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Structure of a decasaccharide isolated by mild acid degradation and dephosphorylation of the lipopolysaccharide of *Pseudomonas fluorescens* strain ATCC 49271

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Abstract

Mild acid degradation of the *Pseudomonas fluorescens* strain ATCC 49271 lipopolysaccharide resulted in a core oligosaccharide containing D-glucose, 2-acetamido-2-deoxy-D-glucose, 2-(L-alanylamino)-2-deoxy-D-galactose, 2-acetamido-2,6-dideoxy-D-glucose (QuiNAc), 2-acetamido-2,6-dideoxy-L-galactose (FucNAc), L-*glycero*-D-*manno*-heptose (Hep), 3-deoxy-D-*manno*-octulosonic acid (Kdo, present in multiple forms), and 5-acetamidino-7-acetamido-3,5,7,9-tetradeoxy-L-*glycero*-D-*galacto*-nonulosonic acid (a di-*N*-acyl derivative of legionaminic acid, Non) as well as *O*-acetyl, *O*-carbamoyl, and phosphate groups, including triphosphate groups. The dephosphorylated (HF) decasaccharide and products of its partial and full *O*-deacylation were studied by methylation analysis, GLC–MS, and ^{1}H NMR spectroscopy, including 1D NOE and 2D shift-correlated spectroscopy (COSY). The core oligosaccharide of *P. fluorescens* strain ATCC 49271 was found to be a decasaccharide (with partially degraded Kdo region) and one O-antigen repeating unit (di-*N*-acyllegionaminic acid, Non) attached. The following structure of the dephosphorylated core oligosaccharide was established:

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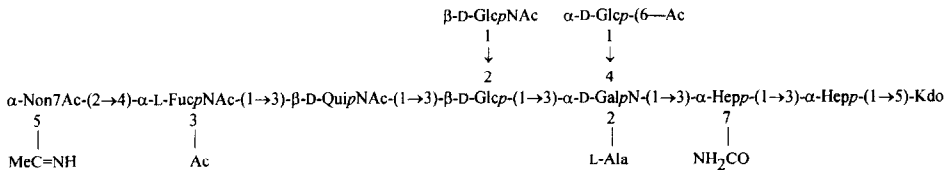


Keywords: Lipopolysaccharide; Core oligosaccharide; *Pseudomonas*; NMR spectroscopy

1. Introduction

Recently, we have described the structural analysis of the core oligosaccharide in the lipopolysaccharide (LPS) of *Pseudomonas fluorescens* strain ATCC 49271 obtained after strong alkaline degradation, whereby two tetraphosphorylated oligosaccharides, a decasaccharide and a tridecasaccharide, were isolated and identified [1]. However, the procedure used for isolation of the core oligosaccharides had the disadvantage that some of the core substituents, such as *O*- and *N*-linked acetyl, *N*-acetimidoyl, *N*-alanyl, and phosphate groups (including pyrophosphate and triphosphate groups), as well as a recently described *O*-carbamoyl group [2], had been removed during the preparation of the core oligosaccharides. In order to determine the position of these groups as well as to complete the core structure of *P. fluorescens* strain ATCC 49271, we report here the structural elucidation of a decasaccharide which was obtained by mild acid hydrolysis of the *P. fluorescens* LPS and subsequent dephosphorylation, thus retaining all *O*- and *N*-linked acyl groups, including the carbamoyl residue.

2. Results and discussion

LPS was isolated as described in the preceding paper [1]. In order to cleave the lipid A moiety from the core oligosaccharides, LPS was degraded with 0.1 M sodium acetate buffer (pH 4.4, 100 °C, 1 h). The carbohydrate portion obtained after centrifugation was subjected to GPC on Sephadex G-50 which revealed a high molecular weight fraction 1 (O-specific polysaccharide) and two oligosaccharide fractions 2 and 3. The major oligosaccharide of fraction 2 (OS) was purified by repeated GPC under the same chromatographic conditions and further analyzed in detail.

The O-specific polysaccharide (fraction 1) was identified as a homopolymer of 5-acetamidino-7-acetamido-3,5,7,9-tetradeoxynonulosonic acid (legionaminic acid [3]) partially (ca. 75%) *O*-acetylated at position 8.

Fraction 2 (OS) was found to be highly phosphorylated (1420 nmol/mg LPS). The ^{31}P NMR spectrum of OS, recorded at pD 4 (Fig. 1), contained mainly signals for monophosphate groups at 1–5 ppm and a triphosphate group at −9.0, −10.7 (*P*-P-*P*), and −21.5 (P-*P*-P) ppm which was first identified in *P. aeruginosa* LPS [4]; signals of low intensity in the region between −4 and −11 ppm may be due to the presence of

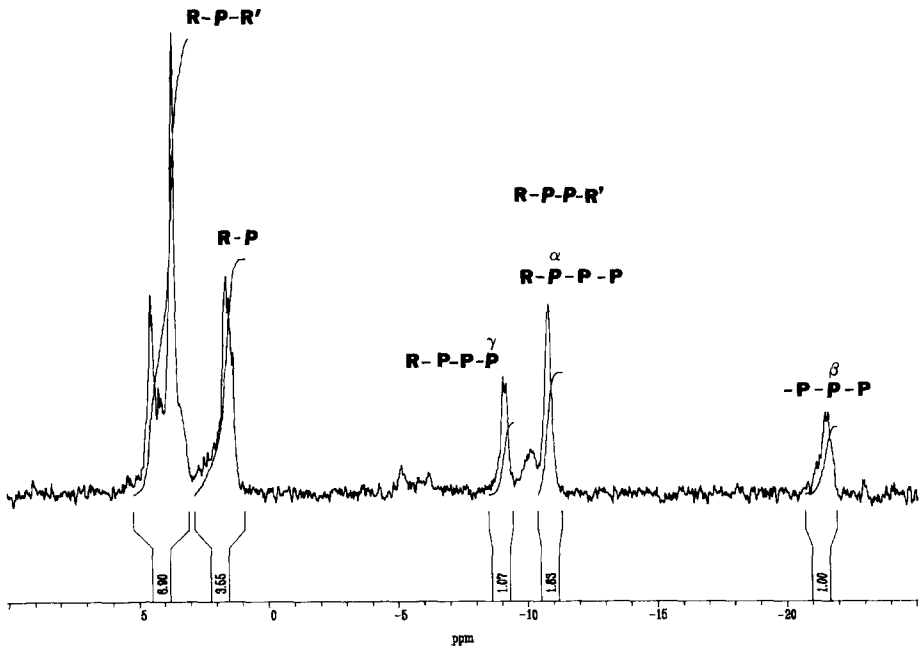


Fig. 1. 145.8-MHz ^{31}P NMR spectrum of the core oligosaccharide (OS) recorded at pD 4.

minor pyrophosphate groups. As judged by the ratio of integral intensities of the main signals, the ratio of monophosphate groups to the triphosphate group was 9.5:1.

OS was dephosphorylated with aq 48% hydrofluoric acid to give OS_{HF}. Sugar analysis of OS_{HF} performed by GLC of acetylated methyl glycosides revealed the presence of glucose, L-*glycero*-D-*manno*-heptose, 2-amino-2-deoxyhexoses, 2-amino-2,6-dideoxyhexoses, and 3-deoxy-D-*manno*-octulosonic acid (Kdo). Acid hydrolysis of OS_{HF} followed by analysis on an amino acid analyzer resulted in identification of almost equal amounts of L-alanine, D-GlcN, D-GalN, 2-amino-2,6-dideoxy-D-glucose (D-quinovosamine, D-QuiN), and 2-amino-2,6-dideoxy-L-galactose (L-fucosamine, L-FucN), the absolute configurations of the monosaccharides being proved by GLC of acetylated (*R*)-2-butyl glycosides [5] or (*R*)-2-octyl glycosides [6].

Mild methanolysis of OS_{HF} (0.5 M HCl/MeOH, 80 °C) gave, in addition to the expected methyl glycosides, two sugar acyl derivatives, namely, GalN(Ala)–OMe and 7-*O*-carbamoyl-L-*glycero*-D-*manno*-heptose (Hep7Cm), which were identified by GLC–MS of the fully acetylated derivatives by comparison with authentic samples obtained from LPS of *Pseudomonas aeruginosa* PAC 605. The structure of the latter compound was additionally proven by GLC–MS analysis of the fully *N,O*-methylated derivative as described [2]. 2-(L-Alanylamino)-2-deoxy-D-galactose [7,8] and 7-*O*-carbamoyl-L-*glycero*-D-*manno*-heptose [2] have been previously identified in LPSs of a number of *P. aeruginosa* rough mutant strains, and thus are components of *P. fluorescens* LPS as well.

In addition to the monosaccharide derivatives, mild methanolysis resulted in methyl

glycosides of a number of disaccharides and trisaccharides, which were identified by GLC–MS (in both chemical-ionization and electron-impact mode) as either acetylated or methylated derivatives, using the published data on the fragmentation routes [9]. These were HexN → Hex-OMe, Hex → HexN(Ala)-OMe, Hep → Hep-OMe, Hep7Cm → Hep-OMe, Hep → Kdo-OMe, Hep → Hep → Kdo-OMe, and Hep7Cm → Hep → Kdo-OMe. These data provided information about the partial sequence of the monosaccharide units in the core and, in particular, showed the presence of a fragment Hep^{II} → Hep^{I} → Kdo with the carbamoyl group attached to Hep^{II}.

Methylation [10] of borohydride-reduced OS_{HF} followed by hydrolysis under the conditions of Stellner et al. [11], and GLC–MS analysis of the derived alditol acetates resulted in the peracetates of 2,3,4,6-Me_4-Glc-ol, 4,6-Me_2-Glc-ol, 2,4,6,7-Me_4-Hep-ol, 2,4,6-Me_3-Hep-ol, 2,4,6-Me_3-Hep7-Me_2Cm-ol, 4-Me-QuiNAcMe-ol, 3-Me-FucNAcMe-ol, 3,4,6-Me_3-GlcNAcMe-ol, and 6-Me-GalNAcMe-ol.

These data revealed the presence of two side chains terminated with Glc and GlcNAc and attached to 2,3-disubstituted Glc and 3,4-disubstituted GalNAc, while QuiNAc and FucNAc are monosubstituted at position 3 and 4, respectively. Both Hep and Hep7Cm are substituted at position 3. Since the amount of 2,4,6,7-Me_4-Hep was found to be about twice as much as the total amount of 2,4,6-Me_3-Hep7-Me_2Cm and 2,4,6-Me_3-Hep, this fact may be accounted for by the partial loss of the *N*,*N*-dimethylcarbamoyl group during methylation analysis of OS_{HF}.

In addition to the partially methylated monosaccharides, a disaccharide-alditol derivative Ac_2Me-HexNAcMe → Ac_2Me_4-Hep-ol was identified, which appeared to be derived from the disaccharide fragment → 3,4)-GalN-(1 → 3)-Hep7Cm after loss of the carbamoyl group. Its identification further contributes to the determination of the monosaccharide sequence in the core.

The ^{1}H NMR spectrum indicated that OS_{HF} is a mixture of related oligosaccharides containing a different number of *O*-acetyl groups (signals at 2.05–2.15 ppm). It was separated by HPLC on reversed-phase C18 to give a di-*O*-acetylated oligosaccharide **1** and a mono-*O*-acetylated oligosaccharide **2**. Treatment of OS_{HF} with aq 12% ammonia resulted in an *O*-deacetylated oligosaccharide **3**. When *O*-deacetylation preceded dephosphorylation, a mixture of **3** with another *O*-deacetylated oligosaccharide **4** was obtained and separated by HPLC on reversed-phase C18. Compound **4** is suggested to be a lactone form of **3** (see below).

The ^{1}H NMR spectra showed that oligosaccharides **1–4** are still heterogeneous because of the occurrence of Kdo at the reducing end in multiple forms. These resulted from the cleavage of its glycosidic linkage and further transformations of the Kdo residue to lactone and anhydro derivatives known to occur during mild acid treatment [12]. This suggestion follows from the splitting within a wide range of the signals for H-1 (4.96–5.13 and 5.40–5.41 ppm) and H-2 (3.94–4.17 ppm) of a heptose residue (Hep^{I}) attached to Kdo and, to a lesser extent, of the signal for H-1 (5.16–5.21 ppm) of the second heptose residue (Hep^{II}) attached to Hep^{I}, which is clearly observed in the 2D COSY spectra (Fig. 2). Only one H-1 signal was present for each of the other monosaccharides, which are remote from the reducing Kdo.

The ^{1}H NMR spectrum of **3** was partially assigned using 2D COSY and H,H-relayed COSY (Table 1). The amino sugars were distinguished by correlation of the protons at

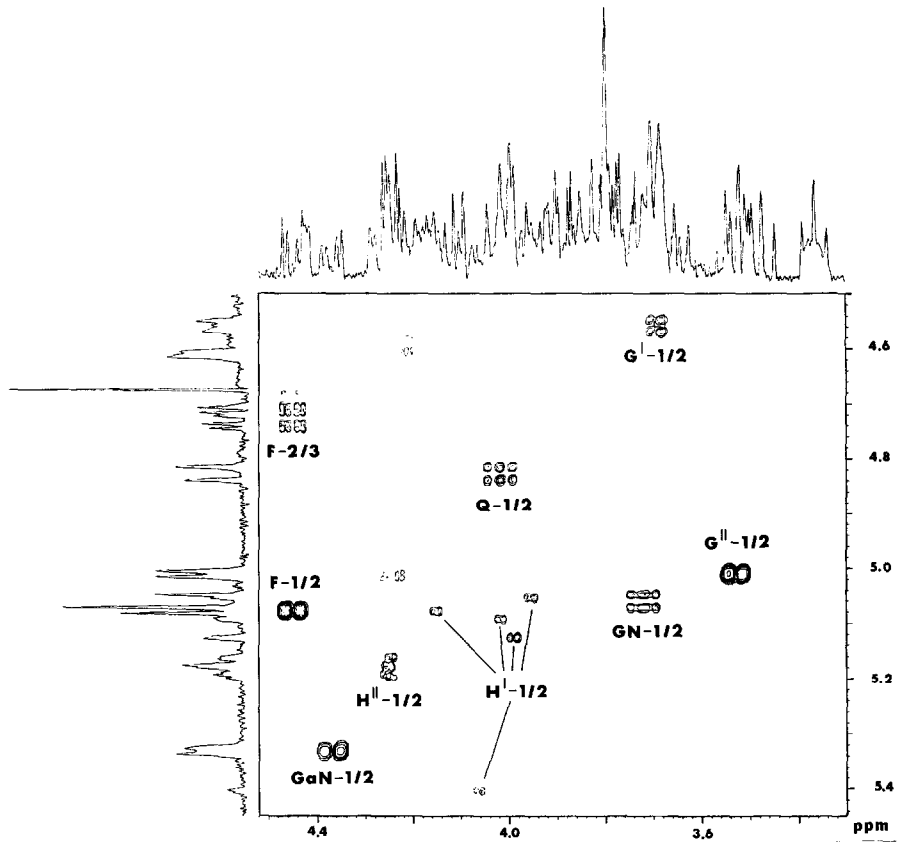


Fig. 2. Part of a 2D COSY spectrum of oligosaccharide **4**. The corresponding parts of the 1D ^{1}H NMR spectrum are displayed along the axes.

the carbons bearing nitrogen to the corresponding carbons in the region 50–58 ppm, as determined from the 2D heteronuclear ^{13}C,^{1}H COSY spectrum. Based on these data and the coupling constant values, it was suggested that **3** contains two residues of glucose [one β-linked (Glc^{I}) and the other α-linked (Glc^{II}), $\delta_{H\text{-}1}$ 4.56 and 5.00, $J_{1,2}$ 7 and 3.5 Hz, respectively], two α-Hep ($J_{1,2}$ ~ 2 Hz for both), β-GlcNAc ($\delta_{H\text{-}1}$ 5.13, $J_{1,2}$ 8.5 Hz; $\delta_{C\text{-}2}$ 56.9), β-QuiNAc ($\delta_{H\text{-}1}$ 4.80, $J_{1,2}$ 8.5 Hz; $\delta_{H\text{-}6}$ 1.40, $J_{5,6}$ 6 Hz; $\delta_{C\text{-}2}$ 57.2; $\delta_{C\text{-}6}$ 18.9), α-GalNAc ($\delta_{H\text{-}1}$ 5.27, $J_{1,2}$ 3.5 Hz; $\delta_{C\text{-}2}$ 51.2), and α-FucNAc ($\delta_{H\text{-}1}$ 5.02, $J_{1,2}$ 4 Hz; $\delta_{H\text{-}6}$ 1.24, $J_{5,6}$ 6.5 Hz; $\delta_{C\text{-}2}$ 50.5; $\delta_{C\text{-}6}$ 17.7). In addition, there is present a residue of 5-acetimidino-7-acetamido-3,5,7,9-tetradeoxy-L-*glycero*-D-*galacto*-nonulosonic acid (di-*N*-acyl derivative of nonulosonic acid, Non) with the axial carboxyl group (i.e., an α-linked residue, characteristic signals for H-3ax, H-3eq, and H-9 at 1.82, 2.80, and 1.15 ppm, respectively, $J_{3ax,3eq} \approx J_{3ax,4} \approx 12$, $J_{3eq,4}$ 4, $J_{8,9}$ 6.5 Hz; C-1,3,5,7,9 at 172.0, 41.0, 53.2, 55.1, and 19.5 ppm, respectively, cf. the published data [3]). This higher sugar represents an *O*-deacetylated repeating unit of the O-specific polysaccharide [13,14] attached to the core. Also, signals for an *N*-alanyl group ($\delta_{H\text{-}3}$ 1.40, $J_{2,3}$ 7 Hz; $\delta_{C\text{-}2}$

Table 1
360-MHz ^{1}H NMR chemical shifts for oligosaccharide **3** in D_2O at pD 4 (δ in ppm) [a]

H-1	H-2	H-3	H-4	H-5	H-6a H-6b	H-7	H-8	H-9
α-Non-(2 →								
		1.82 ax 2.80 eq	3.64	3.69	3.83	3.83	3.90	1.15
→ 4)-α-L-Fuc *p*N-(1 →								
5.02	4.19	3.89	4.30	4.48	1.24			
→ 3)-β-D-Qui *p*N-(1 →								
4.80	4.00	3.65	3.33	3.53	1.40			
β-D-Glc *p*N-(1 →								
5.13	3.71	3.52	3.52	3.31	3.92 3.77			
α-D-Glc p^{II}-(1 →								
5.00	3.53	3.85	3.50	4.18	3.80			
→ 2)-β-D-Glc p^{I}-(1 → 3 ↑								
4.56	3.71	3.71	3.79	3.37	3.94 3.79			
→ 3)-α-D-Gal *p*N-(1 → 4 ↑								
5.27	4.43	4.25	4.41	4.26	3.86			
→ 3)-α-Hep p^{II}-(1 →								
5.16–5.21	4.25	3.98						

[a] Chemical shifts for the *N*-acetyl groups: 1.95, 1.97, 2.00, 2.06, and 2.08 ppm; for the *N*-alanyl group: 1.40 (H-3) and 3.68 ppm (H-2).

51.2; $\delta_{C\text{-}3}$ 18.9) could be identified, whereas signals for Kdo at the reducing end could not be clearly recognized because of the occurrence of this residue in multiple forms [12].

The ^{1}H NMR spectra of **1** and **2** contained signals for an *N*-acetimidoyl group (δ_H 2.19–2.20), four *N*-acetyl groups (δ_H 1.9–2.1 ppm), and two and one *O*-acetyl groups (δ_H 2.05–2.15 ppm), respectively. Signals for the *N*-acetimidoyl group and the *O*-acetyl groups were absent from the spectra of **3** and **4**, but a signal for an additional, fifth *N*-acetyl group appeared. These data suggested that the *N*-acetimidoyl group, which, as in the O-specific polysaccharide [13,14], seems to be attached in the core oligosaccharide to Non, is smoothly converted into an *N*-acetyl group during *O*-deacetylation with aqueous ammonia. Such chemical behavior has been reported for this group in most studied oligosaccharides and polysaccharides [15–17] except for the homopolymer of legionaminic acid, where the complete conversion required much more drastic alkaline treatment [3].

The signal for H-3 of Ala in the spectrum of **3** was shifted from 1.40 ppm at pD 4 to 1.66 ppm at pD 7, thus indicating that the amino group of Ala is not acylated. Hence, the five acetyl groups of **3** are attached to the amino groups of the amino sugars (GlcN, QuiN, FucN, and Non), whose signals shifted only insignificantly on the change of pD.

In the ^{1}H NMR spectrum of **1** and **2**, the signal for H-3 of FucN was shifted downfield to 5.06 ppm compared with its position in the spectrum of **3** at 3.89 ppm. This displacement is caused by the deshielding effect of the *O*-acetyl group and pointed to the location of one of the *O*-acetyl groups at O-3 of FucN. A similar shift of the signal for H-6 of α-Glc from 3.80 ppm in **3** to 4.27 ppm in **1** showed that the second *O*-acetyl group is attached to O-6 of α-Glc. And, finally, the low-field position of the signal for H-3 of FucN at 4.73 ppm in **4**, which contains no *O*-acetyl group, allowed the suggestion that in this case the deshielding is caused by acylation of FucN at O-3 by the carboxyl group of the adjacent Non, resulting in formation of a tricyclic lactone. Formation of the 1,4-dioxane ring also influenced signals of Non (e.g., the signal for H-4 of Non shifted from 3.64 ppm in **3** to 4.25 ppm in **4** and the values of the coupling constants $J_{3ax,3eq}$, $J_{3ax,4}$, and $J_{3eq,4}$ changed from 12, 12, and 4 Hz in **3** to 13.5, 11, and 5.5 Hz in **4**, respectively).

On sequential, selective preirradiation of H-1 of the sugar units in **3**, the following interresidue NOEs were observed: H-1 GalN/H-3 Hep^{II}, H-1 Glc^{I}/H-3 GalN, H-1 Glc^{II}/H-4 GalN, H-1 GlcN/H-2 or H-3 Glc^{I}, H-1 QuiN/H-2 or H-3 Glc^{I} and H-3 Glc^{II}, H-1 FucN/H-3 QuiN. These data showed that two Glc residues are attached to GalN at positions 3 and 4, and GalN is linked to Hep^{II} at position 3. FucN is attached to QuiN at position 3 and is substituted by Non at position 4, as followed from the methylation analysis data (see above) and an NOE on H-4 of FucN appearing as a result of preirradiation of H-3ax of Non.

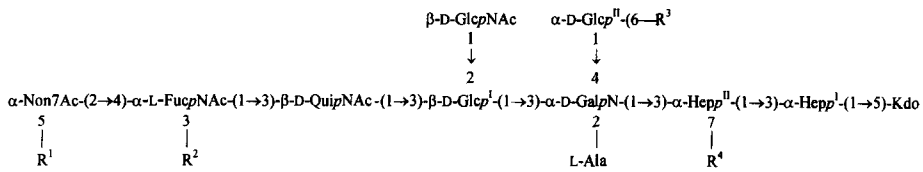
Attachment of GlcN to Glc^{I} showed that this glucose residue is located at the branching point (see the results of methylation analysis) and, hence, Glc^{II} is a lateral sugar residue and the NOE on H-3 of Glc^{II}, caused by preirradiation of H-1 of QuiN, is due to spatial proximity of the protons of the two nonbonded monosaccharide residues. However, it could not be determined whether GlcN is attached to Glc^{I} at position 2 and QuiN at position 3 or vice versa because of the coincidence of the signals for H-2 and H-3 of Glc^{I} in the spectrum. The conclusion in favor of the first variant was reached taking into account the presence of a disaccharide fragment β-D-Glc*p*N-(1 → 2)-β-D-Glc*p*^{I} in a decasaccharide tetraphosphate isolated by strong alkaline degradation of *O*-deacylated LPS of *P. fluorescens* ATCC 49271 [1]. Similarly, the presence in this decasaccharide of a fragment α-Hep*p*^{I}-(1 → 5)-α-Kdo proved substitution of the reducing Kdo in **3** at position 5.

The O-specific polysaccharide (fraction 1) was identified as partially 8-*O*-acetylated (ca. 75%) legionaminic acid [3]. An epitope associated with the *O*-acetylated units is shared with the O-antigen of *Legionella pneumophila* serogroup 1 and seems to be responsible for the serological cross-reactivity between the two microorganisms [13,14], which was observed with both polyclonal antisera [18] and monoclonal antibodies [19].

This nonulosonic acid and that found in the O-specific polysaccharide of the same strain *P. fluorescens* ATCC 49271 [13], which is an *N*- and *O*-acetylated homopolymer of this sugar, have the same relative configuration. It seems that the oligosaccharide

studied represents the core of the *P. fluorescens* lipopolysaccharide with one O-antigen repeating unit attached, and, thus, the two nonulosonic acids should have the same absolute configuration as well. The absolute configuration of the nonulosonic acid in the lipopolysaccharide of *P. fluorescens* [1,13], like that of the acid of *L. pneumophila* serogroup 1 [3], was assumed to be D-*glycero*-L-*galacto*, but should be revised in favor of the L-*glycero*-D-*galacto* configuration as has been demonstrated for the nonulosonic acid in the *Vibrio salmonicida* lipopolysaccharide [20]. The revision originates from the misassignment of the D-*threo* configuration to the fragment C-7–C-8 of the nonulosonic acid made from the correct observation that this fragment is homomorphic to D-threonine [16,21,22]. As a consequence the originally assigned $^{5}C_{2}$ chair conformation of the D-*glycero*-L-*galacto*-nonulosonic acid [3] has to be changed in favor of the $^{2}C_{5}$ chair conformation for the L-*glycero*-D-*galacto*-nonulosonic acid, thus being closely related to the 8-epimeric Neu5Ac (5-acetamido-3,5-dideoxy-D-*glycero*-D-*galacto*-nonulosonic acid). The absolute configuration of the nonulosonic acid should be correspondingly revised also in the O-specific polysaccharides studied before, namely that of *P. aeruginosa* [16], *Salmonella arizonae* [21], *Yersinia ruckeri* [22], and *Vibrio alginolyticus* [23].

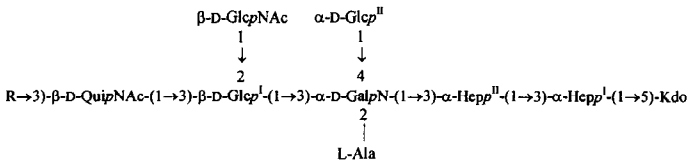
Therefore, on the basis of these data, it was concluded that oligosaccharides **1–4** have the following structures:



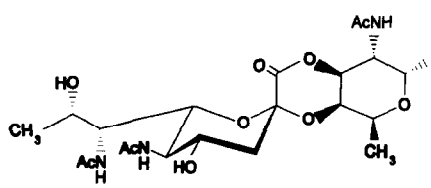
1 R^1 = MeC=NH, R^2 = R^3 = Ac, R^4 = NH_2CO

2 R^1 = MeC=NH, R^2 = Ac, R^3 = H, R^4 = NH_2CO

3 R^1 = Ac, R^2 = R^3 = R^4 = H



4 R =

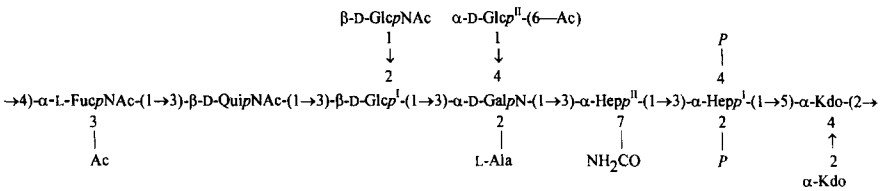


These structures fit in with the structure of the carbohydrate backbone of the core oligosaccharide of *P. fluorescens* ATCC 49271 LPS, which has been established previously [1].

Comparison of the ^{1}H NMR spectra of OS_{HF}, **1**, and **2** with the spectrum of OS showed that, unlike OS_{HF}, OS is homogeneous with respect to the *O*-acetylation pattern, and, hence, one of the *O*-acetyl groups, namely, that at position 6 of Glc^{II}, was partially lost during dephosphorylation of OS with aq 48% hydrofluoric acid.

Analysis of the ^{1}H NMR spectrum of OS using 2D COSY and H,H-relay COSY showed that the signals for all monosaccharide residues in the outer region of the core oligosaccharide (Glc^{I}, Glc^{II}, GlcNAc, GalNAc, QuiNAc, and FucNAc) as well as the signals for Non are essentially at the same positions as in the spectrum of the dephosphorylated oligosaccharide **3**, and, therefore, phosphate groups are attached to the inner core region containing Hep^{I}, Hep^{II}, and Kdo. Location of two of the monophosphate groups at positions 2 and 4 of Hep^{I} was determined earlier in a study of the oligosaccharides obtained by strong alkaline degradation of the *O*-deacylated LPS of *P. fluorescens* strain ATCC 49271 [1]. Attempts to find the exact sites of attachment of other monophosphate groups and the triphosphate group by detailed NMR analysis failed because of a high degree of heterogeneity in the inner core region, which is caused by the occurrence of Kdo in multiple forms and, probably, by nonstoichiometric phosphorylation resulting in the splitting of the signals and their low intensities.

Combining the data obtained by analysis of the products of mild acid degradation (this work) and strong alkaline degradation [1] of LPS, the following structure of the core oligosaccharide of *P. fluorescens* strain ATCC 49271 was established:



where some additional phosphate groups, including a triphosphate group, are attached to the Hep-Kdo region. Position 4 of FucNAc is the site of attachment of the O-specific polysaccharide in S-type LPS or one *O*-deacetylated O-antigen repeating unit in SR-type LPS; Kdo^{I} is linked to position 6 of $GlcN^{II}$ of the lipid A backbone.

The heptasaccharide region of the core of *P. fluorescens*, including the inner core GalN, Glc^{I}, and Glc^{II}, seems to be shared with the LPS of *P. aeruginosa* [2,7,8].

3. Experimental

Chromatography.—GPC was performed on a column (45 × 2.4 cm) of Sephadex G-50 (superfine) using a pyridine–acetate buffer (pH 4.5). HPLC was carried out on a Zorbax ODS C18 column (25 × 1 cm) in aq 10% MeOH. Monitoring was done by a

Knauer differential refractometer. GLC was performed with a Hewlett–Packard Model 5890, Series II chromatograph equipped with a capillary column (30 m × 0.25 mm) of cross-linked SPB*-5 as stationary phase. GLC–MS was carried out with a Hewlett–Packard Model 5985 instrument using electron impact and chemical ionization with ammonia.

NMR spectroscopy.—The NMR spectra were run with a Bruker AM-360 spectrometer for solutions in D_2O at 30 °C with acetone (δ_H 2.225, δ_C 31.45) as internal standard or phosphoric acid (δ_P 0) as external standard. Standard Bruker software was used to obtain 1D NOE and 2D COSY spectra.

Isolation and degradation of LPS.—LPS was isolated as described previously [1]. A solution of LPS (280 mg) in 30 mL of 0.1 M NaOAc buffer (pH 4.4) was heated with boiling water until a lipid precipitate formed, the precipitate (61 mg) was removed by centrifugation, and the supernatant was concentrated in vacuum and fractionated by GPC on Sephadex G-50 (S) to give an O-specific polysaccharide (fraction 1, 21 mg) and two oligosaccharides (fraction 2, 60 mg; and fraction 3, 17 mg). Fraction 2 (OS) was rechromatographed on the same gel.

Sugar and phosphorus analysis.—Neutral and amino sugars were analyzed by GLC as acetylated methyl glycosides obtained by methanolysis with 2 M HCl in MeOH (120 °C, 16 h) followed by acetylation. Amino sugars were analyzed conventionally using an LKB Alpha plus 4151 amino acid analyzer after hydrolysis with 4 M HCl (100 °C, 5 h). Phosphate was determined by the method of Lowry et al. [24], and Kdo by the TBA reaction according to the modified method [25].

Dephosphorylation and O-deacetylation.—OS (30 mg) was treated with 48% hydrofluoric acid at 4 °C for 30 h. After neutralization with cold aq 25% ammonia, the dephosphorylated product (OS_{HF}, 23 mg) was desalted on Sephadex G-50 (S) and fractionated by HPLC on reversed-phase C18 to give oligosaccharides **1** (5.5 mg) and **2** (6.8 mg).

Methylation analysis.—OS_{HF} (1 mg) was reduced with $NaBH_4$ and methylated with MeI in dimethyl sulfoxide in the presence of solid NaOH as described [10]. The methylated product was subjected to acetolysis (0.25 M H_2SO_4 in glacial AcOH, 80 °C, 16 h) followed by hydrolysis with the same mixture diluted with an equal volume of water (80 °C, 8 h) [11]. The derived partially methylated monosaccharides were conventionally reduced with $NaBD_4$, acetylated, and analyzed by GLC–MS.

Partial methanolysis.—OS_{HF} (0.5 mg) was heated with 0.5 M HCl in MeOH at 85 °C for 45 min. After evaporation, the product was acetylated with Ac_2O in pyridine or methylated as described above and studied by GLC–MS.

OS (40 mg) was heated with aq 12% ammonia at 45 °C for 4 h, the solution was freeze-dried, and the *O*-deacetylated product (29 mg) was dephosphorylated as described above and fractionated by HPLC on reversed-phase C18 to give oligosaccharides **3** (10.0 mg) and **4** (6.4 mg). Similar *O*-deacetylation of OS_{HF} (10 mg) afforded **3** (7.8 mg).

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