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Note

Somatic antigens of pseudomonads: structure of the O-specific polysaccharide of *Pseudomonas fluorescens* biovar A strain IMV 472

Yuriy A. Knirel [a,*], Stanislav N. Veremeychenko [b], Galina M. Zdorovenko [b], Alexander S. Shashkov [a], Nikolay A. Paramonov [a], Irina Ya. Zakharova [b], Nikolay K. Kochetkov [a]

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

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

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Pseudomonas fluorescens is a phenotypically heterogeneous microorganism divided into five biovars having undefined taxonomical rank. No serological classification has been elaborated for strains of this bacterium. In our studies of cell-wall lipopolysaccharides of *P. fluorescens*, we have established [1] the structure of the O-specific polysaccaride of biovar A strain IMV 1152. Now we report the structure of the O-specific polysaccharide chain of the lipopolysaccharide of strain IMV 472 belonging to the same biovar A.

The lipopolysaccharide of strain IMV 472 was isolated by the phenol–water procedure [2] and cleaved with dilute acetic acid as described [3], to give the O-specific polysaccharide.

The ^{13}C NMR spectrum of the polysaccharide (Table 1) contained signals for four anomeric carbons at 97.6, 100.8, 102.5, and 103.8 ppm, one carbon bearing nitrogen at 57.0 ppm, three CH_3–C groups (C-6 of 6-deoxy sugars) at 16.3, 17.8, and 18.1 ppm, other sugar carbons in the region 67.7–84.7 ppm, one NAc group (CH_3 at 23.8 ppm, CO at 176.1 ppm), and one OAc group (CH_3 at 22.0 ppm, CO at 174.1 ppm).

Therefore, the polysaccharide has a tetrasaccharide repeating unit, which contains three residues of 6-deoxy sugars and one residue of an *N*-acetylated amino sugar.

* Corresponding author.

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Table 1
^{13}C NMR chemical shifts (δ in ppm) [a]

Sugar unit		C-1	C-2	C-3	C-4	C-5	C-6
O-Specific polysaccharide							
→ 3)-β-L-Rha*p*2Ac-(1 →	**(A)**	100.8	70.2	76.5	71.5	73.7	18.1
→ 4)-α-L-Rha*p*-(1 →	**(B)**	102.5	71.2	70.6	84.7	68.7	17.8
→ 3)-α-D-Fuc*p*-(1 →	**(C)**	97.6	76.7	76.7	73.0	67.7	16.3
2 ↑							
β-D-Glc*p*NAc-(1 →	**(D)**	103.8	57.0	75.4	71.5	76.8	62.6
O-Deacetylated polysaccharide							
→ 3)-β-L-Rha*p*-(1 →	**(A)**	101.7	68.9	80.4	71.6	73.5	18.0
→ 4)-α-L-Rha*p*-(1 →	**(B)**	102.7	71.2	70.6	84.3	68.9	17.9
→ 3)-α-D-Fuc*p*-(1 →	**(C)**	98.0	77.2	77.2	73.1	67.8	16.4
2 ↑							
β-D-Glc*p*NAc (1 →	**(D)**	103.8	57.3	75.3	71.4	77.2	62.3

[a] Additional signals: NAc at 23.8 ppm (Me) and 176.1 ppm (CO), OAc at 22.0 ppm (Me) and 174.1 ppm (CO).

The ^1H NMR spectrum of the polysaccharide (Table 2) accorded with this conclusion. It was completely assigned with the use of sequential, selective spin-decoupling procedures and 2D homonuclear shift-correlated (COSY) spectroscopy. The low-field position at 5.57 ppm of the signal for H-2 of one of the sugar residues (unit **A**) was evidently due to deshielding caused by the OAc group.

As judged by the coupling constants $^3J_{H,H}$, all sugar residues are pyranoid. Two of the 6-deoxy sugars (units **A** and **B**) have the *manno* configuration ($J_{1,2} < 2$ Hz), i.e., are residues of rhamnose. The third 6-deoxy sugar (unit **C**) is α-fucose, as followed from the small values of $J_{1,2}$ 3.7, $J_{3,4}$ 3.5, and $J_{4,5} < 2$ Hz. Large coupling constants $J_{1,2}$, $J_{2,3}$, $J_{3,4}$, and $J_{4,5}$ 8–11 Hz for the fourth residue (unit **D**) proved its *β-gluco* configuration. On preirradiation of H-1 of units **A**–**D**, NOEs on H-3,5 of the same sugar residue were observed only for units **A** and **D**; hence, these units have the β configuration, whereas units **B** and **C** have the α configuration.

Complete assignment of the ^{13}C NMR spectrum of the polysaccharide was performed by using 2D heteronuclear $^{13}C/^1H$ shift-correlated (COSY) spectroscopy. Placing of the signal for C-2 of unit **D** in the region of carbons bearing nitrogen (57.0 ppm) indicated this residue to be 2-acetamido-2-deoxyglucose. The chemical shifts 73.5 and 68.9 ppm for C-5 of units **A** and **B**, respectively, confirmed that the former rhamnose residue is β-linked and the latter α-linked [4].

Preirradiation of H-1 of units **A** and **B** caused NOEs on H-4 of unit **B** and H-3 of unit **C** that indicated the presence of the fragment β-Rha*p*-(1 → 4)-α-Rha*p*-(1 → 3)-α-Fuc*p*. Each of the experiments with preirradiation of H-1 of two other sugar units resulted in more than one significant interresidue NOE (H-1 **C**/H-2 **A** and H-3 **A**; H-1 **D**/H-2 **B** and H-2 **C**) and allowed more than one interpretation. However, consideration of our published data [5,6] on conformation and NOEs in the appropriate model oligosaccharides allowed us to overcome this difficulty.

Table 2
^{1}H NMR data for the O-specific polysaccharide (δ in ppm, J in Hz) [a]

H-1	H-2	H-3	H-4	H-5	H-6
→ 3)-β-L-Rha*p*2Ac-(1 → (unit **A**)					
4.85	5.57	3.81	3.55	3.53	1.36
$J_{1,2} < 2$	$J_{2,3}$2.5	$J_{3,4}$10		$J_{5,6}$5.5	
→ 4)-α-L-Rha*p*-(1 → (unit **B**)					
4.83	3.95	3.83	3.83	3.55	1.26
$J_{1,2} \sim 2$	$J_{2,3} \sim 3$	$J_{3,4} \sim 9.5$	$J_{4,5}$10	$J_{5,6}$6.1	
→ 3)-α-D-Fuc*p*-(1 → (unit **C**)					
	2 ↑				
5.17	3.88	3.97	3.80	4.27	1.14
$J_{1,2}$3.7	$J_{2,3}$10.2	$J_{3,4}$3.5	$J_{4,5} < 2$	$J_{5,6}$6.6	
β-D-Glc*p*NAc-(1 → (unit **D**)					
4.46	3.65	3.49	3.41	3.39	3.91 [b], 3.72 [c]
$J_{1,2}$8.5	$J_{2,3}$10.4	$J_{3,4} \sim 10$		$J_{5,6a} \sim 3$	$J_{5,6b} \sim 6$

[a] Additional signals: CH_3 groups of NAC at 2.06 ppm and OAc at 2.23 ppm.
[b] H-6a, $J_{6a,6b}$ 12 Hz.
[c] H-6b.

In the preferred conformation of α-(1 → 3)-linked disaccharides having different absolute configurations of the constituent monosaccharides and the glycosylated sugar with the *manno* configuration [e.g., α-D-Glc*p*-(1 → 3)-α-L-Rha*p*], H-1 of the glycosylating sugar is in close proximity with both H-2 and H-3 of the glycosylated monosaccharide [5]. As a result, nearly equal NOEs are observed on H-2 and H-3 of the latter sugar unit after preirradiation of H-1 of the former. In contrast, in none of the α-(1 → 2)-linked disaccharides is the distance between H-1 and H-3 small enough for the corresponding NOE to appear.

Taking these data into account, one could conclude that unit **C** is attached to unit **A** at position 3 and the main chain of the polysaccharide has the following structure:

→ 3)-β-Rha*p*-(1 → 4)-α-Rha*p*-(1 → 3)-α-Fuc*p*-(1 →

A **B** **C**

where units **A** and **C** have different absolute configurations.

Theoretical conformational analysis showed that, in the preferred conformation of the model branched trisaccharide β-D-Glc*p*-(1 → 2) [α-L-Rha*p*-(1 → 3)]-β-D-Gal*p*, H-1 of the glucose residue and H-2 of the rhamnose residue are in close proximity, and, as a result, preirradiation of H-1 of the Glc residue causes comparable NOEs on H-2 of the galactose residue and H-2 of the rhamnose residue [6]. These data led to the conclusion that the NOE on H-2 of unit **C**, arising on preirradiation of H-1 of unit **D**, was that between the transglycosidic and anomeric protons, whereas the NOE on H-2 of unit **B** reflected the spatial

proximity of two protons of the nonbonded sugar residues. With the assumption that unit **D** was linked to unit **B** at position 2, the NOE on H-2 of unit **C** could not be accounted for. It is noteworthy that the spatial proximity of H-1 of unit **D** and H-2 of unit **B** requires [6] a definite combination of absolute configurations of units **B**, **C**, and **D**, which must be D, L, L or L, D, D, respectively.

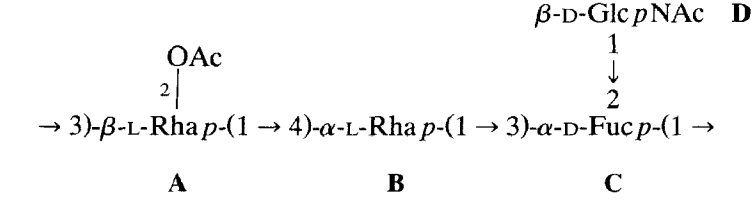
Therefore, some uncertainty in the use of NOE spectroscopy in linkage and sequence analysis, arising from the dependence of NOE on proton proximity rather than on linkage position, can be removed by application of conformational analysis. Moreover, such analysis reveals steric conditions that must be fulfilled in order for additional interresidue NOEs to be observed and thus provides a method for the determination of absolute configurations of sugar constituents by using NOE data.

The substitution pattern of the monosaccharides was confirmed independently by consideration of α-effects of glycosylation [7] on chemical shifts in the ^{13}C NMR spectrum of the polysaccharide. Relatively low-field positions of the signals for C-3 of unit **A** at 76.5 ppm, C-4 of unit **B** at 84.7 ppm, and C-2,3 of unit **C** at 76.7 and 76.7 ppm, respectively, as compared with those for the corresponding free monosaccharides [4,7,8], proved that the polysaccharide is branched, unit **C** is 2,3-disubstituted, units **A** and **B** are substituted at position 3 and 4, respectively, and unit **D** is the lateral sugar residue.

Absolute configurations of the monosaccharides were finally established by calculation of the value of the specific optical rotation by Klyne's rule [9] applied to the *O*-deacetylated polysaccharide. The calculation led to a value close to the experimental one ($[\alpha]_D$ +43° and +48°, respectively) only with the assumption that rhamnose was L and fucose and 2-acetamido-2-deoxyglucose were D. This conclusion accorded with the NOE data discussed above and the glycosylation effects on ^{13}C chemical shifts [10].

Location of the OAc group at C-2 of unit **A** was confirmed by comparison of the ^{13}C NMR spectra of the *O*-deacetylated and initial polysaccharides (Table 2). The displacements of the signals for C-1,2,3 of unit **A** from 101.7, 68.9, and 80.4 ppm to 100.8, 70.2, and 76.5 ppm, respectively, were due to the α- and β-effects of acetylation [11] of the β-rhamnose residue at HO-2.

The data obtained showed that the O-specific polysaccharide of *P. fluorescens* biovar A strain IMV 472 has the following structure:



This structure differs from that established for the O-specific polysaccharide of another strain of biovar A (IMV 1152), which was built up of 6-deoxy hexosamines only [1]. Thus, the same biovar of *P. fluorescens* may include different serological types.

1. Experimental

Optical rotations were measured with a Jasco DIP 360 polarimeter for solutions in water at 28°C.

^{1}H NMR spectra were recorded with a Bruker WM-250 instrument for solutions in D_2O at 74°C. ^{13}C NMR spectra were recorded with a Bruker AM-300 instrument for solutions in D_2O at 80°C. Acetone was used as an internal standard (δ_H 2.23 ppm, δ_C 31.45 ppm). Selective homonuclear spin decoupling, 1D NOE, 2D homonuclear ^{1}H/^{1}H COSY, and 2D heteronuclear ^{13}C/^{1}H COSY spectra were obtained as described earlier [12,13].

Growth of bacteria [14], and isolation of lipopolysaccharide [2] and O-specific polysaccharide [3] were performed as described.

O-Deacetylation. — The polysaccharide (30 mg) was treated with aq 15% ammonia (60°C, 3 h), and the solution was evaporated and freeze-dried to yield the *O*-deacetylated polysaccharide (25 mg).

Acknowledgments

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References

[1] Y.A. Knirel, N.A. Paramonov, A.S. Shashkov, N.K. Kochetkov, G.M. Zdorovenko, S.N. Veremeychenko, and I.Y. Zakharova, *Carbohydr. Res.*, 243 (1993) 205–210.
[2] O. Westphal and K. Jann, *Methods Carbohydr. Chem.*, 5 (1965) 83–91.
[3] Y.A. Knirel, G.M. Zdorovenko, V.M. Dashunin, L.M. Yakovleva, A.S. Shashkov, I.Y. Zakharova, R.I. Gvozdyak, and N.K. Kochetkov, *Bioorg. Khim*, 12 (1986) 1253–1262.
[4] K. Bock and C. Pedersen, *Adv. Carbohydr. Chem. Biochem.*, 41 (1983) 27–65.
[5] G.M. Lipkind, A.S. Shashkov, S.S. Mamyan, and N.K. Kochetkov, *Carbohydr. Res.*, 181 (1988) 1–12.
[6] G.M. Lipkind, A.S. Shashkov, O.A. Nechaev, V.I. Torgov, V.N. Shibaev, and N.K. Kochetkov, *Carbohydr. Res.*, 195 (1989) 27–37.
[7] G.M. Lipkind, A.S. Shashkov, Y.A. Knirel, E.V. Vinogradov, and N.K. Kochetkov, *Carbohydr. Res.*, 175 (1988) 59–75.
[8] P.-E. Jansson, L. Kenne, and G. Widmalm, *Carbohydr. Res.*, 188 (1989) 169–191.
[9] W. Klyne, *Biochem. J.*, 46 (1950) xxii–xxiii.
[10] A.S. Shashkov, G.M. Lipkind, Y.A. Knirel, and N.K. Kochetkov, *Magn. Reson. Chem.*, 26 (1988) 735–747.
[11] P.-E. Jansson, L. Kenne, and E. Schweda, *J. Chem. Soc., Perkin Trans. 2*, (1987) 377–383.
[12] N.A. Kocharova, Y.A. Knirel, A.S. Shashkov, N.K. Kochetkov, and G. Pier, *J. Biol. Chem.*, 264 (1989) 15569–15573.
[13] E.V. Vinogradov, Z. Sidorczyk, A. Swierzko, A. Rozalski, E.D. Daeva, A.S. Shashkov, Y.A. Knirel, and N.K. Kochetkov, *Eur. J. Biochem.*, 197 (1991) 93–103.
[14] G.M. Zdorovenko, S.N. Veremeychenko, and I.Y. Zakharova, *Microbiol. Zh.*, 49 (1987) 12–17.