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Note

Cell wall teichoic acids of streptomycetes of the phenetic cluster '*Streptomyces fulvissimus*'

Alexander S. Shashkov,[a] Galina M. Streshinskaya,[b,*] Sof'ya N. Senchenkova,[a] Yuliya I. Kozlova,[b] Irina V. Alferova,[c] Larisa P. Terekhova[c] and Ludmila I. Evtushenko[d]

[a]*N. D. Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, Leninsky Prospect 47, Moscow 119991, Russia*
[b]*School of Biology, M. V. Lomonosov Moscow State University, Moscow 119899, Russia*
[c]*G. F. Gauze Institute of New Antibiotics, Russian Academy of Medical Sciences, Bol'shaya Pirogovskaya 11, Moscow 119021, Russia*
[d]*All-Russian Collection of Microorganisms (VKM), G. K. Skryabin Institute of Physiology and Biochemistry of Microorganisms, Russian Academy of Sciences, Pushchino, Moscow Region 142290, Russia*

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Abstract—Structures of the anionic polymers of streptomycetes *Streptomyces fulvissimus* VKM Ac-994^T, *Streptomyces longispororuber* VKM Ac-1735^T, *Streptomyces aureoveticillatus* VKM Ac-48^T and *Streptomyces spectabilis* INA 00606 belonging to the phenetic cluster '*S. fulvissimus*' were investigated by chemical and NMR spectroscopic methods. A teichoic acid from the cell wall of *S. spectabilis* INA 00606 was studied in more detail, and this was shown to represent 1,3-poly(glycerol phosphate) substituted with glucosamine (α-D-GlcNAc) and L-glutamic acid (non-stoichiometric substitution). For the first time, glutamic acid is identified as an acyl substituent in teichoic acids of streptomycetes. The polymer chain is built of the following fragments:

1. -1)-*sn*-Gro-(3-P-; 2. -1)-*sn*-Gro-(3-P-; and 3. -1)-*sn*-Gro-(3-P-
2)
↑
α-D-Glc*p*NAc-(1 L-Glu-(1

Cell walls of other streptomycetes of the phenocluster under study contain 1,3-poly(glycerol phosphates) with glucosamine as a glycosyl substituent at O-2 of the glycerol phosphate units and L-glutamic acid and lysine as O-2 acyl substituents. Not all amino sugar residues in the polymers of these strains are N-acetylated, and the content of the glucosamine and lysine residues in the polymers of different strains is not the same. Despite certain quantitative differences in the structures of the polymers, one may consider streptomycetes of the phenocluster '*S. fulvissimus*' as closely related microorganisms, the details of the structures serving as additional criteria for the determination of the species status of a strain under study.

Keywords: NMR spectroscopy; Teichoic acids; Glutamic acid; *Streptomyces*

Teichoic acids are widespread in cell walls of a large group of Gram-positive bacteria belonging to the order Actinomycetales. One of the functions of these cell surface polymers is conferring negative charges to the cell walls. Teichoic acids are characterised by a great diversity of structures.[1,2] The polymeric chains are negatively charged primarily due to the presence of phosphate residues that link repeating units and also due to substituents that increase the negative charge density. Lactic acid,[3] pyruvic acid,[4–7] succinic acid,[4,8] sulfuric acid[9] and additional phosphoric acid[10] residues have been identified as constituents of teichoic acids of actinomycetes. The increase in the negative charge is

* Corresponding author. Tel.: +7 095 9395601; fax: +7 095 9394309; e-mail: streshinskaya@mail.ru

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probably connected with functioning of the anionic polymers, in particular with the activity of autolytic enzymes, which are positively charged proteins[2,11] and, in inactive state, are presumably bound to teichoic acids.

The goal of the present study was structure elucidation of cell wall anionic polymers of streptomycetes belonging to the cluster '*Streptomyces fulvissimus*',[12] viz., *S. fulvissimus* VKM Ac-994^T, *Streptomyces longispororuber* VKM Ac-1735^T, *Streptomyces aureoveticillatus* VKM Ac-48^T and *Streptomyces spectabilis* INA 00606 as well as the search for, and evaluation of, the taxonomic significance of novel, fairly stable phenotypic characteristics. Peculiarities of the polymer structures and their combinations in the cell walls serve as these characteristic features as has been shown for certain strains of streptomycetes and other microorganisms of the order Actinomycetales.[3–8]

Teichoic acid of *S. spectabilis* INA 00606 was studied in most detail. The cell wall of the streptomycete contained 2.3% of teichoic acid-linked phosphorus. Paper electrophoresis (buffer 1) of the cell wall hydrolysate (2 M HCl, 100 °C, 3 h) revealed GroP and $GroP_2$ as the main phosphates together with inorganic phosphate. According to paper chromatographic examination (solvent system 1), the hydrolysate contained glycerol, an amino sugar, which might also result from hydrolysis of the glycan moiety of peptidoglycan, and minor amounts of galactose and mannose. The facts that the cell wall contains teichoic acid-linked phosphorus and the cell wall hydrolysate contains equal amounts of glycerol mono- and bisphosphate suggest the possible presence of 1,3-poly(glycerol phosphate), which is most likely little substituted.[13]

Three successive extractions of the cell wall with 10% trichloroacetic acid (24, 48 and 72 h, 4 °C) afforded three identical, teichoic acid-containing preparations, which were used for subsequent studies.

Acid hydrolyses of these preparations resulted in glycerol mono- and bisphosphates, inorganic phosphate, an amino sugar and glycerol. Alkaline hydrolysis (1 M NaOH, 100 °C, 3 h) afforded, in addition to glycerol phosphates, two alkali-resistant phosphates (E_{GroP} 0.35 and 0.57). The latter was present in trace amounts and its composition has not been established, whereas the former was isolated by preparative paper electrophoresis. Its treatment with phosphomonoesterase (EC 3.1.3.1, Sigma) for 16 h at 37 °C resulted in inorganic phosphate and a glycoside that yielded glycerol and an amino sugar upon acid hydrolysis. The amino sugar was identified as glucosamine by paper chromatography (solvent system 2) by comparison with an authentic sample.

Hydrolysis of the polymer with 48% aqueous hydrofluoric acid (4 °C, 16 h) yielded a glycoside composed of equal amounts of glycerol and glucosamine, its structure was established by NMR spectroscopy (Tables 1 and 3).

Ammonolysis of the polymer revealed small amount of lysine esterifying the teichoic acid chain. The absolute

Table 1. NMR data for the polymer from the cell wall of *Streptomyces spectabilis* INA-00606

Residue of polymer	Proton	δ (TSP 0.0 ppm)	Carbon	δ (acetone 31.45 ppm)
-1)-sn-Gro-(3-*P*-	H-1,1′	3.98; 3.92	C-1	67.7
	H-2	4.06	C-2	70.8
	H-3,3′	3.98; 3.92	C-3	67.7
-1)-sn-Gro-(3-*P*-2)	H-1,1′	4.03; 4.03	C-1	65.7
	H-2	4.06	C-2	77.2
	H-3,3′	4.03; 3.98	C-3	66.0
α-D-Glc*p*NAc-(1	H-1	5.09	C-1	98.3
	H-2	3.94[a]	C-2	55.1[b]
	H-3	3.80	C-3	72.4
	H-4	3.48	C-4	71.5
	H-5	3.93	C-5	73.5
	H-6	3.89	C-6	62.0
	H-6′	3.79		
-1)-sn-Gro-(3-*P*-2)	H-1,1′	4.04; 4.04	C-1	66.15
	H-2	4.35	C-2	76.6
	H-3,3′	4.04; 4.04	C-3	66.15
Glu-(1	H-1,1′		C-1	175.8
	H-2	4.38	C-2	53.4
	H-3,3′	2.23; 1.99	C-3	27.9
	H-4	2.44	C-4	32.9
			C-5	177.5
Terminal resides of the polymer				
sn-Gro-(3-*P*-2)	H-1,1′	3.80; 3.77	C-1	62.4
	H-2	3.89	C-2	78.7
	H-3,3′	3.98; 3.92	C-3	67.4
α-D-Glc*p*NAc-(1	H-1	5.05	C-1	98.2
	H-2	3.92[c]	C-2	55.2[d]
	H-3	3.80	C-3	72.4
	H-4	3.48	C-4	71.5
	H-5	3.93	C-5	73.5
	H-6	3.90	C-6	62.0
	H-6′	3.79		
Products of HF hydrolysis				
Gro-3-*P*	H-1,1′	3.69; 3.61	C-1	63.6
	H-2	3.90	C-2	72.3
	H-3,3′	3.91; 3.91	C-3	67.3
sn-Gro-2)	H-1,1′	3.76; 3.74[e]	C-1	62.8[f]
	H-2	3.76	C-2	80.5
	H-3,3′	3.73; 3.68[e]	C-3	61.9[f]
α-D-Glc*p*NAc-(1	H-1	5.05	C-1	98.4
	H-2	3.93[a]	C-2	55.2[d]
	H-3	3.79	C-3	72.4
	H-4	3.50	C-4	71.4
	H-5	3.87	C-5	73.6
	H-6	3.86	C-6	62.0
	H-6′	3.80		

Minor signal for terminal units: [a]CH_3CON at δ 2.05; [b]CH_3CON at δ 23.6 and 176.5, correspondingly; [c]CH_3CON at δ 2.03; [d]CH_3CON at δ 23.35 and 176.3, correspondingly; [e,f]alternative assignment.

configuration of lysine was determined to have L-configuration. In the other, streptomycetes studied viz. *Streptomyces azureus* and *Streptomyces roseoflavus* var. *roseofungini* it had L-configuration too.[14,15] Identification of the constituents of the polymer as well as elucidation of its total structure were carried out by NMR spectroscopy.

The most intense signals in the ^{13}C NMR spectrum of the polymer at δ 67.7 (d, $^{2}J_{C,P} = 5$ Hz) and at δ 70.8 (t, $^{3}J_{C,P} = 7$ Hz) (Fig. 1, Table 1) corresponded to the carbon atoms of the 1,3-poly(glycerol phosphate) chain. The spectrum also contained less intense signals, some of which were identified as belonging to 2-amino-2-deoxy-α-glucopyranose[15] and 1,3-poly(glycerol phosphate) units bearing this sugar as a substituent at O-2. Three signals at δ 53.4, 32.9 and 27.9 were typical of glutamine (Glu) or glutamic acid (Glu) carbon atoms carrying protons. The low-field region of the spectrum contained three signals for the carbonyl groups (δ 175.8, 176.3 and 177.5). The absence of a signal at δ 174.5 characteristic of $CONH_2$ of glutamine indicated that it was glutamic acid (Glu) that was the acyl substituent.[16]

Table 2. NMR data for the polymer from the cell wall of *Streptomyces fulvissimus* VKM Ac-994

Residue	Proton	δ (TSP 0.0 ppm)	Carbon	δ (acetone 31.4 ppm)
-1)-sn-Gro-(3-*P*-2)	H-1,1′	4.04; 4.04	C-1	56.15
	H-2	4.33	C-2	56.20
	H-3,3′	4.02; 4.02	C-3	56.15
Lys- 1	H-1		C-1	n.d.
	H-2	4.31	C-2	53.8
	H-3	1.72	C-3	27.7
	H-4	1.46	C-4	22.8
	H-5	1.92	C-5	30.8
	H-6	3.02	C-6	40.5
	NH	7.55		
	NH_3^{+} [a]	7.08		

n.d.: not detected.
[a] For the solvent in H_2O–D_2O 9:1.

The region of the resonance of the anomeric protons in the ^{1}H NMR spectrum contained doublets ($J_{1,2} = 4$ Hz) at δ 5.09 and 5.05 (minor). In the high-field region of the spectrum, two singlets at δ 2.05 and 2.03 (minor) for the CH_3CON groups were observed, and the signals for the protons of the methylene groups at δ 2.44, 2.23 and 1.99 were present in a 2:1:1 ratio. Other protons resonated at δ 3.5–4.4 (Table 1).

Assignment of the signals in the ^{1}H NMR spectrum was carried out in part using two-dimensional experiments COSY and TOCSY. These spectra revealed, in particular, the complete spin systems of 2-amino-2-deoxy-α-glucopyranose and glutamic acid. The low-field position of the signal for H-2 of the sugar residue (δ 3.94) suggested the presence of an *N*-acetylamino group at C-2. Hence, the sugar fragment of the polymer is represented by 2-acetamido-2-deoxy-α-glucopyranose. Assignment of all the signals in the ^{1}H NMR spectrum, including those for the protons of O-2 substituted and non-substituted internal and terminal glycerol residues, was carried out with recourse to two-dimensional ^{1}H,

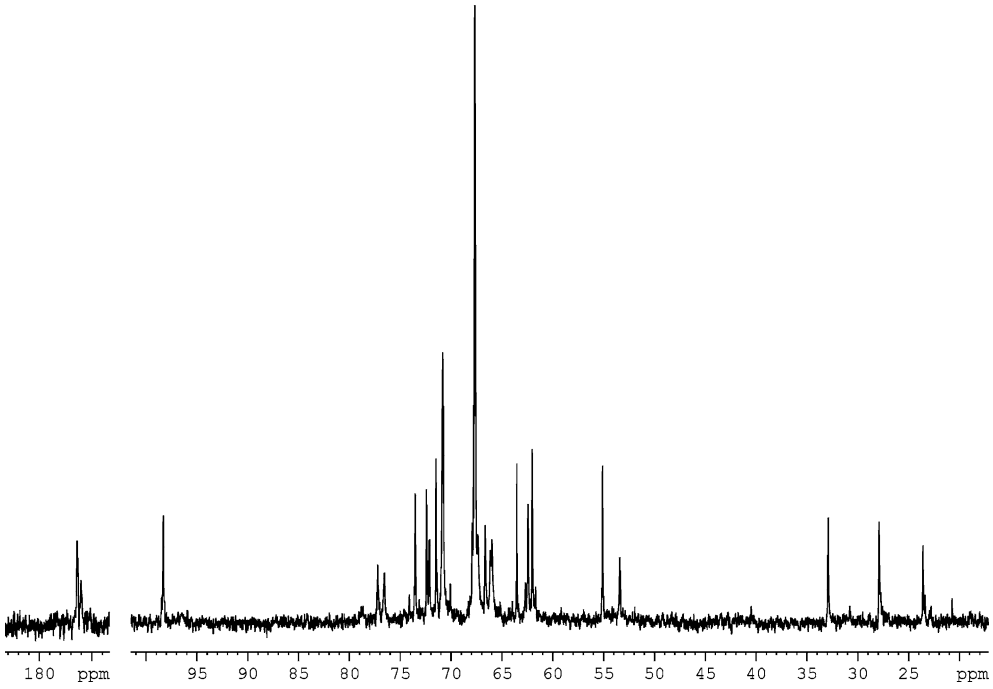

Figure 1. 125 MHz ^{13}C NMR spectrum of the polymer from the cell wall of *Streptomyces spectabilis* INA-00606.

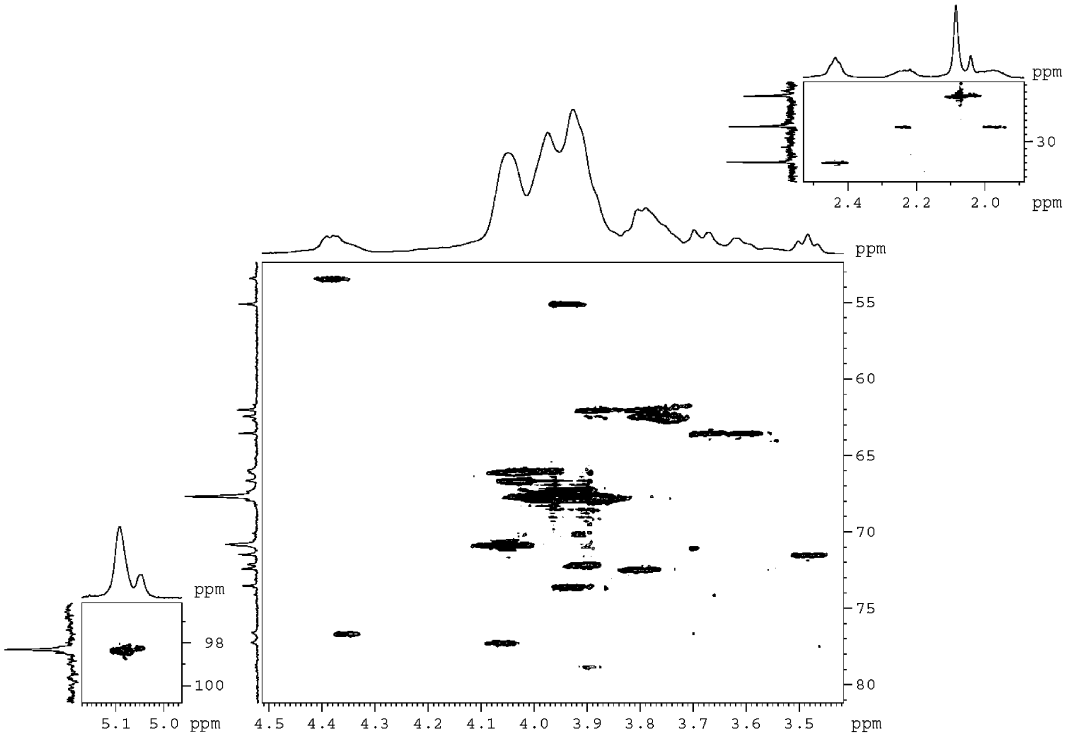

Figure 2. Parts of an ^{1}H, ^{13}C HSQC spectrum of the polymer from the cell wall of *Streptomyces fulvissimus* VKM Ac-994.

^{13}C HSQC technique (Fig. 2). Two low-field signals at δ 77.2 and 76.6 were assigned to C-2 atoms of the substituted glycerol residues. The corresponding signals in the ^{1}H NMR spectrum were found at δ 4.06 and 4.35, respectively. The HMBC spectrum contained a correlation peak δ_H/δ_C 5.09/77.2 (H-1 Glc*p*NAc/C-2 Gro), which proved the signal at δ_C 77.2 to belong to C-2 of glycerol bearing the amino sugar substituent. The low-field position (δ_H 4.35) of a proton linked to the aforementioned carbon atom (δ_C 76.6) is typical of the acylation effect manifested in the ^{1}H NMR spectra, which allowed us to suggest that glutamic acid is an *O*-acyl substituent in some glycerol units. The absolute configuration of glutamic acid and glucosamine were determined and have L- and D-configurations, respectively.

That the L-glutamic acid residue was localised at O-2 of glycerol was proved by recording ^{1}H NMR spectra and two-dimensional TOCSY and ROESY spectra in a mixture H_2O–D_2O 9:1 (Fig. 3). The low-field region of the ^{1}H NMR spectrum contained two doublets of the NH protons at δ 8.28 and 8.16. The correlation peaks in the TOCSY spectrum allow unambiguous assignment of the former signal to the NH group of Glu and of the latter signal to the NH group of α-Glc*p*NAc. In the ROESY spectrum, a correlation peak of the proton of the NH group of Glu with the protons of the –CH_2–O–P– fragment of the glycerol residues (δ 4.04) was observed, apart from trivial intraresidue correlation peaks of this proton with H-2, 3, 4 of Glu. The proton of the NH group of α-Glc*p*NAc in the ROESY spectrum gives distinct correlation peaks with the protons of the methyl group NHAc (a major and a minor for the inner and terminal residues, correspondingly).

The ^{31}P NMR spectrum of the preparation contained one broad major peak at δ +0.6. Correlation peaks δ_H/δ_P 3.98–4.04/0.6 in a two-dimensional ^{1}H, ^{31}P HMQC spectrum corroborate the localisation of the phosphate groups at C-1,3 of the glycerol units.

Integration of well resolved signals in the ^{13}C NMR spectrum allowed estimation of the relative percentage of the substituted and unsubstituted glycerol residues in the preparation and the mean chain length of the polymers. These estimates are as follows: unsubstituted glycerol residues, 68%; glycerol residues with glutamic acid as the O-2 substituent, 16% and glycerol residues with the amino sugar as the O-2 substituent, 16%.

Thus, the results of this study allowed us to establish that the cell wall anionic polymer of *S. spectabilis* is 1,3-poly(glycerol phosphate) partially O-glycosylated with 2-acetamido-2-deoxy-α-D-glucopyranose and partially O-acylated with L-glutamic acid and lysine.

The stepwise extraction of cell walls of streptomycetes *S. fulvissimus* VKM Ac-994^{T} (1.82% of teichoic acid-linked phosphorus), *S. longispororuber* VKM Ac-1735^{T} (1.92% of teichoic acid-linked phosphorus) and *S. aureoveticillatus* VKM Ac-48^{T} (2.1% of teichoic acid-linked phosphorus) with 10% trichloroacetic acid at 4 °C also afforded preparations of polymers, which were investigated by NMR spectroscopy.

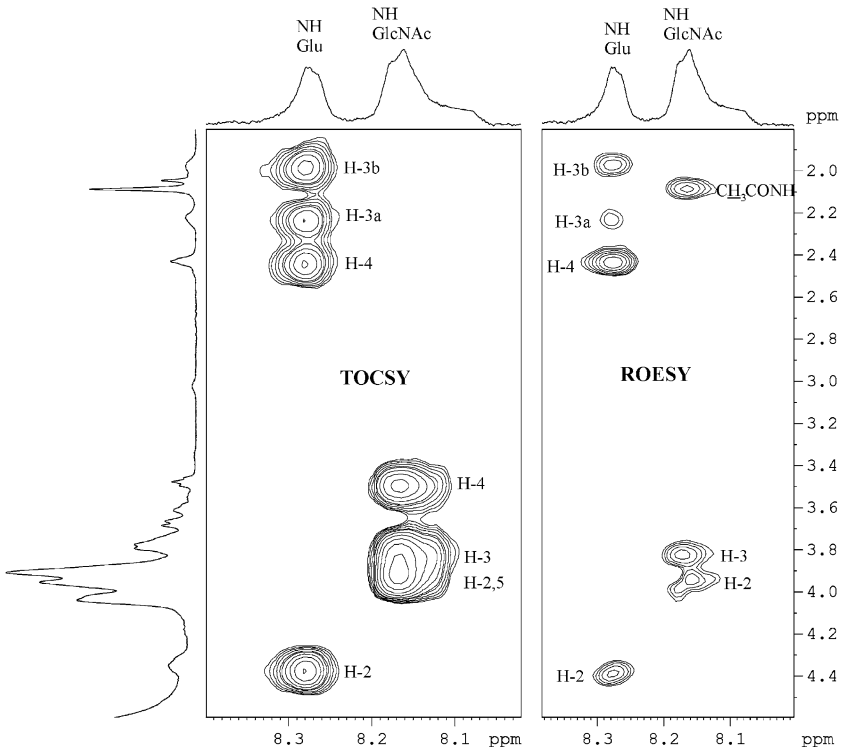

Figure 3. Parts of TOCSY and ROESY spectra of the polymer from the cell wall of *Streptomyces longispororuber* VKM Ac-1735.

The ^{13}C, ^{1}H and ^{31}P NMR spectra of teichoic acid of *S. fulvissimus* VKM Ac-994^{T} (Table 2) contained all the signals characteristic of the polymer of *S. spectabilis* together with a series of minor signals corresponding to lysine residues. Exhaustive analysis of two-dimensional spectra analogous to that described above showed that the lysine residues are localised as acyl substituents at O-2 of glycerol. The following estimates of the relative percentage of different units were obtained by integrating the characteristic signals in the ^{13}C NMR spectrum: unsubstituted 1,3-poly(glycerol phosphate) units, 65%; glycerol residues with glutamic acid as the O-2 substituent, 9%; glycerol residues with lysine as the O-2 substituent, 5% and glycerol residues with the amino sugar as the O-2 substituent, 21%. The polymers contain from 6 to 8 units on an average.

Analysis of 1D and 2D NMR spectra of teichoic acid of *S. longispororuber* VKM Ac-1735^{T} demonstrated that the preparation contained the same structural units as the preparations of *S. spectabilis* and *S. fulvissimus*, however, part of α-D-glucosamine residues is devoid of *N*-acyl substituents, which brings about substantial changes in certain ^{1}H and ^{13}C signals of these residues compared with N-acetylated ones (Table 3). Two-dimensional experiments TOCSY and ROESY for solutions in a mixture H_2O–D_2O 9:1 demonstrated that all the acetyl groups (δ_H 2.09) are linked solely to the α-D-glucosamine residues. The ratio of N-acetylated residues and those with free NH_2 group (3:2) was determined by integrating the peaks of the anomeric protons in the ^{1}H NMR spectrum. Integration of the characteristic signals in the ^{13}C NMR spectrum allowed approximate estimation of different units as follows: unsubstituted 1,3-poly(glycerol phosphate) units, 60%;

Table 3. NMR data for the polymer from the cell wall of *Streptomyces longispororuber* VKM Ac-1735

Residue	Proton	δ (TSP 0.0 ppm)	Carbon	δ (acetone 31.45 ppm)
-1)-sn-Gro-(3-*P*-2)	H-1,1′	4.04; 4.04	C-1	65.9
	H-2	4.06	C-2	77.2
	H-3,3′	4.03; 3.98	C-3	66.6
α-D-Glc*p*NAc-(1→	H-1	5.09; 5.05[a]	C-1	98.5
	H-2	3.95[b]	C-2	55.1[c]
	H-3	3.82	C-3	72.4
	H-4	3.48	C-4	71.5
	H-5	3.96	C-5	73.5
	H-6	3.90	C-6	62.0
	H-6′	3.80		
-1)-sn-Gro-(3-*P*-2)	H-1,1′	4.04; 4.04	C-1	66.0
	H-2	4.35	C-2	77.1
	H-3,3′	4.03; 4.03	C-3	66.5
α-D-Glc*p*NH_3^+-(1→	H-1	5.43; 5.39[a]	C-1	96.2
	H-2	3.36	C-2	55.5
	H-3	3.96	C-3	71.2
	H-4	3.50	C-4	71.2
	H-5	3.96	C-5	73.8
	H-6	3.90	C-6	61.7
	H-6′	3.80		

[a] Minor signals for terminal units.
[b] CH_3CON at δ 2.09.
[c] CH_3CON at δ 23.6 and 176.5, correspondingly.

glycerol residues with glutamic acid as the O-2 substituent, 5%; glycerol residues with lysine as the O-2 substituent, 7% and glycerol residues with the amino sugar as the O-2 substituent, 33%. The amount of a teichoic acid preparation isolated from *S. aureoveticillatus* VKM Ac-48^T was insufficient to perform a complete set of two-dimensional NMR experiments, however, the analysis of signals in one-dimensional ^{13}C NMR and APT spectra allowed revealing all the signals characteristic of the structural units of *S. fulvissimus*. In addition, signals typical of 1,5-poly(ribitol phosphate) with β-glucopyranosyl residues at O-2 of ribitol[3] were also present (about 10% relative to other polymers). Integration of the characteristic signals in the ^{13}C NMR spectrum allowed the following approximate estimation of the structural units as follows: unsubstituted 1,3-poly(glycerol phosphate) units, 54%; glycerol residues with glutamic acid as the O-2 substituent, 9%; glycerol residues with lysine as the O-2 substituent, 7% and glycerol residues with the amino sugar as the O-2 substituent, 30%. The quality of the ^{13}C NMR spectrum precluded determination of the polymer chain mean length.

A characteristic feature of teichoic acids of the streptomycetes under study is the presence of several acyl substituents that esterify the 1,3-poly(glycerol phosphate) chain, viz., lysine and glutamic acid, and acetyl groups as amino sugar substituents. It is for first time that glutamic acid has been identified as an acyl substituent in teichoic acids of streptomycetes. L-Glutamine linked to the D-glucuronic acid residues was revealed in the cell wall lipopolysaccharide of *Serratia marcescens*.[17] Not all amino sugar components of the polymer of *S. longispororuber* are acetylated. The lysine content in the polymers is also different. The cell wall of *S. aureoveticillatus* contains small amount of 1,5-poly(ribitol phosphate) with β-glucopyranosyl substituents along with poly(glycerol phosphate) chains.

1. Experimental

The cultures of streptomycetes were grown on a peptone-yeast medium[18] for 18–20 h on a shaker at 28 °C. The biomass was collected at the logarithmic phase of growth. The cell wall was obtained by centrifugation after disruption of mycelium by sonication in 2% sodium dodecyl sulfate, washed several times with water and freeze dried.[19] The polymers were isolated from the cell walls by repeated extraction with 10% trichloroacetic acid at 2–4 °C for 24, 48 and 72 h, the extracts were separated from cell debris, dialysed against distilled water and freeze dried.

The teichoic acid-linked phosphorus was determined according to a published procedure.[19] The ammonolysis was carried out as described previously.[14]

Descending paper chromatography and electrophoresis were carried out on a Filtrak FN-3 paper (Germany). Electrophoresis was run in a pyridinium acetate buffer, pH 5.6 (buffer 1) to separate phosphates. Paper chromatography was performed in the solvent systems pyridine–benzene–butan-1-ol–water (3:1:5:3, v/v) (solvent system 1) to separate glycerol and monosaccharides and butan-1-ol–AcOH–water (4:1:5, v/v) to separate the amino sugar and amino acids (solvent system 2). Phosphate esters were detected with the molybdate reagent; reducing sugars, with aniline hydrogenphthalate; glycerol and monosaccharides, with 5% $AgNO_3$ in aqueous ammonia; amino sugar and amino acids were detected with a ninhydrin reagent. Acid hydrolysis was carried out with 2 M HCl at 100 °C for 3 h and alkaline hydrolysis, with 1 M NaOH at 100 °C for 3 h. Partial hydrolysis with 48% HF was carried out at 4 °C for 16 h followed by freeze drying through a trap with NaOH. The hydrolysis products were separated on a column (90 × 1.5 cm) with TSK-gel HW-40S (Toyopearl, Japan), elution with 1% AcOH being monitored with a differential refractometer (Knauer, Germany).

The absolute configuration of glucosamine was determined according to a published procedure.[20]

The absolute configuration of glutamic acid was determined in the following way. The preparation of teichoic acids was hydrolysed with 2 M CF_3COOH at 120 °C for 2 h and evaporated with a stream of air. To the dry residue, 15 μl of anhydrous CF_3COOH and 0.2 ml of (+) 2-octanol were added and heated at 120 °C for 16 h. The sample was evaporated under the stream of air followed by acetylation with acetic anhydride–pyridine mixture (1:1 v/v) at 100 °C for 1 h; then, the sample was dried under the stream of air. References were obtained from L-Glu with (+)-2-octanol and (±)-2-octanol. The compounds were studied with gas liquid chromatography using a Hewllet-Packard apparatus (model 5880) and a capillary column Ultra-1 (0.2 mm × 25 m) with OV-1 phase at 120–290 °C and nitrogen was the carrier gas.

For determination of the absolute configuration of lysine, the cell wall was hydrolysed with 2 M CF_3COOH at 120 °C for 2 h; after evaporation the sample was N-acetylated with Ac_2O in aqueous saturated $NaHCO_3$ (30 min 0 °C, then 30 min 20 °C), deionised with IRA-120 resin (H^+-form), then heated with (+)-2-octanol (0.1 ml) and CF_3COOH (0.015 ml) for 12 h at 120 °C, evaporated, and the residue conventionally acetylated was analysed by GLC on a SE-54 capillary column (25 m × 0.2 mm) using the authentic samples prepared from L-lysine with (+)-2-octanol and (±)-2-octanol.

NMR spectra were recorded with a DRX-500 (Bruker, Germany) spectrometer for 2–3% solutions in D_2O at 30 °C with TSP (δ_H 0.00) and acetone (δ_C 31.45) as the internal standards. 1D ^1H NMR spectra

were obtained with a presaturation of the HDO signal for 1 s. 2D spectra were obtained using standard pulse sequences from the Bruker software. A mixing time of 150 and 200 ms was used in 2D TOCSY and NOESY experiments, respectively.

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