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Teichulosonic Acid, an Anionic Polymer of a New Class from the Cell Wall of *Actinoplanes utahensis* VKM Ac-674^T

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Abstract—The cell wall of *Actinoplanes utahensis* VKM Ac-674^T contains two anionic polymers: teichoic acid 1,3-poly(glycerol phosphate) that is widespread in cell walls of Gram-positive bacteria; and a unique teichulosonic acid belonging to a new class of bioglycans described only in microorganisms of the Actinomycetales order. The latter polymer contains residues of di-N-acyl derivative of sialic acid-like monosaccharide – 5,7-diamino-3,5,7,9-tetradeoxy-L-*glycero*-β-L-*manno*-non-2-ulosonic or pseudaminic acid (Pse) which bears the N-(3,4-dihydroxybutanoyl) group (Dhb) at C7. This polymer has irregular structure and consists of fragments of two types, which differ in substitution of the Dhb residues at O4 either with β-D-glucopyranose or with β-Pse residues. Most of the β-Pse residues (~80%) are glycosylated at position 4 with α-D-galactopyranose residues in both types of fragments. The glucose, galactose, and Dhb residues are partly O-acetylated. The structures of the polymers were established by chemical and NMR spectroscopy methods.

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Key words: *Actinoplanes utahensis*, cell wall, anionic polysaccharides, teichoic acids, teichulosonic acids, NMR spectroscopy

Actinoplanes utahensis VKM Ac-674^T is a representative of mycelial sporangium-forming actinomycetes with mobile spores [1]. According to the modern classification of bacteria, the *Actinoplanes* genus belongs to the Micromonosporaceae family of the Actinomycetales order and includes more than 30 species [2]. Most of the known organisms of this genus are mesophiles, aerobes inhibiting various types of soils and plant remains and also deposits of lakes and rivers [1]. Many of them synthesize biologically active substances including antibiotics [1, 3]. Cell walls of the earlier studied *Actinoplanes* contain anionic polymers of various types: poly(glycosyl glycerol phosphates), which differ in components and have been found in *A. philippinensis* VKM Ac-647^T [4] and *A. campanulata* VKM Ac-1319^T [5]; poly(glycosyl 1-phosphate) found in *Actinoplanes* sp. INA 3697 [6]; teichuronic acid found in *A. brasiliensis* INA 3802 [7].

The goal of the present work was to study the anionic polymers of the cell wall of *A. utahensis* VKM Ac-674^T. Along with a teichoic acid of poly(glycerol phosphate) nature, the cell wall of the studied organism contains a polymer of a new class, a di-N-acyl derivative of sialic acid-like monosaccharide – 5,7-diamino-3,5,7,9-tetradeoxy-L-*glycero*-β-L-*manno*-non-2-ulosonic or β-pseudaminic acid (β-Pse).

MATERIALS AND METHODS

Bacterial strain, cultivation, isolation of cell wall, and peptidoglycan and glycopolymer preparations. *Actinoplanes utahensis* VKM Ac-674^T strain was obtained from the All-Russian Collection of Microorganisms (VKM), Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences.

To obtain biomass, cells were grown aerobically in shaker flasks containing peptone–yeast medium [8] at

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28°C. Mycelium was harvested by centrifugation in the mid-logarithmic phase, washed with 0.95% NaCl solution, stored at −18°C, and used for preparing cell walls.

To obtain cell wall preparation, cells were ultrasonicated using a UP100H ultrasonic disintegrator from Hielscher (Germany) (30 kHz, 3-5 times per 2 min in ice water) with addition of SDS (~2% by volume) for removal of possible membrane fragments [9] and subsequent differential centrifugation. Determination of phosphorus forms in the cell wall, obtaining of peptidoglycan, its qualitative analysis and identification of the diaminopimelic acid isomers were described earlier [9, 10].

Preparations of glycopolymers were isolated from the cell wall by three consecutive 24-h extractions with 10% trichloroacetic acid (TCA) at 2-4°C, and the supernatants were dialyzed against distilled water and lyophilized [9]. The obtained 24-, 48-, and 72-h preparations were studied by chemical methods. Then the preparations were pooled. This pooled preparation was studied by NMR spectroscopy after its gel filtration (PS1 preparation).

Acidic hydrolysis. The conditions for acidic hydrolysis of the cell wall, glycopolymer preparations and peptidoglycan, study of the degradation products, and reagents for their detection were described earlier [9].

The pooled glycopolymer preparation was subjected to gel filtration on a 56 × 2.6 cm column with Sephadex G-50 Superfine from Amersham Biosciences (Sweden) in 0.05 M pyridine acetate buffer, pH 4.5; elution was monitored using a differential refractometer from Knauer (Germany). Thus obtained fraction – PS1 preparation – was used for NMR spectroscopy and subsequent isolation of PS2 polymer.

Treating PS1 preparation (55 mg) with 48% HF for 16 h at 4°C with subsequent gel chromatography on a 90 × 1.5 cm column with TSK HW-40S gel from Toyopearl (Japan) in 1% AcOH and monitoring elution using a differential refractometer from Knauer, PS2 polymer (yield 28 mg) was obtained; PS2 was used for NMR spectroscopy and isolation of de-O-acetylated glycopolymer.

For de-O-acetylation of PS2 polymer, the latter (28 mg) was treated with 12.5% NH_4OH (1 ml) for 16 h at 37°C with subsequent chromatography on a column with TSK gel as described above. The isolated polymer fraction (17 mg) was used for NMR spectroscopy and obtaining of disaccharide by degradation according to Smith.

For Smith degradation, de-O-acetylated polymer (17 mg) was treated with 0.1 M $NaIO_4$ (20 ml) for 4 days, then $NaBH_4$ (180 mg) was added and the mixture was incubated overnight at room temperature. Then the mixture was neutralized with AcOH to pH 7, and disaccharide thus obtained was isolated on a column with TSK gel as described above with its subsequent purification by HPLC (Bio-Rad, USA) on a 1 × 25 cm column with Zorbax C18 carrier in 0.02% aqueous TFA with a UV detector (200 nm). The yield of disaccharide was 4.7 mg. The disaccharide was studied by NMR spectroscopy.

The absolute configuration of Glc was determined by GLC of acetylated glycosides with (+)-octane-2-ol as described earlier [11]. The absolute configuration of galactose was taken equal to configuration of this monosaccharide in the polymer with analogous structure [12].

The NMR spectra were recorded using a Bruker Avance 600 spectrometer for solutions in 99.96% D_2O at 30°C. TSP (δ_H 0.0) and acetone (δ_C 31.45) were used as internal standards for the ^1H and ^{13}C spectra, respectively. The 2D NMR spectra were recorded and treated according to the standard Bruker (Germany) procedures. The mixing time for TOCSY was 100 msec, the spin lock time for ROESY 150 msec, HMBC was optimized for spin-coupling constants $J_{H,C}$ 8 Hz.

RESULTS AND DISCUSSION

The cell wall of *A. utahensis* VKM Ac-674^T contains 0.32% phosphorus of organic compounds. Peptidoglycan includes the amino acids usual for *Actinoplanes* (glycine, alanine, glutamic acid) [1] and also three isomers of diaminopimelic acid: meso- (major), 3-hydroxy-, and LL- (minor).

Glycerol mono- and bisphosphates, glycerol, galactose, glucose, and traces of rhamnose and mannose were identified in cell wall hydrolysates (2 M HCl, 100°C, 3 h). Xylose and arabinose typical of the complete cells of *Actinoplanes* [1] were not revealed.

Preparations obtained from the cell wall by extraction with 10% TCA contained the same products of acidic hydrolysis excluding rhamnose and mannose. Electrophoresis showed two polymer fractions: the major one had mobility m_{GroP} = 0.6-0.8, the other – m_{GroP} = 1.22. The content of the latter in the 24-h preparation was minimal and increased in the other preparations (48- and 72-h).

The fraction with mobility m_{GroP} = 1.22 was accumulated by preparative electrophoresis. Its hydrolysis (2 M HCl, 100°C, 3 h) revealed equal amounts of glycerol mono- and bisphosphates: along with mobility of this fraction, this indicates possible presence of 1,3-poly(glycerol phosphate). No monosaccharide was detected in the hydrolysate. Consequently, this polymer is most likely unsubstituted 1,3-poly(glycerol phosphate) [13].

Analysis revealed the identity of the TCA-obtained preparations; this allowed pooling them for NMR spectroscopy. The structure of polymer with mobility m_{GroP} = 0.6-0.8 was established by NMR spectroscopy. A total pooled preparation was chromatographed on a column with Sephadex G-50; the isolated fraction – PS1 preparation – was used for NMR spectroscopic study of the cell wall polymers.

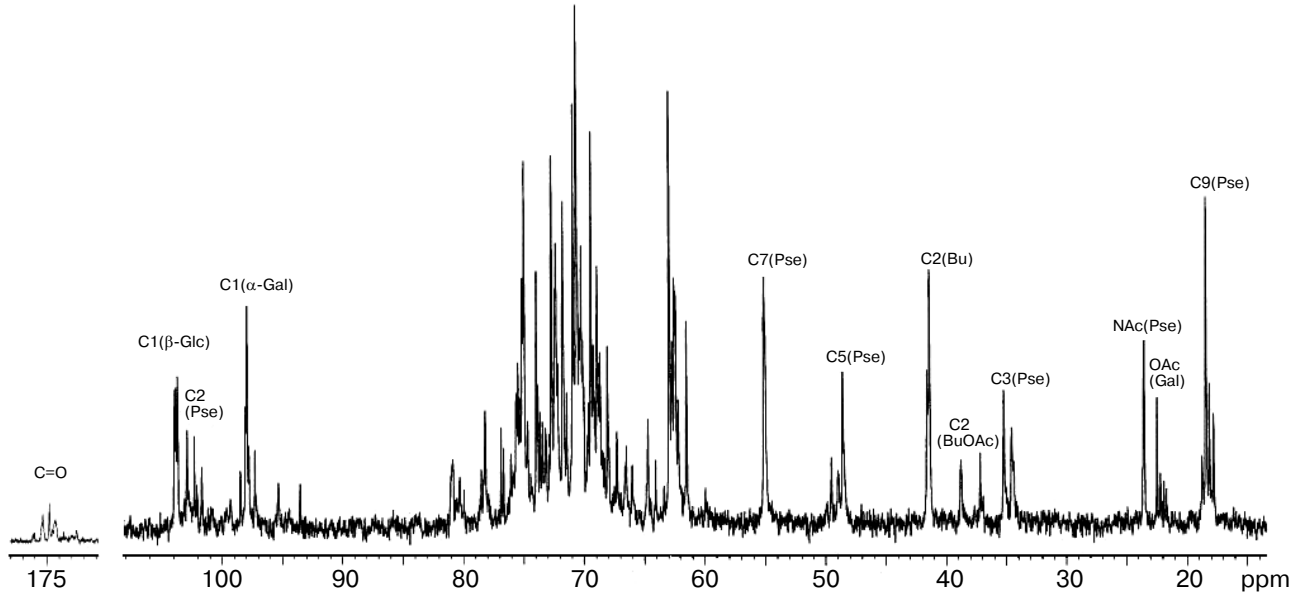


Fig. 1. ^{13}C-NMR spectrum of PS1 glycopolymer preparation from the cell wall of *A. utahensis* VKM Ac-674^T. Arabic numerals indicate the carbon atoms in the residues given in brackets.

The ^{13}C-NMR spectrum of PS1 (Fig. 1) was typical of carbohydrate polymers with irregular structure and contained several signals with various intensities in the resonance area of anomeric carbon atoms. The presence of several signals with various intensities in the area δ_C 21.5-22.5 ppm typical of resonance of the methyl groups of O-acetates also indicated that the polymer has irregular structure.

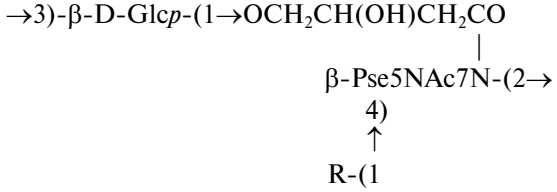
To determine the structure of PS1, this preparation was then treated with HF; this resulted in hydrolysis of phosphodiester bonds and subsequent destruction of 1,3-poly(glycerol phosphate), and PS2 was obtained after gel chromatography. The 1D and 2D NMR spectra of PS2 were recorded to localize O-acetyl groups when spectra of PS2 and de-O-acetylated polymer were compared.

The ^{13}C-NMR spectrum of de-O-acetylated polymer looked more regular (see table), in particular, it had three intense signals at δ_C 103.8, 101.6, and 98.0 ppm in the resonance area of anomeric carbon atoms. Signals of the carbonyl groups were observed in the low-field area at δ_C 175.2, 174.4, 173.0, and 172.1 ppm, while signals of the methyl groups of N-acetates (δ_C 23.6 ppm) and $\underline{C}H_3$-C groups (δ_C 17.6 (minor) and 17.5 ppm) were observed in the high-field area. The other signals were localized in the area δ_C 34.3-80.6 ppm. The APT spectrum (Fig. 2) indicated that a signal in the resonance area of anomeric carbon atoms at δ_C 101.6 ppm belongs to the quaternary C atom. There were also signals of CH_2 groups at δ_C 74.9, 69.1, 66.7, 63.0, 62.1, 41.4, 36.6, and 34.3 ppm.

The ^{1}H-NMR spectrum of de-O-acetylated polymer (table) had two intense doublets at δ_H 5.04 ppm (J = 3.6 Hz) and 4.54 ppm (J = 7.9 Hz) in the resonance area of anomeric protons. A three-proton singlet at 2.06 ppm, doublets (J = 6.0 Hz) at δ_H 1.19 ppm (minor) and 1.17 ppm, and also multiplets at δ_H 2.71, 2.54, 2.40, 2.36, 1.79, and 1.71 ppm were observed in other characteristic resonance areas. Other signals were detected in the area 3.3-4.3 ppm.

Signals in the ^{13}C- and ^{1}H-NMR spectra were attributed by the 2D homonuclear ^{1}H/^{1}H COSY, TOCSY, and ROESY spectra and heteronuclear ^{1}H/^{13}C gHSQC and gHMBC spectra. The homonuclear spectra revealed in the preparation residues of β-D-glucopyranose (β-D-Glc*p*), α-D-galactopyranose (α-D-Gal*p*), 5,7-diamino-3,5,7,9-tetradeoxy-L-*glycero*-β-L-*manno*-non-2-ulosonic acid (β-pseudaminic acid, β-Pse), and 3,4-dihydroxybutyric acid (Dhb). The combined analysis of ROESY and heteronuclear 2D spectra showed that these residues are constituents of two different structural fragments. The correlation peak between H2 of Dhb residues and H7 of β-Pse residues in the ROESY spectrum indicates that Dhb residues are the N-acyl substituent at C7 of β-Pse. In turn, Dhb residues were substituted at O4 either by β-D-Glc*p* residue (fragment I, the table) or by β-Pse residue (fragment II, see table):

fragment I

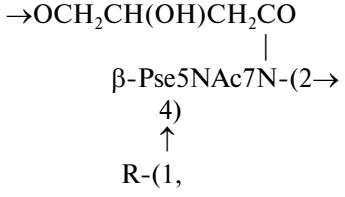


Chemical shifts in the ^{13}C- and ^{1}H-NMR spectra of the fragments of teichulosonic acid from the cell wall of *Actinoplanes utahensis* VKM Ac-674^{T}

Teichulosonic acid, residue		C1 *H1*	C2 *H2* (*H2a,2b*)	C3 *H3* (*H3e,3a*)	C4 *H4* (*H4a,4b*)	C5 *H5*	C6 *H6* (*H6a,6b*)	C7 *H7*	C8 *H8*	C9 *H9*
Fragment I of de-O-acetylated polymer*										
→3)-β-D-Glc*p*-(1→	(***A***)	103.8 *4.54*	73.7 *3.36*	80.6 *4.07*	70.1** *3.46*	77.0 *3.47*	62.1 *3.89, 3.72*			
→O-CH_2-CHOH-CH_2-CO | β-Pse5NAc7NH-(2→ 4) ↑	(***B***)	174.4 172.1	41.4 *2.40* 101.6	69.0*** *4.24* 34.3 *2.71, 1.79*	74.9 *3.95, 3.65* 72.0 *4.10*	48.4**** *4.30*	75.3 *3.84*	55.0 *4.16*	68.9 *4.24*	17.5 *1.17*
α-D-Gal*p*-(1	(***C***)	98.0 *5.04*	69.4 *3.76*	70.7***** *3.67*	70.9 *3.94*	72.8 *4.18*	63.0 *3.74, 3.74*			
Fragment II of de-O-acetylated polymer******										
→O-CH_2-CHOH-CH_2-CO | β-Pse5NAc7NH-(2→	(***D***)	174.4 173.0	41.4 *2.36* 101.2	68.5 *4.17* 36.6 *2.54, 1.71*	69.1 *3.73, 3.53* 67.7 *3.92*	49.2*** *4.19*	75.2 *3.96*	55.0 *4.20*	69.3 *4.19*	17.5 *1.19*
Disaccharide										
β-D-Glc*p*-(1→	(***A***)	103.8 *4.49*	74.3 *3.32*	76.8 *3.50*	70.9 *3.39*	77.1 *3.45*	61.9 *3.91, 3.72*			
→O-CH_2-CHOH-CH_2-CO | β-Pse5NAc7NH	(***B***)	174.2 176.7	41.0 *2.45. 2.41* 97.4	68.6 *4.24* 35.9 *1.94, 1.78* $J_{3,3}$ 13.3 $J_{3a,4}$ 12.8	74.4 *3.95, 3.65* 66.3 *4.17* $J_{3e,4}$ 5.0	50.0 *4.23* $J_{4,5}$ 3.6	71.2 *4.05* $J_{5,6}$ 1.0	54.2 *4.18* $J_{6,7}$ 10.5	68.2 *4.11* $J_{7,8}$ 3.7	16.8 *1.10* $J_{8,9}$ 6.6

* For β-Pse5NAc7NH residue unsubstituted at O4, the chemical shifts coincide with those for β-Pse5NAc7NH residue in structural unit ***D***.
** For 4-O-acetylated (100%) residue of the initial polymer: C3, 4, 5 at δ_C 78.2, 70.6, and 75.1 ppm, respectively, and H3, 4, 5 at δ_H 4.42, 4.83, and 3.65 ppm, respectively.
*** For 3-O-acetylated (~50%) residue of the initial polymer: C2, 3, 4 at δ_C 38.6, 71.8, and 72.2 ppm, respectively, and H2a, 2b, 3, 4a, 4b at δ_H 2.65, 2.50, 5.37, 4.06, and 3.81 ppm, respectively.
**** $CH_3\underline{C}ON$ at δ_C 23.5, 175.5 ppm and δ_H 2.06 ppm.
***** For 3-O-acetylated (~30%) residue of the initial polymer: C2, 3, 4 at δ_C 67.3, 73.9, and 68.9 ppm, respectively, and H2, 3, 4 at δ_H 3.99, 4.94, and 4.08 ppm, respectively.
****** For β-Pse5NAc7NH residue glycosylated at O4, chemical shifts coincide with those for β-Pse5NAc7NH in structural unit ***B***.

fragment II



where R = α-D-Gal*p* (80%) or H (20%).

The type of substitution in fragment I is proved by the presence of correlation peaks H1 (β-D-Glc*p*)/H4a,4b (Dhb) in the ROESY spectrum and also heteronuclear correlation peaks H1 (β-D-Glc*p*)/C4 (Dhb) and H4a,4b (Dhb)/C1 (β-D-Glc*p*) in the HMBC spectrum. In the case of fragment II, chemical shifts of Dhb residue changed significantly compared with those of fragment I (table) due to the different structure of the glycosylating saccharide. Correlation peak H4a (Dhb)/C2 (β-Pse) in the HMBC spectrum corresponds to the β-Pse-(2→4)-Dhb bond. In fragment I β-Pse is the glycosylating residue in relation to the β-D-Glc*p* residue, and existence of the β-Pse-(2→3)-β-D-Glc*p* bond is supported by the presence of the correlation peak H3 (β-D-Glc*p*)/C2 (β-Pse) in the HMBC spectrum. Most (~80%) of the β-Pse residues in both fragments are substituted by α-D-Gal*p* residues at position 4; this is supported by correlation peaks H1 (α-D-Gal*p*)/H4 (β-Pse) in the ROESY spec-

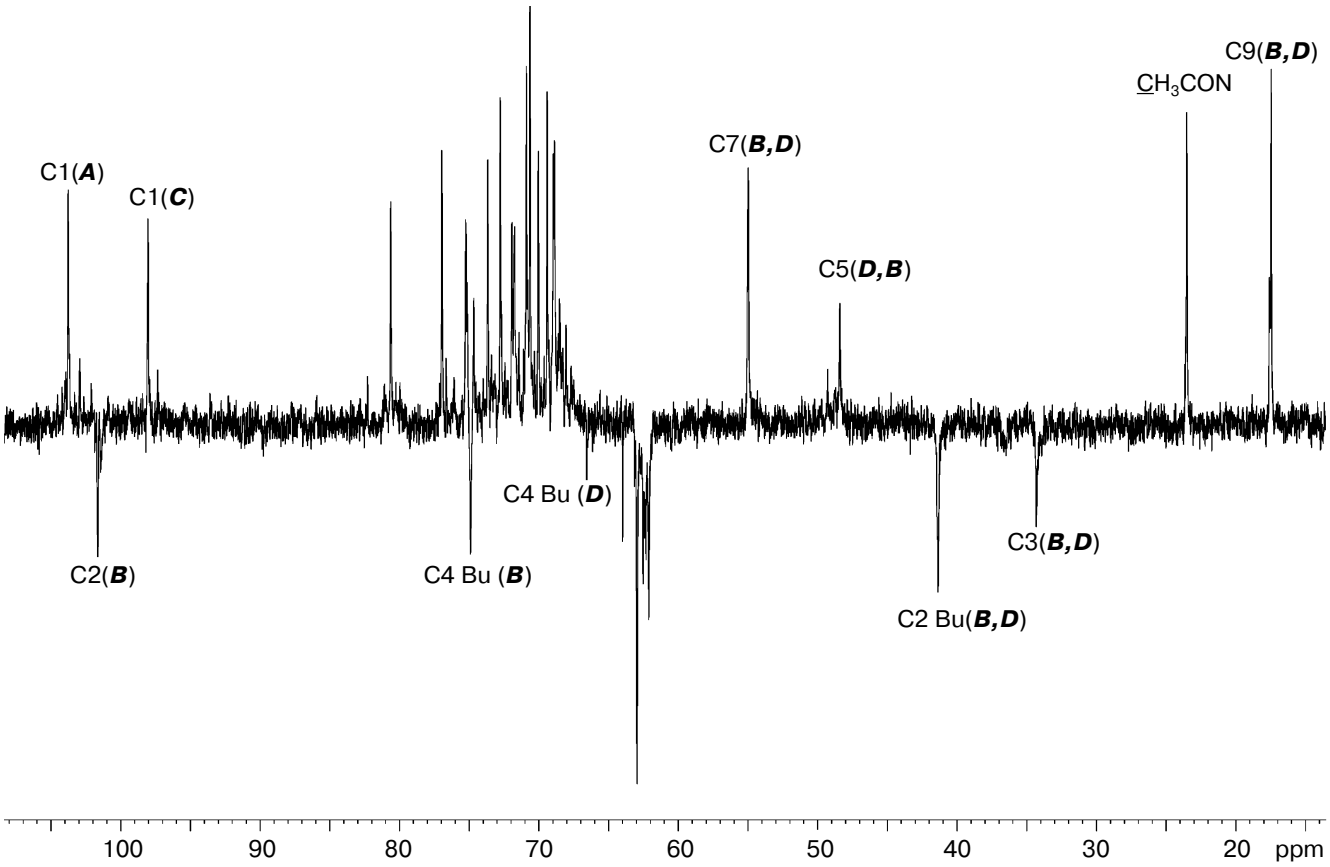
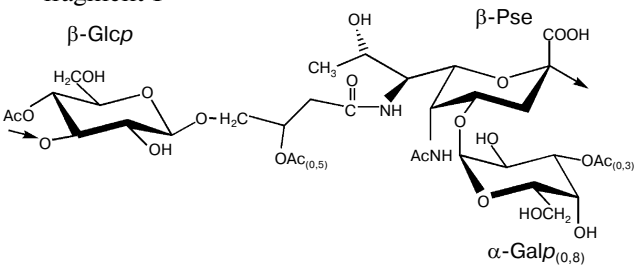


Fig. 2. ^{13}C-APT spectrum of de-O-acetylated teichulosonic acid from the cell wall of *A. utahensis* VKM Ac-674^T. Arabic numerals indicate the carbon atoms in the residues designated by Latin letters in accordance with the table.

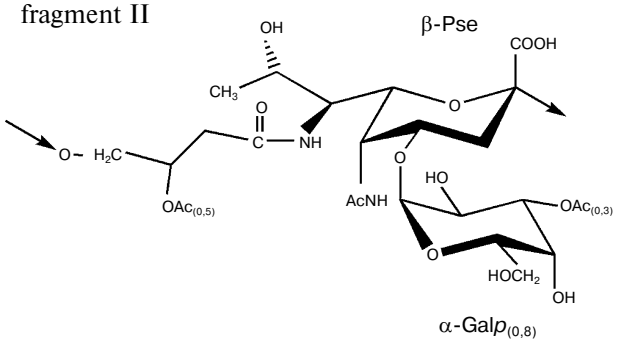
trum and H1 (α-D-Gal*p*)/C4 (β-Pse) in the HMBC spectrum.

Complete attribution of signals in the spectra of the de-O-acetylated polymer allows identification of the positions of the O-acetyl groups in PS1 polymer on comparison of the 1D and 2D spectra of the two preparations. Attribution of signals in subspectra of the O-acetylated residues becomes much easier if the known α- and β-effects of acylation are accounted for. In the ^{1}H spectra, acylation is accompanied by a significant (~1 ppm) low-field shift of signal of the proton at the carbon atom bearing the acetoxyl group (positive α-effect). In the ^{13}C spectrum, the α-effect of acylation is also positive (0-4 ppm), whereas β-effects on the neighboring carbon atoms are negative (from –1 to –4 ppm). As can be seen from comparison of chemical shifts in the residues of de-O-acetylated and PS1 structural fragments (table), the O-acetyl substituents are at C4 of β-Glc*p* residues (100%), at C3 of α-Gal*p* residues (~30%), and at C3 of the 3,4-dihydroxybutyric acid residue (~50%). The complete structure of the fragments of teichulosonic acid from the cell wall of *A. utahensis* VKM Ac-674^T is presented below.

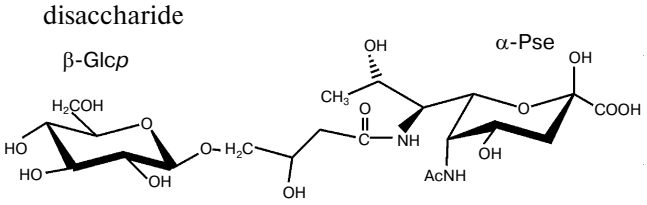
fragment I



fragment II



Chemical shifts of disaccharide obtained by HPLC of the hydrolysate of the de-O-acetylated polymer after its Smith degradation are also presented in the table. It is obvious that a disaccharide is obtained from fragment I as a result of acidic hydrolysis of the products of periodate oxidation of galactose residues. The acid-labile ketoside bond is also broken in this case, and the Pse residue attains energetically more favorable α-anomeric configuration (table) with the equatorial carboxyl group at C2:



So, the NMR data indicate that the polymeric chain is heterogeneous and contains at least two different structural fragments. However, it should be noted that for a mixture of two regular polymers consisting only of fragments I or II, the NMR spectra would not differ from the above discussed spectra of the heterogeneous polymer. Unfortunately, all attempts to solve the problem by chromatography or electrophoresis were unsuccessful.

Description of the earlier unknown natural biopolymers of Gram-positive bacteria along with phosphate-containing compounds (teichoic acids) and teichuronic acid simultaneously present in cell wall became possible due to development of nondestructive experimental methods, mainly high-resolution NMR spectroscopy. Similar to teichoic acids localized on the cell surface, polymers composed of sialic acid-like monosaccharides are named teichulosonic acids [14]. Sialic acids participating in cell–cell and cell–molecule interactions have been found in all types of animal glycoconjugates [15, 16].

The 5,7-diamino-3,5,7,9-tetradeoxy-L-*glycero*-β-L-*manno*-non-2-ulosonic acid identified in the composition of one of the *A. utahensis* cell wall polymers is one of several tens of natural derivatives of 9-carbon acidic ketosaccharides that are found in prokaryotic heteropolysaccharides [14, 15, 17].

Teichulosonic acids of two types have been found in cell walls of microorganisms inhabiting soils and belonging to various genera of the Actinomycetales order (*Brevibacterium*, *Arthrobacter*, *Streptomyces*, and *Kribbella* [18]). The first type is characterized by a polymeric chain consisting of 3-deoxy-D-*glycero*-D-*galacto*-non-2-ulosonic acid (Kdn) residues (>20) [19, 20], whereas the second one is characterized by polymeric chain consisting of residues of di-N-acyl derivatives of 5,7-diamino-3,5,7,9-tetradeoxy-L-*glycero*-β-L-*manno*-non-2-ulosonic acid (Pse) [12, 21]. Polymers with chains consisting of the residues of nonulosonic acids are described only for representatives of the Actinomycetales order.

To date, polymers having β-Pse residues have been identified in cell walls of actinomyces belonging to *Actinoplanes* and *Kribbella* genera. Along with the common structural features, teichulosonic acids of the above mentioned organisms have some distinctions. The main chain of polymer of the *A. utahensis* VKM Ac-674^T cell wall is composed of 3,4-dihydroxybutyrate (for the first time identified in bioglycans (http://www.glyco.ac.ru/bcsdb3/)), β-D-Glc*p*, and β-Pse, whereas O4 of β-Pse is substituted with α-D-Gal*p*. Teichulosonic acid of *Kribbella* is characterized by the presence of 4-hydroxybutyrate, β-D-Gal*p*, and β-Pse in the main chain, and α-D-Gal*p*, α-D-Gal*p*3OMe, α-D-Gal*p*2,3OMe or β-L-Rha*p* have been identified as substituents at O4 of β-Pse. Different strains contain specific sets of monosaccharides in the polymers.

Study of teichulosonic acids revealed some individual structural features typical of bacterial species, as it was for other cell wall anionic polymers. Along with teichulosonic acids, the cell wall usually also contains polymers of another nature [12, 20, 21].

Anionic polymers of teichulosonic acids nature located on the cell surface seem to play an important role in vital activity of the microorganisms, as teichoic acids do [22, 23]. Analogous to function of similar compounds in other organisms, they possibly participate in interaction with plants and other environmental microorganisms.

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