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Structure of the capsular polysaccharide from *Alteromonas sp.* CMM 155

Vladimir A. Zubkov [a], Evgeny L. Nazarenko [a,*], Raisa P. Gorshkova [a], Elena P. Ivanova [a], Alexander S. Shashkov [b], Yury A. Knirel [b], Nicolai A. Paramonov [b], Yury S. Ovodov [a]

[a] *Pacific Institute of Bioorganic Chemistry, Far East Branch of the Russian Academy of Sciences, Vladivostok, Russian Federation*

[b] *N.D. Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, Moscow, Russian Federation*

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Abstract

Capsular polysaccharide (CPS) was obtained by water-saline extraction of the *Alteromonas sp.* CMM 155. On the basis of solvolysis with anhydrous HF and ^{1}H- and ^{13}C-NMR spectral data, including NOE experiments, it was concluded that the capsular polysaccharide had the following structure containing novel *N*-acyl-amino sugar and bacillosamine residues:

→ 3)-α-D-Gal*p*NAc-(1 → 4)-α-L-GalA*p*NAc-(1 → 3)-α-D-Qui*p*NAc4NAc-(1 → 3)-

β-D-Qui*p*4NAlaAc-(1 →

Keywords: *Alteromonas*; Capsular polysaccharide; Oligosaccharide; NMR-spectroscopy

1. Introduction

The structures of the capsular polysaccharides from three strains of *Alteromonas sp.* have been established earlier [1–3]. In the present paper, we report the results of the structural analysis of the capsular polysaccharide from *Alteromonas sp.* CMM 155 from the Collection of Marine Microorganisms (CMM) which is at the disposal of our Institute.

* Corresponding author.

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2. Results and discussions

CPS was extracted from the microorganisms with 0.9% aqueous saline using sonication, and purified by ion-exchange chromatography on DEAE TSK 650 M.

Analysis of the sugar composition of CPS by paper chromatography (PC) indicated the presence of five ninhydrin-positive components. Two of them were identified as alanine and galactosamine by PC and electrophoresis. The latter was isolated by preparative PC and the $[\alpha]_D$ value of its hydrochloride (+72°) indicated the D-configuration, see ref. [4], $[\alpha]_D$ + 91.5°. The D-configuration of alanine was determined using GC of the acetylated (*S*)-(+)-2-butyl ester [5].

The ^{13}C-NMR spectrum of CPS (Fig. 1, Table 1) indicated a tetrasaccharide repeating unit (signals of four anomeric carbons at 97.7, 98.4, 99.6 and 105.0 ppm). There were also present characteristic signals of three CH_3-groups (6-deoxysugars and alanine) at 18.1, 18.2 and 17.7 ppm, 5 signals of *N*-acetyl groups in the region of 23.1–23.8 ppm, 5 carbons bearing nitrogen in the region of 50–58 ppm, one signal of a non-substituted CH_2OH-group at 62.4 ppm and 7 carbonyl groups in the region of 172–176 ppm. In addition, an acetylated alanine residue was identified from ^{1}H and ^{13}C-NMR spectra (see below) [6].

Hence, the tetrasaccharide repeating unit of CPS consisted of four amino sugar residues; one of them represents 2-acetamido-2-deoxy-D-galactose. Moreover, taking into account the number of the CH_3-groups and carbons bearing nitrogen, it may be proposed that two monosaccharides represent 6-deoxy sugars and, at least, one of the sugar residues represents diamino sugar.

Coupling constant values (173–174 Hz) for the signals at 97.7–99.6 ppm and 160 Hz for the signal at 105.0 ppm demonstrated [7] the pyranose form of each sugar; three of them possessed α-configuration and one was a β-glycosidic residue.

A complete assignment of the signals in the ^{1}H-NMR spectrum of CPS was achieved using homonuclear double resonance in a difference mode [8], 2D homonuclear shift-correlated spectroscopy (COSY) and one- and two-step relayed coherence transfer (COSYRCT) (Table 2). As a result, the chemical shifts [9] and $^3J_{H,H}$ values [10]

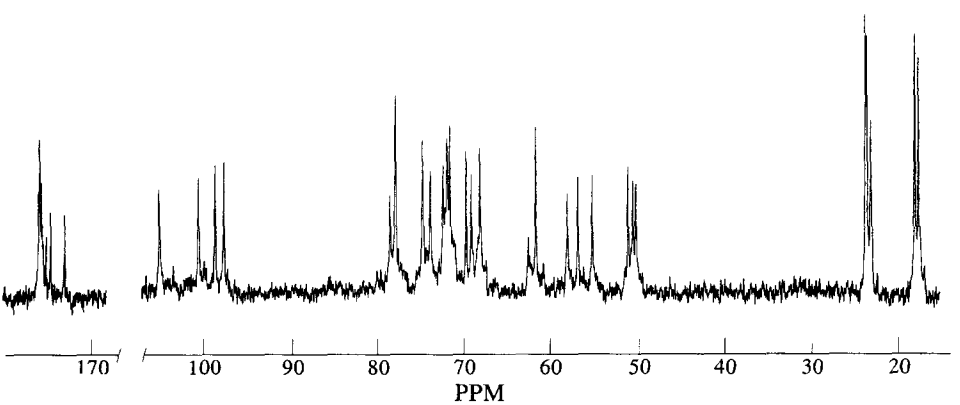


Fig. 1. ^{13}C-NMR spectrum of the CPS from *Alteromonas sp.* CMM 155.

Table 1
^{13}C-NMR data for the CPS and OS-I from *Alteromonas sp.* CMM 155 (δ, ppm), (glycosylation effects in ppm are in parentheses) [a]

Sugar residue	C-1	C-2	C-3	C-4	C-5	C-6
CPS						
A: → 3)-α-D-Gal*p*NAc-(1 →	99.6 [100.4] [b]	50.3	78.8 (10.0)	70.0 (0.1)	71.6	62.4
B: → 4)-α-L-GalA*p*NAc-(1 →	98.4 [98.5]	50.7 [50.4]	70.0 [69.0]	77.5 [77.8]	73.1 [71.8]	175.6 [172.9]
C: → 3)-α-D-Qui*p*NAc4NAc-(1 →	97.7	55.4 (−0.3)	74.7	56.8 (−1.5)	68.3	18.1
D: → 3)-β-D-Qui*p*4NA1aAc-(1 [c] →	105.0	74.1 (−1.0)	77.9	58.1 (0.7)	72.4	18.1
OS-I						
B: α-L-GalA*p*NAc-(1 →	98.3	50.3	68.9	71.1	71.1	175.7
α-L-GalA*p*NAc-(1 →	98.7	50.3	68.9	71.1	71.1	175.7
C: → 3)-α-D-Qui*p*NAc4NAc-(1 →	92.0	56.2 (0.4)	74.1	56.7 (−1.6)	67.6	18.2
→ 3)-β-D-Qui*p*NAc4NAc-(1 →	96.2	58.5 (0)	76.8	56.7 (−1.2)	72.1	18.2

[a] Additional signals assigned to NHAc: in the region of 23.1–23.7 ppm (CH_3) (5 signals for CPS) and 174.0–175.6 ppm (C=O) (5 signals for CPS).
[b] In square brackets the signals at pD 1 are given.
[c] The signals of the *N*-acetylalanine residue are: 174.4 ppm (C=O), 51.0 ppm (CH) and 17.7 ppm (CH_3).

confirmed the pyranose form of all sugar residues. Two sugar residues were determined to possess the *α-galacto*-configuration ($J_{1,2}$ 3.5, $J_{2,3}$ 10, $J_{3,4}$ 3, $J_{4,5}$ < 2 Hz); of two further sugars one had the *α-gluco*-configuration ($J_{1,2}$ 3.5 Hz, $J_{2,3} \sim J_{3,4} \sim J_{4,5}$ 9–10 Hz) and one the *β-gluco*-configuration ($J_{1,2}$ 8 Hz). Of the two sugars possessing the *galacto*-configuration, one represents 2-acetamido-2-deoxy-D-galactose [9]. In addition, H-2 and H-3 signals of the alanine residue at 4.22 and 1.27 ppm were observed in the ^{1}H-NMR spectrum with an integral intensity ratio of 1:3.

The ^{13}C-NMR spectrum of CPS was completely assigned using 2D heteronuclear shift-correlated multiquantum spectroscopy HMQC [11] (Table 1). As a result, the configurations of glycosidic linkages were confirmed and an assignment of each amino sugar was achieved. Units A and B were shown to represent 2-acetamido-2-deoxy-D-galactose and 2-acetamido-2-deoxygalacturonic acid, respectively [1]. This conclusion was confirmed by the analysis of a change of chemical shifts of C-5 and C-6 of unit B dependent on the pD of the CPS solution; acidification to pD 1 resulted in displacement of C-5 and C-6 signals of unit B from 73.1 and 175.6 ppm to 71.8 and 172.9 ppm, respectively. This phenomenon is typical of the uronic acids. The positions of other resonances were changed slightly (less than +0.3 ppm).

[1] The position and multiplicity of H-5 and H-6 of the unit A and H-5 of the unit B were determined from the heteronuclear HMQC spectrum.

Table 2
^{1}H-NMR data for CPS and OS-I from *Alteromonas sp.* KMM 155 (δ, ppm, J, Hz) [a]

Sugar residue	Proton	δ, ppm	J, Hz
CPS			
A: → 3)-α-D-Gal*p*NAc-(1 →	H-1	5.31	$J_{1,2}$ 3,5
	H-2	4,31	$J_{2,3}$ 10,0
	H-3	4.08	$J_{3,4}$ 3,0
	H-4	4.20	$J_{4,5}$ < 2
	H-5 [b]	4.04	
	H-6,6 [b]	3.72	
B: → 4)-α-L-GalA*p*NAc-(1 →	H-1	5.19	$J_{1,2}$ 3,5
	H-2	4.33	$J_{2,3}$ 10,0
	H-3	3.92	$J_{3,4}$ 3,0
	H-4	4.40	$J_{4,5}$ < 2
	H-5 [b]	4.10	
C: → 3)-α-D-Qui*p*NAc4NAc-(1 →	H-1	4.93	$J_{1,2}$ 3,5
	H-2	4.10	$J_{2,3}$ 10,0
	H-3	3.98	$J_{3,4}$ 10,0
	H-4	3.66	$J_{4,5}$ 10,0
	H-5	4.31	$J_{5,6}$ 6,5
	H-6	1.13	
D: → 3)-β-D-Qui*p*4NAlaAc-(1 →	H-1	4.55	$J_{1,2}$ 8,0
	H-2	3.41	$J_{2,3}$ 9,0
	H-3	3.80	$J_{3,4}$ 9,0
	H-4	3.72 [c]	$J_{4,5}$ 9,0
	H-5	3.55	$J_{5,6}$ 6,5
	H-6	1.15	
OS-I			
B: L-GalA*p*NAc-(1 →	H-1	5.20(α)	$J_{1,2}$ 3,5
		5.18(β)	
	H-2	4.20(α)	$J_{2,3}$ 10,0
		4.19(β)	
	H-3	3.80	$J_{3,4}$ 3,0
	H-4	4.30	$J_{4,5}$ < 2
	H-5	4.14	
C: → 3)-α-D-Qui*p*NAc4NAc-(1 →	H-1	5.06	$J_{1,2}$ 3,5
	H-2	4.11	$J_{2,3}$ 10,0
	H-3	3.98	$J_{3,4}$ 10,0
	H-4	3.69	$J_{4,5}$ 10,0
	H-5	4.03	$J_{5,6}$ 6,0
	H-6	1.17	
C: → 3)-β-D-Qui*p*NAc4Ac-(1 →	H-1	4.62	$J_{1,2}$ 8,0
	H-2	3.89	$J_{2,3}$ 10,0
	H-3	3.84	$J_{3,4}$ 10,0
	H-4	3.69	$J_{4,5}$ 10,0
	H-5	3.60	$J_{5,6}$ 6,0
	H-6	1.20	

[a] Additional signals of acetamidoCH_3 in the region of 1.96–2.06 ppm.
[b] Data from heteronuclear HMQC spectrum.
[c] Additional signals of the *N*-acetylalanine residue: 4.22 ppm (H-2, quartet, ^3J 7.0 Hz, 1 H), 1.27 ppm (H-3, doublet, 3 H).

Table 3
NOE data for the CPS from *Alteromonas sp.* CMM 155. The sugar units are designated with capital letters according to the formulae; the numbers refer to protons in the sugar rings

Observed NOE	Irradiated anomeric protons			
	1A	1B	1C	1D
2A	+			
3A				+
2B		+		
4B	+			
2C			+	
3C		+		
2D				+
3D			+	+
5D				+
3D' [a]			+	

[a] CH_3 of *N*-acetylalanine residue

Also, from the results of complete assignment of the resonances in ^{1}H- and ^{13}C-NMR spectra it was established that the 6-deoxy-diamino sugar (unit C) possessing the *α-gluco*-configuration represented 2,4-diamino-2,4,6-trideoxyglucose (bacillosamine) and the 6-deoxy-amino-hexose having *β-gluco*-configuration (unit D) appeared to be 4-amino-4,6-dideoxy-β-glucose (Table 1).

Linkage and sequence analysis of CPS and location of the alanine residue were carried out using a 1D NOE experiment (Table 3). On the successive pre-irradiation of anomeric protons of the GalNAc residue (unit A) at 5.31 ppm and the acetamido uronic acid (unit B) at 5.19 ppm, a significant NOE was observed from the H-4 of unit B at 4.40 ppm and the H-3 of unit C at 3.98 ppm, thus demonstrating the sequential linkages of units as A-(1 → 4)-B-(1 → 3)-C. A sequential saturation of H-1 of units C and D at 4.93 and 4.55 ppm gave NOEs to H-3A (4.08 ppm), H-3D (3.80 ppm) and H-5D (3.55 ppm) indicating the substitution of units A and D at position 3 and confirming β-configuration of unit D.

On irradiation of the anomeric proton of the 2,4-diacetamido-2,4,6-trideoxyglucose residue, a significant NOE was observed on protons of the CH_3-group of the alanine residue (1.27 ppm). Such an effect is possible only in the case of *N*-acylation of an NH_2-group at C-4 by an *N*-acetylalanine residue (CH_3-group of alanine is in close proximity with H-1 of unit C).

These data and the results of the analysis of the NMR spectral data of OS-I (see below) prove that units C and D are 2,4-diacetamido-2,4,6-trideoxy-α-glucose and 4-(*N*-acetylalanyl)amino-4,6-dideoxy-β-glucose, respectively.

Analysis of glycosylation effects obtained from assignment of ^{13}C resonances (Table 1) allowed determination of the absolute configurations of the sugar residues in the repeating unit [12]. Thus, values for the α- and β-glycosylation effect for C-3 and C-4 of the unit A substituted by unit D at C-3 (+10 and +0.1 ppm, respectively) indicated the same absolute (D-) configuration of these residues (the D-configuration of the *N*-acetylgalactosamine residue was determined on the basis of the $[\alpha]_D$ value).

The D-configuration of unit C was estimated from the β-effect of glycosylation values in the unit D (negative for C-2 and positive for C-4). The different absolute configurations of units B and C followed from the negative (-1.5 ppm) β-effect of glycosylation on C-4 of the bacillosamine residue and, hence, the galactosamine uronic acid possessed the L-configuration.

Solvolysis of CPS with anhydrous hydrogen fluoride [13] (20°C, 2 h) afforded oligosaccharide OS-I isolated by gel-permeation chromatography (Scheme 1). A comparative analysis of ^{1}H- and ^{13}C-NMR spectra of CPS and OS-I (Tables 1 and 2) demonstrated that OS-I is a disaccharide containing L-galactosamine uronic acid and di-*N*-acetylbacillosamine residues. This oligosaccharide has an identical ^{13}C-NMR spectrum with disaccharide obtained on HF-solvolysis of the O-specific polysaccharide from *Pseudomonas aeruginosa* serotype 03a,3d [14].

Analysis of the glycosylation effects obtained from ^{13}C-NMR data of OS-I showed a different absolute configuration of the units B and C (Table 1).

Hence, on the basis of the data obtained, the following structure of the capsular polysaccharide from *Alteromonas sp.* CMM 155 was established:

$$\rightarrow 3)\text{-}\alpha\text{-D-Gal}p\text{NAc}^{A}\text{-}(1\rightarrow 4)\text{-}\alpha\text{-L-GalA}p\text{NAc}^{B}\text{-}(1\rightarrow 3)\text{-}\alpha\text{-D-Qui}p\text{NAc4NAc}^{C}\text{-}$$

$$(1\rightarrow 3)\text{-}\beta\text{-D-Qui}p4\text{NAlaAc}^{D}\text{-}(1\rightarrow$$

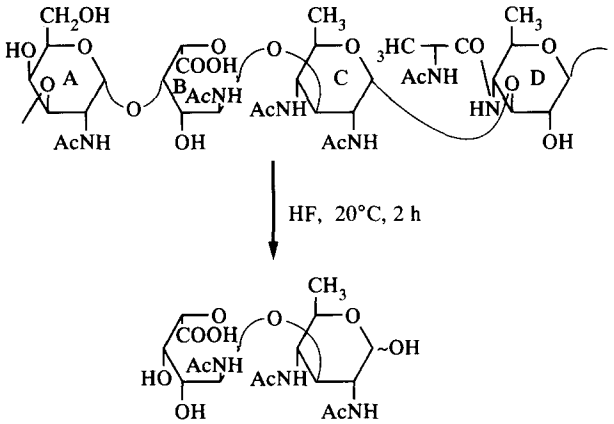
where Qui$p$NAc4NAc is di-*N*-acetyl-D-bacillosamine and Qui$p$4NAlaAc is 4-(*N*-acetyl-D-alanyl)amino-4,6-dideoxy-D-glucose.

It should be noted that the latter *N*-acyl-amino sugar has not been previosly identified in bacterial polysaccharides. The derivatives of 3-amino-3,6-dideoxy-D-glucose *N*-acylated by *N*-acetyl-amino acids are as follows: 3-(*N*-acetyl-D-alanyl)amino-3,6-dideoxy-D-glucose and 3-(*N*-acetyl-L-seryl)amino-3,6-dideoxy-D-glucose identified [15,16] in the O-specific polysaccharides from *Proteus penneri* O14 and *E. coli* O114, respectively. It is interesting that the repeating unit of CPS from *Alteromonas sp.* CMM 155 is composed of four amino sugars; among them such a rare diamino-sugar as di-*N*-acetyl-D-bacillosamine known as the constituent of the O-antigenic polysaccharides from *Pseudomonas aeruginosa* [14], *Pseudomonas aurantiaca* IMB 31 [17], *Vibrio cholerae* O:3, O:5 [18,19] and *Fusobacterium necroforum* [20].

3. Experimental

General methods.—Optical rotations were measured with a Perkin–Elmer 141 polarimeter for solutions in water at 20°C. Solutions were freeze-dried or evaporated in vacuo at 40°C. The growth of bacteria and isolation of CPS were performed as described earlier [1].

Chromatography.—Ascending and descending PC was carried out on Filtrak FN-15 and Whatman 3MM papers in *n*-butanol–pyridine–water 6:4:3. Alkaline silver nitrate and ninhydrin were used for detecting sugars. GC of alditol acetates was performed with a Pye Unicam 104 instrument equipped with a glass column (0.4 × 150 cm) packed with 3% QF–1 on Gas Chrom Q within the temperature range of 180 → 225°C. GC of



Scheme 1. HF-solvolysis of the CPS from *Alteromonas sp.* CMM 155.

acetylated (*S*)-(+)-2-butyl ester of alanine was carried out on the Hewlett–Packard 5890 instrument equipped with capillary column (0.2 mm × 25 m) with Ultra-1 stationary phase within the temperature range of 80 → 290°C. Ion-exchange chromatography of CPS was performed on a column (2.5 × 50 cm) with DEAE TSK 650M eluted with 50 mM tris HCl buffer (pH 7.0). The CPS was eluted by 0.5 M NaCl in the same buffer with monitoring by RIDK 101 refractive index detector. Electrophoresis was performed in 0.025 M pyridine–acetate buffer (pH 4.5) at 30 V/cm.

NMR-spectroscopy.—The ^{13}C-NMR spectra were recorded on a Bruker AM-300 spectrometer for solutions in D_2O at 70°C for the CPS and 20°C for OS-I (internal acetone, δ_C 31.45 ppm). The ^{1}H-NMR spectra were obtained on a Bruker WM-250 instrument for solutions in D_2O at 70°C (internal acetone, δ_H 2.225 ppm). COSY, COSYRCT, HMQC and NOE spectra were obtained as described [3,11].

Complete acid hydrolysis.—CPS (2 mg) was hydrolysed with 2 M trifluoroacetic acid (1 mL, 100°C, 2 h) and the sugars obtained were analysed by PC and GC of the alditol acetates. As a result, galactosamine and alanine were identified in the hydrolysate. On a preparative scale, 20 mg of CPS and 2 mL of acid were used. As a result, 3 mg of galactosamine was isolated by PC and converted into the hydrochloride (0.1 M HCl in methanol, 100°C, 0.5 h); $[\alpha]^{20}_{578}$ + 72° (*c* 0.3, water).

Solvolysis with anhydrous HF.—CPS (100 mg) was treated with anhydrous HF (20°C, 2 h) [13], an excess of HF was removed over NaOH, the resulting mixture was separated on TSK HW-40 (F) to yield OS-I (20 mg) (Scheme 1).

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