1, products (b)).

Analysis of amino components in OS-IV showed the presence of GalN, alanine, and ethanolamine in molar ratios 1 : 1.25 : 0.33. The ESI mass spectrum of OS-IV (Fig. 2a) showed a mixture of core oligosaccharides with the expected monosaccharide composition ($RhaGlc_4GalNHep_2Kdo$) [12] and non-sugar substituents including N-alanyl and O-carbamoyl groups, phosphate, and phosphoethanolamine (*P*Etn) [9, 14], but no O-acetyl groups, which have been detected earlier in the core of some other *P. aeruginosa* strains [6, 11]. Ion peaks for the major compounds with Kdo in an anhydro-form having the mono-isotopic molecular masses 1833.50, 1913.47, 1956.51, and 2036.48 daltons demonstrated a heterogeneity that is associated with different numbers of phosphate groups and the presence or absence of phosphoethanolamine. The total content of *P*Etn-containing compounds in OS-IV was estimated as ~60%.

The ESI mass spectrum of OS-III (Fig. 2b) showed that it differs from OS-IV in the absence of one of the glucose residues and in the attachment to the core of one O-antigen repeating unit. The latter contains one residue each of L-rhamnose, 2-acetamido-2,6-dideoxy-D-glucose (N-acetyl-D-quinovosamine, D-QuiNAc) and 2-acetamido-2-deoxy-L-galacturonic acid (GalNAcA) as well as one O-acetyl group [15, 16]. OS-III was characterized by the same type of heterogeneity as OS-IV (see above) and, in addition, by non-stoichiometric O-acety-

a

b

Fig. 1. Structures of the products of alkaline (a) and mild acid (b) degradations of the LPS of *P. aeruginosa* immunotype 5. Shown are structures of the major compounds in the oligosaccharide mixtures OS-III and OS-IV (OS-IV-3) that contain *P*Etn and the maximum number of phosphate groups. Cm, carbamoyl; Etn, ethanolamine; Rha, rhamnose; Rha2Ac, 2-O-acetylrhamnose; Kdo, 3-deoxy-D-manno-oct-2-ulosonic acid; Hep, L-glycero-D-manno-heptose; ΔHexNA, 2-amino-2-deoxy-D-threo-hex-4-enuronic acid; GalNAcA, 2-acetamido-2-deoxygalacturonic acid; QuiN, 2-amino-2,6-dideoxyglucose. All monosaccharides are in the pyranose form; Glc, GlcN, GalN, and QuiN have the D configuration; Rha, GalNAcA, and Ala have the L configuration.

lation of Rha from the O-antigen oligosaccharide (the mono-isotopic molecular masses were 2221.66, 2301.63, 2344.65, and 2424.63 daltons for the non-O-acetylated compounds and 2263.67, 2343.65, 2386.68, and 2466.64 daltons for the O-acetylated compounds).

OS-IV was further fractionated by high-performance anion-exchange chromatography to give four major oligosaccharides designated as OS-IV-1 to OS-IV-4. From them, OS-IV-3 and OS-IV-4 contained the maximum number of phosphate groups (four and three) and OS-IV-3 contained also ethanolamine, as inferred by ESI MS determination of their molecular masses 2036.47 and 1913.47 daltons, respectively. Accordingly, the ^{1}H-NMR spectrum of OS-IV-3, but not OS-IV-4, showed signals

for ethanolamine at δ 3.32 (CH_2N) and 4.23 (CH_2O), and the ^{31}P-NMR spectrum showed signals for a diphosphate group at δ −10.9 and −11.0. Therefore, it was suggested that OS-IV-3 differs from OS-IV-4 in the presence of phosphoethanolamine that is linked to one of the phosphate groups to give ethanolamine diphosphate.

Each of two other isolated oligosaccharides, OS-IV-1 and OS-IV-2, was a mixture of lower phosphorylated compounds that contains one phosphate group less than OS-IV-3 and OS-IV-4, respectively. Further hydrolysis of the isolated OS-IV-3 under the conditions of mild acid degradation of the LPS converted it into OS-IV-4. These data, together with an earlier finding that alkaline degradation of the LPS produced oligosaccharides mainly with three phosphate groups in the core (OS-I and OS-II; Fig. 1, products (a)) [12], showed that OS-IV-3 is the most representative product in respect to the phosphorylation pattern, whereas most, if not all, lesser phosphorylated products resulted from partial degradation.

The ^{1}H-NMR spectrum of OS-IV-3 was fully assigned using two-dimensional correlation NMR spectroscopy, including 2D ^{1}H,^{1}H COSY and TOCSY experiments (table). The sugar spin systems were assigned by tracing connectivities from H1 and (for Rha) H6 signals, by coupling constant values estimated from the two-dimensional NMR spectra and using published NMR spectroscopic data of OS-II [12]. The signals for both L-glycero-D-manno-heptose residues (Hep^I and Hep^{II}) were split owing to the occurrence of a Kdo residue in multiple forms, including anhydro-forms, at the reducing end of the oligosaccharide.

In the two-dimensional ^{1}H,^{31}P HMQC spectrum of OS-IV-3, the signals for the diphosphate group showed correlations with the signals for the CH_2O group of ethanolamine and H2 of Hep^I at δ_P/δ_H −11.0/4.23 and −10.9/4.66-4.69, respectively. The signals for the monophosphate groups showed no correlation with ethanolamine but with H4 of Hep^I and H6 of Hep^{II} at δ_P/δ_H 1.8/4.43-4.49 and 1.6/4.62-4.67, respectively. The attachment of *PP*Etn at position 2 of Hep^I was confirmed by a two-dimensional ^{1}H,^{31}P HMQC-TOCSY experiment with OS-IV-3, which revealed correlations between P2 and H1 of Hep^I at δ_P/δ_H −10.9/5.32-5.38; 5.72 and between P4 and H3 and H5 of Hep^I at δ_P/δ_H 1.8/4.08-4.25 and 1.8/3.58; 3.75-3.78, respectively. In the ^{1}H,^{31}P HMQC spectrum of OS-IV-4, which contains no *PP*Etn, a P2/H2 correlation for Hep^I was observed at δ_P/δ_H 0.4/4.57-4.61, i.e., was shifted significantly downfield in the ^{31}P dimension and slightly upfield in the ^{1}H dimension owing to the replacement of the diphosphate group for a monophosphate group. The Hep^I P4/H4 and Hep^{II} P6/H6 cross-peaks were essentially at the same positions in the ^{1}H,^{31}P HMQC spectra of OS-IV-3 and OS-IV-4.

The data obtained by mild acid degradation of the LPS were in agreement with the published data of strong alkaline degradation [12]. Particularly, they confirmed the occurrence of two core glyco-forms. One glyco-form is present in those LPS species that have a core substituted with the O-polysaccharide or one O-polysaccharide repeat (structures **3** and **2** in Fig. 3, respectively). The other glyco-form, which differs in the position of the rhamnose residue and the presence of an additional, fourth glucose residue, occurs in the LPS species that lacks any O-antigen (structure **1** in Fig. 3). The occurrence of two isomeric core glyco-forms has been reported earlier in *P. aeruginosa* immunotype 1 (serogroup O6) [6] and in a rough, clinical isolate from a cystic fibrosis

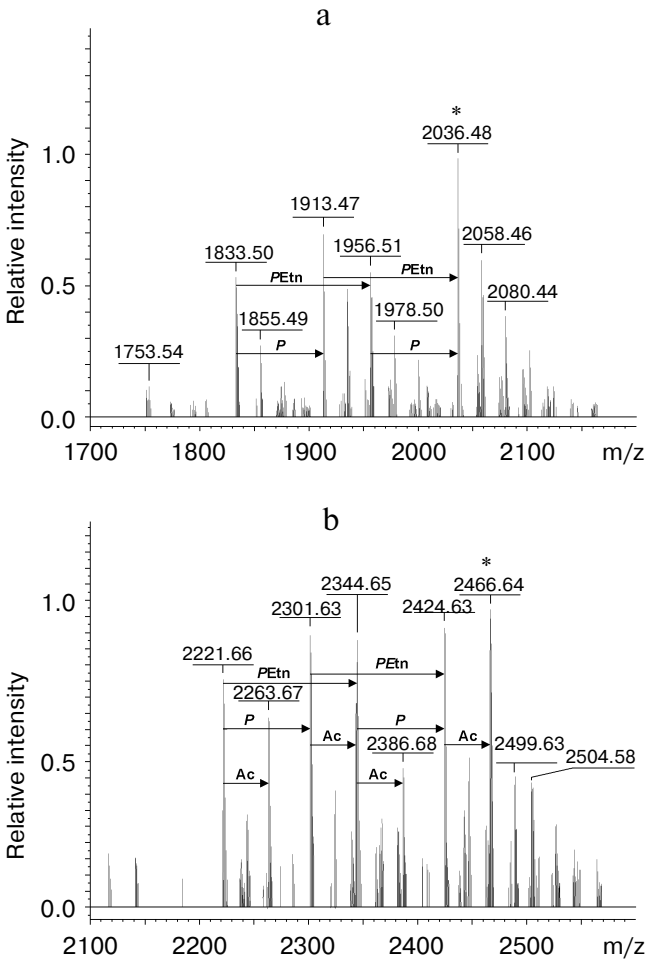

Fig. 2. Negative ion ESI mass spectra of OS-IV (a) and OS-III (b). The spectra were charge deconvoluted and the molecular masses refer to neutral mono-isotopic peaks. Peaks marked by asterisks correspond to the compounds in the oligosaccharide mixtures OS-IV (OS-IV-3) and OS-III that contain *P*Etn and the maximum number of phosphate groups with Kdo in an anhydro-form (for structures see Fig. 1). Compounds having molecular masses higher by 22 and 38 daltons are Na- and K-adducts, respectively.

^{1}H-NMR data of OS-IV-3 (δ, ppm)

Monosaccharide	H1	H2	H3	H4	H5	H6(6a)	H6b
→3)-α-Hep^{I}2*PP*Etn4*P*-(1→	5.32-5.38; 5.72	4.66-4.69	4.08-4.25	4.43-4.49	3.75-3.78; 3.58		
→3)-α-Hep^{II}6*P*7Cm-(1→	5.16-5.18	4.37-4.42	4.02-4.04	4.08-4.11	3.97-4.01	4.62-4.67	
→3,4)-α-GalNAla-(1→	5.38	4.46	4.33	4.29			
→6)-β-Glc^I-(1→	4.58	3.14	3.50	3.29	3.67	3.82	3.89
→6)-α-Glc^{II}-(1→	5.04	3.52	3.85	3.61	4.36	3.79	3.87
→2)-α-Rha-(1→	5.12	4.14	3.93	3.52	3.77	1.33	
α-Glc^{III}-(1→	4.98	3.58	3.70	3.47	3.69	3.84	
β-Glc^{IV}-(1→	4.63	3.36	3.51	3.41	3.46	3.72	3.91

Note: Signals of Hep^I and Hep^{II} are split owing to the occurrence of Kdo^I in multiple forms, whose signals were not clearly detected in the spectrum and not assigned.

patient, *P. aeruginosa* 2192 [11], whereas a core glyco-form that differs also in the number of glucose residues has been found in *P. aeruginosa* PAO1 (serogroup O5) [5]. Whether the fourth glucose residue is present or not, one of the core glyco-forms is not substituted (**1**), whereas the other glyco-form is substituted with the O-polysaccharide (**3**) or one O-polysaccharide repeat (**2**). Based on the finding that the attachment of Rha to either of the positions in the core blocks the attachment to the other position, it could be suggested that there is a competition

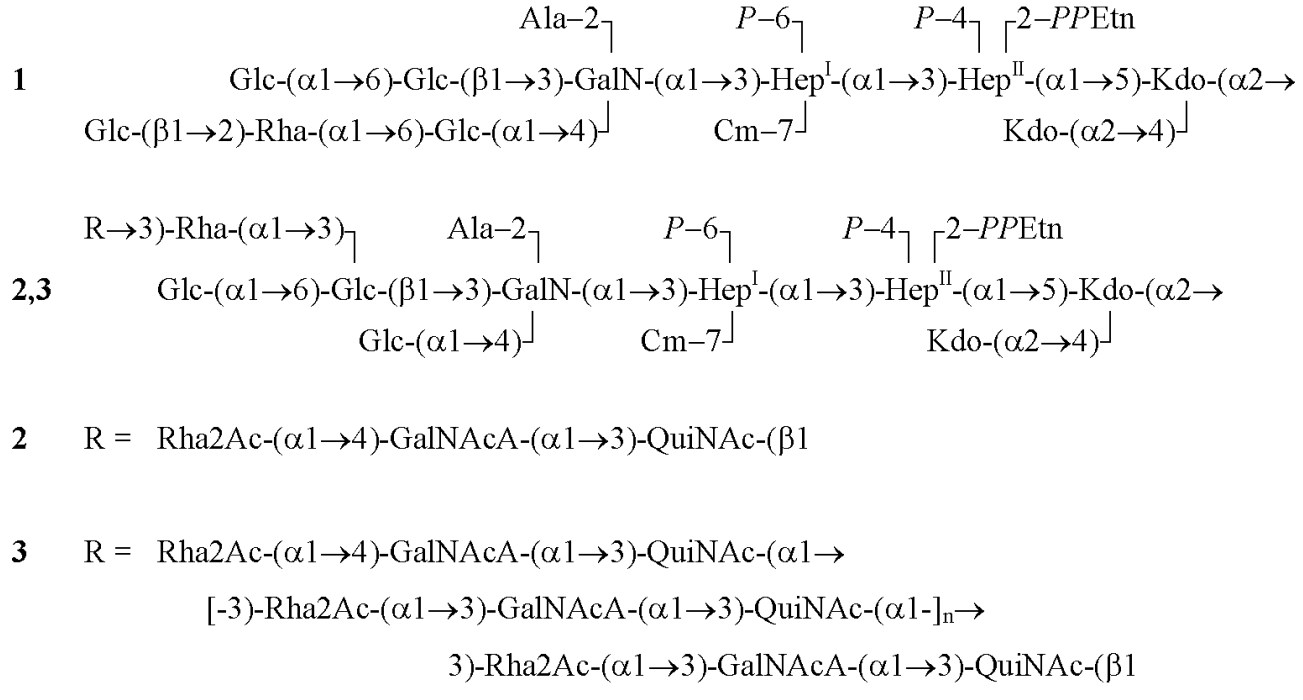

Fig. 3. Structures of the carbohydrate moiety of the R-, SR-, and S-type LPS of *P. aeruginosa* immunotype 5 (**1**-**3**, respectively). For abbreviations, see the legend to Fig. 1. O-Acetylation of Rha in the O-antigen moiety of oligosaccharide **2** is non-stoichiometric.

between the corresponding rhamnosyl transferases that results in an enhanced synthesis of the appropriate core glyco-form. This may provide a mechanism for regulation of the content of the O-polysaccharide-containing LPS species on the cell surface [13].

The LPS core of *P. aeruginosa* is characterized by a high content of phosphate, including two monophosphate groups and one ethanolamine diphosphate group, which are distributed between Hep^{I} and Hep^{II} (Fig. 3). The position of *PP*Etn at O2 of Hep^{I} has been demonstrated in a smooth *P. aeruginosa* strain for the first time in this work and found to be the same as in a *P. aeruginosa* PAO1 (serogroup O5)-derived *algC* mutant having an incomplete core [9]. The degree of substitution of Hep^{I} P2 with *P*Etn is not less than 60% but it is also possible that *P*Etn is present in stoichiometric or almost stoichiometric amounts since the observed non-stoichiometric substitution may result from partial elimination of *P*Etn during mild acid degradation of the LPS.

These and our preliminary data on another smooth strain of *P. aeruginosa* from serogroup O12 indicated that the phosphorylation pattern, including the position of *PP*Etn, is a conserved feature of *P. aeruginosa* LPS. Another conserved feature is the presence of a carbamoyl group at O7 of Hep^{II} [14], whereas the N-alanyl group that is present on GalN in most *P. aeruginosa* strains studied [6, 8, 9, 11], may be replaced with an N-acetyl group [10]. Up to four O-acetyl groups at undefined positions have been reported in the core of a rough clinical isolate *P. aeruginosa* 2192 [11] and several smooth strains [6] but no significant O-acetylation was observed in the core of *P. aeruginosa* immunotype 5 studied in this work.

Therefore, the structural data of the core obtained in this work in combination with published data of the repeating unit of the O-polysaccharide [12, 15, 16] and the mode of its attachment to the core [12] enabled determination of the full structure of the carbohydrate moiety of the *P. aeruginosa* immunotype 5 LPS shown in Fig. 3. The highly heterogeneous LPS consists of three types of species, including those without O-antigen and with a short- or long-chain O-antigen (structures **1-3** in Fig. 3), which are analogous to R-, SR-, and S-type LPS of enterobacteria, respectively.

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