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Structure of an acidic polysaccharide from the agar-decomposing marine bacterium *Pseudoalteromonas atlantica* strain IAM 14165 containing 5,7-diacetamido-3,5,7,9-tetradeoxy-L-*glycero*-L-*manno*-non-2-ulosonic acid

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Abstract—The structure of an acidic polysaccharide from *Pseudoalteromonas atlantica* strain 14165 containing 5,7-diacetamido-3,5,7,9-tetradeoxy-L-*glycero*-L-*manno*-non-2-ulosonic acid (di-*N*-acetylpseudaminic acid, Pse5Ac7Ac) has been elucidated. The polysaccharide was studied by ^{1}H and ^{13}C NMR spectroscopy, including 2D experiments, along with sugar and methylation analyses. After a selective hydrolysis a modified polysaccharide devoid of its side chain could be isolated. It was found that the polysaccharide has pentasaccharide repeating units with following structure:

β-Pse*p*5Ac7Ac-(2→6)
|
→3)-α-D-Gal*p*-(1→6)-α-D-Glc*p*NAc-(1→4)-α-D-Gal*p*A-(1→3)-β-D-Qui*p*NAc-(1→

Keywords: *Pseudoalteromonas atlantica*; Polysaccharides; Structure; NMR spectroscopy; Marine bacteria; 5,7-Diacetamido-3,5,7,9-tetradeoxy-L-*glycero*-L-*manno*-non-2-ulosonic acid; Pseudaminic acid

1. Introduction

The marine aerobic agar-decomposing bacterium *Pseudoalteromonas atlantica* is one of the oldest species of the genus and it was tentatively assigned in 1955 to the genus *Pseudomonas* as *Pseudomonas atlantica.*[1] This bacterium has been intensively studied in regard to its production of agarase that is widely used and commercially available. However, the generic assignment of this species has not been validated. Only several decades later, the taxonomic status of this species was clarified by isolation and characterisation a few new strains belonging to this species (including strain IAM 14165).[2] Consequently, bacteria of this species were considered to be members of *Alteromonas haloplanktis* rRNA cluster.[3] In 1995, based on the analysis of 16S rRNA gene sequences, the genus *Alteromonas* was subjected to taxonomic revision. As a results, the revised *Alteromonas* genus contained only one species, *A. macleodii*, while a new genus *Pseudoalteromonas*, which included the

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rRNA homology group II species or *Alteromonas haloplanktis* rRNA cluster, was formed.[4] Accordingly, the species *Alteromonas atlantica* was transformed to the newly created genus *Pseudoalteromonas.* Herein we present the results of a structural analysis of the acidic antigenic polysaccharide from *Pseudoalteromonas atlantica* IAM 14165.

2. Results and discussion

The lipopolysaccharide (LPS) was obtained from wet bacterial cells by Westphal's procedure[5] with hot phenol–water extraction. Delipidation using mild acidic conditions, followed by ion-exchange chromatography yielded a polysaccharide (PS). From ^{13}C NMR spectroscopy data the phenol phase did not contain carbohydrate biopolymers.

Analysis of the content of the PS using GLC of derived alditol acetates revealed 2-amino-2,6-dideoxyglucose (QuiN), galactose (Gal) and 2-amino-2-deoxyglucose (GlcN). Moreover, methanolysis of the polysaccharide followed by GLC analysis of acetylated methyl glycosides showed the presence of galacturonic acid (GalA). The higher sugar residue 5,7-diacetamido-3,5,7,9-tetradeoxy-L-*glycero*-L-*manno*-non-2-ulosonic acid (Pse5Ac7Ac) was identified by analysis of NMR data obtained (see below). GLC analysis of the acetylated glycosides with (*S*)-2-butanol showed that QuiN, Gal, GlcN and GalA have the D configuration.

The ^{13}C NMR spectrum (Fig. 1, Table 1) of the PS contained five signals in the anomeric region, δ 99.7–103.7 and one of these resonances corresponds to a quaternary (data of the DEPT experiment), hence, a keto sugar is present. The spectrum also contained four signals in the nitrogen bearing carbon region, δ 49.7, 54.9 (double intensity) and 55.7 (C-5 and C-7 of Pse5Ac7Ac, C-2 of GlcN and QuiN, respectively), two signals of CH_3–C groups of deoxy sugars at δ 18.0 and 18.6 (C-6 of QuiN and C-9 of Pse5Ac7Ac, respectively), four methyl signals characteristic of *N*-acetyl groups at 23.3–23.7 (Me) with corresponding carbonyl signals at δ 176.0–176.7 (data of the HMBC experiment), two COOH signals at δ 175.2 and 175.5 (C-6 of GalA and C-1 of Pse5Ac7Ac, respectively; data of the HMBC experiment), two substituted HOCH_2-groups at δ 65.3 and 67.0 (C-6 of Gal and GlcNAc, respectively; data of the DEPT experiment), one signal from a methylene carbon at δ 37.0 (C-3 of Pse5Ac7Ac) and 17 other sugar ring carbons in the region at δ 67.8–82.8. The total number of signals in the spectrum demonstrated the presence in the pentasaccharide repeating unit of a higher, nine-carbon sugar along with four hexose residues. The absence from signals for nonanomeric sugar carbons at a lower field than δ 83 in the ^{13}C NMR spectrum demonstrated the pyranoid form of all sugar residues.[6]

Accordingly, four anomeric signals were found in a low-field region of the ^{1}H NMR spectrum of the PS (Fig. 2, Table 2) at δ 4.72–5.34. A high-field region of the spectrum contained signals for one C–CH_2–C group in a pyranose ring (δ 1.56 and 2.46) and two CH_3–C groups (δ 1.19 and 1.29) of deoxy sugars, as well as signals from four *N*-acetyl groups at δ 1.92–2.09. In the HMBC spectrum, the methylene protons showed cross-peak with the anomeric carbon of the keto sugar

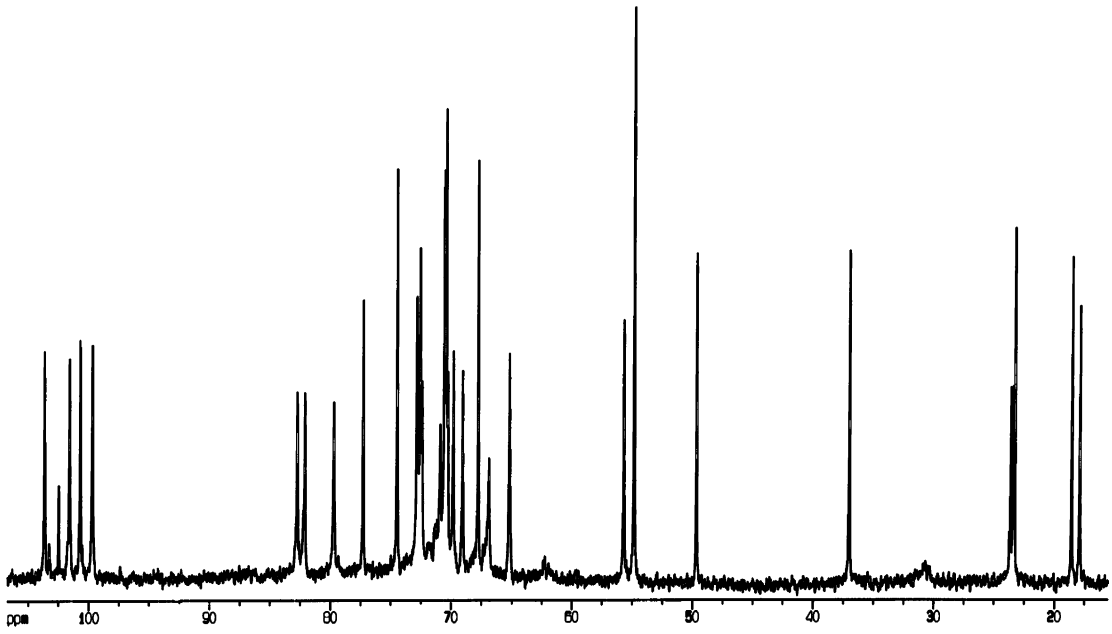

Figure 1. Part of the ^{13}C NMR spectrum of PS from *Pseudoalteromonas atlantica* strain IAM 14165.

Table 1. ^{13}C NMR data (ppm) for the PS[a] and MP[b] of *P. atlantica* 14165 strain

Residue	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9
PS									
→6)-α-D-Glc*p*NAc-(1→	100.6	54.9	72.8	70.9	72.6	67.0			
	[8.8]	[0.1]	[1.1]	[−0.4]	[0.1]	**[5.2]**			
→4)-α-D-Gal*p*A-(1→	101.7	69.8	70.2	82.0	72.5	175.2			
	[8.6]	[0.8]	[0.1]	**[10.4]**	[0.2]	[−1.2]			
→3)-β-D-Qui*p*NAc-(1→	103.7	55.7	82.8	77.3	72.8	18.0			
	[7.8]	[−2.7]	**[7.8]**	[0.6]	[−0.4]	[0.1]			
→3,6)-α-D-Gal*p*-(1→	99.7	69.2	79.9	70.6	70.6	65.3			
	[6.5]	[−0.2]	**[9.8]**	[0.3]	[−0.7]	**[3.3]**			
β-Pse-(2→	175.5	102.9	37.0	67.8	49.7	74.6	54.9	70.2	18.6
			[1.1]	[0.8]	[−0.4]	[2.4]	[0.7]	[2.1]	[2.0]
MP									
→6)-α-D-Glc*p*NAc-(1→	100.5	54.6	72.3	71.0	72.3	67.0			
	[8.7]	[−0.4]	[0.6]	[−0.3]	[−0.2]	**[5.2]**			
→4)-α-D-Gal*p*A-(1→	101.5	69.8	70.1	82.3	72.6	ND[c]			
	[8.4]	[0.8]	[0.2]	**[10.7]**	[0.3]				
→3)-β-D-Qui*p*NAc-(1→	103.5	55.5	82.5	77.1	72.5	17.4			
	[7.6]	[−2.9]	**[7.5]**	[0.4]	[−0.7]	[−0.5]			
→3)-α-D-Gal*p*-(1→	99.5	69.3	79.4	70.3	71.7	62.1			
	[6.3]	[−0.1]	**[9.3]**	[0.0]	[0.4]	[0.1]			

Calculated chemical shift changes compared to the monomers are given in square brackets (bold for positions of substitution).
[a] Chemical shifts for NAc are δ 23.3–23.7 (Me), 176.0–176.7 (CO).
[b] Chemical shifts for NAc are δ 23.2 and 23.5 (Me).
[c] ND = not determined.

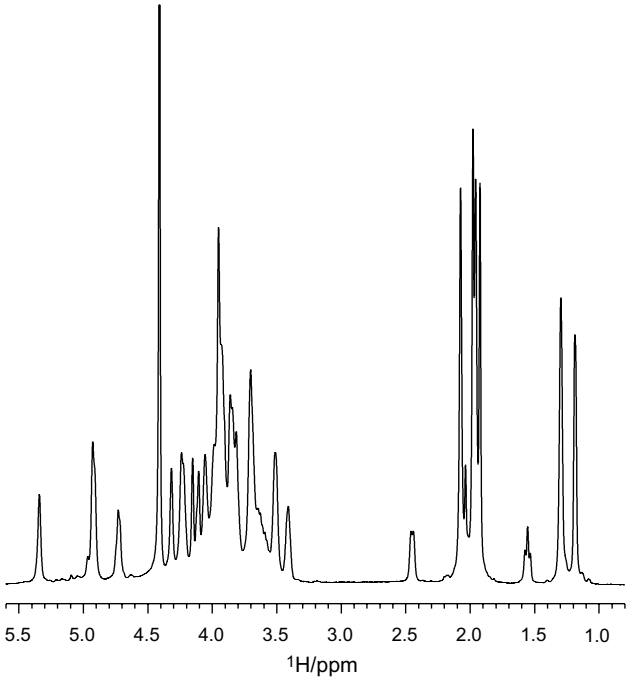

Figure 2. ^{1}H NMR spectrum of PS from *Pseudoalteromonas atlantica* strain IAM 14165.

at δ 102.9, thus demonstrating H-3$_{ax}$ and H-3$_{eq}$ of a 3-deoxy-2-ulosonic acid.

Methylation analysis of the PS resulted in identification of 2,4-di-*O*-methylhexose (derived from Gal), 2-deoxy-3,4-di-*O*-methyl-2-(*N*-methyl)acetamidohexose (derived from GlcN) and 2,6-dideoxy-4-*O*-methyl-2-(*N*-methyl)acetamidohexose (derived from QuiN). Thus, the polysaccharide is branched and contains 6-substituted D-GlcNAc, 3-substituted D-QuiNAc, 3,6-disubstituted D-Gal as well as D-GalA and a nine-carbon sugar.

The ^{1}H and ^{13}C NMR spectra of the PS were assigned using 2D homonuclear COSY, TOCSY, NOESY and ^{1}H,^{13}C HSQC and HMBC experiments (Tables 1 and 2). The TOCSY spectrum showed cross-peaks of H-1 with H-2 up to six for the two sugars with *gluco* configuration and the spin systems of GlcNAc and QuiNAc were assigned on the basis of the characteristic values for their coupling constant. The spin systems of GalA and Gal were identified by H-1/H-2 up to H-5 and H-1/H-2 up to H-4 correlations, respectively, in the COSY and TOCSY spectra. The remaining H-5/H-6a,6b cross-peak in the COSY spectrum was assigned to Gal.

The fifth spin system was identified as a 3,9-dideoxy-non-2-ulosonic acid. A ^{1}H,^{13}C HMQC experiment revealed correlation of protons at carbons bearing nitrogen (H-5 and H-7) to the corresponding carbons (C-5 and C-7) at δ 49.7 and 54.9; hence, a 5,7-diamino-3,5,7,9-tetradeoxy-non-2-ulosonic acid is present.

The coupling constant values for the nine-carbon sugar were similar to those published for pseudaminic acid[7] and different from the data of the other known stereoisomers.[8] Particularly, $J_{3ax,4} \sim 12$ Hz and small $J_{4,5}$ and $J_{5,6}$ values indicated that H-4 occupies the axial position and H-5 the equatorial position, and, hence, the C-4,5,6 fragment has the *lyxo* configuration. A large $J_{6,7}$ value of ~10 Hz showed the *erythro* configuration

Table 2. ^{1}H NMR data (ppm) for the PS[a] and MP[b] of *P. atlantica* 14165 strain

Residue	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b	H-7	H-8	H-9
PS										
→6)-α-D-Glc*p*NAc-(1→	4.91	3.94	3.70	3.62	4.25	3.64	4.06			
	[−0.30]	[0.06]	[−0.05]	[0.13]	[0.39]					
→4)-α-D-Gal*p*A-(1→	5.34	3.86	3.93	4.32	4.24					
	[0.04]	[0.03]	[0.01]	[**0.03**]	[−0.15]					
→3)-β-D-Qui*p*NAc-(1→	4.72	3.81	3.70	3.41	3.51	1.29				
→3,6)-α-D-Gal*p*-(1→	4.93	3.84	3.96	4.12	3.99	3.51	3.91			
	[−0.29]	[0.06]	[**0.15**]	[0.17]	[−0.04]					
β-Pse-(2→			1.56 (ax)							
			[0.01]	3.82	4.15	3.95		3.95	4.05	1.19
			2.46 (eq)	[−0.26]	[−0.11]	[0.20]		[−0.06]	[0.01]	[0.07]
			[0.05]							
MP										
→6)-α-D-Glc*p*NAc-(1→	4.93	3.97	3.73	3.60	4.25	3.73	4.01			
	[−0.28]	[0.09]	[−0.02]	[0.11]	[0.39]					
→4)-α-D-Gal*p*A-(1→	5.36	3.88	3.95	4.32	4.21					
	[0.06]	[0.05]	[0.03]	[**0.03**]	[−0.18]					
→3)-β-D-Qui*p*NAc-(1→	4.76	3.85	3.72	3.45	3.55	1.33				
→3)-α-D-Gal*p*-(1→	4.96	3.88	4.03	4.15	3.94	3.73	3.73			
	[−0.26]	[0.10]	[**0.22**]	[0.20]	[−0.09]					

Calculated chemical shift changes compared to the monomers are given in square brackets.
[a] Chemical shifts for NAc are δ 1.92–2.09.
[b] Chemical shifts for NAc are δ 1.99 and 2.11.

of the C-6,7 fragment (higher sugars with the *threo* configuration are characterised by a small $J_{6,7}$ value of ~2 Hz).[8] A relatively large difference (0.9 ppm) between the chemical shifts of H-3$_{ax}$ and H-3$_{eq}$ is typical of the axial orientation of the carboxyl group in glycosides of 3-deoxy-non-2-ulosonic acids.[8] Therefore, the non-2-ulosonic acid present has the same configuration as pseudaminic acid and is β-linked.

A low-field position of the signals for C-6 of GlcNAc, C-4 of GalA, C-3 of QuiNAc and C-3,6 of Gal at δ 67.0, 82.0, 82.8, 79.9 and 65.3, respectively, revealed the positions of substitution based on a comparison with the corresponding nonsubstituted monosaccharides.[9,10]

The monosaccharide sequence of the hexose pattern was determined by the NOESY experiment, which showed cross-peaks for GlcNAc H-1 to GalA H-4; GalA H-1 to QuiNAc H-3; QuiNAc H-1 to Gal H-3 and Gal H-1 to GlcNAc H-6a,b at δ 4.91/4.32, 5.34/3.70, 4.72/3.96 and 4.93/3.64,4.06, respectively (Fig. 3). The ^{1}H,^{13}C HMBC experiment confirmed the glycosylation pattern and showed inter-residue connectivities: GlcNAc H-1 to GalA C-4; GalA H-1 to QuiNAc C-3; QuiNAc H-1 to Gal C-3 and Gal H-1 to GlcNAc C-6 at δ 4.91/82.0, 5.34/82.8, 4.72/79.9 and 4.93/67.0, as well as Pse5Ac7Ac C-2, Gal H-6b at δ 102.9/3.91. Thus, these data defined the complete sequence of the monosaccharide residues in the repeating unit.

To confirm the substitution of Pse5Ac7Ac at position 6 of the Gal residue the PS was partially hydrolysed under mild acidic conditions. The selective hydrolysis resulted in a modified polysaccharide (MP) lacking

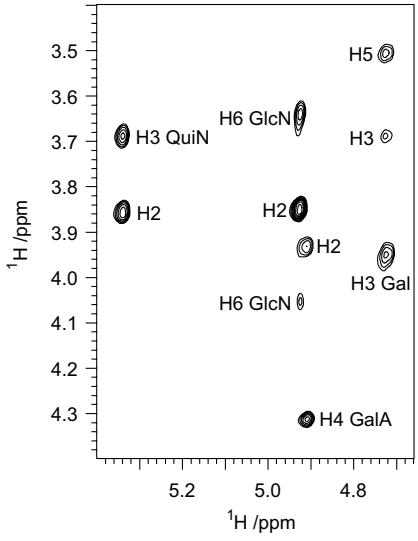

Figure 3. Selected region of the ^{1}H,^{1}H NOESY spectrum from *Pseudoalteromonas atlantica* strain IAM 14165.

Pse5Ac7Ac. An upfield displacement of the signal for C-6 of Gal residue to δ 62.1 in MP (Fig. 4), as compared with its position in the PS at δ 65.3, demonstrated the substitution of Gal residue with Pse5Ac7Ac. The small chemical shift displacement, in the present case ~3 ppm, is typical for substitution with a keto sugar.[11]

These data define the following structure of the polysaccharide from *Pseudoalteromonas atlantica* strain IAM 14165:

β-Pse*p*5Ac7Ac-(2→6)
|
→3)-α-D-Gal*p*-(1→6)-α-D-Glc*p*NAc-(1→4)-α-D-Gal*p*A-(1→3)-β-D-Qui*p*NAc-(1→

5,7-diacetamido-3,5,7,9-tetradeoxy-L-*glycero*-L-*manno*-non-2-ulosonic acid has been identified earlier as the component of the antigenic PS from *Shigella boydii* serogroup 7,[12] *Pseudomonas aeruginosa* serogroups 7, 7b, 7c, 9a, 9b, 9d,[7,13] *Vibrio cholerae* serogroup 2[14] and *Escherichia coli* O136.[15]

3. Experimental

3.1. Bacterial growth and isolation of the polysaccharide

P. atlantica strain IAM 14165 was grown on the modified Yoshimizu–Kimura medium.[16] Wet bacterial cells were extracted with hot aq 45% phenol,[5] the resulting mixture was centrifuged, the aqueous layer dialysed, freed from insoluble contaminations by centrifugation, concentrated in vacuum, and freeze dried to yield 500 mg of the LPS from 20 L of cultural fluid. The LPS was hydrolysed with aq 2% HOAc (100 °C, 2 h) and the lipid A precipitate was removed by centrifugation. The water-soluble portion was concentrated and then fractionated by GPC to give a high-molecular weight polysaccharide.

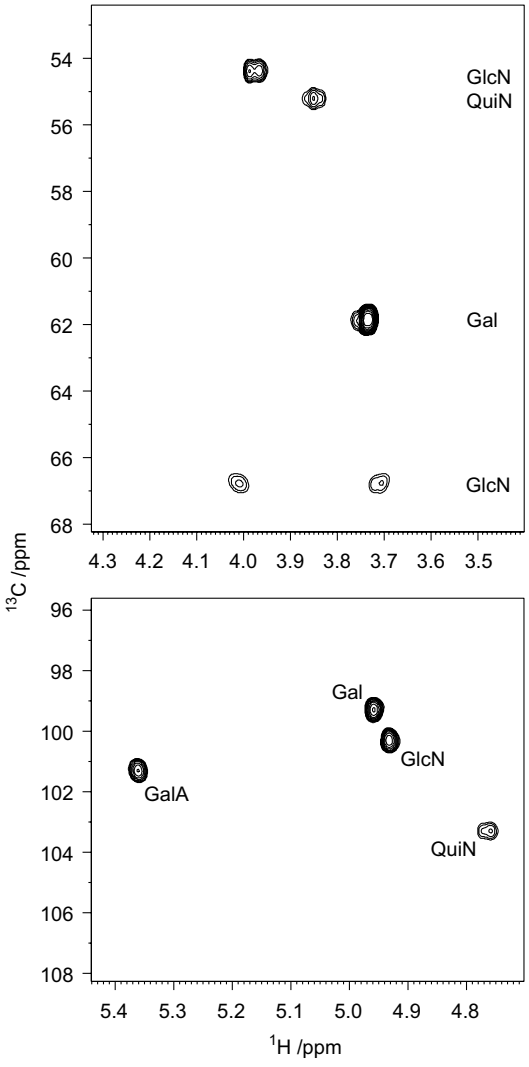

Figure 4. Part of the ^{1}H,^{13}C HSQC spectrum of MP from *Pseudoalteromonas atlantica* strain IAM 14165.

3.2. Anion-exchange chromatography

Anion-exchange chromatography was performed on a column (2.5 × 50 cm) of DEAE TSK 650M eluted with 50 mM Tris–HCl buffer, pH 7.0, and then with aq 0.5 M NaCl in the same buffer and monitored with a RIDK 101 refractometer, which yielded a purified polysaccharide (PS).

3.3. Chemical analyses

The polysaccharide was hydrolysed with 2 M CF_3CO_2H (120 °C, 2 h), monosaccharides were reduced with 0.25 M $NaBH_4$ in aq 1 M ammonia (20 °C, 1 h), acetylated with a 1:1 (v/v) mixture of pyridine and acetic anhydride (120 °C, 30 min) and analysed by GLC. Methanolysis of the polysaccharide (1 mg) was carried out using 1 M HCl–MeOH (85 °C, 16 h), followed by acetylation with Ac_2O in pyridine (120 °C, 30 min), and subsequently analysed by GLC. The absolute configurations of the monosaccharides were determined by GLC of acetylated (*S*)-(+)-2-butyl glycosides according to published methods.[17,18] As the reference for QuiNAc the polysaccharide of *Proteus penneri* 26 containing L-QuiNAc was used.[19]

GLC was performed using a Hewlett–Packard 5890 Series II instrument equipped with an HP fused silica column (0.20 mm × 25 m) using a temperature programme of 180 °C for 1 min followed by 3 °C/min to 250 °C. H_2 was a carrier gas.

Methylation was performed with CH_3I in dimethyl sulfoxide in the presence of sodium methylsulfinylmethanide.[20] Partially methylated monosaccharides were derived by hydrolysis under the same conditions as in sugar analysis, converted into the alditol acetates and analysed by GLC–MS.[21]

GLC–MS was performed on Hewlett–Packard model 5970 mass spectrometer equipped HP-5MS fused silica column (0.20 mm × 25 m). A temperature gradient of 170 °C for 3 min followed by 3 °C/min to 250 °C was used with He as carrier gas.

3.4. Removal of pseudaminic acid

The PS (10 mg) was treated with 3% AcOH (100 °C, 7 h) and the following gel chromatography on a column of

Superdex 30 (Pharmacia, Sweden) in 0.05 M pyridinium acetate buffer (pH 4.5) buffer gave a polysaccharide fraction (MP) devoid of pseudaminic acid (1 mg).

3.5. NMR spectroscopy

Samples were deuterium-exchanged by freeze drying from D_2O and then examined in solutions of D_2O, using sodium 3-trimethylsilyl-[2,2,3,3$^2H_4$]propanoate as internal reference ($\delta_H = 0.00$) and dioxane in D_2O as external reference ($\delta_C = 67.4$). NMR spectra were recorded for PS at 60 °C on a Varian Inova 600 MHz spectrometer and for MP at 45 °C on a Bruker 500 MHz spectrometer. Experiments were performed according to standard pulse sequences and data were processed using software supplied by the manufacturers.

Acknowledgements

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