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Note

Structure of the lipopolysaccharide of *Pseudomonas aeruginosa* O-12 with a randomly *O*-acetylated core region

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Abstract

The lipopolysaccharide of *Pseudomonas aeruginosa* O-12 was studied by strong alkaline and mild acid degradations and dephosphorylation followed by fractionation of the products by GPC and high-performance anion-exchange chromatography and analyses by ESI FT-MS and NMR spectroscopy. The structures of the lipopolysaccharide core and the O-polysaccharide repeating unit were elucidated and the site and the configuration of the linkage between the O-polysaccharide and the core established. The core was found to be randomly *O*-acetylated, most *O*-acetyl groups being located on the terminal rhamnose residue of the outer core region.

Keywords: Lipopolysaccharide; Core structures; O-antigen repeating unit; O-acetylation; *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is an opportunistic human pathogen, which causes severe infections in hosts with weakened defense mechanisms. Based on the immunospecificity of the O-antigens (O-polysaccharide chains of the lipopolysaccharides), strains of *P. aeruginosa* are classified into more than 20 O-serotypes (Ref. 1 and refs cited therein).

The structure of the chemical repeating unit of the O-polysaccharide has been determined in all *P. aeruginosa* O-serotypes.[1,2] However, it could not be ascertained from this structure what are the first and last monosaccharides in the repeating unit, nor which monosaccharide is linked to the LPS core. The chemical repeating unit may be any cyclic permutation of the actual biological repeating unit, that is the properly ordered oligosaccharide, which, after having been preassembled on a lipid carrier, is polymerized into the O-polysaccharide in the O-antigen polymerase (Wzy)-dependent pathway of LPS biosynthesis. Later, the biological repeating unit has been defined in *P. aeruginosa* serotypes O-1, O-5, O-6, O-10, and O-11,[3–7] and the biosynthesis pathways of the O-antigens of *P. aeruginosa* serotypes O-5 and O-6 have been discussed.[8]

The structure of the LPS core has been established in *P. aeruginosa* strains belonging to various O-serotypes[4–7,9] and in a number of rough mutants,[9–12] including a cystic fibrosis isolate, *P. aeruginosa* 2192, having the full core.[11] Peculiar features of the core of *P. aeruginosa* are a high degree of phosphorylation and the presence of various non-sugar groups, including ethanolamine diphosphate, N-linked alanyl (or less commonly acetyl) and O-linked carbamoyl groups. In addition, in some strains *O*-acetyl groups are present in non-stoichiometric amounts in the outer core region at multiple unknown positions.[4,5,11]

Abbreviations: 8eLeg, 5,7-diamino-3,5,7,9-tetradeoxy-L-*glycero*-D-*galacto*-non-2-ulosonic acid (8-epilegionaminic acid); Cm, carbamoyl; Etn, ethanolamine; FucN, 2-amino-2,6-dideoxygalactose; 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-dideoxyglucose.

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In this work, we determined the structure of the biological repeating unit of the *P. aeruginosa* O-12 polysaccharide and demonstrated a random distribution of the *O*-acetyl groups in the LPS core of this strain. The structure of the chemical repeating unit of the O-polysaccharide of *P. aeruginosa* O-12 has been determined[1,2,13] and found to be a linear trisaccharide that contains 2-acetamido-2,6-dideoxy-D-glucose (D-QuiNAc), 2-acetimidoylamino-2,6-dideoxy-L-galactose (L-FucNAm), and 5,7-diacetamido-3,5,7,9-tetradeoxy-L-*glycero*-D-*galacto*-non-2-ulosonic acid (di-*N*-acetyl-8-epilegionaminic acid, 8eLeg5Ac7Ac[14]).

Strong alkaline degradation of the LPS following mild hydrazinolysis resulted in a mixture of oligosaccharide phosphates, which was isolated by GPC on Sephadex G-50 and then fractionated by HPAEC at super-high pH (Fig. 1) to give two major fractions, I_{NaOH} and II_{NaOH}, and a minor fraction III_{NaOH}. The isolated compounds were analyzed by ESI FT-MS and NMR spectroscopy.

Fraction II_{NaOH} was found to contain two core-lipid A backbone oligosaccharide pentakisphosphates (**1** and **3**, Fig. 2) in the ratio ~7:1, respectively, having the same molecular mass 2357.51 Da. They showed an ^{1}H NMR spectrum (Table 1) practically identical to that of the corresponding compounds isolated by HPAEC after strong alkaline degradation of the LPS of *P. aeruginosa* immunotype 4 (O-1).[6] Compounds **1** and **3** are isomers that differ in the position of the terminal rhamnose residue in the outer core region (core glycoforms 1 and 2). In smooth *P. aeruginosa* strains studied,[3–6] glycoform 1 core carries no O-polysaccharide, whereas glycoform 2 core is mainly (Ref. 6 and this work) or completely[3–5] substituted with an O-polysaccharide chain.

Minor fraction III_{NaOH} contained one compound **2** having molecular mass 2211.46 Da. The mass difference of 146.05 Da between compound **2** on the one hand and compounds **1** and **3** on the other hand corresponds to the lack of the rhamnose residue. This was confirmed by the ^{1}H NMR spectrum of **2**, which showed only minor signals for rhamnose. That **2** is not an artifact was confirmed by ESI FT-MS of the products derived by mild acid degradation of the LPS (see below). No

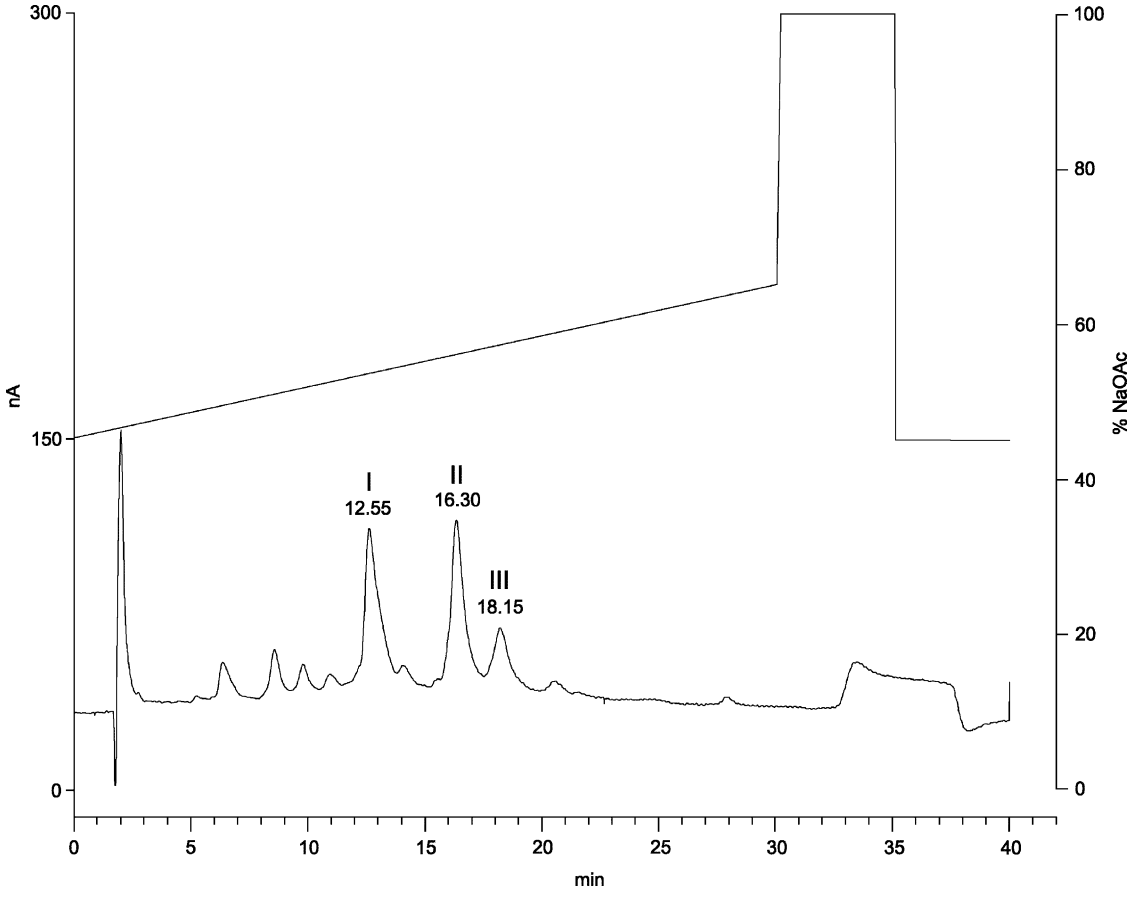

Fig. 1. HPAEC of the alkaline degradation products from the LPS of *P. aeruginosa* O-12. Fractions I, II and III correspond to the core-lipid A backbone oligosaccharide with one O-antigen repeating unit, the unsubstituted core-lipid A backbone oligosaccharide, and the core-lipid A backbone lacking Rha, respectively.

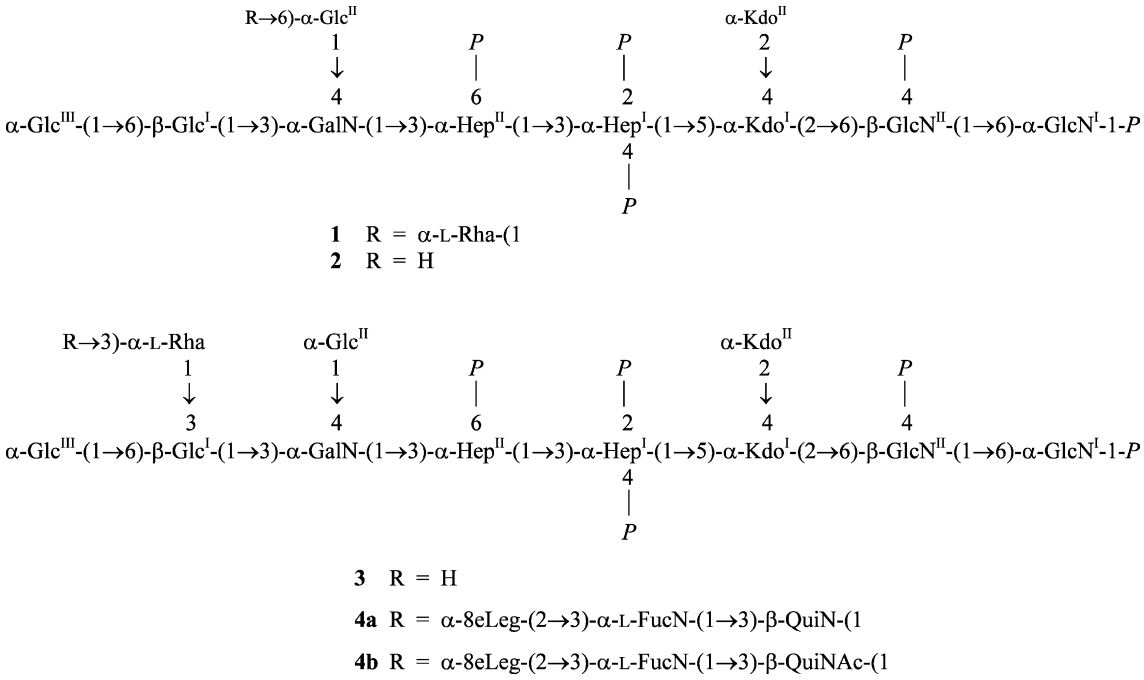

Fig. 2. Structures of the alkaline degradation products from the LPS of *P. aeruginosa* O-12, **1**–**4**. Abbreviations: 8eLeg, 5,7-diamino-3,5,7,9-tetradeoxy-L-*glycero*-D-*galacto*-non-2-ulosonic (8-epilegionaminic) acid; FucN, 2-amino-2,6-dideoxygalactose; Hep, L-*glycero*-D-*manno*-heptose; Kdo, 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.

rhamnose-lacking compound was derived on alkaline degradation under the same conditions from the LPS of other *P. aeruginosa* strains studied earlier.[4–6,11]

Fraction I_{NaOH} contained two compounds **4a** and **4b** in the ratio ~5:1, which have molecular masses 2879.51 and 2921.52 Da, respectively. These values correspond to the core-lipid A backbone oligosaccharide pentakisphosphates bearing one O-antigen repeating unit, which is either fully N-deacetylated **4a** or retains one of the *N*-acetyl groups **4b**. A similar incomplete N-deacetylation was observed on strong alkaline degradation of the LPS of *P. aeruginosa* immunotype 4 (O-1).[6] Compounds **4a**,**b** were evidently derived from a short-chain SR-type LPS, which, together with long-chain S-type and O-antigen-lacking R-type LPS, is typically present in *P. aeruginosa*.[1]

The structure of the core-lipid A backbone moiety in **4** (Fig. 2) was established as described previously[4,5,11] based on the full assignment of the ^{1}H NMR spectrum (Fig. 3, Table 1) using 2D ^{1}H,^{1}H COSY and TOCSY experiments followed by linkage and sequence analyses by 2D ^{1}H,^{13}C HMQC and ROESY experiments and determination of the phosphorylation pattern by a ^{1}H,^{31}P HMQC experiment (Table 2). As a result, a typical glycoform 2 core was identified in **4**, which is identical to that found in the LPS of the other *P. aeruginosa* smooth strains studied.[4–6]

The structure of the O-antigen trisaccharide moiety in **4** (Fig. 2) was determined using a 2D ROESY experiment, which revealed FucN H-1,QuiN H-3 and QuiN H-1,Rha H-3 correlations at δ 5.40/3.73 and 4.81/4.00 in **4a** or δ 5.25/3.80 and 4.72/3.86 in **4b**, respectively. Based on these data combined with the structure of the chemical repeating unit of the O-antigen,[13] it was inferred that 8eLeg is attached at position 3 of FucN and occupies the terminal non-reducing end of the O-antigen trisaccharide. The α configuration of the FucN linkage and the β configuration of the QuiN linkage were established by $J_{1,2}$ values of 3 and 7 Hz, respectively. The α configuration of 8eLeg followed from the axial orientation of the carboxyl group, as shown by a relatively large difference of 0.81 ppm between H-3eq and H-3ax chemical shifts.[13] Compound **4b** differs from **4a** by a significantly lower-field position of the H-2 resonance of QuiN (δ 3.93 vs. 3.05), which demonstrates N-acetylation of QuiN in **4b**.

The data obtained in this work and previously[13,14] define the structure of the biological repeating unit and, thus, the full structure **5** of the O-antigen of *P. aeruginosa* O-12 (Fig. 4). As in the other D-QuiNAc-containing LPS of *P. aeruginosa*,[4–6] this sugar is the first monosaccharide of the O-polysaccharide and is β-linked to the terminal rhamnose residue of the glycoform 2 core. Therefore, the anomeric configuration of D-QuiNAc in the first and interior O-12-antigen repeating units is different, which is in accordance with the involvement of different enzymes, O-antigen polymerase (Wzy) and ligase (WaaL), with connection of the

Table 1
^{1}H NMR data of the alkaline degradation products from the LPS of *P. aeruginosa* O-12 (δ)

Sugar residue	H-1	H-2	H-3	H-4	H-5	H-6(6a)	H-6b(7a)	H-7b
	H-3ax	H-3eq	H-4	H-5	H-6	H-7	H-8(8a)	H-9(8b)
Compound **1**								
→6)-α-D-Glc*p*N^{I}-1-*P*	5.67	3.44	3.93	3.64	4.17	3.79	4.32	
→6)-β-D-Glc*p*N^{II}-(1→	4.88	3.08	3.87	3.76	3.77	3.47	3.72	
→4,5)-α-Kdo*p*^{I}-(2→	2.02	2.22	4.12	4.31	3.69 [a]	3.88	3.64	3.91
α-Kdo*p*^{II}-(2→	1.82	2.10	4.17	4.09	3.73 [a]	4.15	3.81	4.02
→3)-α-Hep*p*^{I}2,4*P*-(1→	5.39	4.55	4.21	4.49	4.35	4.01	3.73	3.96
→3)-α-Hep*p*^{II}6*P*-(1→	5.19	4.42	4.25	4.16	4.02	4.49	3.75	3.80
→3,4)-α-D-Gal*p*N-(1→	5.61	3.84	4.49	4.41	4.24	3.89	3.91	
→6)-β-D-Glc*p*^{I}-(1→	4.68	3.26	3.57	3.27	3.73	3.82	3.91	
→6)-α-D-Glc*p*^{II}-(1→	5.05	3.51	3.77	3.59	4.19	3.79	3.91	
α-D-Glc*p*^{III}-(1→	4.99	3.58	3.71	3.47	3.69	3.79	3.86	
α-L-Rha*p*-(1→	4.81	4.03	3.83	3.46	3.75	1.33		
Compounds **4a**,**b** [b]								
→6)-α-D-Glc*p*N^{I}-1-*P*	5.67	3.44	3.92	3.64	4.17	3.79	4.32	
→6)-β-D-Glc*p*N^{II}-(1→	4.88	3.07	3.87	3.75	3.75	3.50	3.74	
→4,5)-α-Kdo*p*^{I}-(2→	2.03	2.21	4.13	4.31	3.69 [a]	3.91	3.66	3.94
α-Kdo*p*^{II}-(2→	1.81	2.11	4.21	4.09	3.76 [a]	4.15	3.82	4.01
→3)-α-Hep*p*^{I}2,4*P*-(1→	5.39	4.52	4.21	4.47	4.32	4.02	3.75	3.98
→3)-α-Hep*p*^{II}6*P*-(1→	5.21	4.45	4.26	4.18	4.01	4.49	3.76	3.82
→3,4)-α-D-Gal*p*N-(1→	5.61	3.84	4.49	4.42	4.24	3.90	3.91	
→3,6)-β-D-Glc*p*^{I}-(1→	4.68	3.50	3.69	3.55	3.70	3.85	3.93	
α-D-Glc*p*^{II}-(1→	5.06	3.51	3.77	3.52	4.09	3.85	3.88	
α-D-Glc*p*^{III}-(1→	5.02	3.58	3.74	3.42	3.75	3.75	3.89	
→3)-α-L-Rha*p*-(1→	5.19 (5.15)	4.25	4.00 (3.86)	3.65 (3.51)	4.06 (4.01)	1.28 (1.26)		
→3)-β-D-Qui*p*N-(1→	4.81 (4.72)	3.05 (3.93)	3.73 (3.80)	3.36 (3.39)	3.54	1.35 (1.33)		
→3)-α-L-Fuc*p*N-(1→	5.40 (5.25)	3.63 (3.46)	4.16 (4.19)	4.02 (4.00)	4.36 (4.37)	1.22		
α-8eLeg*p*-(2→	1.91	2.72	3.65	3.01	4.22	3.40	4.03	1.42 (1.41)

[a] Tentative assignment.
[b] When different, data of **4b** are given in parentheses. The signal for the NAc group in **4b** is at δ 2.06.

repeating units to each other and transfer of the O-polysaccharide or a single O-antigen repeating unit to the core, respectively.[8]

The LPS was cleaved by mild hydrolysis with aqueous 1% HOAc at 100 °C and a core oligosaccharide mixture was fractionated by GPC on Sephadex G-50 to give two fractions, I_{HOAc} and II_{HOAc}, which correspond to the core with one O-antigen repeating unit and an unsubstituted core, respectively. ESI FT-MS studies, using published data on similar oligosaccharides from the LPS of *P. aeruginosa* immunotype 5,[5] showed that both fractions were mixtures of oligosaccharides that differ in the number of phosphate groups ($P_{2\text{-}4}$) and *O*-acetyl groups ($Ac_{0\text{-}2}$) and in the presence or absence of ethanolamine (Etn). There were present also minor compounds that lack Rha and O-polysaccharide fragments consisting of one to four repeating units. The major compounds in fraction II_{HOAc} were core oligosaccharides $RhaGlc_3(GalNAla)Hep(HepCm)Kdo\mathit{P}_4EtnAc_{0\text{-}1}$ with molecular masses 1892.44 and 1934.45 Da for the non-acetylated compound **6** and its mono-O-acetylated derivative, respectively, as well as the corresponding oligosaccharides with Kdo in an anhydro form(s) (Fig. 5(B)). Molecular masses 2581.75 and 2623.77 Da of the major compounds in fraction I_{HOAc} (Fig. 5(A)) differ from those in fraction II_{HOAc} by 689.3 Da, which corresponds to one O-antigen repeating unit (see structure **5** in Fig. 4).

A mixture of **6** and *O*-acetylated **6** was isolated from fraction II_{HOAc} by HPAEC under neutral conditions. The mixture was studied by NMR spectroscopy, including 2D ^{1}H,^{1}H COSY, TOCSY, ^{1}H,^{31}P HMQC and ^{1}H,^{31}P HMQC-TOCSY experiments, as described by us earlier in studies of the products that were derived in the same way from the LPS of *P. aeruginosa* immunotype 5.[5] The compounds from both strains were found to share a number of structural features, including the same phosphorylation pattern, the presence of an *N*-alanyl group at GalN, an *O*-carbamoyl group at Hep^{II}, and ethanolamine diphosphate (Etn*PP*) at Hep^{I} (Fig. 6). The major distinction of **6** is the presence of three glucose residues rather than four in the *P. aeruginosa*

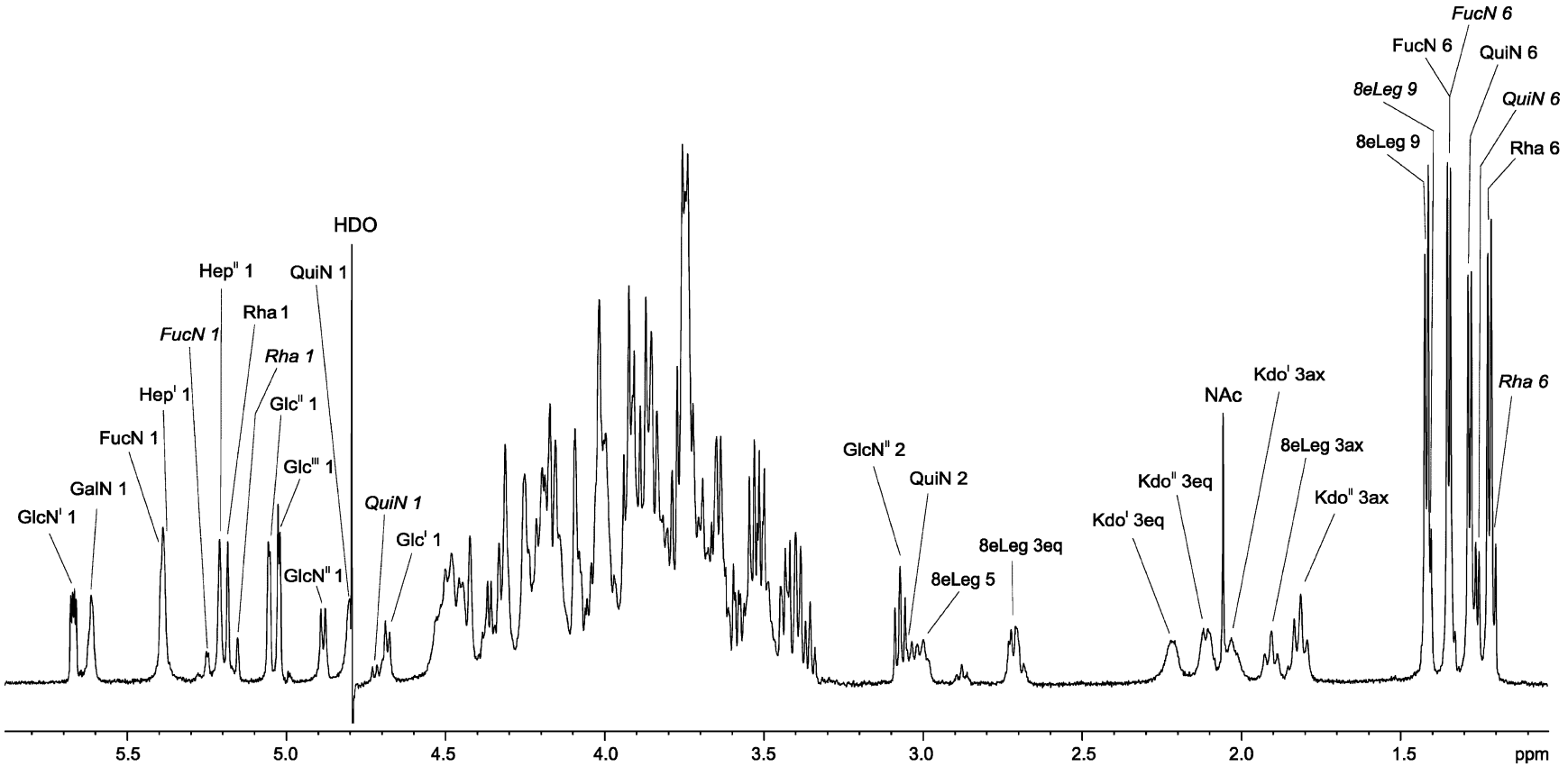

Fig. 3. ^{1}H NMR spectrum of compounds **4a**,**b** obtained by alkaline degradation from the LPS of *P. aeruginosa* O-12. Arabic numerals refer to protons in sugar residues. For abbreviations see legend to Fig. 2. Designations for Rha, QuiN, FucN and 8eLeg in **4a** and **4b** are not italicized and italicized, respectively.

Table 2
^{31}P NMR data of the alkaline degradation products from the LPS of *P. aeruginosa* O-12 (δ)

Sugar residue	P-1	P-2	P-4	P-6
Compound **1**				
→6)-α-D-Glc*p* N^{I}-1-*P*	3.41			
→6)-β-D-Glc*p* N4*P*^{II}-(1→			3.87	
→3)-α-Hep*p* 2,4*P*^{I}-(1→		1.89	4.18	
→3)-α-Hep*p* 6*P*^{II}-(1→				4.60
Compounds **4a,b**				
→6)-α-D-Glc*p* N^{I}-1-*P*	1.94			
→6)-β-D-Glc*p* N4*P*^{II}-(1→			3.04	
→3)-α-Hep*p* 2,4*P*^{I}-(1→		0.38	3.04	
→3)-α-Hep*p* 6*P*^{II}-(1→				3.88

8eLeg*p*5Ac7Ac-(2→3)-α-L-Fuc*p*NAm-(1→3)-α-D-Qui*p*NAc-(1-

[→8)-α-8eLeg*p*5Ac7Ac-(2→3)-α-L-Fuc*p*NAm-(1→3)-α-D-Qui*p*NAc-(1-]$_n$

→8)-α-8eLeg*p*5Ac7Ac-(2→3)-α-L-Fuc*p*NAm-(1→3)-β-D-Qui*p*NAc-(1→

5

Fig. 4. Full structure of the O-polysaccharide of the *P. aeruginosa* O-12 LPS **5**. Abbreviations: 8eLeg5Ac7Ac, 5,7-diacetamido-3,5,7,9-tetradeoxy-L-*glycero*-D-*galacto*-non-2-ulosonic (di-*N*-acetyl-8-epilegionaminic) acid; FucNAm, 2-acetimidoylamino-2,6-dideoxygalactose; QuiNAc, 2-acetamido-2,6-dideoxyglucose.

immunotype 5 LPS.[5] As expected based on the alkaline degradation data of the LPS (see above), **6** represented mainly core glycoform 1, which was contaminated by a small amount (~15%) of the core glycoform 2 isomer.

The products from *P. aeruginosa* O-12 were distinguished also by O-acetylation to a marked degree, whereas the corresponding product from *P. aeruginosa* immunotype 5 contained only a negligible amount of *O*-acetyl groups.[5] On the average, it was ~0.5 *O*-acetyl group per molecule as followed from the ^{1}H NMR spectrum of the mixture (Fig. 7), which showed ~0.5:1:1 ratios of the integral intensities of the CH_3 signals for *O*-acetyl groups (δ 2.1–2.2), alanine (δ 1.54) and rhamnose (δ 1.22–1.35), respectively. Multiple signals for the methyl group of Rha suggested that this sugar residue is *O*-acetylated.

Tracing connectivities using ^{1}H,^{1}H TOCSY experiments with different mixing times (from 50 to 150 ms) enabled assignment, starting from the CH_3 signals, of all ^{1}H NMR resonances for five major types of Rha residues (Table 3). Two of them belong to the non-acetylated Rha residues of core glycoforms 1 and 2 (δ_{H1} 4.81 and 5.15), whose contribution to the total rhamnose was ~50 and 15%, respectively. Three others were from mono-*O*-acetylated Rha residues of core glycoform 1 with an *O*-acetyl group at position 2, 3, or 4 (~10% each), as followed from a low-field displacement by 1.15, 1.21 and 1.44 ppm of the signals for H-2, H-3, and H-4, respectively, as compared with their positions in non-acetylated Rha (Table 3). The remaining minor CH_3 signals (<5% each) may belong to *O*-acetylated Rha residues of core glycoform 2 and/or to di-*O*-acetylated Rha residues of core glycoform 1.

For more exact determination of the maximum number of the *O*-acetyl groups and their distribution, the LPS was degraded with sodium acetate buffer pH 4.2 at 100 °C. ESI FT-MS analysis of the resulting mixture showed that this treatment caused a more significant dephosphorylation as compared with the HOAc treatment but a lesser O-deacetylation. The unsubstituted core was found to carry up to three *O*-acetyl groups (Fig. 8(B)), whereas the core with one O-antigen repeating unit bears no or one *O*-acetyl group (Fig. 8(A)). Dephosphorylation of the mixture with aqueous 48% HF caused partial cleavage of rhamnose, which resulted in a marked increase of the content of Rha-lacking compounds. The ESI mass spectrum of the dephosphorylated mixture confirmed that the full unsubstituted core contains up to three *O*-acetyl groups and showed that the Rha-lacking core and core with one O-antigen repeating unit contain no more than one *O*-acetyl group (Fig. 9).

Therefore, the majority of the *O*-acetyl groups are located on the rhamnose residue. This conclusion is in accordance with our previous finding that *O*-acetyl groups are located in the outer core region of the LPS of a *P. aeruginosa* cystic fibrosis isolate.[11] The exact position of the *O*-acetyl groups beyond rhamnose remains unknown but it can be suggested that, like those on Rha, they are distributed randomly all over the sites in the outer core that are accessible for O-acetyl transferase(s). Recently, a random O-acetylation of a lateral 6-deoxy-L-talose residue has been reported in the O-polysaccharide of *Aeromonas hydrophila* O-34.[15] Comparison of the resonance region of the *O*-acetyl groups in the ^{1}H NMR spectra showed that the O-acetylation pattern is similar in all *P. aeruginosa* strains studied, whereas the degree of O-acetylation varies from negligible, as in *P. aeruginosa* immunotype 5,[5] to four and more *O*-acetyl groups, as in rough cystic fibrosis isolate, *P. aeruginosa* 2192.[11]

1. Experimental

1.1. Bacterial strains, growth and isolation of lipopolysaccharides

P. aeruginosa O-12, strain 170023, was from the Hungarian National Collection of Medical Bacteria (National Institute of Hygiene, Budapest). 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,

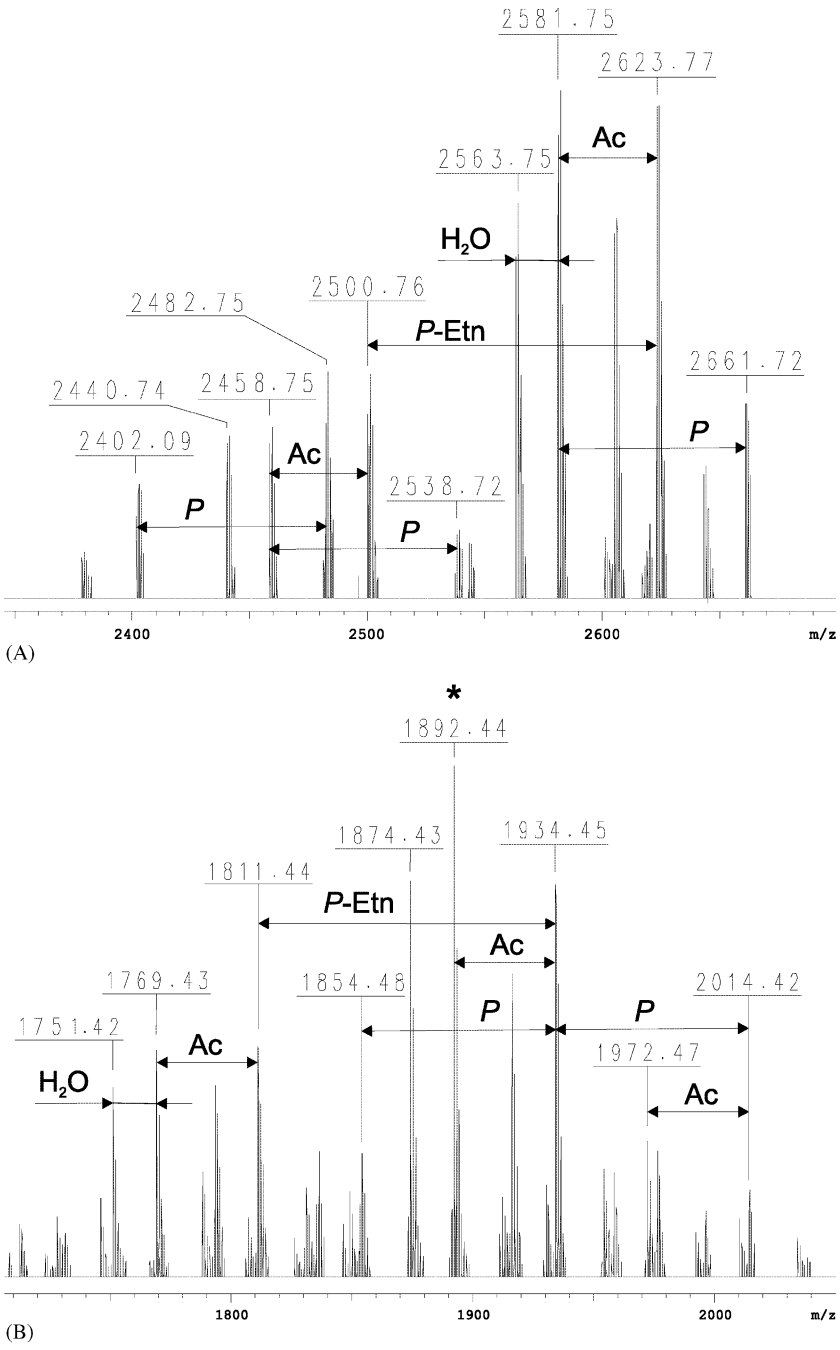

Fig. 5. Parts of ESI mass spectra of fractions I_{HOAc} (A) and II_{HOAc} (B) obtained by mild acid degradation with aqueous 1% HOAc from the LPS of *P. aeruginosa* O-12. The shown regions represent all essential ion peaks found in the complete spectrum, except for peaks for oligomers ($n = 1$–4) of the O-antigen repeating unit with *m/z* 707.3, 1396.6, 2085.9 and 2775.2. Mass peak for compound **6** is shown by asterisk.

washed with acetone and dried. LPS was isolated from dry bacterial cells by extraction with aq 45% phenol (30 min) at 65–68 °C.[16] Cells were removed by centrifugation, the supernatant was dialyzed against distilled 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.

1.2. Alkaline degradation

LPS (200 mg) was treated with anhyd hydrazine (4 mL) at 37 °C for 1 h, diluted with cold 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 9 h, cooled, acidified with conc HCl to pH 5.5, extracted twice with

α-L-Rha-(1→6)-α-Glc
1 P PPEtn
↓ | |
4 6 2
α-Glc-(1→6)-β-Glc-(1→3)-α-GalN-(1→3)-α-Hep-(1→3)-α-Hep-(1→5)-α-Kdo
2 7 4
| | |
L-Ala Cm P

6

Fig. 6. Structure of the non-O-acetylated glycoform 1 core **6** obtained by mild acid degradation from the LPS of *P. aeruginosa* O-12. Abbreviations: Cm, carbamoyl; Etn, ethanolamine, Hep; L-*glycero*-D-*manno*-heptose; Kdo, 3-deoxy-D-*manno*-oct-2-ulosonic acid. All monosaccharides are in the pyranose form and have the D configuration unless stated otherwise.

CH_2Cl_2, and the aqueous layer 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}, using a Knauer differential refractometer for monitoring of the elution. The isolated oligosaccharide mixture (27.9% of the LPS weight) was fractionated by HPAEC on a semi-preparative CarboPac PA1 column (250 × 9 mm, Dionex) using a linear gradient of 0.4–0.6 M NaOAc in 0.1 M NaOH at flow rate 1 mL min^{-1} for 90 min. Two milliliter fractions were collected and analyzed by HPAEC with pulsed amperometric detection (Dionex) 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 a flow rate of 1 mL min^{-1} for 30 min. After desalting on a column (40 × 2.6 cm) of Sephadex G-50 (S), two major fractions, I_{NaOH} and II_{NaOH}, and a minor fraction III_{NaOH}, having retention times 12.55, 16.30, and 18.15 min in analytical HPAEC, were isolated in yields 6.8, 6.1 and 2.6% of the initial oligosaccharide mixture weight, respectively.

1.3. Mild acid degradation

1.3.1. Protocol A. LPS (200 mg) was dissolved in aq 1% HOAc and heated for 3 h at 100 °C. The precipitate was removed by centrifugation, and the supernatant was fractionated by GPC on a column (40 × 2.6 cm) of Sephadex G-50 (S) as described above. A polysaccharide and two oligosaccharides, I_{HOAc} and II_{HOAc}, were isolated in yields 23.2, 20.0 and 10.2% of the LPS weight. Oligosaccharide fraction II_{HOAc} was fractionated by HPAEC on a semi-preparative CarboPac PA1 column using a linear gradient of 0.02–0.52 M NaOAc in water at flow rate 1.5 mL min^{-1} for 140 min. Three-milliliter fractions were collected and analyzed by HPAEC on an analytical CarboPac PA1 column (Dionex) using the same eluant at 1.5 mL min^{-1} for 30 min; before pulse amperometric detection the eluate was mixed (3:1) with 1.5 M NaOH. After desalting on

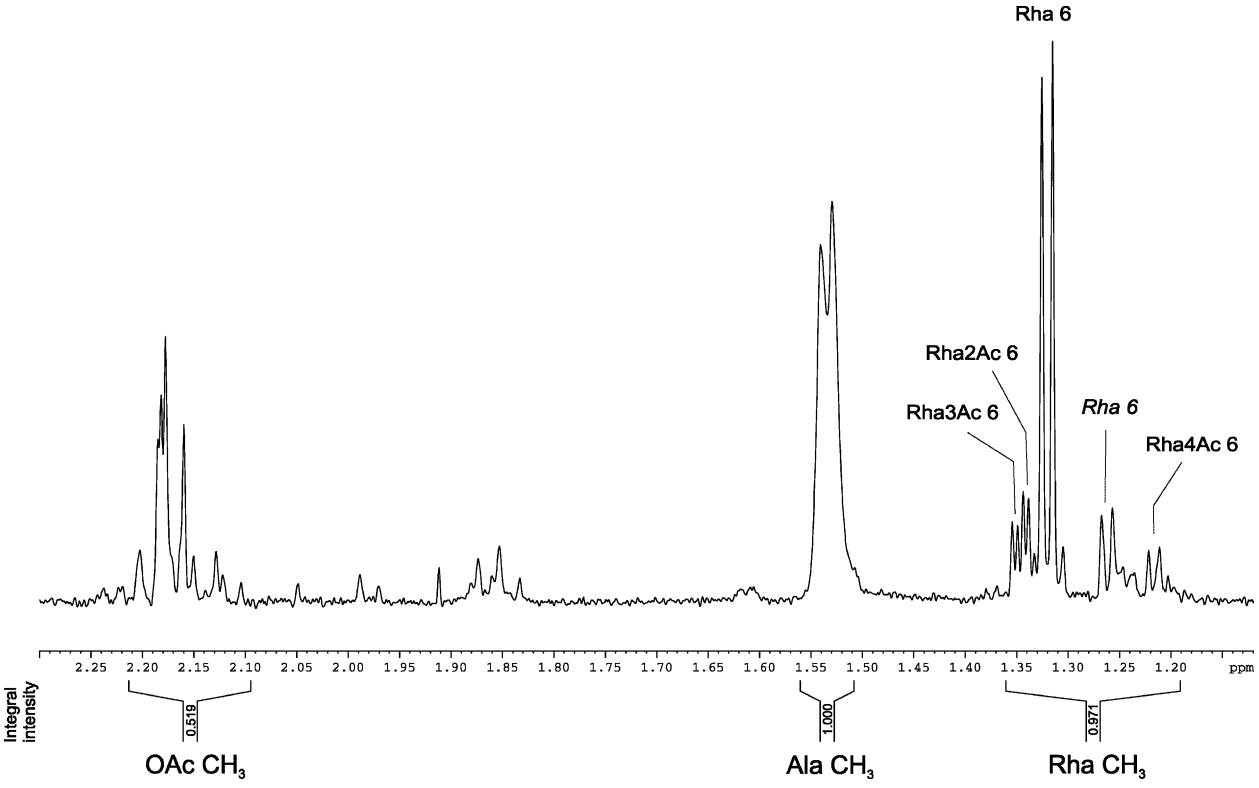

Fig. 7. Part of a ^1H NMR spectrum of the products derived by the mild acid degradation from the LPS of *P. aeruginosa* O-12. Shown is the region of the CH_3 signals. Designations for Rha of glycoform 1 and 2 core are not italicized and italicized, respectively.

Table 3
^{1}H NMR data of the rhamnose residue in the products obtained by mild acid degradation with aqueous 1% HOAc from the LPS of *P. aeruginosa* O-12 (δ)

Sugar residue	H-1	H-2	H-3	H-4	H-5	H-6
Glycoform 1 core						
α-L-Rha	4.81	4.02	3.83	3.45	3.75	1.32
α-L-Rha2Ac	4.84	5.17	4.02	3.50	3.82	1.34
α-L-Rha3Ac	4.83	4.17	5.04	3.66	3.88	1.35
α-L-Rha4Ac	4.85	4.07	4.04	4.89	3.91	1.22
Glycoform 2 core						
α-L-Rha	5.15	4.04	3.80	3.47	3.99	1.26

The major signals for the OAc groups are at δ 2.160, 2.178, 2.182 and 2.185.

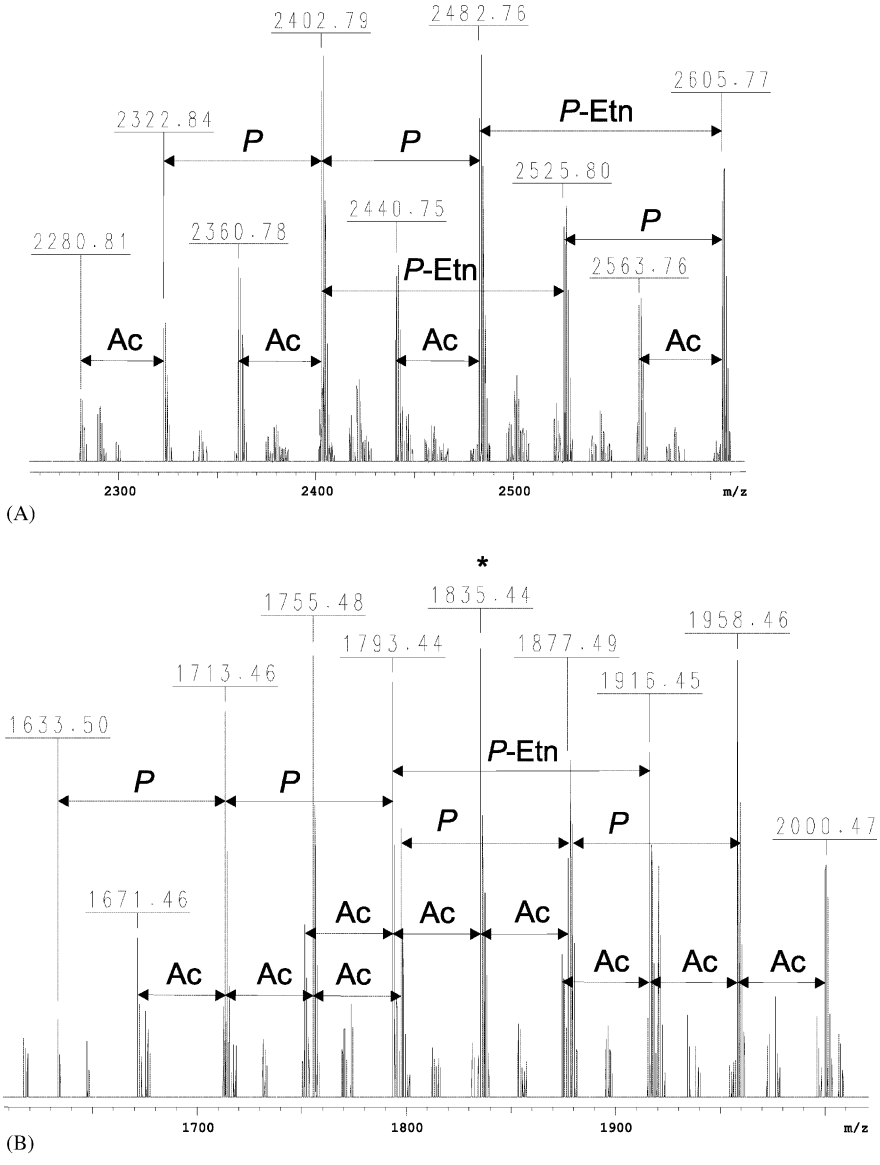

Fig. 8. Parts of an ESI mass spectrum of the products obtained by mild acid degradation at pH 4.2 from the LPS of *P. aeruginosa* O-12. Parts A and B show mass peaks for the core with one O-antigen repeating unit and the unsubstituted core, respectively. The spectrum showed also mass peaks for oligomers of the O-antigen repeating unit, which are outside the ranges shown. The major mass peak for the compound $RhaGlc_3(GalNAla)Hep(HepCm)anhKdoP_3Ac_2$ is shown by an asterisk.

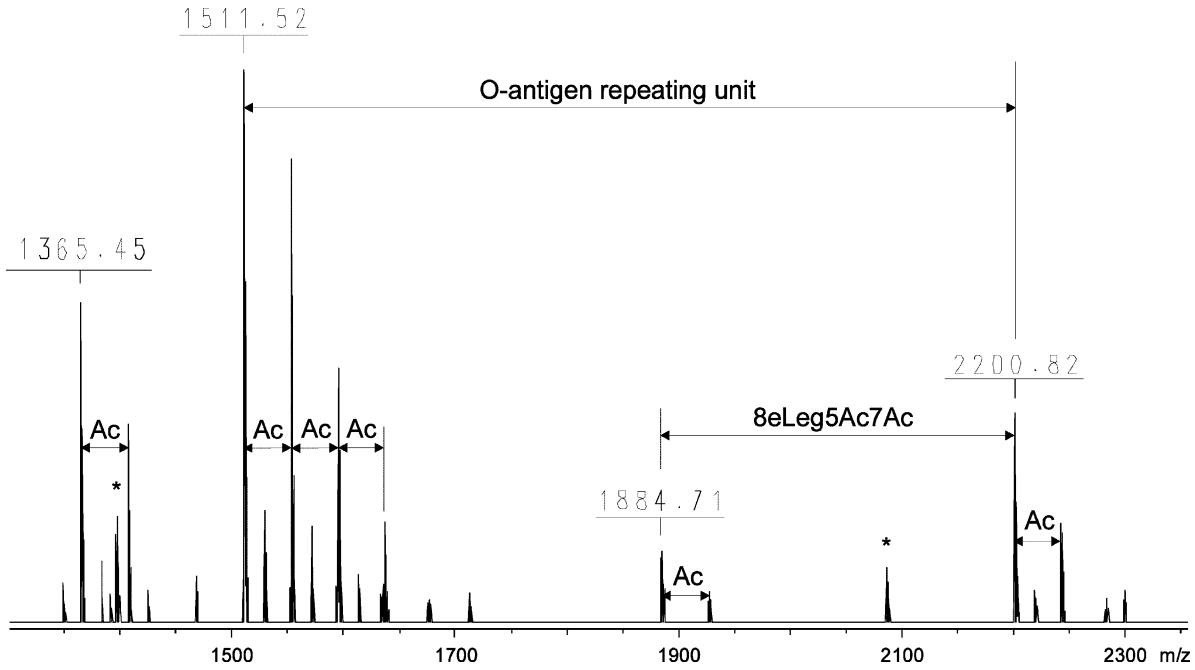

Fig. 9. Part of an ESI mass spectrum of the products obtained by mild acid degradation at pH 4.2 from the LPS of *P. aeruginosa* O-12 followed by dephosphorylation with aqueous 48% HF. Major mass peaks at *m/z* 1511.52, 1365.45 and 2200.82 belong to the dephosphorylated non-O-acetylated compounds: the unsubstituted core $RhaGlc_3(GalNAla)Hep(HepCm)anhKdo$, the core lacking Rha, and the core with one O-antigen repeating unit, respectively. Mass peaks for oligomers ($n = 2$ and 3) of the O-antigen repeating unit are shown by an asterisk.

Sephadex G-50 (S), the product having retention time 17.7 min in analytical HPAEC (a mixture of **6** and O-acetylated **6**) was isolated in a yield 3.8% of the fraction II_{HOAc} weight.

1.3.2. Protocol B. LPS (200 mg) was dissolved in 0.1 M sodium acetate buffer pH 4.2 and heated at 100 °C for 2 h. The precipitate was removed by centrifugation, the supernatant fractionated by GPC on a column (80 × 2.6 cm) of Sephadex G-50 (S) in pyridinium acetate buffer pH 4.5 (4 mL Py and 10 mL HOAc in 1 L water) at 30 mL h^{-1}. Poly- and oligosaccharide fractions were isolated in yields 33.3 and 22.4% of the LPS weight.

1.4. Dephosphorylation

The oligosaccharide (2 mg) obtained by degradation of the LPS with 0.1 M sodium acetate buffer pH 4.2 (Protocol B) was dissolved in aq 48% HF (100 μL) and kept at 4 °C for 48 h, the solution was dried under diminished pressure, using a cartridge with solid NaOH to absorb HF, the residue was dissolved in water and lyophilized.

1.5. 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 aq 85% H_3PO_4 (δ_P 0.0) as reference. Prior to the measurements, the samples were lyophilised 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.

1.6. 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 30:30:0.01 2-propanol-water-triethylamine at a concentration of ~20 ng μL^{-1} and sprayed with a flow rate of 2 μL min^{-1}.

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References

1. Knirel, Y. A. *CRC Crit. Rev. Microbiol.* **1990**, *17*, 273–304.
2. Knirel, Y. A.; Vinogradov, E. V.; Kocharova, N. A.; Paramonov, N. A.; Kochetkov, N. K.; Dmitriev, B. A.; Stanislavsky, E. S.; Lányi, B. *Acta Microbiol. Hung.* **1988**, *35*, 3–24.
3. Sadovskaya, I.; Brisson, J.-R.; Thibault, P.; Richards, J. C.; Lam, J. S.; Altman, E. *Eur. J. Biochem.* **2000**, *267*, 1640–1650.
4. Bystrova, O. V.; Shashkov, A. S.; Kocharova, N. A.; Knirel, Y. A.; Lindner, B.; Zähringer, U.; Pier, G. B. *Eur. J. Biochem.* **2002**, *269*, 2194–2203.
5. Bystrova, O.V.; Lindner, B.; Moll, H.; Kocharova, N.A.; Knirel, Y.A.; Zähringer, U.; Pier, G.B. *Biochemistry (Moscow)* in press.
6. Bystrova, O. V.; Lindner, B.; Moll, H.; Kocharova, N. A.; Shashkov, A. S.; Knirel, Y. A.; Zähringer, U.; Pier, G. B. *Carbohydr. Res.* **2003**, *338*, 1801–1806.
7. Dean, C. R.; Datta, A.; Carlson, R. W.; Goldberg, J. B. *J. Bacteriol.* **2002**, *184*, 323–326.
8. Rocchetta, H. L.; Burrows, L. L.; Lam, J. S. *Microbiol. Mol. Biol. Rev.* **1999**, *63*, 523–553.
9. Sadovskaya, I.; Brisson, J.-R.; Lam, J. S.; Richards, J. C.; Altman, E. *Eur. J. Biochem.* **1998**, *255*, 673–684.
10. Sanchez-Carballo, P. M.; Rietschel, E. T.; Kosma, P.; Zähringer, U. *Eur. J. Biochem.* **1999**, *261*, 500–508.
11. Knirel, Y. A.; Bystrova, O. V.; Shashkov, A. S.; Kocharova, N. A.; Senchenkova, S. N.; Moll, H.; Lindner, B.; Zähringer, U.; Hatano, K.; Pier, G. B. *Eur. J. Biochem.* **2001**, *268*, 4708–4719.
12. Kooistra, O.; Bedoux, G.; Brecker, L.; Lindner, B.; Sanchez-Carballo, P.; Haras, D.; Zähringer, U. Carbohydro Res., accepted for publication.
13. Knirel, Y. A.; Vinogradov, E. V.; Shashkov, A. S.; Dmitriev, B. A.; Kochetkov, N. K.; Stanislavsky, E. S.; Mashilova, G. M. *Eur. J. Biochem.* **1987**, *163*, 627–637.
14. Tsvetkov, Y. E.; Shashkov, A. S.; Knirel, Y. A.; Zähringer, U. *Carbohydr. Res.* **2001**, *331*, 233–237.
15. Knirel, Y. A.; Shashkov, A. S.; Senchenkova, S. N.; Merino, S.; Tomas, J. M. *Carbohydr. Res.* **2002**, *337*, 1381–1386.
16. Westphal, O.; Jann, K. *Methods Carbohydr. Chem.* **1965**, *5*, 83–91.