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Structural studies of the O-antigenic polysaccharide from an *Aeromonas caviae* strain

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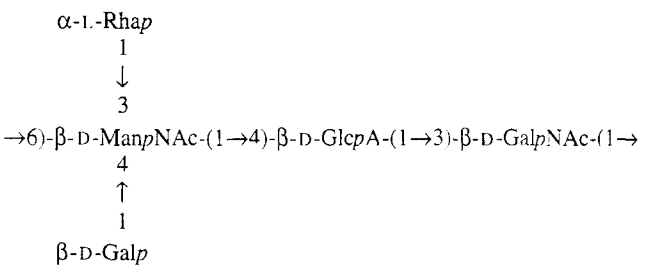
Abstract

The structure of the O-antigenic polysaccharide from a strain of *Aeromonas caviae*, isolated from the stools of a patient with diarrhoea, has been investigated. Sugar analysis, methylation analyses, and a uronic acid degradation together with NMR spectroscopy were the principal methods used. The sequence of the sugar residues could be determined by NOESY and HMBC experiments. It is concluded that the polysaccharide is composed of pentasaccharide repeating units with the following structure:



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1. Introduction

Organisms belonging to the genus *Aeromonas* are putative agents of diarrhoea in humans [1,2]. Strains of *Aeromonas caviae*, isolated from the stools of patients with diarrhoea, cross-reacted with an invasive diarrhoeal pathogen of humans, *Shigella boydii* type 5 [3]. The cross-reactivity was found to be due to the O-antigens of the cell-wall lipopolysaccharide (LPS). The majority of the cross-reacting *A. caviae* isolates produced several virulence-related properties [3]. To further characterise the cross-reactivity of *A. caviae*, elucidation of its O-antigenic structures is important. Information about the structure of the O-antigen may also be valuable for determining the diarrhoeagenic role of the bacteria. In this paper, we report structural studies of the O-antigenic polysaccharide from one of the cross-reacting isolates, *A. caviae*, strain 11212.

2. Results and discussion

Treatment of the LPS with aq 1% AcOH for 1 h at 100 °C yielded a polysaccharide (PS). Purification was performed on a Bio-Gel P-2 column where the polysaccharide was eluted shortly after the void volume. It was further purified by ion-exchange chromatography on a DEAE-Sepharose column. A hydrolysate of the PS contained rhamnose, glucose, galactose, 2-amino-2-deoxymannose, 2-amino-2-deoxygalactose, and heptose in the ratios 8:2:21:36:32:1. The minor amounts of glucose and heptose were attributed to the core. NMR spectra indicated that the two amino sugars were *N*-acetylated (see below). Determination of the absolute configuration of the sugars revealed that all sugars had the D configuration except for rhamnose which had the L configuration. From this analysis glucuronic acid was also identified as its acetylated (+)-2-butyl glycoside (+)-2-butyl ester, and was shown to have the D configuration. From the methylation analysis and NMR spectra, discussed below, it is evident that all sugars are pyranoid. Methylation analysis of the PS (Table 1, column A) showed that the repeating unit contains terminal rhamnose, terminal galactose, 3-substituted *N*-acetylgalactosamine, and 3,4,6-trisubstituted *N*-acetylmannosamine. After carboxyl-reduction of the methylated polysaccharide, the analysis revealed, in addition to the components observed in the methylation analysis, a derivative from a 4-substituted glucuronic acid (Table 1, column B).

The ^{1}H NMR spectrum (Fig. 1) showed seven signals in the anomeric region of which five, at δ 4.99, 4.78, 4.59, 4.52, and 4.30, originated from anomeric protons (vide infra). The ^{1}H NMR spectrum also contained, inter alia, signals for a methyl group at δ 1.25 and for two *N*-acetyl groups at δ 2.07. The ^{13}C NMR spectrum contained five signals in the anomeric region and also signals for a methyl group at δ 17.1, for methyl groups of *N*-acetyl groups at δ 22.8 and 23.6, for two carbons carrying nitrogen at δ 50.3 and 52.0, for two hydroxymethyl groups at δ 61.5 and 61.9, and for three carbonyl groups at δ 175.1, 175.5, and 175.6. The chemical shifts of the signals from protons and

Table 1
Methylation analysis of *Aeromonas caviae* 11212 polysaccharide and derivatives

Sugar	t_R [a]	Detector response (%)		
		A	B	C
2,3,4-Rha [b]	0.78	15	17	31
2,3,4,6-Gal	1.04	47	34	35
2,3-Glc	1.54		27	
2,3,4,6-GalNAc [c]	1.99			34
2,4,6-GalNAc	2.35	18	11	
2-ManNAc	2.89	20	11	

[a] Key: t_R, retention time of methylated alditol acetates relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol (1.00) and hexa-*O*-acetyl-D-glucitol (2.00) on an HP-5 capillary column using the temperature program 180 °C (1 min) to 250 °C at 3 °C/min. A, methylated polysaccharide; B, methylated and carboxyl-reduced polysaccharide; C, uronic acid degraded, methylated polysaccharide.
[b] 2,3,4-Rha = 2,3,4-Tri-*O*-methyl-L-rhamnose, etc.
[c] Trideuteriomethyl at O-3.

carbons together with $J_{H\text{-}1,H\text{-}2}$ and $J_{H\text{-}1,C\text{-}1}$ values for the anomeric protons are given in Table 2.

The assignments of the signals were performed using homo- and hetero-nuclear two-dimensional NMR techniques and showed a pentasaccharide repeating unit. The sugar residues are labelled **A** to **E** with respect to the decreasing chemical shift of their

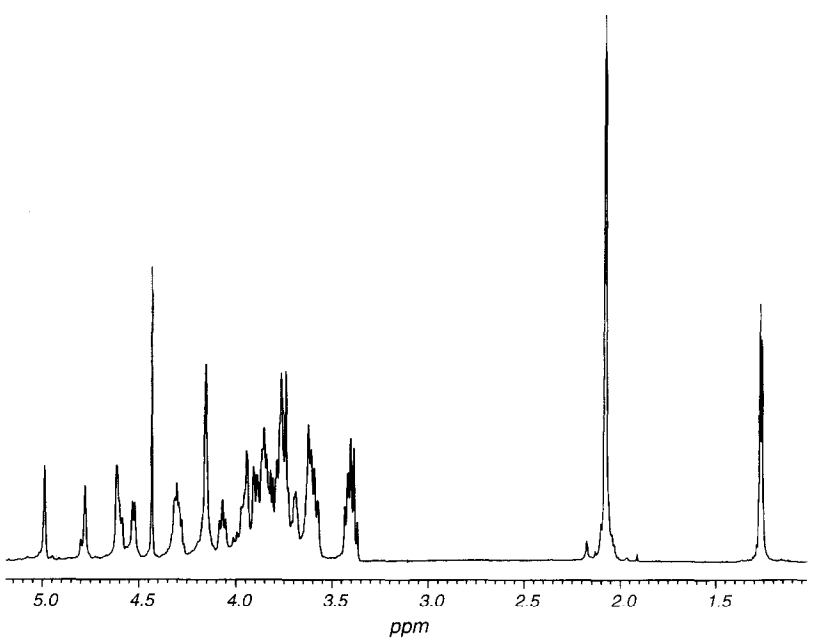

Fig. 1. The ^{1}H NMR spectrum at 600 MHz of the native *A. caviae* strain 11212 O-antigen polysaccharide.

Table 2
Chemical shift (δ, ppm) of the signals in the ^1H and ^{13}C NMR spectra [a] of *Aeromonas caviae* 11212 O-antigen polysaccharide

Sugar residue	$^1H/^{13}C$								
	1	2	3	4	5	6		NAc	CO
α-L-Rha*p*-(1 →	4.99 (4) [b]	3.76	3.90	3.39	4.28	1.26			
A	97.0 [172]	70.9	70.8	73.4	68.8	17.1			
→ 3,4,6)-β-D-Man*p*NAc-(1 →	4.78 (5) [b]	4.61	3.97	3.85	3.61	4.15	4.15	2.07	
B	99.9 [165]	50.3	74.0	71.7	75.2	68.1		22.8 [c]	175.6
→ 3)-β-D-Gal*p*NAc-(1 →	4.59 (8)	4.07	3.85	4.15	3.69	3.76	3.86	2.07	
C	102.2 [162]	52.0	81.2	68.5	76.0	61.9		23.6 [c]	175.1
→ 4)-β-D-Glc*p*A-(1 →	4.52 (7.8)	3.40	3.60	3.72	3.75				
D	104.6 [162]	73.4	74.7	81.5	76.4	175.5			
β-D-Gal*p*-(1 →	4.30 (7)	3.41	3.58	3.94	3.62	3.79	3.81		
E	103.6 [162]	72.3	73.4	69.3	75.7	61.5			

[a] $J_{H\text{-}1,H\text{-}2}$ values are given in Hz in parentheses and $J_{H\text{-}1,C\text{-}1}$ values in Hz in square brackets.
[b] Valuc of $\nu_{1/2}$.
[c] Assignments are interchangeable.

anomeric proton signals. The assignment of a spin system to a specific sugar residue and substitution pattern as determined from the methylation analysis was performed as follows.

Residues **A** and **B** showed small $J_{H\text{-}1,H\text{-}2}$ values as expected for sugars with the *manno* configuration. The upfield chemical shift of the C-2 signal in residue **B**, δ 50.3, demonstrated that **B** is the 3,4,6-trisubstituted D-Man*p*NAc residue which has the β-anomeric configuration as shown by the value of $J_{H\text{-}1,C\text{-}1}$ (165 Hz) and two intra-residue NOEs, between the anomeric proton and H-3 and H-5 (Table 3, Fig. 2). Residue **A** was then assigned to the terminal L-Rha*p* group, corroborated by the chemical shifts of the H-6 and C-6 signals. The value of $J_{H\text{-}1,C\text{-}1}$ (172 Hz) indicated that **A** is α-linked.

For residues **C**, **D**, and **E** the values of $J_{H\text{-}1,H\text{-}2}$ all exceeded 7 Hz, showing *β-gluco / galacto* configurations. The chemical shift, δ 52.0, for the C-2 signal of residue **C** revealed that this is the 3-substituted β-D-Gal*p*NAc residue. Values of $^3J_{H,H}$, the downfield chemical shift of the C-4 signal in residue **D** (δ 81.5), and the resemblance between the chemical shifts of the signals for C-2–C-6 of residue **E** and those of β-D-galactopyranose [4] made it possible to identify these residues as 4-substituted β-D-Glc*p*A and terminal β-D-Gal*p*, respectively.

In order to obtain sequence information, the polysaccharide was subjected to a uronic acid degradation [5]. Dried methylated polysaccharide was treated with sodium methylsulfinylmethanide in dimethyl sulfoxide, methylated with trideuteriomethyl iodide, and hydrolysed. The mixture of methylated sugars was analysed by GLC–MS (Table 1, column C). 2-Deoxy-4,6-di-*O*-methyl-2-(*N*-methylacetamido)-3-*O*-trideuteriomethyl-D-galactose was derived from the 3-substituted 2-acetamido-2-deoxy-D-galactopyranosyl

Table 3
Observed NOEs from the anomeric protons of the PS from *Aeromonas caviae* 11212

Residue	Anomeric proton	NOE to proton	Residue, atom
	δ	δ	
α-L-Rha *p*-(1 →	4.99	4.61	**B**, H-2
A		3.97	**B**, H-3
		3.76	**A**, H-2
→ 3,4,6)-β-D-Man *p*NAc-(1 →	4.78	4.61	**B**, H-2
B		3.97	**B**, H-3
		3.72	**D**, H-4
		3.61	**B**, H-5
→ 3)-β-D-Gal *p*NAc-(1 →	4.59	4.15	**B**, H-6
C		3.85	**C**, H-3
		3.69	**C**, H-5
→ 4)-β-D-Glc *p*A-(1 →	4.52	4.15	**C**, H-4
D		3.85	**C**, H-3
		3.75	**D**, H-5
		3.60	**D**, H-3
β-D-Gal *p*-(1 →	4.30	4.15	**B**, H-6
E		3.85	**B**, H-4
		3.62	**E**, H-5
		3.58	**E**, H-3

residue, since O-3 which had been linked to the uronic acid was liberated on degradation of the uronic acid. This demonstrates disaccharide element **1**:

→4)-β-D-Glc*p*A-(1→3)-β-D-Gal*p*NAc-(1→

D **C**

1

Information on the sequence of the sugar residues was also gained from a NOESY experiment (Table 3, Fig. 2) and a ^{1}H-detected HMBC experiment (Table 4), establishing inter-residue through-space-proximity and through-bond correlations, respectively. In the spectrum from the HMBC experiment an inter-residue connectivity between H-1 in residue **D** and C-3 in residue **C** was found. Two inter-residue NOEs in the NOESY spectrum, between H-1 in residue **D** and H-3 and H-4 in residue **C**, were also found and these results further confirmed structure element **1**.

The uronic acid degradation also gave information about the sugar linked to the 4-position of the glucuronic acid. This sugar is liberated through the initiating elimination reaction and subsequently degraded [5]. From the analysis of a hydrolysate of the uronic acid degradation product, it is obvious that the 3,4,6-trisubstituted 2-acetamido-

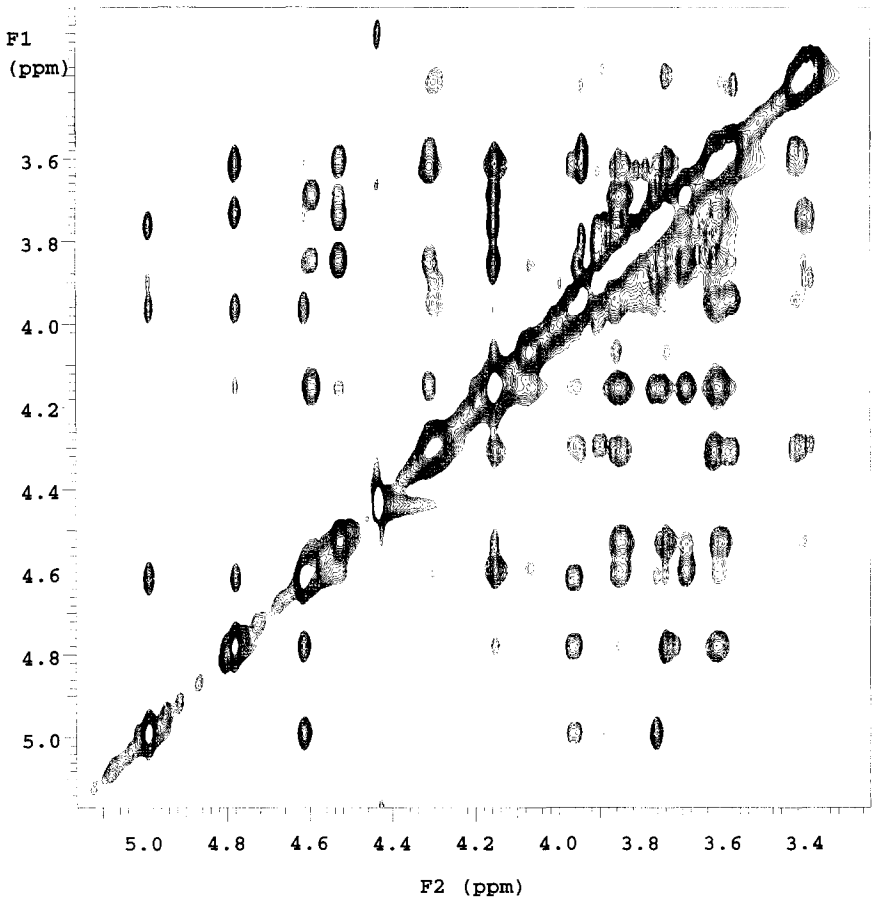

Fig. 2. Part of the $^{1}H,^{1}H$ NOESY spectrum of the native *A. caviae* strain 11212 O-antigen polysaccharide.

2-deoxymannose residue **B** is the sugar linked to the glucuronic acid residue **D**. In the analysis its derivative was not detected by GLC and the second disaccharide element **2** is thus defined as:

→3,4,6)-β-D-Man*p*NAc-(1→4)-β-D-Glc*p*A (1→

B D

2

Two inter-residue three-bond connectivities from the HMBC spectrum, between the anomeric proton in residue **B** and C-4 in residue **D**, and between the anomeric carbon in **B** and H-4 in **D**, as well as one NOE between H-1 in residue **B** and H-4 in **D**, also define the structural element **2** above.

Table 4
Heteronuclear two- and three-bond connectivities observed in the HMBC experiment for the anomeric atoms of the PS from *Aeromonas caviae* 11212

Sugar residue	Anomeric atom		Connectivity to		Residue, atom
	δ_H	δ_C	δ_C	δ_H	
α-L-Rha*p*-(1 →	4.99		74.0		**B**, C-3
A			~ 70.9		**A**, C-2/**A**, C-3
			68.8		**A**, C-5
		97.0		3.97	**B**, H-3
→ 3,4,6)-β-D-Man*p*NAc-(1 →	4.78		81.5		**D**, C-4
B			50.3		**B**, C-2
		99.9		4.61	**B**, H-2
				3.72	**D**, H-4
→ 3)-β-D-Gal*p*NAc-(1 →	4.59		68.1		**B**, C-6
C		102.2		4.07	**C**, H-2
→ 4)-β-D-Glc*p*A-(1 →	4.52		81.2		**C**, C-3
D		104.6		3.40	**D**, H-2
β-D-Gal*p*-(1 →	4.30		71.7		**B**, C-4
E		103.6		3.85	**B**, H-4
				3.41	**E**, H-2

The 3-substituted 2-acetamido-2-deoxygalactose residue **C** shows one inter-residue three-bond connectivity in the HMBC spectrum, between the anomeric proton and C-6 in the branchpoint residue **B**. The (1 → 6) linkage is also identified by one inter-residue NOE, between the anomeric proton in residue **C** and one or two of the protons at position 6 of residue **B**. Thus, the structural element **3** is defined as:

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

C **B**

3

Two heteronuclear three-bond connectivities, between H-1 in residue **A** and C-3 in residue **B**, and between C-1 in **A** and H-3 in **B**, together with two inter-residue NOEs, between the anomeric proton in residue **A** and H-2 and H-3 in residue **B**, define the fourth disaccharide element:

α-L-Rha*p*-(1→3)-β-D-Man*p*NAc-(1→

A **B**

4

Over the fifth glycosidic linkage, two inter-residue connectivities from the HMBC spectrum are observed, between C-1 in residue **E** and H-4 in residue **B**, and between H-1 and C-4 of the same residues. One NOE is also observed between the anomeric proton of residue **E** and H-4 in residue **B**, and the structural element **5** is established as follows:

β-D-Gal*p*-(1→4)-β-D-Man*p*NAc-(1→

E **B**

5

From the combined results it is concluded that the polysaccharide from *Aeromonas caviae*, strain 11212, is composed of pentasaccharide repeating units with the structure:

α-L-Rha*p*
A 1
↓
3
→6)-β-D-Man*p*NAc-(1→4)-β-D-Glc*p*A-(1→3)-β-D-Gal*p*NAc-(1→
B 4 **D** **C**
↑
1
β-D-Gal*p*
E

The cross-reactivity between *A. caviae*, strain 11212 and *Shigella boydii* type 5 may be explained by the similarities of their O-antigens in the region of the branchpoint residue. The *S. boydii* type 5 O-antigen contains a β-D-mannopyranosyl residue substituted in position 3 by an α-L-rhamnopyranosyl group, at position 4 by a 2-substituted β-D-galactopyranosyl residue, and at position 6, partially by an *O*-acetyl group [6].

3. Experimental

General methods.—Gel permeation chromatography was performed on a Bio-Gel P-2 column (Bio-Rad Laboratories, Richmond, CA, USA). Column effluents were monitored using a differential refractometer (Waters, Milford, MA, USA). Ion-exchange chromatography was performed on a DEAE-Sepharose column (3 × 20 cm; Pharmacia, Uppsala, Sweden) irrigated with water. The PS was eluted with a gradient starting from 0.1 M NaCl and ending with M NaCl. Alditol acetates, partially methylated alditol acetates, and acetylated 2-butyl glycosides were separated on a DB-225 fused-silica column (0.25 mm × 30 m; J&W Scientific, Folsom, CA, USA), using a temperature program of 180 °C for 1 min followed by 4 °C/min to 210 °C, or an HP-5 fused-silica column (0.20 mm × 25 m; Hewlett–Packard, Palo Alto, CA, USA), using a temperature

program of 180 °C for 1 min followed by 3 °C/min to 250 °C. H_2 was used as carrier gas. To separate the two diastereomers of acetylated rhamnose 2-butyl glycosides, the HP-5 column was used with a temperature program of 140 °C for 10 min followed by 0.5 °C/min to 250 °C. Both columns were fitted to a Hewlett–Packard model 5890 series II gas chromatograph equipped with flame-ionisation detectors. GLC–MS analyses were performed on a Hewlett–Packard model 5970 mass spectrometer equipped with an HP-5MS fused-silica column (0.20 mm × 25 m). A temperature program of 170 °C for 3 min followed by 3 °C/min to 250 °C was used with He as carrier gas.

NMR spectroscopy.—NMR spectra of solutions in D_2O were recorded at 60 °C and pD 7, using JEOL GSX-270, JEOL ALPHA-400, and Varian Unity+ 600 instruments. Data processing was performed using standard JEOL software, VNMR (Varian), or Felix 2.05 (Biosym Technologies, San Diego, CA, USA). Chemical shifts are reported in ppm relative to sodium 4,4-dimethyl-4-sila(2,2,3,3-$^2H_4$)pentanoate (TSP, δ_H 0.00) or acetone (δ_C 31.00) as internal reference. Double quantum-filtered correlated spectroscopy (DQF-COSY), relayed COSY, and TOCSY experiments were used to assign the proton signals and performed according to standard pulse sequences. For assignment of the ^{13}C chemical shifts, heteronuclear single quantum coherence with gradient enhancement [7] and DEPT experiments were used. For sequence information, a two-dimensional nuclear Overhauser effect spectroscopy (NOESY) experiment with a mixing time of 200 ms, and a heteronuclear multiple-bond connectivity (HMBC) [8] experiment, with a 60-ms delay for the evolution of long-range connectivities, were used.

Bacteria and cultivation.—*A. caviae* strain 11212 was subcultured on horse-blood agar plates and incubated at 37 °C for 24 h. Colonies from these plates were used for inoculation of trypticase soy broth supplemented with 0.5% (w/v) yeast extract (GIBCO, Paisley, Scotland, UK). A 35-L fermentor containing 20 L of medium was inoculated with 5 L of a late logarithmic phase culture. The bacteria were grown at a constant pH of 7.2, agitated at 175 rpm, and aerated at 8 L/min. The bacteria were killed in the early stationary phase by adding formaldehyde to a final concentration of 1% and then kept at 4 °C for 18 h. The cells were harvested by centrifugation at 5000 × *g* for 1 h, and freeze-dried. The total yield was 42 g dry weight (2.1 g/L).

Isolation of LPS and PS.—The LPS was extracted from the membrane by the hot phenol–water method [9]. The LPS was subsequently suspended in aq 1% AcOH and kept at 100 °C for 1 h. The precipitate was removed by centrifugation and the pH of the solution adjusted to neutral. The solvent was evaporated, and the polysaccharide was applied to a column of Bio-Gel P-2, using aq 1% butanol as irrigant, and eluted shortly after the void volume.

Sugar analysis.—The PS was hydrolysed with 4 M HCl at 100 °C for 2 h. After reduction with sodium borohydride and acetylation, the samples were analysed by GLC. The absolute configuration of the sugars present in the PS from *A. caviae* strain 11212 was determined essentially as devised by Leontein et al. [10], but with (+)-2-butanol.

Methylation analysis.—The analyses were performed according to Hakomori [11] using sodium methylsulfinylmethanide in dimethyl sulfoxide. The methylated compounds were recovered by use of Sep-Pak C_{18} cartridges (Millipore Corporation, Milford, MA, USA) [12]. The purified methylated samples were then hydrolysed (4 M

HCl, 100 °C, 2 h), and the sugars converted into partially methylated alditol acetates and analysed by GLC–MS.

Carboxyl-reduction of methylated polysaccharide.—Dried, methylated polysaccharide (1 mg) was dissolved in dried tetrahydrofuran (0.5 mL) and lithium borohydride (20 mg) was added. The solution was kept at 80 °C for 2 h and excess of lithium borohydride was decomposed with aq 1% AcOH. $CHCl_3$ (3 mL) was added and the solution was washed five times with water (~ 1 mL).

Uronic acid degradation.—The uronic acid degradation of the polysaccharide [13,14] was performed as follows. To a solution of methylated polysaccharide (1 mg) in dried dimethyl sulfoxide (0.5 mL) were added a trace of toluene-*p*-sulfonic acid and 2,2-dimethoxypropane (40 μL) to eliminate any water present. Sodium methylsulfinylmethanide in dimethyl sulfoxide (2 M, 0.3 mL) was added, and the mixture was agitated in an ultrasonic bath for 30 min and kept at room temperature for 15 h. Trideuteriomethyl iodide (0.25 mL) was added with external cooling and the mixture agitated for 30 min in an ultrasonic bath. Excess of trideuteriomethyl iodide was removed by flushing with nitrogen and the solution was diluted with water and added to a Sep-Pak C_{18} cartridge. After washing, the material was eluted with acetonitrile. The product was hydrolysed with 2 M trifluoroacetic acid for 15 h at 100 °C and the mixture of methylated products was analysed.

Acknowledgements

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