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Carbohydrate Research 337 (2002) 2255–2261

CARBOHYDRATE RESEARCH

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Cell wall anionic polymers of *Streptomyces* sp. MB-8, the causative agent of potato scab

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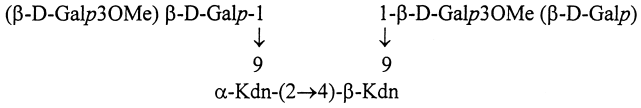
Received 13 March 2002; accepted 26 May 2002

Dedicated to Professor Horton on the occasion of his 70th birthday

Abstract

The cell wall of *Streptomyces* sp. MB-8 contains a major teichoic acid, viz., 1,3-poly(glycerol phosphate) substituted with *N*-acetyl-α-D-glucosamine (the degree of substitution is 60%), a minor teichoic acid, viz., non-substituted poly(glycerol phosphate), and a family of Kdn (3-deoxy-D-*glycero*-D-*galacto*-non-2-ulopyranosonic acid)-containing oligomers of the following general structure:



The composition of the oligomers was established using MALDI-TOF mass spectroscopy. The present study provides the second example of the identification of Kdn as a component of cell wall polymers of streptomycetes, which are the causative agents of potato scab.

Keywords: NMR spectroscopy; MALDI-TOF MS; Teichoic acids; Kdn; 3-*O*-Methylgalactose; *Streptomyces*

Anionic carbohydrate-containing polymers are the cell wall components of Gram-positive bacteria, that are covalently linked to muramic acid residues of the peptidoglycan by phosphodiester bonds. Teichoic and teichuronic acids, sugar-1-phosphate polymers, and anionic polysaccharides are the most widespread polymers.[1,2] In the majority of cases, the anionic polymers confer negative charges to the bacterial cell walls, which is vital for their physiological activity. Structural studies of these compounds may contribute to the understanding of the mechanisms of their functioning associated with ion-exchange processes, the activities of autolytic enzymes, etc.[2] Cell surface polymers also play an important role in pathogenesis of various diseases, as they determine the specificities of bacterial cell interactions with other biological systems.[3]

Recently, we have reported that the cell wall of a streptomycete strain isolated from potato infected with scab contained a polymer of 3-deoxy-D-*glycero*-D-*galacto*-non-2-ulopyranosonic acid (Kdn) together with another (minor) polymer, viz., glycerolteichoic acid. [4]

The aim of the present work was to study cell wall anionic polymers of another streptomycete species, viz.,

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0008-6215/02/$ - see front matter
PII: S0008-6215(02)00188-X

Streptomyces sp. MB-8, which is also the causative agent of potato scab.

The cell wall of *Streptomyces* sp. MB-8 contained about 1.6% of teichoic acid-linked phosphorus. Glycerol mono- and bisphosphates, inorganic phosphate, glycerol, galactose, 3-*O*-methylgalactose (madurose), and glucosamine were identified in acid hydrolysate.

The anionic polymers were extracted from the cell wall with cold 10% trichloroacetic acid. This preparation yielded the same acid hydrolysis products as the purified cell wall itself. Electrophoresis of this preparation in buffer A revealed the presence of three fractions, which were isolated by preparative electrophoresis and investigated separately.

The electrophoretic mobility relative to that of glycerol phosphate (m_{GroP}) of fraction 1 was equal to 1.3. Acid hydrolysis resulted in glycerol mono- and bisphosphates and inorganic phosphate. The formation of the same products alongside diglycerol trisphosphate upon alkaline hydrolysis suggested the fraction 1 to represent unsubstituted poly(glycerol phosphate).[5]

Fraction 2 had a electrophoretic mobility (m_{GroP}) of 0.82. Glycerol mono- and bisphosphates, inorganic phosphate, glycerol, and glucosamine were detected upon acid hydrolysis.

Hydrolysis of this fraction with 40% aqueous hydrofluoric acid yielded glycerol, inorganic phosphate, and a glycoside G1. The latter was isolated by preparative paper chromatography. Glycoside G1 was neutral as followed from electrophoresis in buffer A, chromatographic mobility in system B relative to glycerol (R_{Gro}) was equal to 0.7, and could be stained with alkaline $AgNO_3$ but not with ninhydrin or aniline phthalate. Glycoside G1 contained equimolar amounts of glycerol and *N*-acetylglucosamine, whose acetyl group was identified by NMR spectroscopy (vide infra). No formaldehyde was formed upon periodate oxidation, and no reducing groups were present in G1. Hence, the glucosaminyl residue existed in a pyranose form and was linked to glycerol at position 2 by a glycosidic bond. That the configuration of the glycosidic bond was α followed from the analysis of the NMR spectra (vide infra). Thus, the glycoside G1 was determined as 2-acetamido-2-deoxy-α-D-glucosaminyl-(1 → 2)-glycerol.

Alkaline hydrolysate of the polymer comprised of glycerol, mono- and bisphosphates, inorganic phosphate, and a phosphate ester, which produced glycerol, mono- and bisphosphates, and glucosamine upon acid hydrolysis and was not stained with ninhydrin. Apparently, this phosphate ester represents diglycerol phosphate bearing a phosphate group and *N*-acetylglucosamine residue at both O-2.[5]

These data altogether allow us to suggest that the fraction 2 contains 1,3-poly(glycerol phosphate) partially substituted with *N*-acetyl-α-D-glucosamine.

Fraction 3 had $m_{GroP} = 0.56$ and stained grey with the molybdate reagent. Galactose and 3-*O*-methylgalactose were identified upon acid hydrolysis together with trace amounts of degradation products of the teichoic acid present in the fraction 2. It thus follows that the teichoic acid does not represent the major component of this fraction.

The non-fractionated polymer preparation and fractions 2 and 3 were further investigated using NMR spectroscopy.

1. Non-fractionated preparation

The anomeric carbon resonance region of its ^{13}C NMR spectrum contained two intense signals of hexopyranoses (δ 98.2 and 104.8) in the ratio 1:3 and two minor signals at δ 96.6, 98.3. Two signals at δ 39.9 and 40.3 characteristic of C-3 of deoxynonulosonic acids, an intense signal of a carbon bearing nitrogen at δ 55.0, and a signal typical of *N*-acetyl group (CH_3CON, δ 23.3) were also present in the spectrum. The APT spectrum[6] allowed identification of non-protonated anomeric carbon atoms (C-2) of the nonulosonic acid (δ 96.6 and 98.3), the signals of the $–CH_2O–$ groups at δ 61.9, 62.3, 65.4, 65.9, 66.6, 67.7, 70.1 and 73.0, and two signals of the CO groups (δ 174.7 and 176.1).

In the ^1H NMR spectrum of the non-fractionated preparation, signals typical of the axial and equatorial protons at C-3 of the nonulosonic acid α-anomer (δ 1.72, triplet; δ 2.59, doublet of doublets) and the β-anomer (δ 1.84, triplet; δ 2.23, doublet of doublets) were clearly observed. The spectrum contained also an intense signal of the CH_3CON group at δ 2.05 and minor signals at δ 2.10 and 2.11 belonging apparently to the CH_3COO groups. The resonance of the *O*-methyl group (an intense signal at δ 3.42) was present. Three intense signals in the anomeric proton resonance region were observed at δ 4.40 and 4.41 ($J_{1,2}$ ca. 8 Hz) and at δ 5.06 ppm ($J_{1,2}$ ca. 3 Hz).

This spectral region contained also minor signals of non-anomeric protons attached to carbon atoms bearing *O*-acetyl groups (the HSQC spectrum).

2. Fraction 2

The ^{13}C NMR spectrum of this preparation contained signals at δ 98.2 and 104.8 (3:1) in the anomeric carbon resonance region. Thus, the hexose-containing polymer with the low-field position of the anomeric carbon might be considered as the major component of this preparation. Assignment of the resonances in the ^{13}C NMR spectrum has led to identification of the major polymer in this preparation as 1,3-poly(glycerol phosphate) partially substituted with *N*-acetyl-α-D-glucosamine at position 2 (cf. Ref. 7) (Tables 1 and 2).

Table 1
^{13}C NMR data (δ, ppm) for the teichoic acid (fraction 2) from the cell wall of *Streptomyces* sp. MB-8

Residue	Carbon							
	C-1	C-2	C-3	C-4	C-5	C-6	CH_3	CO
-1)-sn-Gro-(3-*P*	67.7	70.8	67.7					
-1)-sn-Gro-(3-*P*-	66.6	77.1	65.9					
2)								
↑								
α-D-Glc*p*NAc-(1	98.2	55.0	72.3	71.4	73.0	61.9	23.3	176.1

Table 2
^{1}H NMR data (δ, ppm) for teichoic acid (fraction 2) from the cell wall of *Streptomyces* sp. MB-8

Residue	Proton						
	H-1	H-1'	H-2	H-3	H-3'		
-1)-sn-Gro-(3-*P*-	3.98	3.98	3.92	3.92	3.92		
-1)-sn-Gro-(3-*P*-	4.05	4.01	4.36	4.08	4.00		
2)							
↑	H-1	H-2	H-3	H-4	H-5	H-6	H-6'
α-D-Glc*p*NAc-(1	5.10	3.95	3.82	3.50	3.86	3.91	3.80

Table 3
^{13}C NMR data (δ, ppm) for the glycoside G1 (α-D-Glc*p*NAc-(1 → 2)-sn-Gro)

Residue	Carbon							
	C-1	C-2	C-3	C-4	C-5	C-6	CH_3	CO
Gro	62.8	80.5	61.9					
2)								
↑								
α-D-Glc*p*NAc-(1	98.2	55.1	72.3	71.4	73.8	61.9	23.3	176.1

Table 4
^{1}H NMR data (δ, ppm) for the glycoside G1 (α-D-Glc*p*NAc-(1→2)-sn-Gro)

Residue	Proton		Coupling constant, (Hz)	
Gro	H-1	3.73*)	$J_{1,2}$	2
2)	H-1'	3.73*)	$J_{1,1}$	11
	H-2	3.76	$J_{1',2}$	3
↑	H-3	3.72*)	$J_{2,3}$	4
	H-3'	3.68*)	$J_{2,3'}$	5
			$J_{3,3'}$	12
α-D-Glc*p*NAc-(1	H-1	5.04	$J_{1,2}$	4
	H-2	3.93	$J_{2,3}$	11
	H-3	3.76	$J_{3,4}$	9
	H-4	3.49	$J_{4,5}$	9
	H-5	3.87	$J_{5,6}$	2
	H-6	3.86	$J_{6,6'}$	12
	H-6'	3.78	$J_{5,6'}$	5
	NAc	2.03		

*) Alternative assignment

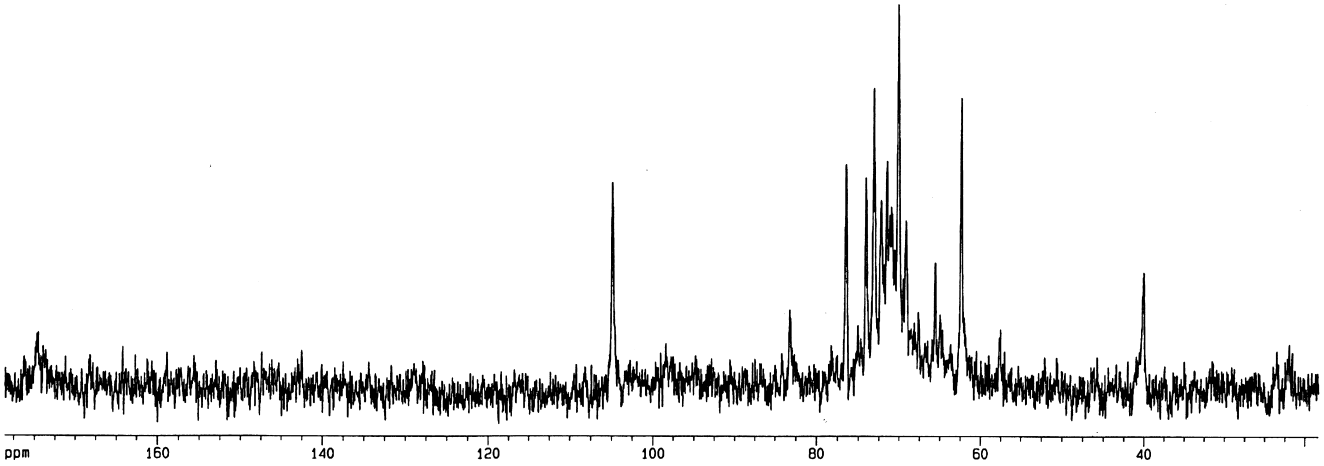


Fig. 1. ^{13}C NMR spectrum of fraction 3 from the cell wall of *Streptomyces* sp. MB-8. The spectrum contains minor signals arising from contamination with teichoic acids.

The structure of the polymer was also confirmed by the analysis of the NMR spectra of the glycoside obtained upon hydrolysis of the non-fractionated preparation with hydrofluoric acid (Tables 3 and 4).

3. Fraction 3

The anomeric carbon resonance region of its ^{13}C NMR spectrum (Fig. 1, Table 5) contained a signal of a hexopyranose at δ 104.8, signals of non-protonated carbon atoms at δ 96.6 and 98.3. The ^{1}H NMR spectrum of this fraction (Table 6), like that of the original preparation, contained signals of H-3_{eq} and H-3_{ax} of both α- and β-anomeric forms of the nonulosonic acid. The ^{1}H and ^{13}C NMR spectra of the fraction 3 were partially assigned using analysis of the 2D spectra (COSY, TOCSY, and HSQC). It was established that this fraction contains reducing terminal β-Kdn residues substituted at positions 9 and 4, α-Kdn residues substituted at position 9, and terminal non-reducing β-galactopyranose and 3-*O*-methyl-β-galactopyranose residues. The glycosylation effect for C-9 of the Kdn

residues (+9 ppm) was typical of the substitution with β-aldopyranose, while the glycosylation effect for C-4 of β-Kdn was typical of the substitution with ketopyranose.[4] Minor signals in the low-field region of the ^{1}H MNR spectrum (δ 5.1 and 5.4) were shown to belong to non-anomeric protons attached to carbon atoms bearing *O*-acetyl groups (the respective signals in the ^{13}C NMR spectrum were observed at δ 71–74).

In order to simplify the spectra and to determine the substitution pattern and the sequence of the monosaccharide residues, we performed de-*O*-acetylation of the fraction 3 and then investigated the resulting product using 2D homonuclear and heteronuclear NMR spectroscopy (COSY, TOCSY, ROESY, HSQC, and HMBC).

The ROESY spectrum contained cross-peaks of H-1's of β-Gal and 3-O-Me-β-Gal with H-9 of the α- and β-Kdn residues, which suggested the presence of the (1→9) linkages of the hexopyranoses with Kdn. This was corroborated by the low-field position of C-9 in the ^{13}C NMR spectrum. Yet another proof of the (1→9) linkages of the hexopyranose residues with Kdn was obtained from the analysis of the HMBC spectrum. This contained the Gal H-1/Kdn C-9 and 3-*O*-Me-Gal H-1/Kdn C-9 correlation peaks.

Table 5
^{13}C NMR data (δ, ppm) for de-*O*-acetylated Kdn-oligomer (fraction 3) from the cell wall of *Streptomyces* sp. MB-8

Residue	Carbon								
	C-1	C-2	C-3	C-4	C-5	C-6	OMe		
β-D-Gal*p*3OMe-(1→	104.8	71.4	83.0	65.4	76.3	62.3	57.4		
β-D-Gal*p*-(1→	104.8	72.1	73.9	69.9	76.3	62.3			
	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9
→9)-β-Kdn	174.7	96.6	40.3	70.1	71.6	72.9	69.5	70.8	73.0
4)									
↑									
→9)-α-Kdn-(2	-	98.3	39.9	70.6	71.0	73.9	69.5	70.2	72.4

Table 6
^{1}H NMR data (δ, ppm) for de-*O*-acetylated Kdn-oligomer (fraction 3) from the cell wall of *Streptomyces* sp. MB-8

Residue	Proton[a]								
	H-1	H-2	H-3	H-4	H-5	H-6,6'	OMe		
β-D-Gal*p*3OMe-(1→	4.41	3.58	3.36	4.21	3.69	3.77	3.42		
β-D-Gal*p*-(1→	4.40	3.52	3.64	3.92	3.69	3.77			
	H-3a	H-3e	H-4	H-5	H-6	H-7	H-8	H-9	H-9'
→9)-β-Kdn	1.84	2.23	4.03	3.59	3.96	3.91	3.89	4.16	3.85
4)									
↑									
→9)-α-Kdn-(2	1.72	2.59	3.64	3.57	3.81	3.93	4.03	4.22	3.81

The Kdn C-4 chemical shift value is characteristic of (2→4)-linked Kdn residues (δ 70.1 for the β-anomer[4] and δ 70.6 for the α-anomer).

Thus, the fraction 3 contained oligomeric chains built of α-(2→4)-linked Kdn residues with β-Kdn at the reducing end. The major part of the Kdn units carry galactopyranose or 3-*O*-methylgalactopyranose substituents (2:1) at O-9, some of them bear *O*-acetyl groups in various positions.

Comparative analysis of the ^{13}C NMR spectra of the original and de-*O*-acetylated polymer preparations and the APT spectrum of the latter showed that the de-*O*-acetylated product is devoid of the peak of the CH_2 group at δ 71.1, while this peak was present in the spectrum of the original preparation. This suggests the localisation of some acetyl groups at O-8 of Kdn. Analysis of the COSY, TOCSY, and HSQC spectra of the *O*-acetylated polymer confirms that the acetyl groups are attached to some O-8's of the Kdn residues.

The localisation of the acetyl groups at some O-7's of Kdn and O-2's of Gal was established analogously.

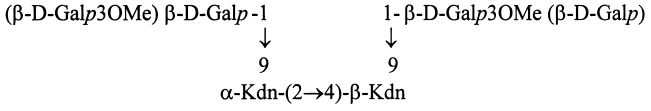
The matrix-assisted laser-desorption ionisation (MALDI) mass spectrum (positive mode) of the oligosaccharide from fraction 3 using a time-of-flight (TOF) instrument and 2,5-dihydroxybenzoic (gentisic) acid as a matrix showed two series of ions with *m*/*z* from 804 to 902 and from 411 to 509.

The first series contained the peaks of molecular ions corresponding to the structures with the following compositions: *m*/*z* 804 (Kdn–Kdn–Gal–Gal), 818 (Kdn–Kdn–Gal–MeGal), 832 (Kdn–Kdn–MeGal–MeGal), 846 (Kdn–Kdn–Gal–Gal–Ac), 860 (Kdn–Kdn–Gal–MeGal–Ac), 874 (Kdn–Kdn–MeGal–MeGal–Ac), 888 (Kdn–Kdn–Gal–Gal–Ac–Ac), and 902 (Kdn–Kdn–Gal–MeGal–Ac–Ac).

This fact allows to confirm that the oligomer family from fraction 3 contains two Kdn residues, which are statistically substituted by galactose and/or 3-*O*-methylgalactose. These oligosaccharides can carry up to two *O*-acetyl groups.

The second group of peaks (*m*/*z* 411–509) corresponds to disaccharide fragments, which probably represent the tetrasaccharide degradation products formed upon (2→4)-bond cleavage.

Thus, the cell wall of the streptomycete species under study contains a major teichoic acid, viz., 1,3-poly(glycerol phosphate) substituted with *N*-acetyl-α-D-glucosamine (the degree of substitution is 60%), a minor teichoic acid, viz., non-substituted poly(glycerol phosphate), and a family of Kdn-containing oligomers of the following general structure:



Kdn-containing oligomers are mainly present in animal tissues as the glycoconjugate constituents.[3] This monosaccharide has been identified as a component of a bacterial heteropolysaccharide from *Klebsiella ozanae* K4,[8] a trisaccharide from *Sinorhizobium fredii*,[9] and of a cell wall homopolymer of *Streptomyces* sp. VKM Ac-2090.[4] The present study provides yet another example of the identification of Kdn as a component of cell wall polymers of streptomycetes which are the causative agents of potato scab.

4. Experimental

The biomass of *Streptomyces* sp. MB-8 was accumulated by growing the culture aerobically in liquid medium to the end of the exponential phase in shaking flasks at 26 °C as described by Naumova et al.[10] The mycelium was harvested by centrifugation and washed with 0.95% NaCl, this was used for cell wall preparation.

A native cell wall was obtained from crude mycelium by fractional centrifugation after preliminary disruption by sonication, and purified using 2% sodium dodecyl sulfate to avoid possible contamination with membrane compounds, including lipoteichoic acids.

Isolation of teichoic acid, analytical methods, acid and alkaline hydrolyses, hydrolysis with HF, identification of glucosamine, as well as structural analysis of the glycoside G1 have been described elsewhere.[7,11]

Descending paper chromatography and electrophoresis were carried out on a Filtrak FN-12, and FN-13 paper (Germany), preliminary washed with 2 M acetic acid and distilled water to neutral reaction.

To separated phosphate esters, electrophoresis was carried out in pyridine–acetate buffer, pH 5.6 (buffer A).[7] The following solvent systems were used for descending paper chromatography: 3:1:5:3 pyridine–benzene–butane-1-ol–water (B) for separation of glycerol, glycosides, and monosaccharides; and 5:5:1:3 pyridine–EtOAc–AcOH–water (C) for the separation of amino sugars.

De-*O*-acetylation of preparations was performed by treating them with 10% aqueous ammonia for 20 h at 20 °C. The ammonium was evaporated under the stream of air.

Teichoic acid and phosphate esters were detected with the molybdate reagent; amino sugars were detected with ninhydrin; reducing sugars with aniline phthalate; glycerol, glycoside, and monosaccharides were detected with 5% $AgNO_3$ in aqueous ammonium.

NMR spectra were recorded with a DRX-500 (Bruker, Germany) spectrometer for 2–3% solutions in D_2O at 30 °C with acetone (δ 2.225 for ^1H and 31.45 for ^{13}C) as the internal standard. 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.

Mass spectrometric analysis (MALDI-TOF MS) of oligosaccharides of fraction 3 were carried out in the positive reflection mode using a KOMPACT MALDI 4 (Kratos Analytical) mass spectrometer. For all samples, 2,5-dihydroxybenzoic (gentisic) acid was used as a matrix.

Acknowledgements

The present work was supported by grants from INTAS No. 96-1571, No. 01-2040 and the Russian Foundation for Basic Research No. 01-04-48769.

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