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Note

Somatic antigens of pseudomonads: structure of the O-specific polysaccharide of *Pseudomonas fluorescens* IMV 2366 (biovar C)

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Dedicated to Professor Derek Horton on the occasion of his 70th birthday

Abstract

The O-specific polysaccharide of *P. fluorescens* IMV 2366 was studied by sugar and methylation analyses along with ^{1}H and ^{13}C NMR spectroscopy, including 2D gsCOSY, TOCSY, gsNOESY, H-detected $^{1}H,^{13}C$ gsHSQC, HMQC–TOCSY, and gsHMBC experiments. The polysaccharide contains L-rhamnose, 2-acetamido-2,6-dideoxy-D-galactose (D-FucNAc) and 3-acylamido-3,6-dideoxy-D-glucose (D-Qui3NAcyl, where Acyl is 3-hydroxy-2,3-dimethyl-5-oxoprolyl). The structure **1** of the polysaccharide was found to be similar to the structure **2** of a 6-deoxy-L-talose (L-6dTal)-containing O-specific polysaccharide of a non-classified *P. fluorescens* strain, 361, studied earlier [Khomenko, V. A.; Naberezhnykh, G. A.; Isakov, V. V.; Solov'eva, T. F.; Ovodov, Y. S.; Knirel, Y. A.; Vinogradov, E. V. *Bioorg. Khim.* **1986**, *12*, 1641–1648; Naberezhnykh, G. A.; Khomenko, V. A.; Isakov, V. V., El'kin, Y. N.; Solov'eva, T. F.; Ovodov, Y. S. *Bioorg. Khim.* **1987**, *13*, 1428–1429].

→2)-β-D-Qui*p*3NAcyl-(1→3)-α-L-Rha*p*-(1→3)-α-D-Fuc*p*NAc-(1→ **1**

→4)-β-D-Qui*p*3NAcyl-(1→3)-α-L-6dTal*p*4Ac-(1→3)-α-D-Fuc*p*NAc-(1→ **2**

Keywords: *Pseudomonas fluorescens*; Bacterial polysaccharide, structure; Lipopolysaccharide; O-Antigen; 3-Acylamido-3,6-dideoxy-D-glucose; 3-Hydroxy-2,3-dimethyl-5-oxoprolyl group

Strains of *Pseudomonas fluorescens* are divided into five biovars having undefined taxonomical rank.[1,2] They are heterogeneous by genotypic and phenotypic characters, including the lipopolysaccharide structure. The structures of the O-specific polysaccharide chains of the lipopolysaccharides of a number of *P. fluorescens* strains belonging to biovars A and B have been established (Ref. 3 and references cited therein). Here we report the structure of the O-specific polysaccharide of *P. fluorescens* IMV 2366 from biovar C.

The O-specific polysaccharide was obtained by mild-acid degradation of the lipopolysaccharide isolated by the phenol–water procedure.[4] The ^{1}H NMR spectrum of the polysaccharide (Fig. 1) contained, inter alia, signals for three anomeric protons at δ 4.81 (d, $J_{1,2}$ 7 Hz), 4.82 (nonresolved), and 5.62 (d, $J_{1,2}$ 2.5 Hz); five CH_3–C groups at δ 1.23, 1.27, 1.30 (all d, J 6–7 Hz), 1.36, and 1.47 (both s); one C–CH_2–C group (AB system) at δ 2.42 and 2.71 (both d, J 17.2 Hz); and one *N*-acetyl group at δ 2.05 (s). The ^{13}C NMR spectrum of the polysaccharide (Fig. 2) contained signals for three anomeric carbons at δ 96.1, 103.0, and 105.2; three nitrogen-bearing carbons at δ 45.5, 48.8, and 57.3; two quaternary carbons at δ 71.7 and 78.9 (data of a DEPT 135 experiment), six CH_3–C groups at δ 16.3, 17.5, 17.8, 18.8, 23.1, and 23.3; three CO groups at δ 174.7, 175.4, and 179.7; and ten other carbons in the region δ 68.1–81.2.

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These data showed that the polysaccharide has a repeating unit containing three 6-deoxy sugars and an unusual non-sugar substituent. Further studies and comparison with published data of the O-specific polysaccharide of *P. fluorescens* 361[5,6] showed that this substituent is an *N*-linked 3-hydroxy-2,3-dimethyl-5-oxoprolyl group.

Acid hydrolysis of the polysaccharide followed by GLC–MS of the derived alditol acetates[7] revealed rhamnose, 2-acetamido-2,6-dideoxygalactose (FucNAc), and 3-acetamido-3,6-dideoxyglucose (Qui3NAc) in the ratios 1.3:1:0.1. The small content of Qui3NAc could be accounted for by a poor cleavage of the *N*-acyl group from 3,6-dideoxy-3-(3-hydroxy-2,3-dimethyl-5-oxoprolylamino)glucose (Qui3NAcyl) during acid hydrolysis. Indeed, the corresponding 3-hydroxy-2,3-dimethyl-5-oxoprolyl-carrying acetylated alditol (Fig. 3) was detected by an $[M + H]^+$ ion at m/z 489 in the chemical-ionization mass spectrum. In addition, the spectrum showed the presence of three related

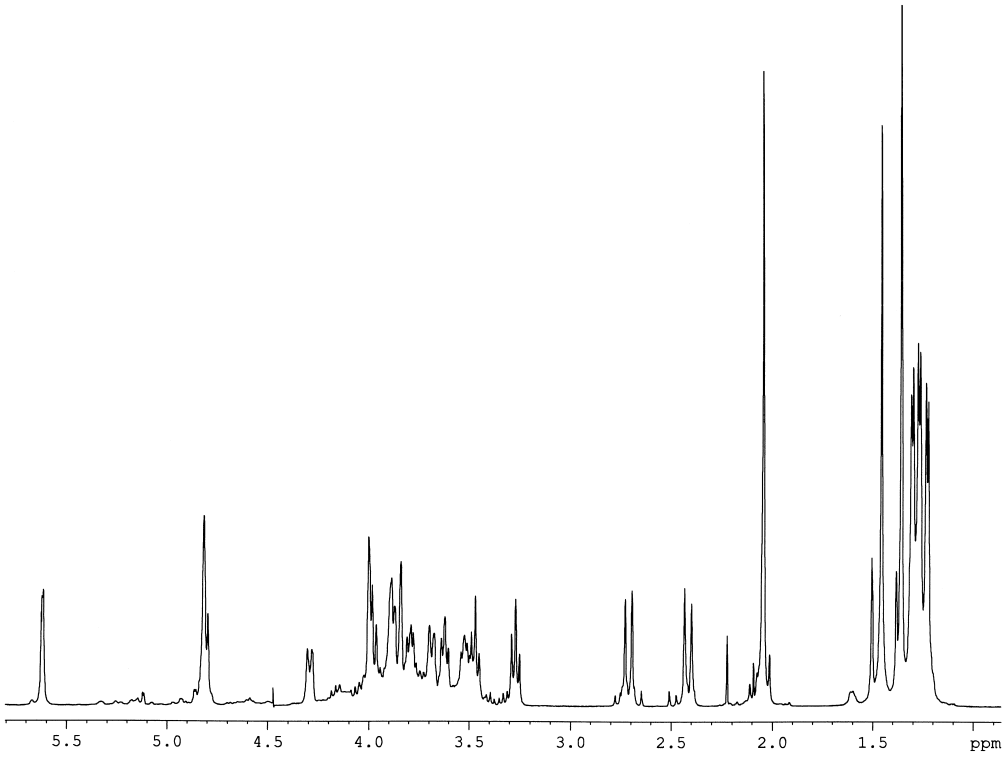

Fig. 1. 500-MHz ^{1}H NMR spectrum of the O-specific polysaccharide.

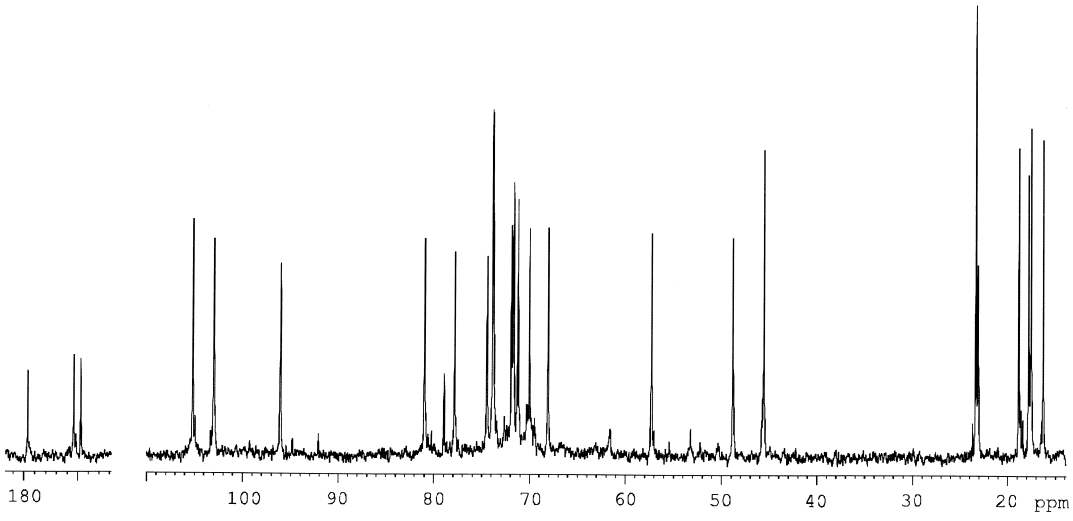

Fig. 2. 125-MHz ^{13}C NMR spectrum of the O-specific polysaccharide.

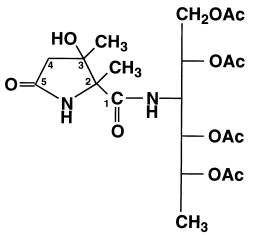

Fig. 3. Structure of the acetylated alditol derived from 3,6-dideoxy-3-(3-hydroxy-2,3-dimethyl-5-oxoprolylamino)glucose (Qui3NAcyl).

compounds, which were characterized by $[M+H]^+$ ions at m/z 471, 513, 531 and were tentatively assigned to the products of dehydratation and enolization/O-acetylation of the acyl group in the course of the sample preparation.

GLC of the acetylated (*R*)-2-octyl glycosides[8] showed that rhamnose has the L configuration and FucN the D configuration. The D configuration of Qui3N was determined by analysis of the glycosylation effects in the ^{13}C NMR spectrum of the polysaccharide (see below). These data showed that the polysaccharide has a trisaccharide repeating unit containing one residue each of L-rhamnose, D-FucNAc, and D-Qui3NAcyl.

Methylation analysis of the polysaccharide revealed 3-substituted rhamnose, 3-substituted FucNAc, and 2-substituted Qui3NAc. The partially methylated 6-deoxy amino sugars were identified by electron-impact MS of the corresponding alditol acetates, which showed ion peaks for the characteristic primary fragments C-1,2 and C-2,3,4,5,6 at m/z 158 and 288 for FucNAc or C-1,2,3 and C-3,4,5,6 at m/z 230 and 216 for Qui3NAc. Derivatives from Qui3NAcyl were not studied.

The ^1H NMR spectrum of the polysaccharide was assigned using 2D gsCOSY and TOCSY experiments. In the TOCSY spectrum, there were cross-peaks for H-1 with H-2 of Rha, H-2,3,4 of FucN and H-2,3,4,5,6 of Qui3N as well as for H-6 with H-2,3,4,5 of Rha, H-5 of FucN and H-1,2,3,4,5 of Qui3N. Then, the ^{13}C NMR spectrum was assigned using ^1H,^{13}C gsHSQC and HMQC–TOCSY experiments. On the basis of the ^1H and ^{13}C NMR chemical shift data (Table 1; compare published data[5,9–11]) and characteristic coupling constant values, the three sugar spin systems were assigned to α-Rha*p*, α-Fuc*p*N, and β-Qui*p*3N. The β configuration of the glycosidic linkage of Qui*p*3N was confirmed by H-1,H-3 and H-1,H-5 correlations in the gsNOESY spectrum of the polysaccharide.

The structure of the acyl substituent at Qui3N was confirmed by the gsNOESY and gsHMBC experiments. The NOESY spectrum displayed a cross peak between the CH_3-2 and CH_3-3 signals at δ 1.47/1.36, thus showing the close proximity of the methyl groups. The CH_3-3 signal gave cross-peaks with H-4a and H-4b at δ 1.36/2.42 and at 1.36/2.71. The HMBC experiment revealed connectivities of CH_3-2 at δ_H 1.47 with C-1, C-2, and C-3 at δ_C 175.4, 71.7, and 78.9; CH_3-3 at δ_H 1.36 with C-2, C-3, and C-4 at δ_C 71.7, 78.9, and 45.5; H-4a at δ_H 2.42 with C-2, C-3, and C-5 at δ_C 71.7, 78.9, and 179.7; and H-4b at δ_H 2.71 with CH_3-3 and C-5 at δ_C 23.3 and 179.7, respectively. A large $J_{4a,4b}$ geminal coupling of 17.2 Hz also confirmed a five-membered cyclic structure with an exocyclic carbonyl group.[12]

Relatively low-field positions of the signals for C-3 of Rha, C-3 of FucN, and C-2 of Qui3N at δ 81.2, 77.9, and 74.5, respectively, as compared with their positions in the spectra of the corresponding nonsubstituted monosaccharides,[5,9,11] were in agreement with the substitution pattern determined by methylation analysis. This was further confirmed by gsNOESY and gsHMBC experiments (Table 2), which also revealed the monosaccharide sequence in the repeating unit. In the HMBC spectrum, the C-1 signal of the 3-hydroxy-2,3-dimethyl-5-oxoprolyl group at δ 175.4 correlated with H-3 of Qui3N at δ 3.99, thus confirming the location of the acyl group.

The D configuration of Qui3N followed from a relatively small β-effect on C-2 of Rha in the disaccharide fragment β-Qui*p*3NAcyl-(1→3)-L-Rha*p*. Its value of −0.3 ppm, as determined by comparison with the data for α-rhamnopyranose,[9] is characteristic of different absolute configurations of the constituent sugars, whereas in case of the same absolute configuration, the effect on C-2 would be negative and >2.5 ppm by the absolute value.[13] On the basis of the data obtained, it was concluded that the O-specific polysaccharide of *P. fluorescens* IMV 2366 has structure **1**.

→2)-β-D-Qui*p*3NAcyl-(1→3)-α-L-Rha*p*-(1→3)-α-D-Fuc*p*NAc-(1→ (**1**)

The structure **1** is similar to the structure **2** of a 6-deoxy-L-talose (L-6dTal)-containing O-specific polysaccharide of a non-classified *P. fluorescens* strain, 361, studied earlier.[5,6]

→4)-β-D-Qui*p*3NAcyl-(1→3)-α-L-6dTal*p*4Ac-(1→3)-β-D-Fuc*p*NAc-(1→ (**2**)

Both polysaccharides contain a unique *N*-acyl group, 3-hydroxy-2,3-dimethyl-5-oxoprolyl, which can be considered as a derivative of pyroglutamic acid. Two more derivatives of pyroglutamic acid, 3-hydroxy-3-methyl-5-oxoprolyl and 2,4-dihydroxy-3,3,4-trimethyl-5-oxoprolyl groups, have been found in the O-specific polysaccharides of *Vibrio cholerae*[14] and *Vibrio anguillarum*,[15] respectively. The (*R*,*R*) configuration has been determined for the former, whereas the absolute configuration of the two other naturally occurring derivatives remains unknown. In all cases, the acyl groups are attached to 6-deoxy amino sugars: D-Qui3N (this work), D-Fuc3N[14] or D-Qui4N,[15] which often carry unusual *N*-acyl substituents,[16,17]

Table 1
500-MHz ^{1}H NMR and 125-MHz ^{13}C NMR chemical shifts of the O-specific polysaccharide (δ in ppm)

Component	Sugar atoms						Acyl atoms						
	H-1	H-2	H-3	H-4	H-5	H-6	H-2	H-4a	H-4b	CH_3-2	CH_3-3		
→2)-β-D-Qui*p*3NAcyl-(1→	4.81	3.62	3.99	3.27	3.53	1.30		2.42	2.71	1.47	1.36		
→3)-α-L-Rha*p*-(1→	4.82	4.00	3.88	3.47	3.79	1.23							
→3)-α-D-Fuc*p*NAc-(1→	5.62	4.30	3.69	3.84	3.89	1.27	2.05						
	C-1	C-2	C-3	C-4	C-5	C-6	C-1	C-2	C-3	C-4	C-5	CH_3-2	CH_3-3
→2)-β-D-Qui*p*3NAcyl-(1→	105.2	74.5	57.3	73.8	73.9	17.8	175.4 (175.7)	71.7 (78.2)	78.9 (71.6)	45.5 (45.8)	179.7 (179.7)	18.8 (18.4)	23.3 (23.2)
→3)-α-L-Rha*p*-(1→	103.0	71.2	81.2	71.7	70.0	17.5							
→3)-α-D-Fuc*p*NAc-(1→	96.1	48.8	77.9	71.9	68.1	16.3	174.7	23.1					

Data of the 3-hydroxy-2,3-dimethyl-5-oxoprolylamino group in the O-specific polysaccharide of *P. fluorescens* 361[5] are given in parentheses.

Table 2
Homonuclear (NOESY) and $^{1}H,^{13}C$ heteronuclear (HMBC) interresidue connectivities for the anomeric signals in the O-specific polysaccharide

Sugar residue	δ_{H-1}	δ_{C-1}	δ_{H}	δ_{C}	Connectivity to
NOESY data					
→3)-α-D-Fuc*p*NAc-(1→	5.62		3.62		Qui3NAcyl H-2
			4.81		Qui3NAcyl H-1
→2)-β-D-Qui*p*3NAcyl-(1→	4.81		3.88		Rha H-3
			5.62		FucNAc H-1
→3)-α-L-Rha*p*-(1→	4.82		3.69		FucNAc H-3
HMBC data					
→3)-α-D-Fuc*p*NAc-(1→	5.62			74.5	Qui3NAcyl C-2
		96.1	3.62		Qui3NAcyl H-2
→2)-β-D-Qui*p*3NAcyl-(1→	4.81			81.2	Rha C-3
		105.2	3.88		Rha H-3
→3)-α-L-Rha*p*-(1→	4.82			77.9	FucNAc C-3
		103.0	3.69		FucNAc H-3

1. Experimental

P. fluorescens IMV 2366 was from the collection of the D.K. Zabolotny Institute of Microbiology and Virology (Kiev, Ukraine). Growth of the bacterium[18] and isolation of the lipopolysaccharide[4] and O-specific polysaccharide[19] were performed as described.

The polysaccharide (1 mg) was hydrolyzed with 2 M CF_3CO_2H at 120 °C for 2 h. The resulting sugars were conventionally converted into the alditol acetates[7] and analyzed by GLC on a Hewlett–Packard model 5880 chromatograph equipped with a DB-5 column, using a temperature gradient of 160 °C (1 min) to 290 °C at 3 °C/min, and by GLC combined with electron-impact MS and chemical-ionization MS using ammonia as the reactant gas on a Hewlett–Packard 5989A instrument equipped with an HP-5 column. Qui3N was identified by comparison with the authentic sample from the O-specific polysaccharide of *Hafnia alvei* 1216.[20]

For determination of the absolute configurations of the monosaccharides, the hydrolysate from 2 mg polysaccharide was applied to a column of an Amberlite IR-120 cation-exchange resin (H^+-form). Neutral sugars were eluted with water and amino sugars were eluted with aq 5% ammonia. The amino fraction was *N*-acetylated with Ac_2O in satd aq $NaHCO_3$, and both fractions were converted into the acetylated (*R*)-2-octyl glycosides and analyzed by GLC as described.[8]

Methylation of the polysaccharide was performed with CH_3I in dimethyl sulfoxide in the presence of sodium methylsulfinylmethanide.[21] Partially methylated monosaccharides were derived by hydrolysis with 2 M CF_3CO_2H as in sugar analysis, converted into the alditol acetates and analyzed by GLC–MS.

^{1}H and ^{13}C NMR spectra were obtained with a Bruker DRX-500 instrument equipped with a *z*-gradient unit in $^{2}H_2O$ at 45 °C. Acetone was used as internal standard (δ_H 2.225, δ_C 31.45). 2D NMR experiments were performed using standard Bruker software, and the XWINNMR 2.1 program was used to acquire and process the NMR data. A mixing time of 100 and 200 ms was used in 2D TOCSY and gsNOESY experiments, and a delay of 60 ms for evolution of long-range connectivities in a gsHMBC experiment.

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