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Note

A new neutral polymer from the cell wall of actinomycete *Kineosporia aurantiaca* VKM Ac-702^T

Elena M. Tul'skaya,[a,*] Sof'ya N. Senchenkova,[b] Ludmila I. Evtushenko,[c] Alexander S. Shashkov[b] and Irina B. Naumova[a,†]

[a]*School of Biology, M. V. Lomonosov Moscow State University, Moscow 119899, Russia*
[b]*N. D. Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, Leninsky Prospect, 47, Moscow 119991, Russia*
[c]*G. K. Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, Pushchino, Moscow Region 142290, Russia*

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Abstract—The major cell wall polymer of *Kineosporia aurantiaca* VKM Ac-702^T a representative of the suborder *Frankineae*, is a galactomannan with a repeating unit of the following structure: →3)-β-D-Gal*p*-(1→6)-β-D-Man*p*-(1→4)-β-D-Man*p*-(1→3)-β-D-Gal*p*-(1→4)-β-D-Man*p*-(1→4)-β-D-Man*p*-(1→ that has not been reported so far. This was established using chemical degradation methods and NMR spectroscopy. The polysaccharide identified in the present study provides the second example of neutral galactomannans in actinomycete cell walls. The cell wall of *K. aurantiaca* VKM Ac-702^T also contains a minor teichoic acid, viz., 1,3-poly(glycerol phosphate) partially substituted with α-glucosamine residues, only part of which are *N*-acetylated.

Keywords: Galactomannan; NMR spectroscopy; Actinomycetes; *Kineosporia*

Anionic carbohydrate-containing polymers occur in cell walls of many Gram-positive bacteria and play an important role in vital functions of a bacterial cell.[1] Among them, teichoic and teichuronic acids, sugar-1-phosphates polymers, and anionic polysaccharides are the most widespread.[1,2] Structural studies of these cell wall components have revealed numerous novel biomolecules,[3] but it is evident that their diversity is still to be described. In addition, it is noteworthy that recent studies have demonstrated that the primary structures of the cell wall anionic polymers can be valuable chemotaxonomic markers of actinomycete species.[3]

In this work we present the results of identification of the cell wall carbohydrate polymers of actinomycete *Kineosporia aurantiaca* VKM Ac-702^T, a representative of the suborder *Frankineae*.[4] The organism was previously reported to contain a teichoic acid of glycerol phosphate nature,[5] but no structural studies of this and other cell wall polymers have been performed in this strain and other representatives of the suborder *Frankineae*.

The main acid hydrolysis products of the cell wall of *K. aurantiaca* VKM Ac-702^T containing about 1.09% of teichoic acid-linked phosphorus were, apart from peptidoglycan components, galactose and mannose and, in addition, small amounts of glycerol mono- and bisphosphates, inorganic phosphate, glycerol, and glucosamine.

Carbohydrate-containing polymers were extracted from the cell wall with cold 10% trichloroacetic acid. The yield was ca. 10–13% of the cell wall dry weight. This preparation afforded the same acid hydrolysis products as the cell wall itself. Electrophoresis in buffer A revealed the presence of two fractions, which were isolated by preparative electrophoresis and investigated separately.

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

[†] Professor Irina B. Naumova (1931–2003) was the leader of our scientific group and this work was performed with her participation.

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Fraction 1 (minor) had the electrophoretic mobility relative to that of glycerol phosphate (m_{GroP}) of 0.93 and stained blue with the molybdate reagent. Acid hydrolysis resulted in glycerol mono- and bis-phosphates, inorganic phosphate and glycerol, as well as a small amount of glucosamine. This suggests that fraction 1 contains a glycerol teichoic acid. Due to the small amount of this fraction, no NMR spectroscopic studies were carried out. However, the ^{1}H and ^{13}C NMR spectra of a non-fractionated preparation, in addition to major signals for galactose and mannose (see below), contained minor signals whose chemical shifts coincided with those of a cell wall teichoic acid from *Streptomyces rutgersensis*.[6] The latter polymer was shown to represent 1,3-poly(glycerol phosphate) with a non-stoichiometric amount of partially *N*-acetylated α-glucosaminyl residues.

Subsequent chemical transformations corroborated this suggestion.

The nature and structure of the alkaline hydrolysis products are particularly important for the elucidation of the structure of the native polymer.[7] The alkaline hydrolysate of fraction 1 comprised glycerol, its mono- and bis-phosphates, inorganic phosphate, and two phosphate esters, one of which was ninhydrin positive, and the other was ninhydrin negative. Acid hydrolysis of these two esters yielded the same products, viz., glycerol mono- and bis-phosphates, and glucosamine. The molar ratios Gro:P:$GlcNH_2$ were found to be equal to 2:2:1; phosphomonoesterase (EC 3.1.3.1, Sigma) liberated only 50% of their total phosphorus. Apparently, they represent glucosamine-containing glycerol phosphodiesters[6,7] with the *N*-acetylated amino group in one of them.

Treatment of fraction 1 with 47% HF (20 °C, 24 h) yielded two glycosides with mobilities R_{Gro} 0.4 (ninhydrin positive) and R_{Gro} 0.7 (ninhydrin-negative) in system B. Further studies of these glycosides as described previously[6] have shown that both of them contain equimolar amounts of glucosamine and glycerol, do not yield formaldehyde upon periodate oxidation and contain no reducing groups. This suggests the pyranose forms of the glucosamine residues attached to O-2 of glycerol.

These data altogether allow us to conclude that fraction 1 contains 1,3-poly(glycerol phosphate) partially substituted with α-glucosamine residues, only part of which are *N*-acetylated. The ^{13}C NMR spectrum of the whole polymer contained several minor signals in the region of δ 95–98. That is why the strict quantitative estimation of teichoic acid was difficult; its approximate proportion is 15–20% relative to the neutral polymer.

Fraction 2 (major) was electrophoretically neutral, and could be stained with alkaline $AgNO_3$. Galactose and mannose were detected upon acid hydrolysis in a molar ratio ~1:2. The absolute D-configurations of mannose and galactose were determined by GLC following their conversion into acetylated (*S*)-octan-2-yl glycosides and comparison with octan-2-yl D-glycopyranosides.[8] These results showed that this fraction is most probably a neutral polysaccharide. The structure of this polymer was completely established using NMR spectroscopy.

The ^{13}C NMR spectrum of the neutral polysaccharide (Fig. 1) contained signals for six anomeric carbon atoms at δ 101.4, 101.6, 102.5 (the signal of double integral intensity), 104.1, and 104.3. Other signals were located in the region of δ 61.6–82.6, suggesting the absence of amino sugars and uronic acids in the repeating unit of the polysaccharide. The APT spectrum (Attached Proton Test)[9] showed that the carbon atoms that resonate at δ 70.2, 62.3 (the signal of double intensity), 61.7 (the signal of double intensity), and 61.6 each carry two protons. Thus, one of the six sugar residues was substituted at C-6 and the other five had non-substituted CH_2OH groups.

The ^{1}H NMR spectrum of the polysaccharide contained six signals in the anomeric region at δ 4.48–4.91 characteristic of pyranoses with β-configuration of the anomeric center. The other ^{1}H signals were located in the region of δ 4.17–3.5 (Table 1). ^{1}H NMR spin systems of the polysaccharide were assigned by ^{1}H, ^{1}H COSY, and TOCSY experiments. The analysis of the 2D spectra revealed four residues with β-Man*p* configuration and two residues with β-Gal*p* configuration (Table 1). β-Configuration of the Gal*p* residues was in agreement with coupling constants for the anomeric signals ($J_{1,2}$ = 8 Hz), and β-configuration of the Man*p* residues was confirmed by the chemical shifts of their H-5's at δ 3.52–3.60.[10]

^{1}H, ^{13}C HSQC spectra revealed substitution of three out of four β-Man*p* residues (*C*, *E*, *F*, Table 2) at position 4 according to the downfield shifts of their C-4's as compared with that of the parent β-Man*p*[11] and substi-

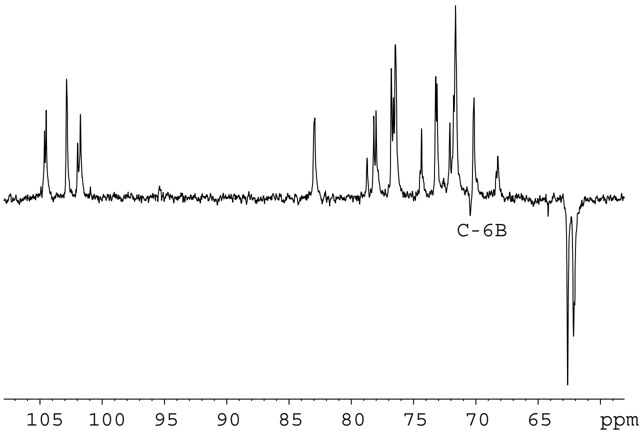

Figure 1. An APT spectrum of fraction 2 from the cell wall of *Kineosporia aurantiaca* VKM Ac-702^T.

Table 1. ^{1}H NMR data for the polysaccharide (Fraction 2) from the cell wall of *Kineosporia aurantiaca* VKM Ac-702^T (δ, ppm, relative to acetone, δ_H 2.225)

Residue	Proton						
	H-1	H-2	H-3	H-4	H-5	H-6	H-6′
→3)-β-D-Gal*p*-(1→ (*A*)	4.52	3.69	3.85	4.17	3.74	3.82	3.73
→6)-β-D-Man*p*-(1→ (*B*)	4.74	4.08	3.66	3.67	3.74	4.23	3.93
→4)-β-D-Man*p*-(1→ (*C*)	4.89	4.17	3.80	3.83	3.50	3.88	3.75
→3)-β-D-Gal*p*-(1→ (*D*)	4.48	3.69	3.83	4.17	3.77	3.82	3.73
→4)-β-D-Man*p*-(1→ (*E*)	4.76	4.12	3.80	3.82	3.60	4.02	3.83
→4)-β-D-Man*p*-(1→ (*F*)	4.91	4.17	3.80	3.85	3.50	3.88	3.75

Table 2. ^{13}C NMR data for the polysaccharide (Fraction 2) from the cell wall of *Kineosporia aurantiaca* VKM Ac-702^T (δ, ppm, relative to acetone, δ_C 31.45)[a]

Residue	Carbon					
	C-1	C-2	C-3	C-4	C-5	C-6
→3)-β-D-Gal*p*-(1→ (*A*)	104.3	71.3	82.6 (+8.5)	69.85 (−0.15)	76.1	62.3
→6)-β-D-Man*p*-(1→ (*B*)	101.6	71.7	73.95	67.8	76.4	70.2
→4)-β-D-Man*p*-(1→ (*C*)	102.5 (+7.6)	71.3	72.85 (−1.5)	78.4	76.1	61.7 (−0.6)
→3)-β-D-Gal*p*-(1→ (*D*)	104.1	71.4	82.6 (+8.5)	69.85 (−0.15)	76.25	62.3
→4)-β-D-Man*p*-(1→ (*E*)	101.4	71.3	72.7 (−1.9)	77.55	76.4	61.6 (−0.7)
→4)-β-D-Man*p*-(1→ (*F*)	102.5 (+7.6)	71.3	72.85	77.8	76.1	61.7

[a] The glycosylation effects important for the determination of the relative (in terms of the same or different) absolute configuration[12] of the residues of the disaccharide fragments are given in parentheses.

tution of one of the β-Man*p* residues (*B*) at position 6 (a downfield chemical shift of C-6 (δ 70.2) in comparison with that of β-Man*p*, δ 62.3). The substitution of the β-Gal*p* residues (*A* and *D*) at position 3 was concluded on the basis of the downfield shift of C-3's (δ 82.6) in comparison with the resonance of C-3 of the parent β-Gal*p* (δ 74.1).[10]

The monosaccharide sequence in the repeating unit of the polysaccharide was established based on the analysis of the ^{1}H, ^{1}H ROESY and ^{1}H, ^{13}C HMBC spectra (Fig. 2). The ^{1}H, ^{1}H ROESY spectrum contained, inter alia, the following inter-residue correlation peaks: H-1(*A*)/H-6(*B*), H-1(*A*)/H-6′(*B*); H-1(*B*)/H-4(*C*), H-1(*B*)/H-6(*C*), weak; H-1(*C*)/H-3(*D*); H-1(*D*)/H-4(*E*), H-1(*D*)/H-6(*E*), weak; H-1(*E*)/H-4(*F*), H-1(*E*)/H-6(*F*), weak; and H-1(*F*)/H-3(*A*). The ^{1}H,^{13}C HMBC spectrum displayed the following inter-residue correlation peaks: H-1(*A*)/C-6(*B*) and H-6(*B*)/C-1(*A*); H-1(*B*)/C-4(*C*) and H-4(*C*)/C-1(*B*); H-1(*C*)/C-3(*D*) and H-3(*D*)/C-1(*C*); H-1(*D*)/C-4(*E*) and H-4(*E*)/C-1(*D*); H-1(*E*)/C-4(*F*) and H-4 (*F*)/C-1(*E*); and H-1(*F*)/C-3(*A*) and H-3(*A*)/C-1(*F*). Both ^{1}H, ^{1}H ROESY and ^{1}H, ^{13}C HMBC spectra proved the following sequence of the repeating unit of the polysaccharide:

→3)-β-D-Gal*p*-(1→6)-β-D-Man*p*-(1→4)-β-D-Man*p*-(1→3)-β-D-Gal*p*-(1→4)-β-D-Man*p*-(1→4)-β-D-Man*p*-(1→.

The same (D-) configuration of each of the monosaccharide residues was unequivocally confirmed by the sign and values of the determining glycosylation effects in the ^{13}C NMR spectrum of the polysaccharide (see Table 2 and Refs. 11 and 12).

Thus, there are at least two carbohydrate-containing polymers in the cell wall of *K. aurantiaca* VKM Ac-702^T. The minor polymer is 1,3-poly(glycerol phosphate) partially substituted with α-glucosamine residues, only part of which are *N*-acetylated. Teichoic acids of the same structure were found in cell walls of streptomycetes very often.[6,13] The main polymer is a neutral polysaccharide, a galactomannan with a new previously unknown structure. This is rather unusual, since neutral polysaccharides are seldom encountered in cell walls of streptomycetes and relative organisms, whose main components are rather anionic carbohydrate-containing polymers. A galactomannan with a different structure has been identified in fungal cell walls,[14] and (1→6)-linked galactan, in the snail *Helix pomatia*[15] and in *Streptomyces* sp. BKM Ac-2125.[16] The present study provides yet another example of the identification of a neutral galactomannan as a component of cell wall polymers of actinomycetes.

1. Experimental

The biomass of *K. aurantiaca* VKM Ac-702^T was accumulated by growing the culture aerobically in liquid medium to the middle of the exponential phase in shaking flasks at 26 °C as described by Naumova et al.[17] The mycelium was harvested by centrifugation and washed with 0.95% NaCl, and used for cell wall preparation. A native cell wall was obtained from crude mycelium by fractional centrifugation after preliminary disruption

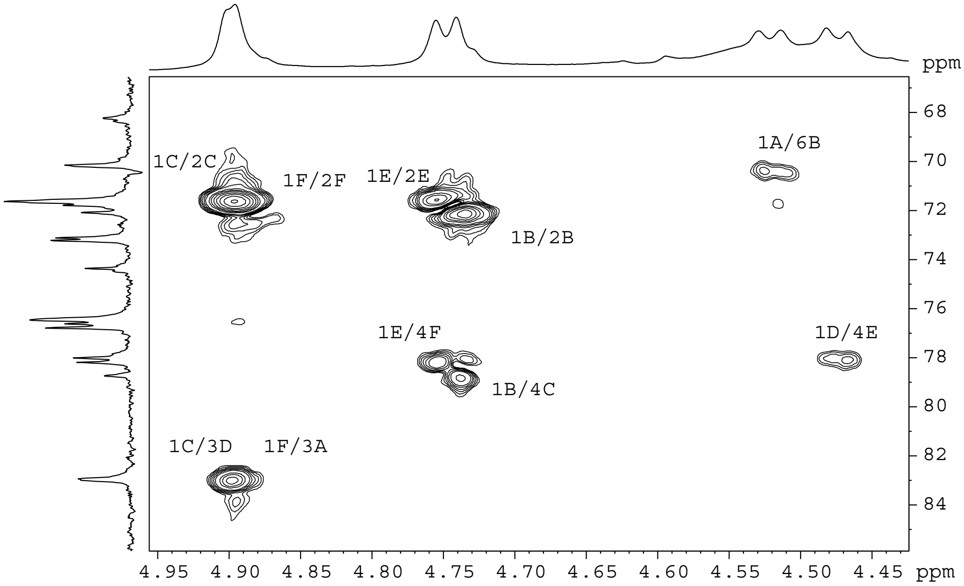

Figure 2. Part of an HMBC spectrum of fraction 2 from the cell wall of *Kineosporia aurantiaca* VKM Ac-702^T. The corresponding portions of the ^{1}H and ^{13}C NMR spectra are shown along the axes. Arabic numerals before and after the flash refer to protons and carbon atoms, respectively, of the residues A–F.

by sonication, and purified using 2% sodium dodecyl sulfate to avoid possible contamination with membrane compounds, including lipoteichoic acids. Isolation of teichoic acid, acid and alkaline hydrolyses, identification of glucosamine, investigation of glycosides as well as analytical methods have been described elsewhere.[6,18]

Descending paper chromatography and electrophoresis were carried out on a Filtrak FN-3, and FN-13 paper (Germany), washed with 2 M acetic acid and distilled water to neutral reaction. For separated cell wall polymers and phosphate esters, electrophoresis was carried out in pyridine-acetate, pH 5.6 (buffer A).[6] The following systems of solvents were used for descending paper chromatography: pyridine–benzene–butane-1-ol–water (3:1:5:3 v/v) (B) for separation of glycerol, glycosides and monosaccharides; and pyridine–ethyl acetate–acetic acid–water (5:5:1:3 v/v) (C) for separation of amino sugars.

Teichoic acid and phosphate esters were detected with the molybdate reagent; amino sugars were detected with ninhydrin; glycerol and monosaccharides were detected with 5% $AgNO_3$ in aqueous ammonium. Determination of the molar ratios glycerol:phosphorus:glucoseamine as well as galactose:mannose were described earlier.[6] The absolute configuration of Gal and Man was determined by a modified method.[8] Dry polysaccharide from fraction 2 (1 mg) was hydrolyzed with 2 M trifluoroacetic acid for 2 h at 120 °C. The acid was evaporated under a stream of air, then toluene was added to, and removed from, the residue. Anhydrous trifluoroacetic acid (15 μL) and (+)-octan-2-ol (0.2 mL) were added and the mixture was heated for 16 h at 120 °C. The volatiles were removed with a stream of air, and the residue was treated with Ac_2O (0.1 mL) and pyridine (0.1 mL) for 1 h at 100 °C. Following drying with a stream of air, the residue was analyzed by gas–liquid chromatography using a Hewlett–Packard 5890A gas chromatograph and a capillary column Ultra-1 (0.2 mm × 25 m) with OV-1 phase at 180–290 °C with nitrogen as the carrier gas. Authentic D-glycosides used as the standards were prepared from (+)-octan-2-ol and (±)–racemate.

NMR spectra were recorded with a DRX-500 (Bruker, Germany) spectrometer for 2–3% solutions in D_2O at 30 °C with acetone (δ_H 2.225, δ_C 31.45) as the internal standard. Pre-saturation of the HDO signal (1 s) was used in the accumulation of the ^{1}H NMR spectra. Two-dimensional spectra were obtained using standard pulse sequences from the Bruker software. A mixing time of 200 ms were used in ROESY experiments. A 60 ms delay was used for the evolution of long-range connectivities in ^{1}H, ^{13}C HMBC experiments.

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