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Note

Structure of the O-polysaccharide and serological cross-reactivity of the lipopolysaccharide of *Providencia alcalifaciens* O32 containing *N*-acetylisomuramic acid

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Abstract—The O-polysaccharide was obtained by mild acid degradation of the lipopolysaccharide of *Providencia alcalifaciens* O32 and studied by sugar and methylation analyses, solvolysis with triflic acid, ^{1}H and ^{13}C NMR spectroscopy, including two-dimensional ^{1}H,^{1}H COSY, TOCSY, ROESY, H-detected ^{1}H,^{13}C HSQC and HMBC experiments. It was found that the polysaccharide has a branched tetrasaccharide repeating unit containing 2-acetamido-3-*O*-[(*S*)-1-carboxyethyl]-2-deoxy-D-glucose (D-GlcNAc3*S*lac, *N*-acetylisomuramic acid) with the following structure:

→3)-α-L-Fuc*p*NAc-(1→3)-α-D-Glc*p*NAc-(1→6)-α-D-Glc*p*NAc3*S*lac-(1→
4
↑
1
β-D-Glc*p*

Serological studies with O-antisera showed antigenic relationships between *P. alcalifaciens* O32 and O29 as well as several other *Providencia* and *Proteus* strains sharing putative epitopes on the O-polysaccharides.
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Keywords: *Providencia alcalifaciens*; O-Antigen; Lipopolysaccharide; Polysaccharide structure; Isomuramic acid

The genus *Providencia* of the *Enterobacteriaceae* family consists of six species: *Providencia alcalifaciens*, *Providencia heimbachae*, *Providencia rettgerii*, *Providencia rustigianii*, *Providencia stuartii* and *Providencia vermicola*.[1,2] Being a component of the normal intestinal flora, in favourable conditions bacteria *P. stuartii* can cause urinary tract infections; *P. alcalifaciens* and *P. rustigianii* can cause various infections, including traveller's diarrhoea.[1] As in other Gram-negative bacteria, the serological O-specificity of *Providencia* is defined by the fine structure of the O-antigen (O-polysaccharide), which is a part of the lipopolysaccharide (endotoxin). The serological classification scheme of *P. alcalifaciens*, *P. rustigianii* and *P. stuartii* includes 62 O-serogroups.[3] At present, more than 25 O-polysaccharide structures have been established with the aim of the elucidation of the molecular basis of the serological classification of *Providencia* strains. In this paper, we report on a new structure of the O-polysaccharide of *P. alcalifaciens* O32.

Mild acid degradation of the lipopolysaccharide of *P. alcalifaciens* O32 gave a high-molecular-mass

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polysaccharide eluted from a Sephadex G-50 column immediately after the void volume. Sugar analysis using GLC of the acetylated alditols revealed Glc, GlcN, 2-amino-2,6-dideoxygalactose (FucN) and 2-amino-3-*O*-(1-carboxyethyl)-2-deoxyglucose (muramic or isomuramic acid, GlcN3lac). The last sugar was identified as the (*S*)-isomer (isomuramic acid) using an amino acid analyzer. The D configuration of GlcN and Glc and the L configuration of FucN were determined by GLC of the acetylated glycosides with (+)-2-octanol; the absolute configuration of GlcN3lac was confirmed by NMR spectroscopy (see below). Methylation analysis, including GLC-MS of the partially methylated alditol acetates derived by hydrolysis of the methylated polysaccharide, revealed 3-substituted FucN, 3,4-disubstituted GlcN, terminal Glc and 6-substituted GlcN3lac.

The ^{13}C NMR spectrum (Fig. 1) indicated a regular structure of the O-polysaccharide. It contained signals for four sugar residues, including those for four anomeric carbons at δ 98.1 (2C), 100.9, and 102.8 (the overlap of two signals at δ 98.1 was later resolved using ^{1}H,^{13}C HSQC). There were also signals for three nitrogen-bearing carbons at δ 49.8, 54.5 and 55.3, other oxygen-bearing sugar-ring carbons, sugar C–CH_2OH groups (C-6), and lactic acid (lac) C-2 in the region δ 60.7–82.8 as well as two C–CH_3 groups at δ 16.8 and 20.5 (FucN C-6 and lac C-3). The spectrum also contained signals for three *N*-acetyl groups at δ 174.4, 174.7, 175.2 (all CO), 23.3, 23.7 and 23.7 (all CH_3) as well as a carboxyl group (lac C-1) at δ 183.4 (compare published data[4,5]). There were no signals in the region δ 82–88, except for the one at δ 82.8, which was later assigned to C-3 of GlcN in GlcN3lac; hence, all sugar residues are in the pyranose form.[6,7] The ^{1}H NMR spectrum of the O-polysaccharide contained signals for four anomeric protons at δ 4.53, 4.81, 5.01 and 5.08, three *N*-acetyl groups at δ 1.97, 2.00 and 2.11, two C–CH_3 groups at δ 1.20 and 1.30, and other protons in the region δ 3.02–4.81.

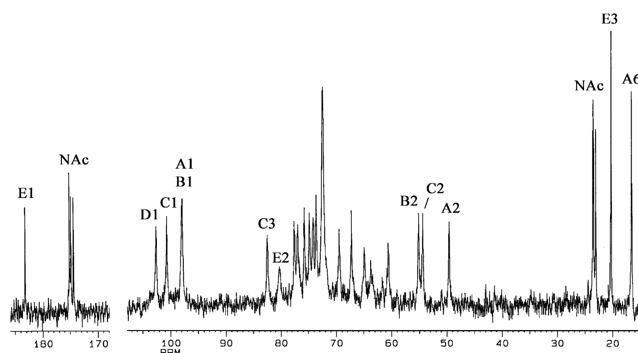

Figure 1. ^{13}C NMR spectrum of the O-polysaccharide of *P. alcalifaciens* O32.

The ^{1}H and ^{13}C NMR spectra of the O-polysaccharide were assigned using ^{1}H,^{1}H COSY, TOCSY, ROESY, ^{1}H,^{13}C HSQC (Fig. 2) and ^{1}H,^{13}C HMBC experiments (Tables 1 and 2). Spin systems of three amino sugars were distinguished by correlations between protons at nitrogen-bearing carbons (H-2) and the corresponding carbons (C-2) at δ 4.26/49.8, 4.22/55.3 and

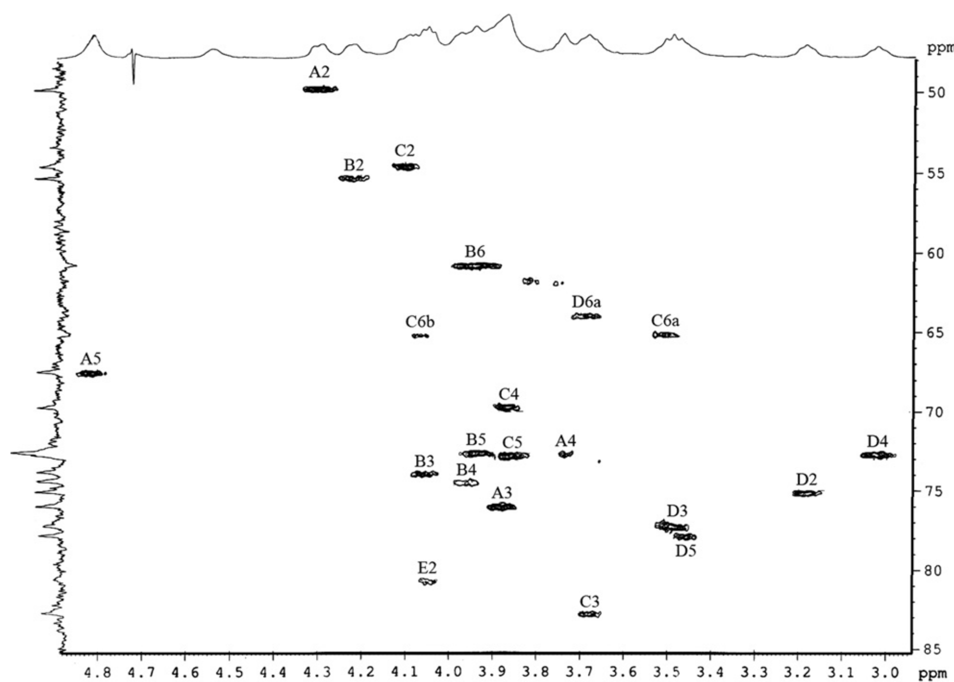

Figure 2. Part of a ^{1}H,^{13}C HSQC spectrum of the O-polysaccharide of *P. alcalifaciens* O32. The corresponding parts of the ^{1}H NMR and ^{13}C DEPT spectra are shown along the axes. Arabic numerals refer to atoms in sugar residues denoted by capital letters as shown in Tables 1 and 2.

Table 1. ^{1}H NMR data (δ, ppm)

Sugar residue		H-1	H-2	H-3	H-4	H-5	H-6a	H-6b
O-Polysaccharide **1**								
→3)-α-L-Fuc*p*NAc-(1→	**A**	5.08	4.26	3.87	3.72	4.81	1.20	
→3,4)-α-D-Glc*p*NAc-(1→	**B**	4.81	4.22	4.06	3.95	3.93	3.94	3.94
→6)-α-D-Glc*p*NAc-(1→	**C**	5.01	4.10	3.68	3.86	3.84	3.50	4.07
β-D-Glc*p*-(1→	**D**	4.53	3.19	3.48	3.02	3.45	3.69	3.91
(*S*)-lac	**E**		4.05	1.30				
Disaccharide								
→3)-α-L-Fuc*p*NAc		5.10	4.29	4.01	3.90	4.22	1.19	
→3)-β-L-Fuc*p*NAc		4.60	3.95	3.83	3.84	3.76	1.24	
α-D-Glc*p*NAc-(1→		5.03[a]	4.06	3.68	3.55	3.73	3.84	3.75
(*S*)-lac			4.06	1.29				

Additional chemical shifts for the *N*-acetyl groups are δ 1.97, 2.00 and 2.11 in the O-polysaccharide **1**; δ 1.98 and 2.08 in the disaccharide.
[a] H-1a; H-1b at δ 4.98.

Table 2. ^{13}C NMR data (δ, ppm)

Sugar residue		C-1	C-2	C-3	C-4	C-5	C-6
O-Polysaccharide **1**							
→3)-α-L-Fuc*p*NAc-(1→	**A**	98.1	49.8	76.0	72.5	67.4	16.8
→3,4)-α-D-Glc*p*NAc-(1→	**B**	98.1	55.3	73.9	74.5	72.8	60.7
→6)-α-D-Glc*p*NAc-(1→	**C**	100.9	54.5	82.8	69.7	72.5	65.0
β-D-Glc*p*-(1→	**D**	102.8	75.1	77.2	72.9	77.8	64.0
(*S*)-lac	**E**	183.4	80.7	20.5			
Disaccharide							
→3)-α-L-Fuc*p*NAc		92.5	50.4	74.9	72.5	67.7	16.9
→3)-β-L-Fuc*p*NAc		96.5	54.2	78.1	71.7	72.1	16.9
α-D-Glc*p*NAc-(1→		100.2[a]	54.4	81.8	70.6	74.1	62.0
(*S*)-lac		182.3	80.3	20.4			

Additional chemical shifts for *N*-acetyl groups are δ 175.2, 174.7, 174.4 (all CO), 23.3 and 23.7 (both CH_3) in the O-polysaccharide **1**; δ 175.8 (2 CO), 23.4 and 23.6 (both CH_3) in the disaccharide.
[a] C-1a; C-1b at δ 100.4.

4.10/54.5. The remaining spin system of Glc was unambiguously assigned by correlations of H-1 with protons from H-2 to H-5 in the TOCSY spectrum and by those between all neighboring protons in the COSY spectrum. The ^{13}C NMR chemical shifts of Glc determined using the ^{1}H,^{13}C HSQC spectrum were characteristic of an unsubstituted β-glucopyranose residue[6,7] and defined the β configuration and the lateral position of Glc in the polysaccharide. The signals for FucN were found by correlations between H-1, H-2, H-3 and H-4 in the COSY and TOCSY spectra, by those of H-6 with C-5 and C-4 in the HMBC spectrum, and between all carbons and the attached protons in the HSQC spectrum. Cross-peaks between H-2 and all other protons of one of the GlcN residues and between H-2 and H-1, H-3 and H-4 of the second GlcN residue were present in the TOCSY spectrum and assigned using the COSY spectrum. The corresponding H/C correlations were assigned directly whereas the H-5/C-5 and C-6/H-6 cross-peaks of the second GlcN residue were found as the remaining cross-peaks in the HSQC spectrum; thus, the signal assignment for this sugar was completed. GlcN and GlcN3lac were distinguished by the position of the C-6 signals at δ 60.7 and 65.0, respectively, taking into account the methylation analysis data, which showed that only GlcN3lac is 6-substituted (see above); as a result, the C-6 resonance of GlcN3lac is shifted downfield.[7] The H-1 signals of the amino sugars were poorly resolved, and, therefore, their α configuration was established based on the chemical shift data, particularly, by the positions of the signals for H-1 at δ 4.81–5.08, C-1 at δ 98.1–100.9, C-5 at δ 67.4 (FucN) and 72.5–72.8 (GlcN).[6,7]

The ROESY spectrum of the polysaccharide showed cross-peaks between the following anomeric protons and protons at the linkage carbons: α-FucN H-1,α-GlcN H-3 at δ 5.08/4.06, α-GlcN H-1,α-GlcN3lac H-6a and H-6b at δ 4.81/3.50 and 4.81/4.07, α-GlcN3lac H-1,α-FucN H-3 at δ 5.01/3.87 and β-Glc H-1,α-GlcN H-4 at δ 4.53/3.95. These data are in agreement with the substitution pattern of the monosaccharides and demonstrated their sequence in the repeating unit. The lac H-2 signal showed a correlation with a GlcN H-3 signal at δ 4.05/3.68, which confirmed the assignment of

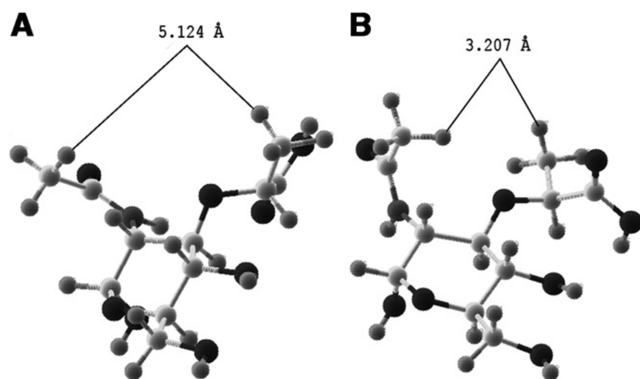

Figure 3. Spatial models for D-GlcpNAc3*R*lac (A) and D-GlcpNAc3*R*-lac (B) generated by conformational search using the force field MM3-1996. Indicated are the calculated distances between methyl groups of the lactic acid residue and the *N*-acetyl group.

the GlcN3lac protons and the structure of the lac ether. A cross-peak between lac H-3 and the *N*-acetyl group of GlcN3lac at δ 1.30/2.08 in the ROESY spectrum confirmed the absolute configuration of lac. In fact, this could appear only in case of the (*S*)-configuration of lac as the distance between the two methyl groups was calculated by molecular modelling in the MM3-1996 force field as ~3.2 Å for GlcpNAc3*S*lac and ~5.1 Å for GlcpNAc3*R*lac (Fig. 3).

Therefore, the O-polysaccharide of *P. alcalifaciens* O32 has the structure **1** shown in Chart 1. In order to independently confirm this structure, solvolysis of the O-polysaccharide with triflic acid[8] was performed, which, among other products, afforded a disaccharide. The ^{1}H and ^{13}C NMR spectra of the disaccharide showed signals for α-D-GlcpNAc3lac, α-L-FucpNAc and β-L-FucpNAc in the ratios ~1:0.5:0.5. They were assigned using COSY, NOESY and ^{1}H,^{13}C HSQC experiments (Tables 1 and 2), and the data obtained were in full agreement with the expected α-D-GlcpNAc3lac-(1→3)-L-FucNAc disaccharide structure.

The carbohydrate backbone of the O-polysaccharide of *P. alcalifaciens* O32 is structurally identical to the O-polysaccharide of *P. alcalifaciens* O29[9] **2** (Chart 1), and the only difference between the two is the lack of lac from the latter. Rabbit polyclonal O-antisera against *P. alcalifaciens* O32 and *P. alcalifaciens* O29 reacted strongly with the homologous LPSs in enzyme-immunosorbent assay (EIA) but cross-reacted only weakly (Table 3), thus indicating the importance of *N*-acetylisomuramic acid residue for manifesting of the immunospecificity. In Western blot (Fig. 4), both O-antisera recognized slow and fast migrating bands of the homologous LPSs, which correspond to high- and low-molecular-mass LPS species with and without the O-polysaccharide chain, respectively. Anti-*P. alcalifaciens* O32 serum recognized also high- and low-molecular-mass LPS species of *P. alcalifaciens* O29, whereas anti-*P. alcalifaciens* O29 serum reacted with high molecular-mass LPS species of *P. alcalifaciens* O32 only.

No cross-reactivity in EIA and Western blot was observed between either O-antiserum and other *Providencia* and *Proteus* LPSs, except for weakly reacting LPSs of several strains (Table 3, Fig. 4). The cross-reactivity of anti-*P. alcalifaciens* O32 serum with the LPS of *Proteus penneri* 26 (O31) could be due to the presence of a common epitope associated with a α-D-GlcpNAc-(1→6)-α-D-GlcpNAc disaccharide (Chart 1). The cross-reactive LPSs of *Proteus vulgaris* O39 and O42 share a putative epitope associated with a α-L-FucpNAc-(1→3)-α-D-GlcpNAc disaccharide. Finally, the serological relationship of the LPSs of *P. alcalifaciens* O32 and *P. rustigianii* O16 could be substantiated by the presence of similar acidic sugars: *N*-acetylisomuramic acid in the former and *N*-acetylmuramic acid in the latter polysaccharide (Chart 1), which, most likely, occupies the non-reducing end of the polysaccharides. Similar results were obtained when anti-*P. alcalifaciens* O29 serum was tested with the same LPSs, except for that no cross-reactivity was observed in Western blot with high-molecular-mass species of the LPS of *P. rustigianii* 16. Low-molecular-mass species of this LPS were reactive, thus suggesting the occurrence of a common epitope(s) on the LPS cores of *P. alcalifaciens* O29 and *P. rustigianii* O16, whose structures remain to be determined.

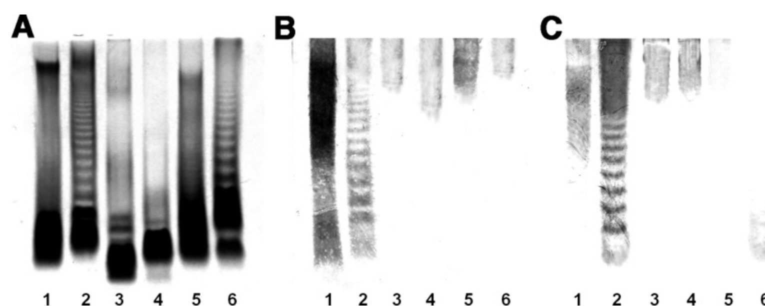

Figure 4. Sodium deoxycholate polyacrylamide gel electrophoresis (A), western blot with O-antisera against *P. alcalifaciens* O32 (B) and *P. alcalifaciens* O29 (C) of the LPSs of *P. alcalifaciens* O32 (lane 1), *P. alcalifaciens* O29 (lane 2), *Proteus penneri* 26 (O31) (lane 3), *Proteus vulgaris* O39 (lane 4), *Proteus vulgaris* O42 (lane 5) and *Providencia rustigianii* O16 (lane 6).

Chart 1. Structures of the O-polysaccharides of *P. alcalifaciens* O32 (**1**), *P. alcalifaciens* O29[9] (**2**), *Proteus penneri* 26 (O31)[16] (**3**), *Proteus vulgaris* O39[17] (**4**), *Proteus vulgaris* O42 (Perepelov, A. V.; Knirel, Y. A.; Rozalski, A., unpublished data) (**5**) and *Providencia rustigianii* O16[18] (**6**).

Table 3. Serological reactivity of anti-*P. alcalifaciens* O32 and anti-*P. alcalifaciens* O29 sera with *Providencia* and *Proteus* LPSs in EIA (reciprocal titers)

LPS from	O-Antiserum against	
	P. alcalifaciens O32	*P. alcalifaciens* O29
P. alcalifaciens O32	128,000	4000
P. alcalifaciens O29	8000	512,000
Proteus penneri 26 (O31)	1000	1000
Proteus vulgaris O39	1000	<1000
Proteus vulgaris O42	2000	1000
P. rustigianii O16	1000	<1000

1. Experimental

1.1. Bacterial strain, isolation and degradation of the lipopolysaccharide

P. alcalifaciens O32:H11, strain 1853/49 obtained from the Hungarian National Collection of Medical Bacteria (National Institute of Hygiene, Budapest), was cultivated under aerobic conditions in tryptic soy broth supplemented with 0.6% yeast extract. The bacterial mass was harvested at the end of the logarithmic growth phase, centrifuged, washed with distilled water and lyophilized. The LPS in a yield of 2.9% of dry bacterial weight was isolated by phenol–water extraction[10] followed by dialysis of the extract without layer separation and removal of impurities by treatment with cold aqueous 50% CCl_3CO_2H; the supernatant was dialyzed and freeze-dried.

A portion of the LPS (191 mg) was heated with 2% acetic acid for 4 h at 100 °C and the carbohydrate-containing supernatant was fractionated on a column (60 × 2.5 cm) of Sephadex G-50 in 0.05 M pyridinium acetate buffer. The yield of the polysaccharide was 15% of the LPS weight.

1.2. Chemical methods

For sugar analysis, the polysaccharide was hydrolyzed with 10 M HCl (80 °C, 30 min), the products were reduced with an excess of $NaBH_4$ (2 h, 20 °C), acetylated with a 1:1 Ac_2O–Py mixture (100 °C, 1 h) and analyzed by GLC on a Hewlett–Packard HP 5890 chromatograph equipped with an Ultra-2 column (Hewlett–Packard) using a temperature gradient from 160 (3 min) to 290 °C at 5 °C min^{-1}. Muramic acid was identified using a Biotronik LC-2000 amino acid analyzer and standard sodium citrate buffers.

For determination of the absolute configurations of the monosaccharides, the polysaccharide was hydro-

lyzed with 10 M HCl as above, N-acetylated (400 μL $NaHCO_3$, 60 μL Ac_2O, 0 °C, 1 h), subjected to (+)-2-octanolysis[11] [100 μL (+)-2-octanol, 15 μL CF_3CO_2H, 120 °C, 16 h], acetylated and analyzed by GLC as in sugar analysis.

Methylation of the polysaccharide was performed according to the Hakomori procedure,[12] the products were recovered using a Sep-Pak cartridge. Partially methylated monosaccharides were derived by hydrolysis with 10 M HCl (80 °C, 30 min), converted into the alditol acetates and analyzed by GLC–MS on a Termo-Quest Finnigan model Trace series GC 2000 instrument equipped with an EC-1 column (0.32 mm × 30 m) using a temperature gradient from 150 °C (2 min) to 250 °C at 10 °C min^{-1}.

For solvolysis, the polysaccharide (14.5 mg) was treated with triflic acid for 16 h at 1 °C; after neutralization with 5% aq NH_3, the products were evaporated and fractionated on a TSK HW-40 column in water to give a disaccharide (0.8 mg).

1.3. NMR spectroscopy and molecular modelling

Samples were freeze-dried twice from a ^2H_2O soln and dissolved in 99.96% ^2H_2O with internal TSP (δ_H 0) and external acetone (δ_C 31.45) as references. ^1H and ^{13}C NMR spectra were recorded at 30 °C using a Bruker DRX-500 NMR spectrometer and XwinNMR Bruker software on SGI Indy/Irix 5.3 workstation. Mixing time of 300 ms and spin-lock time of 30 ms were used in ROESY and TOCSY experiments, respectively. Other NMR experimental parameters were essentially as described.[13]

The spatial modelling was performed by Serena PCModel 7.0 Software on an AMD64 PC.

1.4. Serological techniques

Rabbit polyclonal antisera against *P. alcalifaciens* O32 and *P. alcalifaciens* O29 were obtained by immunization of New Zealand white rabbits with heat-killed bacteria.[14] Enzyme-immunosorbent assay with LPS as antigen, sodium deoxycholate polyacrylamide gel electrophoresis and Western blot were performed as described.[15]

Acknowledgements

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