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# Somatic antigens of pseudomonads: Structure of the O-specific polysaccharide of *Pseudomonas fluorescens* biovar B, strain IMV 247

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**Abstract**

The O-specific polysaccharide of *Pseudomonas fluorescens* biovar B, strain IMV 247, was studied by acid hydrolysis, GLC–MS and $^{1}$H and $^{13}$C NMR spectroscopy, including 1D and 2D NOE, 2D hybrid TOCSY and ROESY (TORO), and 2D H-detected heteronuclear multiple-bond correlation (HMBC) experiments. The polysaccharide was found to contain L-rhamnose, 3,6-dideoxy-3-[(*S*)-3-hydroxybutyramido]-D-glucose (D-Qui3NHb), 2-acetamido-2,4,6-trideoxy-4-[(*S*)-3-hydroxybutyramido-D-glucose (D-QuiNAc4NHb) and 2-acetamido-2-deoxy-D-galacturonic acid (D-GalNAcA). Partial acid hydrolysis of the polysaccharide resulted in a non-reducing GalNAcA → QuiNAc4NHb disaccharide with the 3-hydroxybutyryl group glycosylated intramolecularly by the QuiN4N residue. The following structure of the tetrasaccharide repeating unit of the polysaccharide was established: → 4)-α-D-Gal*p*NAcA-(1 → 3)-α-D-Qui *p*NAc4NHb-(1 → 2)-β-D-Qui *p*3NHb-(1 → 2)-α-L-Rha *p*-(1 → . 

*Keywords:* *Pseudomonas fluorescens*; Bacterial polysaccharide; Lipopolysaccharide; (*S*)-3-Hydroxybutyrate; 3-Amino-3,6-dideoxy-D-glucose; 2,4-Diamino-2,4,6-trideoxy-D-glucose; 2-Amino-2-deoxy-D-galacturonic acid

## 1. Introduction

Strains of *Pseudomonas fluorescens* are divided into five biovars having undefined taxonomic rank [1]. Gram negative bacteria are commonly classified on the basis of serological specificity of O-antigens (polysaccharide chains of lipopolysaccharides), however, no such classification has been elaborated for *P. fluorescens*. Recently, we have found that the O-specific polysaccharides of three strains of *P. fluo-*

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*rescens* belonging to the same biovar A have quite different structures [2–4]. Now we report on the structure of the O-specific polysaccharide of *P. fluorescens* strain IMV 247, which belongs to biovar B.

## 2. Results and discussion

The O-specific polysaccharide was obtained by mild acid degradation of the lipopolysaccharide of *P. fluorescens* strain IMV 247 followed by GPC.

Acid hydrolysis of the polysaccharide followed by GLC–MS of the derived alditol acetates [5] revealed rhamnose, 3-amino-3,6-dideoxyglucose (Qui3N), 3,6-dideoxy-3-(3-hydroxybutyramido)glucose (Qui3NHb), and a disaccharide Qui3N → Rha. It was not excluded that Qui3N and Qui3N → Rha partially occurred in the hydrolysate in the *N*-acetylated form. Qui3NHb was identified by comparison of the retention time and the mass spectrum with those for the authentic sample from the O-specific polysaccharide of *Hafnia alvei* strain 1216 [6]. The partial disaccharide structure was suggested based on the MS data which were interpreted taking into account the identification of Rha and Qui3N as monosaccharides in the polysaccharide hydrolysate and using published data on the MS fragmentation pathways for acetylated disaccharides [7]. GLC of acetylated (*R*)-2-octyl glycosides [8] showed that rhamnose has the L and Qui3N the D configuration, and GLC of trifluoroacetylated (*R*)-2-octyl esters by the modified method [9] demonstrated (*S*)-3-hydroxybutyrate.

Partial acid hydrolysis of the polysaccharide resulted in a mixture of monosaccharides and oligosaccharides, from which Qui3NHb and an acidic disaccharide (**1**) were isolated in the pure state by HPLC on reversed phase C18 as the only compounds containing the 3-hydroxybutyryl group. The structure of Qui3NHb was established by $^1$H NMR data (Table 1), and the 3-hydroxybutyryl group was confirmed to have the (*S*) configuration. Disaccharide **1** was found to be composed of the two other monosaccharide constituents of the polysaccharide: 2-amino-2-deoxygalacturonic acid (GalNA) and 2,4-diamino-2,4,6-trideoxyglucose (bacillosamine, QuiN4N). The absence of these monosaccharides from the hydrolysate of the polysaccharide was evidently accounted for by the acid resistance of disaccharide **1** under both mild and strong hydrolysis conditions. The elucidation of the structure of **1** as an intramolecular glycoside is described below.

The $^{13}$C NMR spectrum of the polysaccharide (Fig. 1; Table 2) was typical of a regular polymer having a tetrasaccharide repeating unit. It contained signals for four anomeric carbons at 96.1–105.8 ppm, four carbons bearing nitrogen at 50.6–57.9 ppm, three $CH_3$–C groups of 6-deoxyhexoses (C-6) at 17.7–18.1 ppm, one COOH group (C-6) of an uronic acid at 174.0 ppm, and 12 other sugar carbons in the region 68.5–81.1 ppm. In addition, signals for two *N*-acetyl and two *N*-(3-hydroxybutyryl) groups were present ($CH_3$ and CO of both groups at 23.7–23.9 and 174.9–175.6 ppm, respectively; CH–OH and $CH_2$ of the 3-hydroxybutyryl groups at 66.0 and 46.1–46.3 ppm, respectively). The absence of signals from the region 82–88 ppm characteristic for furanosides [10] showed that all four monosaccharide residues are in the pyranosidic form.

The $^1$H NMR spectrum of the polysaccharide (Table 1) contained, inter alia, signals for four anomeric protons at 4.66 (d, $J_{1,2}$ 7.5 Hz), 5.12 (d, $J_{1,2}$ 3.5 Hz), 5.39 (s, $J_{1,2} < 2$ Hz), and 5.57 ppm (d, $J_{1,2}$ 3.5 Hz),

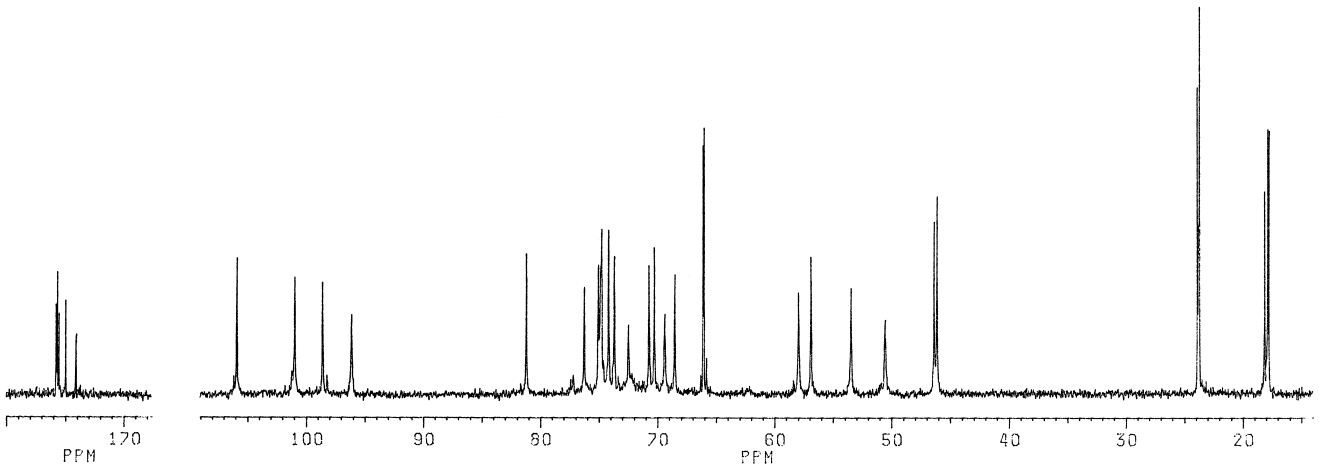



Fig. 1. 75 MHz $^{13}$C NMR spectrum of the O-specific polysaccharide.

Table 1
$^1$H NMR data ($\delta$ in ppm, $J$ in Hz). (*S*)-Hb, 3-hydroxybutyryl group

| Monosaccharide residue | Sugar | | | | | | Ac | Hb | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | H-1 | H-2 | H-3 | H-4 | H-5 | H-6 | H-2 | H-2a | H-2b | H-3 | H-4 |
| $\alpha$-D-Qui *p*3NHb | 5.19 | 3.61 | 4.04 | 3.17 | 3.94 | 1.24 | | 2.31 | 2.73 | 4.23 | 1.28 |
| | $J_{1,2}$ 3.5 | $J_{2,3}$ 10 | $J_{3,4}$ 10 | $J_{4,5}$ 10 | $J_{5,6}$ 6 | | | $J_{2a,3}$ 8 | $J_{2a,2b}$ 15 | $J_{2b,3}$ 5 | $J_{3,4}$ 6 |
| $\beta$-D-Qui *p*3NHb | 4.69 | 3.29 | 3.83 | 3.19 | 3.57 | 1.28 | | 2.31 | 2.73 | 4.23 | 1.28 |
| | $J_{1,2}$ 8 | $J_{2,3}$ 10 | $J_{3,4}$ 10 | $J_{4,5}$ 10 | $J_{5,6}$ 6 | | | $J_{2a,3}$ 8 | $J_{2a,2b}$ 15 | $J_{2b,3}$ 5 | $J_{3,4}$ 6 |
| Disaccharide **1** | | | | | | | | | | | |
| $\alpha$-D-Gal*p*NAcA-(1 → | 5.17 | 4.19 | 3.95 | 4.34 | 4.40 | | 2.05[a] | | | | |
| | $J_{1,2}$ 3.5 | $J_{2,3}$ 10 | $J_{3,4}$ 3 | $J_{4,5} < 2$ | | | | | | | |
| → 3)-$\alpha$-D-Qui *p*NAc4NHb-(1 → | 5.29 | 4.68 | 4.33 | 4.47 | 4.15 | 1.24 | 2.00[a] | 2.31 | 2.73 | 4.23 | 1.28 |
| | $J_{1,2}$ 6.5 | $J_{2,3}$ 7 | $J_{3,4}$ 7 | $J_{4,5}$ 4.5 | $J_{5,6}$ 6.5 | | | $J_{2a,3}$ 9 | $J_{2a,2b}$ 17.5 | $J_{2b,3}$ 5 | $J_{3,4}$ 6 |
| O-specific polysaccharide **2** | | | | | | | | | | | |
| → 4)-$\alpha$-D-Gal*p*NAcA-(1 → | 5.12 | 4.16 | 3.80 | 4.26 | 4.06 | | 1.96 | | | | |
| | $J_{1,2}$ 3.5 | $J_{2,3}$ 11 | $J_{3,4}$ 3 | $J_{4,5} < 2$ | | | | | | | |
| → 3)-$\alpha$-D-Qui *p*NAc4NHb-(1 → | 5.57 | 3.99 | 3.72 | 3.75 | 3.62 | 1.10 | 1.93 | 2.27 | 2.21 | 4.11 | 1.16 |
| | $J_{1,2}$ 3.5 | $J_{2,3}$ 9 | $J_{3,4}$ 9 | $J_{4,5}$ 9 | $J_{5,6}$ 6 | | | $J_{2a,3}$ 8 | $J_{2a,2b}$ 15 | $J_{2b,3}$ 5 | $J_{3,4}$ 6 |
| → 2)-$\beta$-D-Qui *p*3NHb-(1 → | 4.66 | 3.38 | 3.86 | 3.07 | 3.39 | 1.14 | | 2.42 | 2.34 | 4.21 | 1.19 |
| | $J_{1,2}$ 7.5 | $J_{2,3}$ 9.5 | $J_{3,4}$ 9.5 | $J_{4,5}$ 9.5 | $J_{5,6}$ 6 | | | $J_{2a,3}$ 8 | $J_{2a,2b}$ 15 | $J_{2b,3}$ 5 | $J_{3,4}$ 6 |
| → 2)-$\alpha$-L-Rha*p*-(1 → | 5.39 | 3.96 | 3.72 | 3.20 | 3.55 | 1.14 | | | | | |
| | $J_{1,2} < 2$ | $J_{2,3}$ 3.5 | $J_{3,4}$ 9 | $J_{4,5}$ 9 | $J_{5,6}$ 6 | | | | | | |

[a]Assignment could be interchanged.

Table 2
75 MHz $^{13}C$ NMR chemical shifts ($\delta$ in ppm). Hb, (*S*)-3-hydroxybutyryl group

| Monosaccharide residue | Sugar | | | | | | Ac | | Hb | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | C-1 | C-2 | C-3 | C-4 | C-5 | C-6 | C-1 | C-2 | C-1 | C-2 | C-3 | C-4 |
| Disaccharide **1** | | | | | | | | | | | | |
| α-D-Gal*p*NAcA-(1 → | 99.6 | 51.0 | 68.7 | 70.9 | 72.9 | [a] | [a] | 23.5[b] | | | | |
| → 3)-α-D-Qui *p*NAc4NHb-(1 → | 85.3 | 56.3 | 80.6 | 61.9 | 68.0 | 20.4 | [a] | 23.3[b] | [a] | 39.2 | 72.9 | 22.0 |
| O-specific polysaccharide **2** | | | | | | | | | | | | |
| → 4)-α-D-Gal*p*NAcA-(1 → | 98.6 | 50.6 | 69.4 | 76.2 | 72.4 | 174.0 | 175.7 | 23.7 | | | | |
| → 3)-α-D-Qui *p*NAc4NHb-(1 → | 96.1 | 53.5 | 74.7 | 57.9 | 68.5 | 17.8 | 175.4 | 23.7 | 174.9 | 46.3 | 66.0 | 23.7 |
| → 2)-β-D-Qui *p*3NHb-(1 → | 105.8 | 75.1 | 56.9 | 74.7 | 74.1 | 17.7 | | | 175.6 | 46.1 | 66.0 | 23.7 |
| → 2)-α-L-Rha*p*-(1 → | 100.9 | 81.1 | 70.7 | 73.7 | 70.2 | 18.1 | | | | | | |

[a]Not found.
[b]Assignment could be interchanged.

five $CH_3$–C groups of three 6-deoxyhexoses and two *N*-(3-hydroxybutyryl) groups at 1.10–1.19 ppm (all d, *J* 6 Hz), two $CH_2$ groups at 2.21, 2.27 (both m), 2.34, and 2.42 ppm (both dd), and two *N*-acetyl groups at 1.93 and 1.96 ppm (both s).

The $^1H$ NMR spectrum was assigned using se-

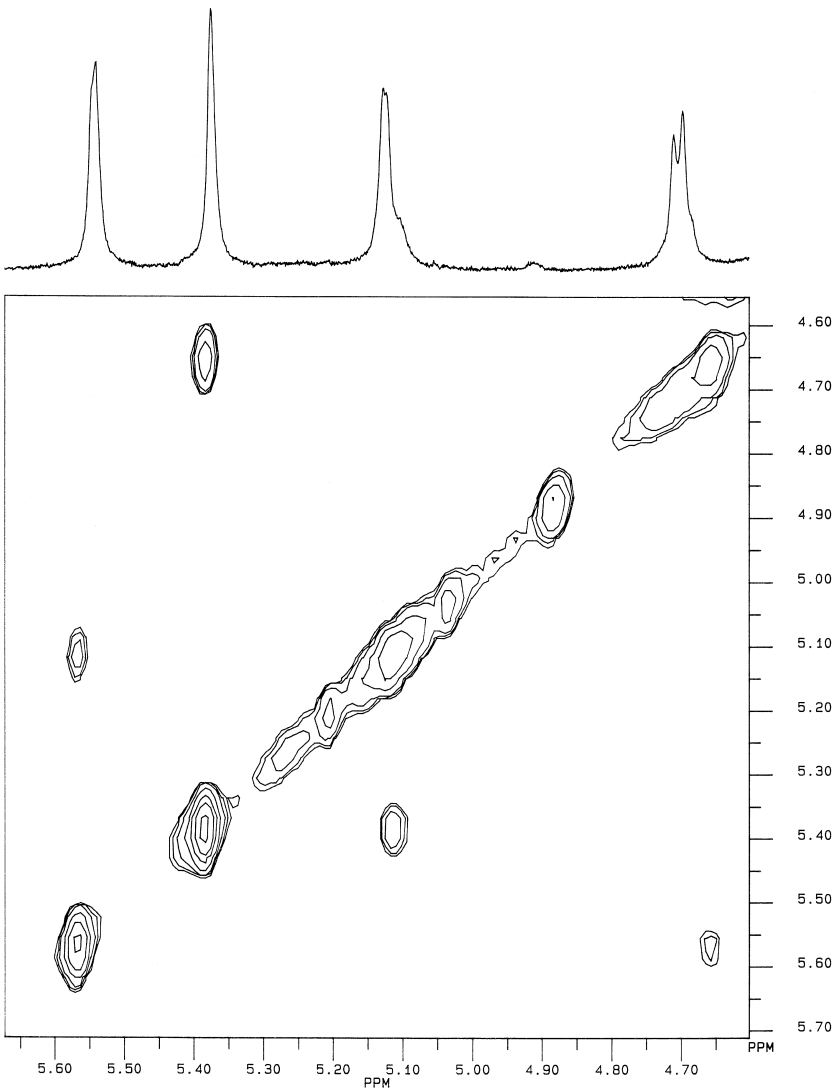



Fig. 2. Part of a 500-MHz 2D hybrid TOCSY–ROESY (TORO) spectrum of the O-specific polysaccharide. The corresponding part of the $^1H$ NMR spectrum is displayed along the horizontal axis.

quential, selective spin-decoupling [11], 2D correlation spectroscopy (COSY), and COSY with one- and two-step relayed coherence transfer. With the $^{1}H$ NMR spectrum assigned, the $^{13}C$ NMR spectrum of the polysaccharide was interpreted using a heteronuclear $^{13}C$, $^{1}H$ COSY experiment.

On the basis of the $^{1}H$ and $^{13}C$ chemical shifts (Tables 1 and 2) and the coupling constant values (Table 1), four sugar spin systems were assigned to residues of α-Rha, α-GalNA, α-QuiN4N, and β-Qui3N. Four amino groups of the amino sugars are acylated by two acetyl groups and two 3-hydroxybutyryl groups. Identification of both Qui3NAc and Qui3NHb in the hydrolysate of the polysaccharide seems to result from partial removal during acid hydrolysis of the *N*-(3-hydroxybutyryl) group from Qui3NHb followed by *N*-acetylation.

In 1D NOE experiments with sequential, selective pre-irradiation of H-1 of all sugar residues, together with intraresidue NOEs on H-2 of the α-linked monosaccharides and H-3,5 of the β-linked Qui3N, the following interresidue NOEs between transglycosidic protons were observed: Rha H-1, GalNA H-4; GalNA H-1, QuiN4N H-3; QuiN4N H-1, Qui3N H-2; Qui3N H-1, Rha H-2. In accordance with the (1 → 2)-linkages, correlations between two pairs of the anomeric protons H-1 QuiN4N, H-1 Qui3N (weak) and H-1 Qui3N, H-1 Rha were revealed. Two additional interresidue cross-peaks QuiN4N H-1, Rha H-3 (weak) and QuiN4N H-1, Rha H-4 (medium) were present and interpreted as a result of spatial proximity of the non-bonded monosaccharide residues; the latter correlation is the expected one for the predominant conformer [12] of the fragment α-QuiN4N-(1 → 2)β-Qui3N-(1 → 2)-Rha, provided that QuiN4N and Qui3N have the same absolute configuration.

A 2D rotating-frame NOE spectroscopy (ROESY) experiment confirmed the 1D NOE data and revealed some additional interresidue correlations. One of them, between QuiN4N H-5 and Qui3N H-2, is typical of α-(1 → 2)-linked disaccharides. Another correlation, between QuiN4N H-6 and Hb H-3 at δ 1.10/4.11, suggested that the second 3-hydroxybutyryl group is attached at N-4 of QuiN4N.

The linear character of the polysaccharide and the monosaccharide sequence was confirmed by use of a 2D hybrid experiment combining TOCSY and ROESY (TORO [13]). The correlations between the anomeric protons shown in Fig. 2 appeared as a result of stepwise transfer of H-1 magnetization by the TOCSY mechanism to other protons of the same sugar residue and by the ROESY mechanism to H-1 of the glycosylating sugar residue. Thus, the NOE, ROESY and TORO data allowed determination of the full monosaccharide sequence in the repeating unit of the polysaccharide which is shown below.

Relatively low-field positions of the signals for Rha C-2, GalNA C-4, and QuiN4N C-3 at 81.1, 76.2, and 74.7 ppm, respectively, as compared with their positions in the spectra of the corresponding free monosaccharides [10,14,15], were due to the α-effects of glycosylation (> 5 ppm), and confirmed the substitution pattern of the sugar residues determined by the NOE experiments. An unexpectedly small α-effect of glycosylation (1 ppm) on Qui3N C-2 may be accounted for by the strong spatial interaction of H-2 Qui3N and H-5 QuiN4N, which was revealed by the ROESY experiment (see above) and resulted in an upfield shift of the signal for Qui3N C-2 [16].

The absolute configurations of GalNA and QuiN4N, which were not released by acid hydrolysis, were determined using the known regularities in α- and β-effects of glycosylation on $^{13}C$ chemical shifts [16–18]. A relatively small effect (+4.3 ppm) on QuiN4N C-1 in the disaccharide fragment α-QuiN4N-(1 → 2)-Qui3N indicated the same absolute configurations of QuiN4N and D-Qui3N, i.e., the D configuration of QuiN4N, since in the case of their different absolute configurations, the effect on C-1 would be significantly larger (about 7 ppm [18]). A relatively large β-effect on C-2 (−2.2 ppm) and a relatively small β-effect on C-4 (+0.4 ppm) of QuiN4N in the disaccharide fragment α-GalNA-(1 → 3)-QuiN4N were characteristic for the same absolute configurations of GalNA and D-QuiN4N, i.e., for the D configuration of GalNA (in the case of the different absolute configurations the effects on C-2 and C-4 would be about +0.3 and −1.7 ppm, respectively [14]).

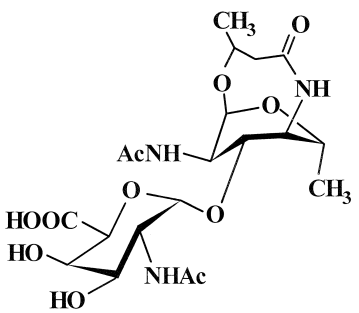
The sites of attachment of the 3-hydroxybutyryl groups were finally confirmed using 2D H-detected heteronuclear multiple-bond correlation (HMBC). In the HMBC spectrum, there were present cross-peaks of C-1 of the 3-hydroxybutyryl groups with Qui3N H-3 and QuiN4N H-4 at $\delta_C/\delta_H$ 175.6/3.86 and 174.9/3.75, respectively, that pointed to the location of these groups at Qui3N N-3 and QuiN4N N-4. Hence, the acetyl groups are attached to N-2 of GalNA and QuiN4N.

On the basis of these data, it was concluded that the O-specific polysaccharide of *P. fluorescens* biovar B, strain 247 has the following structure: → 4)-α-D-Gal*p*NAcA-(1 → 3)-α-D-Qui*p*NAc4NHb-(1 → 2)-β-D-Qui*p*3NHb-(1 → 2)-α-L-Rha*p*-(1 → .

The O-specific polysaccharide has a unique structure different from those of other studied *P. fluorescens* strains [2–4,19,20]. The three amino sugars present are uncommon in nature and have been found previously in a few bacterial polysaccharides, particularly in O-antigens of some other pseudomonads [19–22]. Noteworthy is also the occurrence in the repeating unit of the polysaccharide of two 3-hydroxybutyryl groups, both having the (*S*) configuration.

The $^{1}H$ and $^{13}C$ NMR spectra of the disaccharide **1** showed that it contains GalNA, QuiN4N, two *N*-acetyl groups and one *N*-(3-hydroxybutyryl) group (Tables 1 and 2). The spectra were assigned using 2D COSY and H-detected $^{1}H$, $^{13}C$ heteronuclear multiple-quantum coherence (HMQC) experiments. The coupling constant values for GalNA fitted well with the *galacto* configuration, while those for QuiN4N were far from those expected for the *gluco* configuration [23], and the $^{1}H$ and $^{13}C$ chemical shifts for this residue differed significantly from the corresponding data for QuiNAc4NHb in the spectra of the polysaccharide (Tables 1 and 2). Only one series of the signals was present for both monosaccharide residues in the spectra of **1** and, thus, this disaccharide has no free reducing end. Therefore, it was suggested that **1** is an intramolecular bicyclic glycoside of the 3-hydroxybutyryl group, and QuiN4N in **1** exists in a form different from $^{4}C_{1}$. This was confirmed by a 2D NOESY experiment which, together with an interresidue correlation GalNA H-1, QuiN4N H-3 at $\delta$ 5.17/4.33, revealed a correlation between QuiN4N H-1 and Hb H-3 at $\delta$ 5.29/4.23. Significant displacements to $\delta$ 39.2, 72.9 and 20.4 of the signals for the 3-hydroxybutyryl group in **1**, as compared with their positions at $\delta$ 46.3, 66.0 and 23.7, respectively, in the spectrum of the polysaccharide (Table 2), were in accord with the glycosylation of this group at O-3 as well.

Therefore, the disaccharide **1** has the following structure:




**1**

Formation of an intramolecular glycoside of the sort has been reported previously for an *N*-(3-hydroxybutyryl) derivative of QuiN4N in a disaccharide and a trisaccharide from the O-specific polysaccharide of *P. aeruginosa* IID 1001 (ATCC 27577), for which the structure of QuiNHb4NAc has been proposed [24]. Surprisingly, the NMR data reported for the bicyclic derivatives of this sugar [24] and QuiNAc4NHb in **1** are similar and may indicate that QuiNHb4NAc in the *P. aeruginosa* IID 1001 polysaccharide has been identified erroneously and, in fact, this antigen contains QuiNAc4NHb. In particular, glycosylation of the 3-hydroxybutyryl group at N-2 should not cause such strong distortion of the $^{4}C_{1}$ conformation in QuiN4N as followed from the dramatic changes in the $^{13}C$ chemical shifts and the coupling constant values. Therefore, the disaccharide from the *P. aeruginosa* IID 1001 polysaccharide seems to be a diastereomer of disaccharide **1** differing in the absolute configuration of GalNAcA only. The presence of QuiNAc4NHb has been reported in the O-specific polysaccharides of a number of *P. aeruginosa* strains serologically related to IID 1001 [14,22].

## 3. Experimental

*P. fluorescens* strain IMV 247 was from the collection of the Institute of Microbiology and Virology, Academy of Sciences of Ukraine. Growth of the bacterium [25], isolation of lipopolysaccharide [26], and isolation of O-specific polysaccharide [27] were performed as described.

The polysaccharide (1 mg) was hydrolyzed with 2 M $CF_3COOH$ at 120 °C for 2 h, monosaccharides were conventionally converted into alditol acetates [5] and analyzed by GLC–MS using a Hewlett-Packard Model 7985 instrument operating at 70 eV and equipped with a capillary column of cross-linked SPBTM-5. The mass-spectral data for 3-*O*-(3-acetamido-2,4-di-*O*-acetyl-3,6-dideoxy-$\beta$-D-glucopyranosyl)-1,2,4,5-tetra-*O*-acetyl-L-rhamnitol (*m/z*) are: 485, 442, 317, 303, 272, 243, 215, 200, 184, 170, 153, 144, 143, 140, 111, 101, 95, 83, 69.

The absolute configurations of Rha and Qui3N were determined by GLC of acetylated (*R*)-2-octyl glycosides as described [8]. For determination of the absolute configuration of 3-hydroxybutyric acid, the polysaccharide, Qui3NHb or disaccharide **1** (2–3 mg of each) were hydrolyzed with 2 M $CF_3COOH$ as in the sugar analysis, the hydrolysate was concentrated

to dryness, the residue co-concentrated thrice with water, heated with (*R*)-2-octanol (0.2 mL) in the presence of $CF_3COOH$ (20 $\mu$L) for 8 h at 120 °C, concentrated as above, trifluoroacetylated with $(CF_3CO)_2O$ (0.2 mL) for 16 h at 4 °C and analyzed by GLC using authentic (*R*)-2-octyl (*R*)- and (*S*)-3-trifluoroacetoxybutyrates as references.

Partial hydrolysis of the polysaccharide (30 mg) was performed with 10 M HCl at 80 °C for 0.5 h, products were fractionated by GPC on a column ($1.6 \times 80$ cm) of TSK HW-40 (*S*) in water followed by HPLC on a column ($7.1 \times 250$ mm) of reversed phase C18 using aqueous 0.04% $CF_3COOH$ as eluent and monitoring with a Knauer differential refractometer to give Qui3NHb (5.2 mg) and disaccharide **1** (3.4 mg).

$^1H$ and $^{13}C$ NMR spectra were obtained with Bruker AM-300, AM-500 and DRX-500 instruments in $D_2O$ at 50 or 60 °C. Acetone was used as internal standard ($\delta_H$ 2.225, $\delta_C$ 31.45). Selective spin-decoupling, 1D NOE (in the difference mode), and 2D NMR experiments were performed using standard Bruker software as described [11,28].

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