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Structures of decasaccharide and tridecasaccharide tetraphosphates isolated by strong alkaline degradation of *O*-deacylated lipopolysaccharide of *Pseudomonas fluorescens* strain ATCC 49271

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Abstract

Mild hydrazinolysis of *Pseudomonas fluorescens* strain ATCC 49271 lipopolysaccharide (LPS) followed by strong alkaline degradation and purification by anion-exchange HPLC resulted in two phosphorylated oligosaccharides (**1** and **2**). On the basis of compositional analysis and ^{1}H, ^{13}C, and ^{31}P NMR spectroscopy, including 2D correlation spectroscopy (COSY), 2D rotating frame NOE spectroscopy (ROESY), and 2D inverse mode H-detected heteronuclear $^{1}H-^{13}C$ and $^{1}H-^{31}P$ correlation spectroscopy, the following two structures (**1** and **2**) could be identified

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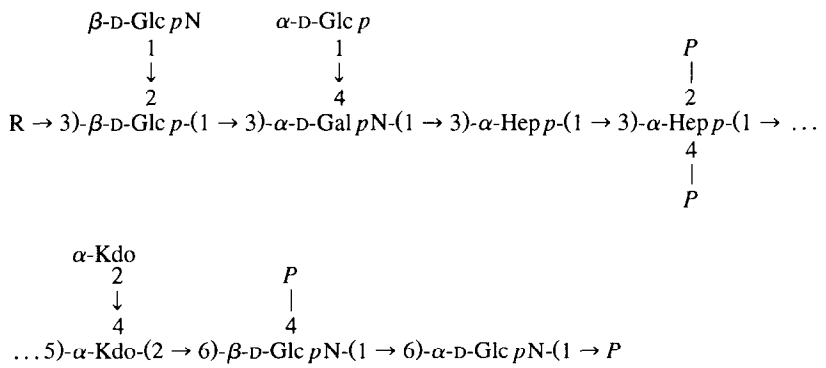


1 R = H
2 R = α-Non-(2 → 4)-α-L-Fuc *p*N-(1 → 3)-β-D-Qui *p*N-(1

where Hep is L-*glycero*-D-*manno*-heptose, Kdo is 3-deoxy-D-*manno*-octulosonic acid, Non is 5,7-diamino-3,5,7,9-tetradeoxy-D-*glycero*-L-*galacto*-nonulosonic acid, and *P* is phosphate.

Decasaccharide **1** and tridecasaccharide **2** represent an incomplete core and the complete core carrying one O-antigen repeating unit, respectively. Both are attached to the lipid A backbone but, due to their degradation protocol, they lack *N*- and *O*-acyl substituents, including *N*- and *O*-acetyl groups, the 5-*N*-acetimidoyl group of Non, the 2-*N*-alanyl group of GalN, and the 7-*O*-carbamoyl group of Hep as well as diphosphate, triphosphate, and, probably, some of the monophosphate groups that are present in the intact core oligosaccharide.

Keywords: Lipopolysaccharide; Core oligosaccharide; *Pseudomonas*; Structure; Degradation; NMR spectroscopy

1. Introduction

Lipopolysaccharides (LPSs) are the well-known endotoxins of Gram-negative bacteria bearing, in their O-specific polysaccharide, specific antigens useful for serodiagnosis, especially in various human pathogenic strains. Since it is well known that there is a good correlation between the structure of the LPS O-chain and the virulence of the bacteria, the structure of the LPS has been considered as a factor mediating pathogenicity [1]. On the other hand, the clinical importance of *Pseudomonas fluorescens* is relatively low due to its inability to grow at body temperature [2]. However, since it became evident that the LPS of *P. fluorescens* cross-reacts with a monoclonal antibody raised against *Legionella pneumophila* serogroup 1, which is known as the most frequent causative agent of "Legionnaires' disease" [3], we started serological and chemical investigations on both strains, aiming to identify the serological epitopes involved in the O-chains.

During preliminary serological investigations we found that the bacterium *P. fluorescens* strain ATCC 49271 cross-reacted with *Legionella pneumophila* serogroup 1 (strain Philadelphia 1) and both LPSs are recognized by a monoclonal antibody (mAb 3/1) specific to the O-polysaccharide chain of the LPS [4,5]. The cross-reactivity was found to be based on the similarity of the structures of the O-specific polysaccharides of

these bacteria, both of which are α-(2 → 4)-linked homopolymers of 5-acetamidino-7-acetamido-3,5,7,9-tetradeoxy-D-*glycero*-L-*galacto*-nonulosonic acid *O*-acetylated at position 8 {completely in *L. pneumophila* [6] or partially (ca. 75%) in *P. fluorescens* [7]}.

We here describe the structural analysis of the core region of the *P. fluorescens* LPS which has resulted in the elucidation of the structures of two phosphorylated oligosaccharides containing incomplete and complete core (**1** and **2**), respectively.

2. Results and discussion

LPS was isolated by a modified method of Gałanos et al. [8] with diethyl ether used instead of water for precipitating LPS from the organic layer [6]. After *O*-deacylation by mild treatment with anhyd hydrazine (37°C), the LPS was degraded with 8 M potassium hydroxide (100°C, 24 h). This approach has been successfully applied for preparation of phosphorylated core-lipid A backbone tetrasaccharides from the LPS of *Salmonella enterica* serovar Minnesota and *Escherichia coli* Re-mutants [9]. A higher concentration of alkali (8 M instead of 4 M used earlier [9]) and a longer treatment (24 h instead of 5 h in ref. [9]) served to improve *N*-deacylation. The carbohydrate portion was separated by GPC on Sephadex G-50 (S) to give the two oligosaccharides (**1** and **2**) and a small amount of a polymeric material which was eluted first from the GPC column. Each of the oligosaccharides was purified by anion-exchange HPLC on CarboPac PA1.

Sugar analysis of the oligosaccharide **1**, which was eluted third from Sephadex G-50, revealed the presence of D-glucose, L-*glycero*-D-*manno*-heptose (Hep), D-GlcN, D-GalN, and 3-deoxy-D-*manno*-octulosonic acid (Kdo) in the ratios 2:2:3:1:2. In addition, **1** contains 4 mol of phosphate and, hence, is a decasaccharide tetraphosphate.

The 360-MHz ^{1}H NMR spectrum of **1** measured in D_2O at pD 4 (Fig. 1) was well resolved and contained signals for eight anomeric protons in the region δ 4.7–5.8. Two of the signals belong to α-linked ($J_{1,2}$ 3.5 Hz) and three to β-linked ($J_{1,2}$ 8–8.5 Hz) pyranoses with axial H-2, and the most low field signal (δ 5.76) belonged to H-1 of an α-glycosyl phosphate ($J_{1,2}$ 3.5 Hz, $J_{1,P}$ 6 Hz). The two remaining signals belong to heptopyranoses, one of them being an unresolved doublet and the second a narrow doublet ($J_{1,2}$ 2 Hz). There were also present signals for two 3-deoxy groups (H-3) at δ 2.28 and 2.14 (equatorial) and δ 2.12 and 1.91 (axial) typical of α-pyranoid Kdo [10,11]. Signals for other sugar protons were in the region 3.1–4.6 ppm.

The presence of the glycosyl phosphate [α-Glc*p*N-(1 → *P*, see below] showed that **1** is a product of the expected type [9], containing a core oligosaccharide attached to a phosphorylated lipid A backbone.

At pD 4 the oligosaccharide **1** was found to be unstable at the elevated temperature (33°C) used for NMR measurements. Therefore, further spectra were measured at pD 7, although resolution was impaired.

The 500-MHz ^{1}H NMR spectrum of **1** at pD 7 was assigned using 2D shift-correlated spectroscopy (COSY, Fig. 2) and, on the basis of the chemical shifts and the coupling constant values (Table 1), the ten sugar spin systems were ascribed to α- and β-glucopyranoses (Glc^{II} and Glc^{I}, respectively), 2-amino-2-deoxy-α-glucopyranosyl

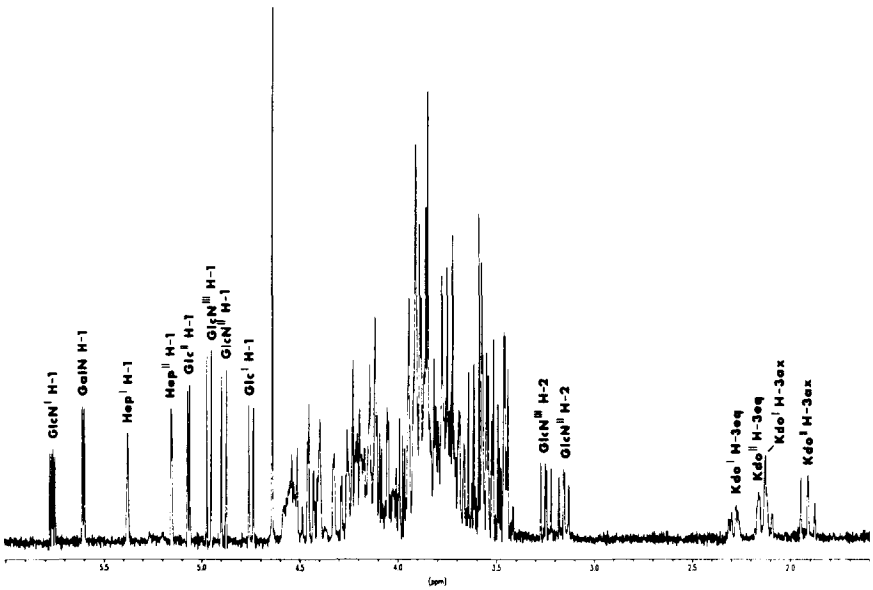


Fig. 1. 360-MHz ^{1}H NMR spectrum of oligosaccharide **1**. For designation of sugar residues see text.

phosphate (GlcN^{I}), two 2-amino-2-deoxy-β-glucopyranoses (GlcN^{II} and GlcN^{III}), 2-amino-2-deoxy-α-galactopyranose (GalN), two α-heptopyranoses (Hep^{I} and Hep^{II}), and two α-Kdo*p* (Kdo^{I} and Kdo^{II}) (the monosaccharide residues are enumerated starting from the reducing end of the oligosaccharide, see the structure of **1** shown below).

The ^{13}C NMR spectrum of **1** was analyzed by 2D H-detected heteronuclear ^{1}H–^{13}C correlation using the inverse mode and, although not completely assigned, employed for the elucidation of the structure. Thus, the assignment of the spin systems of the amino sugars (except for GlcN^{III}) was confirmed by correlation of the protons at the carbons bearing nitrogen (H-2) to the corresponding carbons in the region δ 52–59 observed in the non-decoupled spectrum of **1**. The cross-peaks H-2/C-2 of the amino sugars were present in the spectrum at the following δ_H/δ_C: 3.44/55.8 for GlcN^{I}; 3.02/57.0 for GlcN^{II}; 3.80/51.1 for GalN; no cross-peak H-2/C-2 (expected at δ_H/δ_C 3.14/57.9) was observed for GlcN^{III} because of the considerable broadening of the signal for H-2 (see also footnote to Table 1). The values of the coupling constants $^1J_{H\text{-}1,C\text{-}1}$ 173 and 170 Hz, determined from the non-decoupled HMQC spectrum for Hep^{I} and Hep^{II} ($\delta_{C\text{-}1}$ 99.2 and 104.2, respectively), confirmed the α configuration of both heptose residues. Similar values of 170–173 Hz for GlcN^{I}, GalN, and Glc^{II} ($\delta_{C\text{-}1}$ 92.3, 95.7, and 100.7, respectively) and smaller values of ca. 160 Hz of $^1J_{H\text{-}1,C\text{-}1}$ for GlcN^{II}, GlcN^{III}, and Glc^{I} ($\delta_{C\text{-}1}$ 101.0, 104.8, and 104.8, respectively) were in agreement with the α configuration of the former group of the monosaccharides and the β configuration of the latter.

The coupling of the signals for H-2 and H-4 of Hep^{I} to phosphorus ($J_{H,P}$ 10 Hz for both), revealed from the ^{1}H NMR spectrum of **1**, showed that two phosphate groups were located at positions 2 and 4 of this heptose residue. The H–P coupling for the fourth phosphate group, which is probably located in the lipid A backbone at position 4

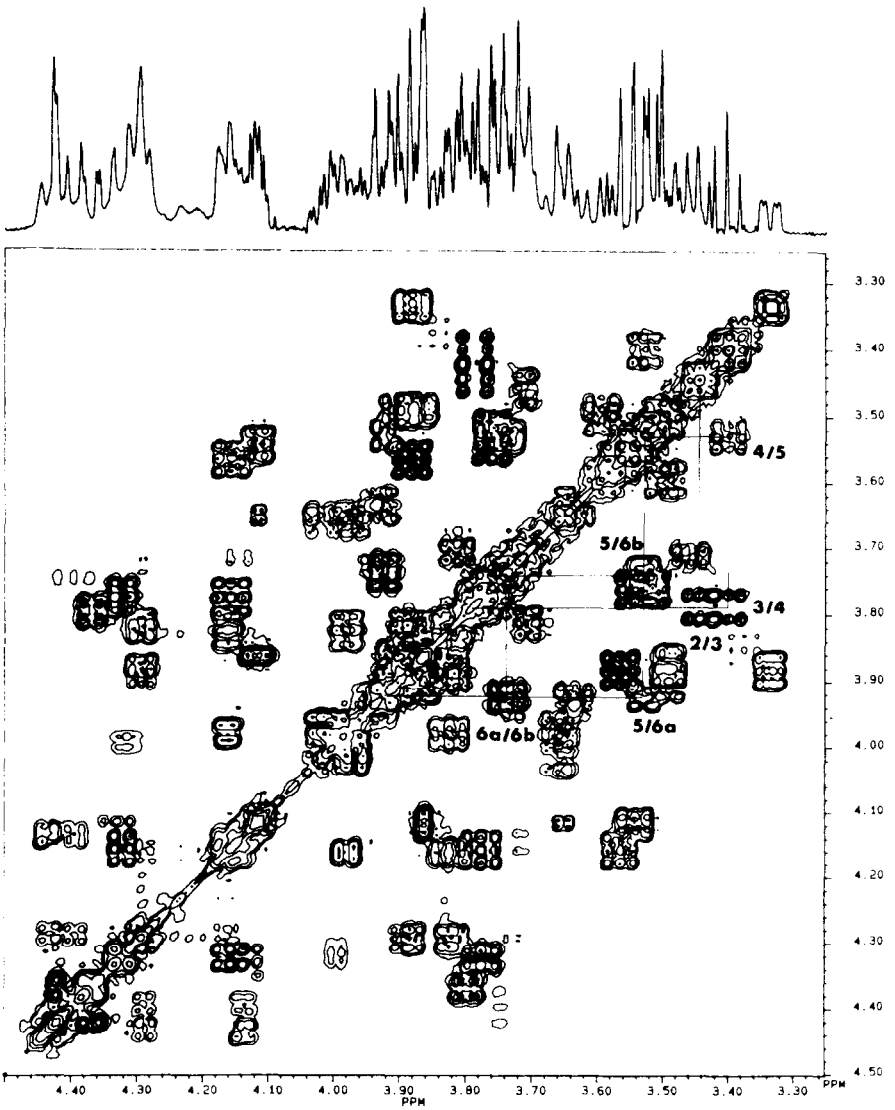


Fig. 2. Part of a 2D COSY spectrum of oligosaccharide **1**. The corresponding part of the 1D ^{1}H NMR spectrum is displayed along the horizontal (f_2) axis. Tracing connectivities for H-2–H-6 Glc^{I} is shown as an example.

of GlcN^{II}, was not clearly observed because of coincidence of the signals for H-4,5,6a of GlcN^{II}.

In accordance with the presence of 4 mol of phosphate, the ^{31}P NMR spectrum of **1** contained signals for four monophosphate groups in the region δ 2.3–5.1 (Fig. 3). The 2D inverse mode H-detected heteronuclear ^{1}H–^{31}P correlation spectrum of **1** (Fig. 3) contained the cross-peaks H/P at the following δ_H/δ_P: 5.63/2.45 and 3.34/2.45 for

Table 1
500-MHz ^{1}H NMR data for oligosaccharide **1** in D_2O at pD 7 (δ in ppm, J in Hz)

H-1	H-2	H-3eq H-3ax	H-4	H-5	H-6a H-6b	H-7a H-7b	H-8a H-8b
β-D-*Glc*p*N*^{III}-(*1*→							
4.76	3.14 [a]	3.50	3.60	3.49	3.92 3.87		
$J_{1,2}$ 8	$J_{2,3}$ 10	$J_{3,4}$ 10	$J_{4,5}$ 9	$J_{5,6a}$ 3	$J_{5,6b}$ 4 $J_{6a,6b}$ 12		
α-D-*Glc*p^{II}-(*1*→							
5.04	3.52	3.76	3.55	4.12	3.86		
$J_{1,2}$ 3.5	$J_{2,3}$ 10	$J_{3,4}$ 9.5	$J_{4,5}$ 9.5	$J_{5,6}$ 6			
→*2*)-*β*-D-*Glc*p^{I}-(*1*→							
4.75	3.45	3.79	3.40	3.53	3.93 3.73		
$J_{1,2}$ 8	$J_{2,3}$ 9	$J_{3,4}$ 9.5	$J_{4,5}$ 9.5	$J_{5,6a}$ 2	$J_{5,6b}$ 8 $J_{6a,6b}$ 12.5		
→*3*)-*α*-D-*Gal*p*N*-(*1*→ *4* ↑							
5.56	3.80	4.37	4.42	4.29	3.88 3.82		
$J_{1,2}$ 3	$J_{2,3}$ 11	$J_{3,4}$ 2.5	$J_{4,5}$ < 1	$J_{5,6a}$ 7.5	$J_{5,6b}$ 7.5 $J_{6a,6b}$ 12.5		
→*3*)-*α*-*Hep*p^{II}-(*1*→							
5.18	4.29	4.31	4.00	~ 4.0	3.97	4.03 3.66	
$J_{1,2}$ 3.5					$J_{6,7a}$ 3 $J_{6,7b}$ 8.5	$J_{7a,7b}$ 11.5	
→*3*)-*α*-*Hep*p^{I}-(*1*→							
5.41	4.44	4.14	4.42	4.29	4.17	3.99 3.83	
$J_{1,2}$ < 1	$J_{2,3}$ 3 $J_{2,P}$ 10	$J_{3,4}$ 10	$J_{4,5}$ 10 $J_{4,P}$ 10	$J_{5,6}$ < 1	$J_{6,7a}$ 2.5	$J_{6,7b}$ 10 $J_{7a,7b}$ 12	
α-*Kdo*^{II}-(*2*→							
		2.08 (eq) 1.78 (ax)	4.32	4.12	3.65	4.02	
		$J_{3eq,4}$ 5 $J_{3ax,4}$ 12 $J_{3eq,3ax}$ 12.5	$J_{4,5}$ 3	$J_{5,6}$ < 1	$J_{6,7}$ 9		
→*5*)-*α*-*Kdo*^{I}-(*2*→ *4* ↑							
		2.25 (eq) 1.97 (ax)	4.22	4.29	3.73	3.94	3.64
		$J_{3eq,4}$ 4.5 $J_{3ax,4}$ 12 $J_{3eq,3ax}$ 12.5	$J_{4,5}$ 3	$J_{5,6}$ < 1	$J_{6,7}$ 9		
→*6*)-*β*-D-*Glc*p*N*^{II}-(*1*→							
4.82	3.02	3.81	3.70	3.71	3.71 3.46		
$J_{1,2}$ 8.5	$J_{2,3}$ 10	$J_{3,4}$ 9					

Table 1 (continued)

H-1	H-2	H-3eq H-3ax	H-4	H-5	H-6a H-6b	H-7a H-7b	H-8a H-8b
→ 6)-α-D-*Glc*p*N*^{I}-(1 →							
5.63	3.34	3.89	3.57	4.16	4.33 3.77		
$J_{1,2}$ 3.5 $J_{1,P}$ 7	$J_{2,3}$ 10 $J_{2,P}$ 1	$J_{3,4}$ 10	$J_{4,5}$ 10	$J_{5,6a}$ 2	$J_{5,6b}$ 10 $J_{6a,6b}$ 12		

[a] The H-1/H-2 and H-2/H-3 correlations appeared in the COSY spectrum at pD 4 but, because of broadening of the signal for H-2, not at pD 7.

H-1/P-1 and H-2/P-1 of GlcN^{I}, respectively; 3.69/4.3 for H-4/P-4 of GlcN^{II}; 4.44/2.3 and 4.42/5.1 for H-2/P-2 and H-4/P-4 of Hep^{I}, respectively. These data allowed the substitution pattern of the phosphate groups to be determined unambiguously.

Linkage and sequence analysis of **1** was performed using rotating frame NOE spectroscopy (ROESY, Fig. 4). As expected, H-1 GlcN^{I} exhibited no interresidue correlation, and H-1 GlcN^{II} gave a cross-peak with H-6b GlcN^{I}, thus confirming that these two sugar residues build up the lipid A backbone. The presence of a cross-peak H-1 GlcN^{III}/H-2 Glc^{I} indicated the (1 → 2) linkage between these monosaccharide units. The mode of substitution of Glc^{I} was confirmed by the low-field shift of the signal for C-2 Glc^{I} to δ 84.2 in the ^{13}C NMR spectrum of **1**, which is caused by glycosylation by a β-pyranose at position 2 [12].

Both H-1 Glc^{I} and H-1 Glc^{II} correlate with H-3 and H-4 GalN, the cross-peaks H-1 Glc^{I}/H-3 and H-1 Glc^{II}/H-4 being much stronger than the other two; the minor

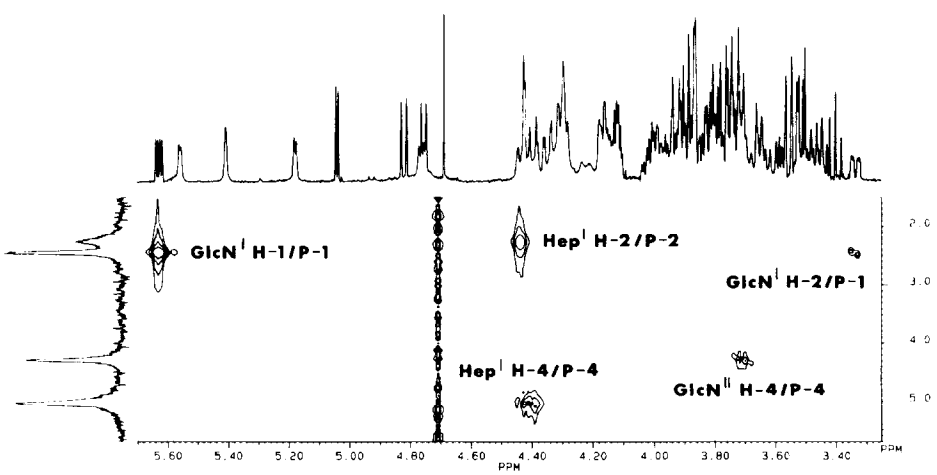


Fig. 3. Two-dimensional ^{1}H–^{31}P HMQC spectrum of oligosaccharide **1**. The corresponding parts of the 1D ^{31}P NMR spectrum and the 1D ^{1}H NMR spectrum are displayed along the vertical (f_1) and horizontal (f_2) axes, respectively.

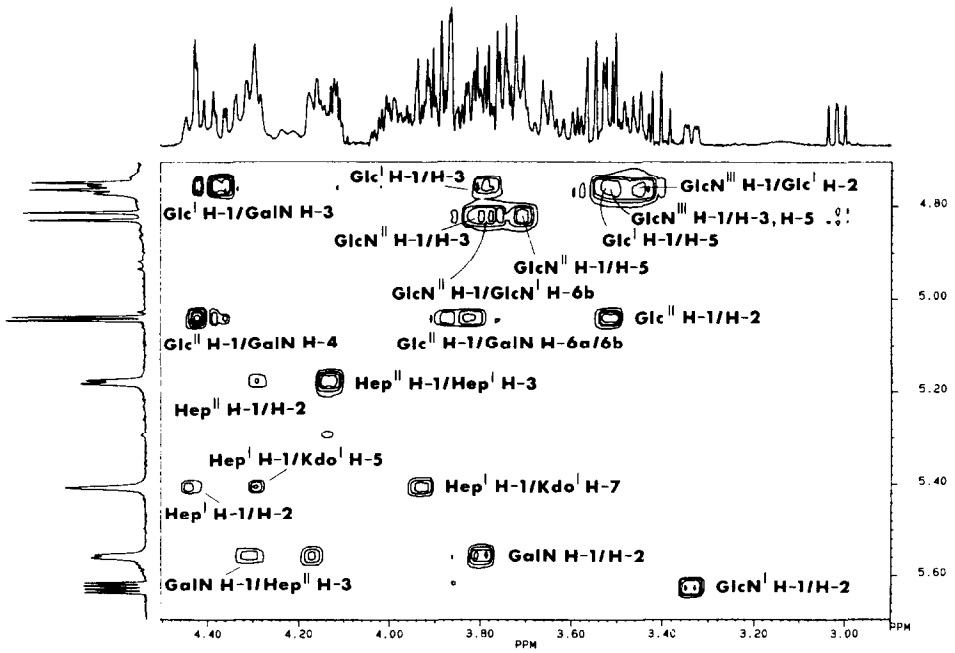
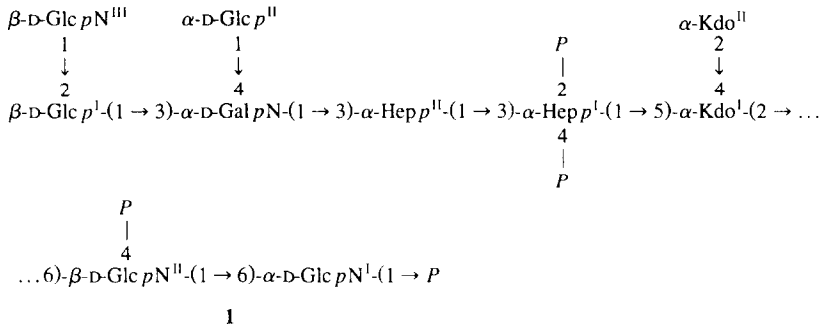


Fig. 4. Part of a 2D ROESY spectrum of oligosaccharide **1**. The corresponding parts of the 1D ^{1}H NMR spectrum are displayed along the axes.

cross-peaks are probably due to spin diffusion. That Glc^{II} substitutes GalN at position 4 is confirmed by correlation of H-1 Glc^{II} with H-6 GalN, which is typical of β-(1 → 4)-linked disaccharides [13], while correlation H-1 Glc^{II}/H-4 GalN would not be observed in a (1 → 6)-linked disaccharide fragment. Therefore, Glc^{I} and Glc^{II} are attached at positions 3 and 4 of GalN, respectively. Correlations H-1 GalN/H-3 Hep^{II} and H-1 Hep^{II}/H-3 Hep^{I} suggested the linear sequence and the (1 → 3) linkages between the three sugar residues. The presence of a cross-peak for H-1 GalN and another proton (at δ/δ 5.56/4.17), which may belong to H-6 Hep^{I}, most probably reflects the spatial proximity of the protons belonging to the non-bonded sugar units.

Two interresidue correlations with H-5 Kdo^{I} and H-7 Kdo^{I} are exhibited by H-1 Hep^{I}. However, correlation between two other protons of these monosaccharide units, namely, H-3ax Kdo^{I} and H-5 Hep^{I}, which is characteristic of these monosaccharides connected by the α-(1 → 5) linkage [14], allowed an unambiguous choice between the two possible modes of substitution of Kdo^{I}. The connectivity of H-6 Kdo^{II} and H-3eq Kdo^{I} proved the α-(2 → 4) linkage between the two Kdo residues [11]. Finally, the site of attachment of Kdo^{I} followed from the chemical shift (δ 64.0) for C-6 GlcN^{II} characteristic of GlcN phosphorylated at position 4 and substituted by Kdo at position 6 [6,12].

Therefore, on the basis of these data, it was concluded that the oligosaccharide **1** is a decasaccharide tetraphosphate having the following structure:



According to sugar and phosphate analysis and NMR data, the oligosaccharide **2**, eluted from Sephadex G-50 before the oligosaccharide **1**, is a tridecasaccharide tetraphosphate which differs from the decasaccharide **1** in the presence of residues of 2-amino-2,6-dideoxy-D-glucose (quinovosamine, QuiN), 2-amino-2,6-dideoxy-L-galactose (fucosamine, FucN), and 5,7-diamino-3,5,7,9-tetradeoxy-D-*glycero*-L-*galacto*-nonulosonic acid (Non), the last sugar representing the *N,O*-deacylated repeating unit of the O-antigen [7].

Although the ^{1}H NMR spectrum of **2** was not sufficiently resolved at either pD 4 or 7 for the complete assignment to be performed (probably because of the large number of free amino groups), the spin systems of the three additional sugars were recognized. The signals for three methyl groups (all d, J 6 Hz) were at δ 1.27 (H-6 FucN) and 1.39 (H-6 QuiN and H-9 Non), and the signals for the methylene group (H-3 Non) at δ 1.84 (t, $J_{3ax,3eq} \approx J_{3ax,4}$ 12 Hz, H-3ax) and 2.76 (dd, $J_{3eq,4}$ 4 Hz, H-3eq). The relatively large difference between the chemical shifts for H-3eq and H-3ax (0.92 ppm) is diagnostic of the axial orientation of the carboxyl group [6,10] and, therefore, as in the O-specific polysaccharide [4], Non is α-linked. The signals for two new anomeric protons appeared at δ 5.00 (d, $J_{1,2}$ 8.5 Hz, H-1 QuiN) and 5.49 (d, $J_{1,2}$ 3.5 Hz, H-1 FucN), and a significant shift from δ 4.76 in the spectrum of **1** to δ 5.09 in the spectrum of **2** was observed for the signal of H-1 GlcN^{III}, while the positions of the other signals changed by not more than 0.12 ppm. The coupling constant values $J_{1,2}$ 8.5 and 3.5 Hz showed that QuiN is β-linked and FucN α-linked.

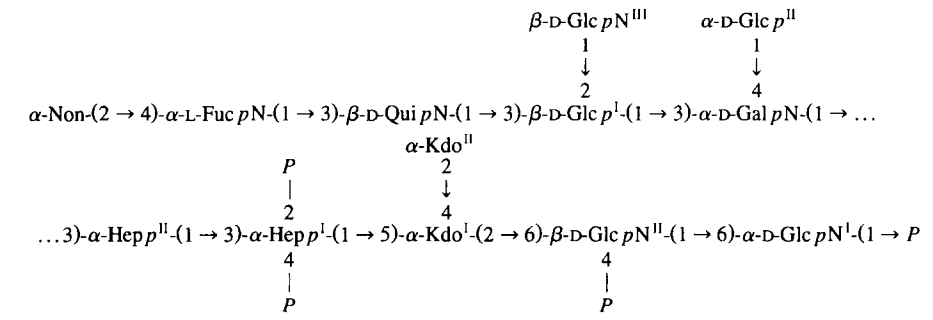
The ^{31}P NMR spectrum of **2** contained signals for four monophosphate groups at δ 0.87, 2.17, 3.67, and 3.91 which gave, in the 2D heteronuclear ^{1}H–^{31}P correlation spectrum, the same cross-peaks H/P as were observed in the spectrum of **1**. Therefore, in **2** the four phosphate groups occupy the same positions as in **1**.

The patterns of the correlation peaks in the ROESY spectra of **1** and the corresponding decasaccharide fragment of **2** were also similar, except that the cross-peak H-1 GlcN^{III}/H-2 Glc^I shifted from δ/δ 4.76/3.45 in **1** to 5.09/3.79 in **2**. The correlation peaks H-1 QuiN/H-3 Glc^I at δ/δ 5.00/4.18 and H-1 FucN/H-3 QuiN at δ/δ 5.49/3.87 indicated that QuiN is attached at position 3 of Glc^I and is itself substituted at position 3 by FucN. Substitution of Glc^I at position 3 in **2** accounts for the above-mentioned displacement of the cross-peak H-1 GlcN^{III}/H-2 Glc^I.

A relatively low-field position at δ 4.39 of the signal H-4 FucN in the ^{1}H NMR spectrum of **2**, compared with its position in the spectrum of the corresponding unsubstituted monosaccharide (e.g., at δ 3.83 [15]), allowed the suggestion that position

4 of FucN is the site of attachment of Non. This suggestion, in particular, and the presence of a trisaccharide fragment α-Non*p*-(2 → 4)-α-L-Fuc*p*N-(1 → 3)-β-D-Qui*p*N-(1 → attached at position 3 of Glc^{I}, in general, were confirmed independently by elucidation of the structure of the core oligosaccharide obtained by mild acid hydrolysis of the *P. fluorescens* ATCC 49271 LPS [16].

Therefore, the oligosaccharide **2** has the following structure:



2

The decasaccharide tetraphosphate **1** corresponds to an incomplete core of the LPS, and the tridecasaccharide tetraphosphate **2** to the complete core with one repeating unit of the O-specific polysaccharide (Non) attached. Both oligosaccharides are linked to the lipid A backbone and lack *N*- and *O*-acyl substituents, including *N*- and *O*-acetyl groups, the 5-*N*-acetimidoyl group of Non, the 2-*N*-alanyl group of GalN, and the 7-*O*-carbamoyl group of Hep^{II}, as well as diphosphate, triphosphate, and, probably, some of the monophosphate groups that are present in the intact LPS [16]. The O-specific polysaccharide chain appeared to be significantly destroyed during the strong alkaline degradation of LPS.

The structure of a nonasaccharide fragment of **1** (without $GlcN^{III}$) is shared with the *P. aeruginosa* LPS [17,18], while, except for the Kdo region, no similarity is observed between the core structures of *P. fluorescens* ATCC 49271 and *L. pneumophila* serogroup 1 [5]. Interestingly, according to our unpublished data, the structure of lipid A of *P. fluorescens* ATCC 49271 also much resembles that of *P. aeruginosa* [19].

3. Experimental

Chromatography.—GPC was performed on a column (45 cm × 2.4 cm) of Sephadex G-50 (superfine) using a pyridine–acetate buffer (pH 4.5) with monitoring by a Knauer differential refractometer. Anion-exchange HPLC was carried out on a column (25 cm × 0.4 cm) of CarboPac PA1 (Dionex, USA) in a gradient of NaOAc (0.3 → 0.7 M) in 0.1 M NaOH and monitored by a Hewlett–Packard pulse amperometric detector. GLC was performed with a Hewlett–Packard Model 5890 chromatograph equipped with a capillary column (30 m × 0.25 mm) of cross-linked SPB™-5 as stationary phase.

NMR spectroscopy.—The NMR spectra were recorded with a Bruker AM-360 (1D) and a Bruker AM-500 (1D and 2D) spectrometer for solutions in D_2O at pD 7 at 33°C

with acetone (δ_H 2.225, δ_C 31.45) as internal standard or aq 80% H_3PO_4 (δ_P 0) as external standard.

In 2D COSY the time-domain data matrix (2 K × 512 points) was multiplied before the magnitude-mode Fourier transformation by the sine-bell function, zero-filled in t_1 and the resulting spectra were subjected to symmetrization. 2D ROESY was performed in the phase-sensitive mode using the method of time-proportional phase incrementation [20] and the pulse sequence proposed by Rance [21]; the time-domain data matrix (2 K × 256 points) was zero-filled, multiplied by a squared cosine-bell function, and Fourier transformed. The 2D H-detected non-decoupled heteronuclear $^1H-^{13}C$ correlation spectrum was obtained via heteronuclear zero and double coherence using the inverse mode [22]; the matrix (2 K × 128 points) was zero-filled in t_1, multiplied by a squared cosine-bell function, and transformed in the magnitude mode. The 2D H-detected heteronuclear $^1H-^{31}P$ correlation spectrum was obtained using the Wagner preparatory sequence for suppression of unwanted signals; the matrix (4 K × 256 points) was zero-filled in t_1, multiplied by a sine-bell function shifted by $\pi/2$ in t_1 and $\pi/4$ in t_2, and transformed in the magnitude mode.

Chemical analysis.—Neutral and amino sugars were analyzed by GLC as acetylated methyl glycosides obtained after dephosphorylation with 48% HF (4°C, 24 h) and methanolysis with 2 M HCl in MeOH (120°C, 16 h) followed by acetylation. Amino sugars were analyzed conventionally after hydrolysis of the sample (4 M HCl, 100°C, 5 h) using an LKB Alpha plus 4151 amino acid analyzer. Phosphate was determined by the method of Lowry et al. [23], and Kdo by the TBA reaction according to the modified method [24]. Absolute configurations of monosaccharides were determined by GLC of acetylated (+)-2-butyl glycosides [25] or (+)-2-octyl glycosides [26].

Bacterial strain and isolation of LPS.—Strain *P. fluorescens* ATCC 49271 was grown at pH 7.1 and 25°C for 17 h, treated with Proteinase K (30 min), then with aq 1% phenol, and cells were removed by ultracentrifugation (35,000 rpm).

Wet cells from two batches (371 g) were digested with DNase and RNase (37°C, 18 h) and then with Proteinase K (37°C, 12 h), dialyzed against distilled water for 54 h, and freeze-dried. LPS was isolated from the dried digested cells (50.5 g) by a modified phenol–$CHCl_3$–petroleum ether method as described previously [5,6]; the yield was 2% (w/w) of the wet cell weight.

O-*Deacylation and alkaline degradation of LPS.*—LPS (700 mg) was treated with anhyd hydrazine (20 mL, 2 h at 20°C, then 1 h at 37°C), the solution was poured into cold acetone, and ether was added for better precipitation of LPS. The precipitate was separated by centrifugation, washed twice with ether, and dried in air to give *O*-deacylated LPS (605 mg).

O-Deacylated LPS (600 mg) was dissolved in 8 M KOH (4 mL), flushed with N_2 with stirring (1 h), and heated in a sealed tube (100°C, 24 h). The solution was cooled, neutralized with 8 M HCl, and extracted with CH_2Cl_2 (2 × 3 mL), and the aqueous layer was fractionated on Sephadex G-50. The oligosaccharide products (the second fraction, 43 mg; and the third fraction, 23 mg) were separately purified by HPLC on CarboPac PA1 and desalted using a column (5 cm × 1.8 cm) of AG 50-X4 (H^+) resin which was washed first with water and then with aq 5% ammonia [27] to give **2** (12.5 mg) and **1** (7.7 mg), recovered from the aq ammonia and water eluate, respectively.

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