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Note

Somatic antigens of pseudomonads: Structure of the O-specific polysaccharide of the reference strain for *Pseudomonas fluorescens* (IMV 4125, ATCC 13525, biovar A)

Yuriy A. Knirel [a,*], Galina M. Zdorovenko [b], Nikolay A. Paramonov [a], Stanislav P. Veremeychenko [b], Filip V. Toukach [a], Alexander S. Shashkov [a]

[a] *N.D. Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, Leninsky Pr. 47, 117913 Moscow, Russian Federation*

[b] *D.K. Zabolotny Institute of Microbiology and Virology, National Academy of Sciences of Ukraine, ul. Zabolotnogo 156, Kiev 143, Ukraine*

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Strains of *Pseudomonas fluorescens* are divided into five biovars having undefined taxonomic rank [1]. No serological classification of this bacterium based on the specificity of O-antigens (outer-membrane lipopolysaccharides) has been elaborated. Recently [2,3], we have shown that the O-specific polysaccharide chains of the lipopolysaccharides of two strains of *P. fluorescens* belonging to the same biovar A have quite different structures. We now report the structure of the O-specific polysaccharide of *P. fluorescens* strain IMV 4125 (ATCC 13525), which is the reference strain for the species and also belongs to biovar A.

The lipopolysaccharide was isolated by the phenol–water procedure [4] and cleaved with dilute acetic acid to give the O-specific polysaccharide. Sugar analysis after full acid hydrolysis of the polysaccharide revealed the presence of L-rhamnose and 3-amino-

* Corresponding author.

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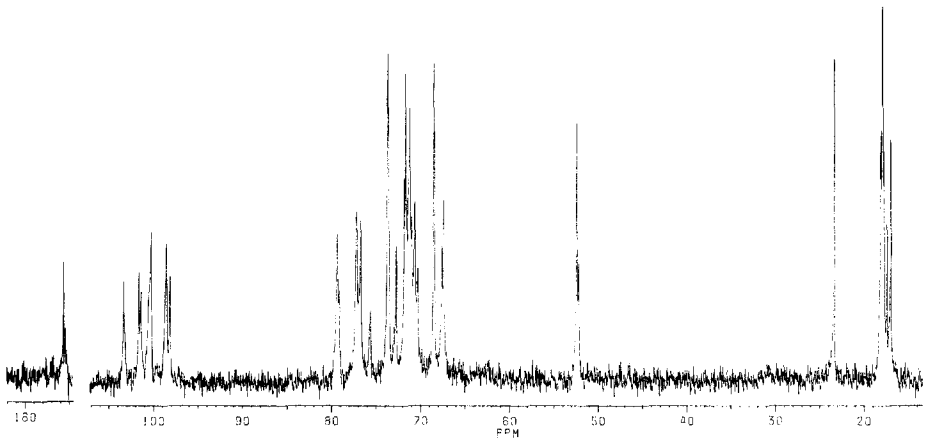


Fig. 1. ^{13}C NMR spectrum of the O-specific polysaccharide.

3,6-dideoxy-D-galactose (Fuc3N) which were identified by GLC and GLC–MS of alditol acetates [5,6] and GLC of acetylated glycosides with (*S*)-2-octanol [7].

The ^{13}C NMR spectrum of the polysaccharide (Fig. 1) contained a number of signals with different intensities, pointing to the absence of strict regularity or the presence of more than one polysaccharide. There were present, inter alia, signals for C-6 of rhamnose at δ 17.7–18.1 and C-6 of Fuc3N at δ 16.9–17.3 (intensity ratio 2.6:1), for C-3 of Fuc3N at 52.1–52.3, and *N*-acetyl groups at δ 23.3 (CH_3) and 175.5–175.8 (CO). Therefore, Fuc3N in the polysaccharide is *N*-acetylated.

Accordingly, the ^1H NMR spectrum of the polysaccharide (Fig. 2) contained signals for H-6 of rhamnose and Fuc3NAc at δ 1.20–1.35 (superposition of a number of doublets, $J_{5,6}$ 6–6.5 Hz), and for *N*-acetyl groups at δ 2.05–2.06 (singlets). As judged by the ratio of the intensities of the signals for H-6 and NAc, the ratio of rhamnose and Fuc3NAc was 2.5:1.

Methylation analysis of the polysaccharide, including GLC–MS of derived alditol acetates, led to the identification of 2,4-di-*O*-methylrhamnose, 3,4-di-*O*-methylrhamnose, and 4-*O*-methylrhamnose in the ratios 1:1.5:2.5, as well as 3,6-dideoxy-2,4-di-*O*-methyl-3-(*N*-methylacetamido)galactose. The mass spectrum of the latter was identical to that of the same derivative of Fuc3NAc derived from the O-specific polysaccharide of *Pseudomonas syringae* pv. *tabaci* IMV 223 [8]. These data suggested that the polysaccharide is branched, with lateral Fuc3NAc residues, the rhamnose residues at the branching points are 2,3-disubstituted, and the remaining rhamnose residues are 2- or 3-substituted, all the monosaccharides occurring in the pyranoid form.

Smith degradation of the polysaccharide afforded a number of oligosaccharide products, which were fractionated by gel-permeation chromatography on TSK HW-40 followed by HPLC on reversed-phase C18. As a result, three main fractions were isolated in comparable amounts, two of which (fractions 1 and 3) were pure compounds **1** and **2**, and the third (fraction 2) was a mixture of oligosaccharide–alditols.

Table 1
^{13}C NMR data (in ppm) [a]

	C-1	C-2	C-3	C-4	C-5	C-6
Trisaccharide–glycerol **1**						
α-D-Fuc*p*3NAc-(1 →	98.6	67.2	52.5	71.7	68.4	16.5
→ 2)-α-L-Rha*p*-(1 → (I)	101.1	77.5	70.3	73.2	70.8	17.9
→ 3)-α-L-Rha*p*-(1 → (II)	100.6	71.5	79.7	72.7	70.9	17.9
→ 2)-Gro	62.7	79.9	61.5			
Tetrasaccharide–glycerol **2**						
α-D-Fuc*p*3NAc-(1 → (I)	98.5	67.2	52.4	71.7	68.4	16.5
α-D-Fuc*p*3NAc-(1 → (II)	98.5	67.3	52.3	71.6	68.5	17.1
→ 2)-α-L-Rha*p*-(1 → (I)	100.6	77.3	71.0	73.6	70.7	17.9
→ 3)-α-L-Rha*p*-(1 → (II)	97.9	77.2	77.2	73.1	70.9	17.6
2 ↑						
→ 2)-Gro	62.6	79.9	61.6			
O-Specific polysaccharide of *Pseudomonas fluorescens* IMV 4125						
Oligosaccharide unit **3**						
α-D-Fuc*p*3NAc-(1 →	98.5	67.3	52.3	71.6	68.4	16.9
→ 3)-α-L-Rha*p*-(1 →	100.2	77.2	76.6	73.6	70.8	18.0
2 ↑						
→ 3)-α-L-Rha*p*-(1 →	103.2	71.3	79.3	72.7	70.8	17.9
→ 2)-α-L-Rha*p*-(1 →	101.5	79.3	71.2	73.6	70.9	17.9
Oligosaccharide unit **4**						
α-D-Fuc*p*3NAc-(1 → (I)	98.4	67.3	52.3	71.6	68.4	16.9
α-D-Fuc*p*3NAc-(1 → (II)	98.0	67.5	52.1	71.8	68.4	17.3
→ 3)-α-L-Rha*p*-(1 → (I)	100.3	77.1	76.6	73.6	70.8	18.1
2 ↑						
→ 3)-α-L-Rha*p*-(1 → (II)	101.3	79.0	77.1	73.6	71.0	17.9
2 ↑						
→ 2)-α-L-Rha*p*-(1 →	101.5	79.3	71.2	73.6	70.9	17.9
O-Specific polysaccharide of *Pseudomonas tabaci* IMV 223 [b]						
α-D-Fuc*p*3NAc-(1 →	98.3	67.2	52.3	71.5	68.4	16.8
→ 3)-α-L-Rha*p*-(1 →	100.2	77.0	76.5	73.5	70.9	17.9
2 ↑						
→ 3)-α-L-Rha*p*-(1 →	103.0	71.1	79.2	72.6	70.5	17.7
→ 2)-α-L-Rha*p*-(1 →	101.5	79.1	71.1	73.6	70.5	17.7

[a] Assignment of the signals with the difference between chemical shifts < 1 ppm could be interchanged. Chemical shifts for NAc are δ 23.3 (Me) and 175.5–175.8 (CO).
[b] Data from ref. [8].

Sugar analysis and the ^{13}C and ^{1}H NMR spectra (Tables 1 and 2) showed that **1** contains two residues of rhamnose and one residue each of Fuc3NAc and glycerol, and that **2** differs from **1** in the presence of an additional Fuc3NAc residue. Therefore, **1** and **2** are a trisaccharide–glycerol and a tetrasaccharide–glycerol, respectively.

Table 2
^{1}H NMR data (in ppm) [a]

	H-1	H-2	H-3	H-4	H-5	H-6
Trisaccharide–glycerol **1**						
α-D-Fuc*p*3NAc-(1 →	4.95	3.79	4.18	3.73	4.38	1.15
→ 2)-α-L-Rha*p*-(1 → (I)	5.12	4.05	3.85	3.52	3.83	1.27
→ 3)-α-L-Rha*p*-(1 → (II)	4.92	4.05	3.92	3.54	3.82	1.25
Tetrasaccharide–glycerol **2**						
α-D-Fuc*p*3NAc-(1 → (I)	4.93	3.78	4.18	3.71	4.36	1.14
α-D-Fuc*p*3NAc-(1 → (II)	5.00	3.77	4.19	3.72	4.27	1.20
→ 2)-α-L-Rha*p*-(1 → (I)	5.22	4.05	3.86	3.56	3.77	1.28
→ 3)-α-L-Rha*p*-(1 → (II) 2 ↑	5.07	4.04	4.00	3.68	3.81	1.25

[a] Chemical shifts for NAc are δ 2.00–2.02.

The ^{1}H NMR spectra of **1** and **2** were analysed using 2D correlation spectroscopy (COSY), COSY with one-step relayed coherence transfer, and sequential, selective spin decoupling, and the signals for the sugar units were completely assigned (Table 2). The coupling constants $J_{1,2}$ 3.5–4 Hz showed that the Fuc3NAc residues are α-linked. The α configuration of the rhamnosidic linkages followed from the chemical shifts of the signals for H-5 (δ 3.77–3.83; cf. δ 3.86 for the H-5 resonance in α-Rha, but 3.39 in β-Rha [9]).

In the 2D rotating-frame NOE (ROESY) spectrum of **1**, there were present interresidue cross-peaks Fuc3NAc H-1,Rha I H-1,2 at δ 4.95/5.12 and 4.95/4.05, respectively, and Rha I H-1,Rha II H-3 at δ 5.12/3.92. In addition, intraresidue cross-peaks H-1,2 were observed for all three sugar residues, which were in accord with the configuration of their glycosidic linkages. Rha II H-1 at δ 4.92 gave only a weak interresidue cross-peak near δ 3.7, which could be assigned to the Rha II H-1,Gro H-2 correlation.

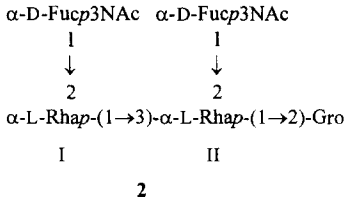
These data suggested that the trisaccharide–glycerol **1** has the following structure:

α-D-Fuc*p*3NAc
1
↓
2
α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→2)-Gro
I II

1

Similarly, a 2D ROESY experiment with **2** revealed the interresidue correlations between Fuc3NAc I H-1,Rha I H-1,2 (cross-peaks at δ 4.93/5.22 and 4.93/4.05, respectively), Fuc3NAc II H-1,Rha II H-1,2 (δ 5.00/5.07 and 5.00/4.04, respectively),

and Rha I H-1,Rha II H-3 at δ 5.22/4.00. These data pointed to the following structure of the tetrasaccharide–glycerol **2**:



2

The structures of **1** and **2** were in accord with the ^{13}C NMR data (Table 2), which were tentatively assigned using published data for the corresponding monosaccharides and glycosylation effects [8–10] and for structurally related carbohydrates [8,11–13].

As judged by the ^{13}C NMR spectrum, fraction 2 was a mixture of several compounds, among which **1** and an oligosaccharide–rhamnitol were identified. The latter displayed characteristic signals at δ 61.9 and 20.2, respectively, typical of C-1 and C-6 of a 2-substituted rhamnitol residue, which resulted evidently from overhydrolysis in the course of Smith degradation.

Analysis of the ^{1}H NMR spectrum of the polysaccharide (Fig. 2) using 2D COSY showed, in particular, that the signals for H-5 were in the region δ 3.75–3.90 for rhamnose residues and δ 4.30–4.40 for Fuc3NAc residues (there were present cross-peaks for Rha H-5,H-6 at δ 3.75–3.90/1.22–1.34 and for Fuc3NAc H-5,H-6 at δ 4.30–4.40/1.20–1.28). Therefore, all monosaccharide residues in the polysaccharide are α-linked, including the rhamnose residues which are oxidised during Smith degradation (cf. the published chemical shifts for H-5 3.86 in α-Rha and δ 4.20 in α-Fuc, but δ 3.39 in β-Rha and δ 3.79 in β-Fuc [9]).

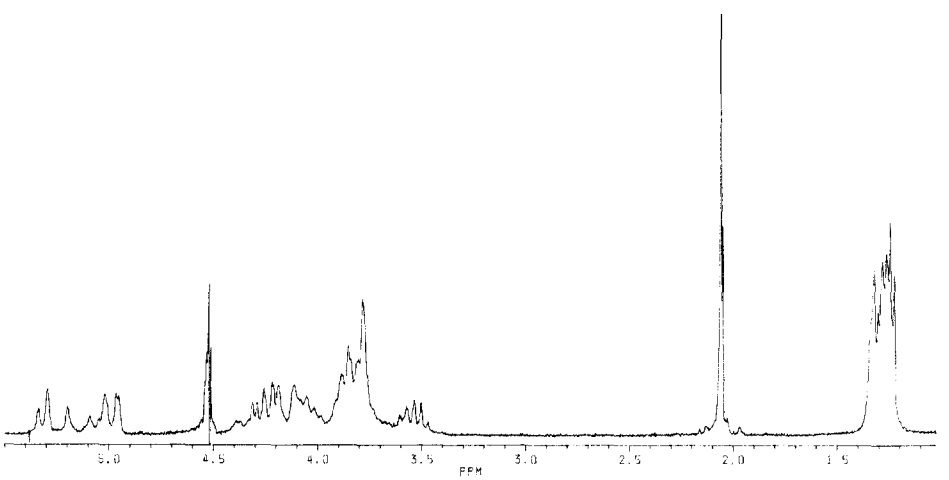


Fig. 2. ^{1}H NMR spectrum of the O-specific polysaccharide.

Assignment of the ^{13}C NMR spectrum of the polysaccharide (Table 2) was performed using the data for the oligosaccharide–glycerols **1** and **2** and the O-specific polysaccharide of *Pseudomonas syringae* pv. *tabaci* IMV 223, which is built up of tetrasaccharide repeating units **3** [8]. The resonance pattern for the unit **3** could easily be recognised in the spectrum, and the rest of the signals could be assigned to the unit **4** differing from the unit **3** in the presence of the second Fuc3NAc residue (cf. the structures of the Smith-degraded products **1** and **2**).

α-D-Fuc*p*3NAc
1
↓
2
→3)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→2)-α-L-Rha*p*-(1→

3

α-D-Fuc*p*3NAc α-D-Fuc*p*3NAc
1 1
↓ ↓
2 2
→3)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→2)-α-L-Rha*p*-(1→
I II

4

Therefore, it can be concluded that the O-specific polysaccharide of the reference strain for *P. fluorescens* (IMV 4125, ATCC 13525) is not strictly regular and consists mainly of two types of oligosaccharide repeating units having the structures **3** and **4**, or that there are two structurally related regular polysaccharides built up of the repeating units **3** and **4**. As judged by the ratio of rhamnose and Fuc3NAc determined by sugar analysis and ^{1}H and ^{13}C NMR spectroscopy, the ratio of the partially methylated derivatives of rhamnose revealed in methylation analysis, and the ratio of the oligosaccharide–glycerols obtained by Smith degradation, the tetrasaccharide repeating unit **3** is slightly predominant and, by various estimations, amounts to 60% of the total.

While the repeating unit **4** is unique for the polysaccharide studied, the repeating unit **3**, as mentioned above, has been reported previously in the O-antigen of *Pseudomonas syringae* pv. *tabaci* IMV 223 [8]. An isomeric repeating unit with the same main chain and Fuc3NAc attached at position 3 of the 2-substituted rhamnose residue has been found in the O-specific polysaccharide of *P. syringae* pv. *tomato* 140(R) [11]. Both these are strictly regular polysaccharides, while structural heterogeneity has been reported for another group of structurally related O-specific polysaccharides of *P. syringae*, which are built up of pentasaccharide repeating units containing four rhamnose residues and one residue of Fuc3NAc [12–14]. However, their heterogeneity is of another sort associated with different positions of substitution of the rhamnose residues in the main chain and different sites of attachment of Fuc3NAc. In addition to *P.*

syringae, structurally heterogeneous O-antigens have been found also in *P. aeruginosa* and a number of *Burkholderia* (former *Pseudomonas*) species [15] but have not been reported hitherto for *P. fluorescens*.

No structural similarity is observed between the O-specific polysaccharide of the reference strain of *P. fluorescens* biovar A and those of two other *P. fluorescens* biovar A strains studied by us earlier [2,3].

1. Experimental

Isolation of the O-specific polysaccharide.—*P. fluorescens* strain IMV 4125 (ATCC 13525) was grown as described [16]. The lipopolysaccharide was extracted from dry bacterial cells by the phenol–water procedure [4] and degraded with aq 1% AcOH at 100 °C for 3 h. After removal of a lipid precipitate, the carbohydrate portion was fractionated by gel-permeation chromatography on a column (70 × 3.5 cm) of Sephadex G-50 in pyridinium acetate buffer (pH 4.5) to give the O-specific polysaccharide eluted close to the void volume of the column.

Sugar and methylation analysis.—The polysaccharide was hydrolysed with 2 M CF_3CO_2H (120 °C, 2 h), and sugars were conventionally converted into alditol acetates [5] and analysed by GLC on a Hewlett–Packard Model 5971 A chromatograph equipped with a glass capillary column (12 m × 0.2 mm) of HP-1 as stationary phase, using a temperature program of 150–270 °C at 8 °C/min, and by GLC–MS on a Hewlett–Packard Model 7985 instrument operating at 70 eV and equipped with a capillary column of cross-linked SPBTM-5.

Methylation was carried out by the Hakomori method [17]. The methylated products were hydrolysed as in the sugar analysis and the resulting partially methylated monosaccharides were analysed by GLC–MS as alditol acetates under the conditions described above. Mass spectra of partially methylated alditol acetates were interpreted using published data [18].

Smith degradation.—The polysaccharide (20 mg) was oxidised with 0.1 M $NaIO_4$ (1.5 ml) for 40 h at ambient temperature in the dark, and the excess of the oxidant was reduced with a drop of ethylene glycol. The product was reduced conventionally with an excess of $NaBH_4$, desalted by gel filtration on a column (1.6 × 80 cm) of TSK HW-40 (S), hydrolysed with aq 2% AcOH, reduced with $NaBH_4$, then desalted and fractionated on TSK HW-40 (S). The oligosaccharides obtained were fractionated by HPLC on a semi-preparative reversed-phase C18 column in water to give trisaccharide–glycerol **1** (1.4 mg, fraction 1), an intermediate mixed fraction 2 (1.1 mg), and tetrasaccharide–glycerol **2** (1.3 mg, fraction 3).

NMR spectroscopy.—The ^1H NMR and ^{13}C NMR spectra were obtained with a Bruker AM-300 instrument in D_2O at 50 °C (^1H) or 60 °C (^{13}C) for the polysaccharide and 60 °C (^1H) or 30 °C (^{13}C) for oligosaccharides. Acetone was used as internal standard (δ_H 2.225, δ_C 31.45). Selective spin-decoupling was carried out by difference mode according to the modified procedure [19]. 2D COSY and 2D ROESY experiments were performed using standard Bruker software. A mixing time of 200 ms was used in the ROESY experiment.

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