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Note

NMR-based identification of cell wall anionic polymers of *Spirilliplanes yamanashiensis* VKM Ac-1993^T

Alexander S. Shashkov,[a] Galina M. Streshinskaya,[b] Lyudmila I. Evtushenko,[c] Irina B. Naumova[b,*]

[a]*N.D. Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, Leninsky Prospect 47, Moscow 117334, Russia*

[b]*School of Biology, M.V. Lomonosov Moscow State University, Moscow 119899, Russia*

[c]*Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, Pushchino, Moscow, 142292, Russia*

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Abstract

The cell wall of *Spirilliplanes yamanashiensis* VKM Ac-1993^T contains four anionic polymers, viz., three teichoic acids and a sugar-1-phosphate polymer. The following are the structures of the teichoic acids: poly[-6-β-D-glucopyranosyl-(1→2)-glycerol phosphate] (PI), 1,3-poly(glycerol phosphate) bearing *N*-acetyl-α-D-glucosamine residues at O-2 (70%) (PII), and poly[-6-*N*-acetyl-α-D-glucosaminyl-(1→2)-glycerol phosphate] (PIII). The repeating unit of the fourth polymer (PIV) has the structure of -6-α-D-Glc*p*NAc-(1→6)-α-D-Glc*p*NAc-1-*P*- with a 3-*O*-methyl-α-D-mannopyranosyl residues at position 3 of some 6-phosphorylated *N*-acetylglucosamine residues (50%). Polymers PI, PIII and PIV have not hitherto been found in prokaryotic cell walls. © 2001 Published by Elsevier Science Ltd.

Keywords: NMR spectroscopy; Teichoic acids; Sugar-1-phosphate polymer; 3-*O*-Methylmannose; *Spirilliplanes*

Teichoic acids are widespread as cell wall components of Gram-positive bacteria and have been found in many actinomycete taxa.[1] Teichoic acids are involved in various cellular processes in Gram-positive bacteria, including control of the Mg^{2+} ion concentration in the wall–membrane complex, regulation of the activities of autolytic enzymes, phage reception and immunogenicity. In addition, the interest in these polymers stems from their taxonomic significance for Gram-positive bacteria, especially actinomycetes.[1]

The aim of the present work was to reveal teichoic acids in representatives of recently described actinomycete taxa. Here we report results of our studies of cell wall anionic polymers of *Spirilliplanes yamanashiensis* VKM Ac-1993^T, the type species of a new genus belonging to the family *Micromonosporaceae* (the order *Actinomycetales*).[2]

The cell wall of *S. yamanashiensis* contained about 3% of an organically bound phosphate, most of which can be attributed to extractable polymers and detected in the acid hydrolysate as glycerol and aminosugar phosphates. The polymers (PREP-1) were extracted from the cell wall with trichloroacetic acid, dialyzed

* Corresponding author. Fax: +7-095-9394309.

E-mail address: naumova@microbiol.bio.msu.su (I.B. Naumova).

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and freeze-dried. Acid hydrolysis with 2 M HCl afforded glycerol, glucose, glucosamine, 3-*O*-methylmannose, glycerol mono- and diphosphates, and inorganic phosphate. Partial acid-hydrolysis (0.1 M HCl) resulted in *N*-acetylglucosamine phosphates. Upon alkaline hydrolysis, only glycerol mono- and diphosphates, alongside glucosamine-containing glycerol phosphodiesters were detected. The fact that acid and alkaline hydrolyses produced different phosphoric esters might suggest the presence of several anionic polymers with different positions of the phosphodiester and glycosidic bonds.[3] The formation of *N*-acetylglucosamine phosphates upon mild-acid hydrolysis indicated the presence of an acid-labile polymer(s) of the sugar-1-phosphate type,[4] and the formation of glycerol phosphodiesters upon alkaline hydrolysis showed the presence of 1,3-poly(glycerol phosphate) chains partially substituted with *N*-acetylglucosamine.[5,6] No other polymer type could undergo such alkaline degradation.

The ^{13}C NMR spectrum of the PREP-1 (Fig. 1) contained, inter alia, four signals in the anomeric carbon resonance region (95.4–103.8 ppm), an intense signal of a carbon bearing nitrogen (55.1 ppm), and signals of *N*-acetyl groups at 23.5 (CH_3CON) and 176.0 (CH_3CON) ppm. Attached-proton test[7] demonstrated that all peaks in the region 62–68 ppm belonged to the $-CH_2O-$ groups, whereas those in the region 70–81 ppm belonged to –CHO– groups.

Three signals at 1.1, 3.0 and 3.3 ppm were present in the ^{31}P NMR spectrum.

The ^1H NMR spectrum contained four signals of the anomeric protons of about equal intensities at 4.62 ($J_{1,2}$ 8 Hz), 4.93, 5.09 ($J_{1,2}$ 4 Hz for both) and 5.49 ppm (a broadened signal).

The 1D spectra were assigned using analysis of 2D homonuclear ^1H, ^1H COSY, TOCSY and rotating-frame NOE (ROESY) spectroscopy, as well as 2D heteronuclear ^1H, ^{13}C HSQC and ^1H, ^{31}P HMQC spectroscopy.

Table 1
^1H NMR data (δ, ppm) for the teichoic acids (PREP-2) from the cell wall of *S. yamanashiensis* VKM Ac-1993^T

Residue	Proton									
	H-1a	H-1b	H-2	H-3a	H-3b	H-4	H-5	H-6a	H-6b	CH_3
Polymer I (PI)										
*sn*Gro-(3-PO_3^--[a] 2) ↑	3.77	3.77	4.03	4.05	4.00					
β-Glc*p*-(1 6) |	4.62		3.33	3.53		3.52	3.59	4.18	4.08	
Polymer II (PII)										
-1)-*sn*Gro-(3-PO_3^--	3.98	3.98	4.05	3.92	3.92					
-1)-*sn*Gro-(3-PO_3^--[b] 2) ↑	4.03	4.03	4.05	3.97	3.97					
α-Glc*p*NAc-(1	5.09		3.94	3.81		3.50	3.93	3.90	3.80	2.05
Polymer III (PIII)										
snGro-(3-PO_3^--[a] 2) ↑	3.77	3.77	4.04	4.13	4.13					
α-Glc*p*NAc-(1 6 |	4.93		3.97	3.79		3.59	3.88	4.14	4.14	2.07

[a] The ^{31}P NMR signal is at 3.3 ppm.

[b] The ^{31}P NMR signal is at 3.0 ppm.

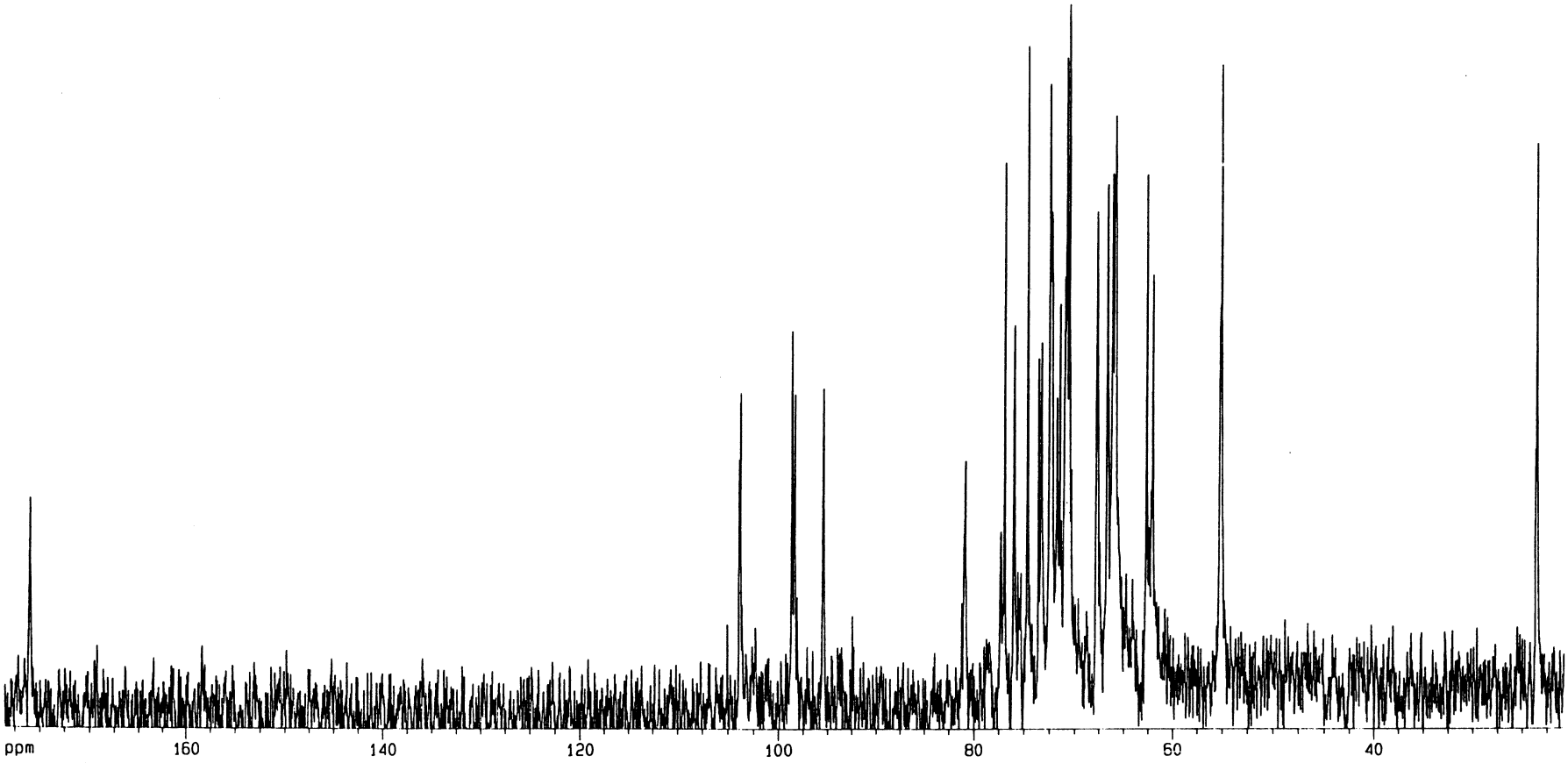

Fig. 1. ^{13}C NMR spectrum of the mixture of anionic polymers (PREP-1) from the cell wall of *S. yamanashiensis* VKM Ac-1993^{T}.

Table 2
^{13}C NMR data (δ, ppm) for the teichoic acids (PREP-2) from the cell wall of *S.* yamanashiensis VKM Ac-1993^T

Residue	Carbon							
	C-1	C-2	C-3	C-4	C-5	C-6	CH_3	CON
Polymer I (PI)								
*sn*Gro-(3-PO_3^--[a] 2) ↑	62.65	80.90	66.20					
β-Glc*p*-(1 6) |	103.80	74.60	77.30	70.80	76.00	66.20		
Polymer II (PII)								
-1)-*sn*Gro-(3-PO_3^--	67.70	70.80	67.70					
-1)-*sn*Gro-(3-PO_3^--[b] 2) ↑	66.70	77.30	66.20					
α-Glc*p*NAc-(1	98.30	55.10	72.50	71.70	73.55	62.00	23.50	176.00
Polymer III (PIII)								
snGro-(3-PO_3^--[a] 2) ↑	62.65	80.90	65.80					
α-Glc*p*NAc*p*-(1 6 |	98.60	55.10	72.50	71.50	72.40	65.85	23.50	176.00

[a] The ^{31}P NMR signal is at 3.3 ppm.

[b] The ^{31}P NMR signal is at 3.0 ppm.

The polymers could not be separated by ion-exchange chromatography or gel filtration and preparative electrophoresis. Taking into account a possible presence of sugar-1-phosphate groups, PREP-1 was subjected to hydrolysis with 0.1M HCl to give polymeric (PREP-2) and oligomeric (PREP-3) fractions separated by preparative electrophoresis. All the preparations were investigated using NMR spectroscopy.

As a result, a number of polymers were revealed in PREP-1 and three polymers were identified in PREP-2 (Tables 1 and 2). The repeating unit of one of them (P1) is built up of β-Glc*p* and glycerol residues. Glycerol is substituted with β-Glc*p* at position 2 as followed from the presence of a β-Glc*p* H-1, Gro H-2,3 cross-peak in the ROESY spectrum and a low-field position at 80.9 ppm of the signal for Gro C-2 in the ^{13}C NMR spectrum. That the phosphate group connects Gro O-3 and β-Glc*p* O-6 was established from the COSY and ^1H, ^{31}P HMQC spectra and confirmed by a low-field position at 66.2 ppm of the signals of the corresponding carbons (Gro C-3 and β-Glc*p* C-6) in the ^{13}C NMR spectrum (Table 2).

The second polymer (PII) was identified as 1,3-poly(glycerol phosphate) in which about 70% of the glycerol residues carry an α-Glc*p*-NAc substituent at O-2[5]. Assignment of the ^{13}C NMR spectrum of PII (Table 2) was confirmed by 2D homonuclear and heteronuclear spectroscopy.

The repeating unit of the third polymer (PIII) includes α-Glc*p*NAc and glycerol residues. The substitution of the latter with the α-Glc*p*NAc residue at position 2 evidenced from the presence of an α-Glc*p*NAc H-1, Gro H-2,3 cross-peak in the ROESY spectrum and a low-field position at 80.9 ppm of the signal for Gro C-2 in the ^{13}C NMR spectrum. The location of the phosphate group between Gro O-3 and α-Glc*p*NAc O-6

Table 3
^{1}H NMR data (δ, ppm) for the oligosaccharides (OI and OII) of PREP-3 obtained from partial hydrolysis of PREP-1 from the cell wall of *S. yamanashiensis* VKM Ac-1993^T

Residue	Proton							
	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b	OMe
Oligomer I (OI)								
PO_3^--6)-α-D-Glc*p*NAc-(1→ [a]	4.92	3.98	3.80	3.57	3.88	4.12	4.12	
→6)-α-D-Glc*p*NAc	5.21	3.89	3.74	3.63	4.00	4.03	3.69	
→6)-β-D-Glc*p*NAc	4.75	3.68	3.57	3.60	3.60	3.99	3.77	
Oligomer II (OII)								
α-D-Man*p*-3-OMe-(1→	5.35	4.26	3.43	3.73	3.60	3.84	3.81	3.43
→3)- α-D-Glc*p*NAc-(1→ 6 | PO_3^-	4.89	4.08	3.94	3.75	3.88	4.12	4.12	
→6)-α-D-Glc*p*NAc	5.24	3.89	3.74	3.63	4.01	4.00	3.69	
→6)-β-D-Glc*p*NAc	4.75	3.68	3.57	3.60	3.61	4.00	3.77	

[a] The ^{31}P NMR signal at 1.1 ppm.

Table 4
^{13}C NMR data (δ, ppm) for the oligosaccharides (OI and OII) of PREP-3 obtained from partial hydrolysis of PREP-1 from the cell wall of *S. yamanashiensis* VKM Ac-1993^T

Residue	Carbon						
	C-1	C-2	C-3	C-4	C-5	C-6	OMe
Oligomer I (OI)							
PO_3^--6)-α-D-Glc*p*NAc-(1→ [a]	98.20	54.80	72.15	70.75	72.15	65.15	
→6)-α-D-Glc*p*NAc	92.15	55.30	72.15	71.00	71.50	66.80	
→6)-β-D-Glc*p*NAc	96.40	57.90	75.35	70.75	75.50	66.75	
α-D-Man*p*-3-OMe-(1→	102.10	67.10	80.90	66.40	74.35	61.95	57.40
Oligomer II (OII)							
→3)- α-D-Glc*p*NAc-(1→ 6 | PO_3^-	98.10	53.10	78.10	71.40	72.15	64.80	
→6)-α-D-Glc*p*NAc	92.15	55.30	72.15	71.00	71.50	66.80	
→6)-β-D-Glc*p*NAc	96.40	57.90	75.35	70.75	75.50	66.75	

[a] The ^{31}P NMR signal at 1.1 ppm.

followed from the presence of the corresponding correlation peaks in the ^{1}H, ^{31}P HMQC spectrum and was confirmed by downfield shifts to 65.8 and 65.85 ppm of the signals of the corresponding carbons in the ^{13}C NMR spectrum (Table 2).

An NMR spectroscopic investigation of PREP-3 revealed the presence of di- and trisaccharides having the structures shown in Tables 3 and 4. The ratio of the integral intensities of the signals from di- and trisaccharides was $\approx$2:1.

Elucidation of the oligosaccharide structures enabled proposing the following structure of the repeating unit of the initial acid-labile polymer (PIV): -6-α-Glc*p*NAc-(1→6)-α-D-Glc*p*NAc-1-*P*- (OI). Presumably, some *N*-acetylglucosamine residues phosphorylated at O-6 carry a 3-*O*-methyl-α-D-mannose substituent at position 3 (OII), though the presence of two polymers with linear and branched repeating units cannot be ruled out.

Earlier, we have shown that the cell walls of *Actinoplanes* species from the same family *Micromonosporaceae* contain several anionic polymers.[8–11] However, the presence of four anionic polymers in the same cell wall has not been documented so far.

1. Experimental

The culture of *S. yamanashiensis* VKM Ac-1993^T (= IFO 15828), was grown on a pepton–yeast medium[12] for 24–30 h on a shaker at 28 °C. Biomass was collected at the logarithmic phase of growth. The cell walls were obtained by ultrasound disintegration of mycelium in 2% sodium dodecyl sulfate, washed several times with water, and freeze-dried.[5] To isolate polymers, cell walls were extracted twice with 10% trichloroacetic acid at 2–4 °C for 24 h each time; the extracts were separated from cell debris, dialyzed against distilled water, freeze-dried and purified by ion-exchange chromatography.[8]

Descending chromatography and electrophoresis were performed on Filtrak FN-13 paper. Electrophoresis was performed in pyridine–acetate buffer (pH 5.6) to separate phosphate esters.[5] Paper chromatography was performed in the solvent system of 3:1:5:3 pyridine–benzene–butanol–water (v/v) to separate glycerol and monosaccharides. Phosphoric esters were detected with the molybdate reagent; reducing sugars with aniline phthalate; and glycerol and monosaccharides with 5% $AgNO_3$ in aq ammonia. Acid hydrolysis was carried out with 2 M HCl for 3 h at 100 °C and 0.1 M HCl for 30 min at 100 °C. Alkaline hydrolysis was performed with 1 M NaOH for 3 h at 100 °C.

NMR spectra were recorded with a DRX-500 (Bruker, Germany) spectrometer for 2–3% solutions in D_2O at 30 °C with acetone (2.225 ppm for ^1H and 31.45 ppm for ^{13}C) as an internal standard. 1D ^1H NMR spectra were obtained with a pre-saturation of the HDO signal for 1 s. 2D spectra were obtained using standard pulse sequences from the Bruker software.

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