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Note

The structure of an acidic trisaccharide component from a cell wall polysaccharide preparation of the green alga *Tetraselmis striata* Butcher

Burkhard Becker [a,*], Jos P.M. Lommerse [b], Michael Melkonian [a], Johannis P. Kamerling [b], Johannes F.G. Vliegenthart [b]

[a] *Botanisches Institut, Universität zu Köln, Gyrhofstr. 15, D-50931 Köln, Germany*

[b] *Bijvoet Center, Department of Bio-Organic Chemistry, Utrecht University, P.O. Box 80.075, NL-3508 TB Utrecht, Netherlands*

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In most green algae, the cell wall consists of microfibrils (mainly cellulose) embedded in a matrix consisting of glycoproteins and polysaccharides [1]. As one of the few exceptions, the prasinophytes possess structures of distinct size and shape, called scales, in multiple layers on their cell surface [2]. In the genera *Tetraselmis* and *Scherffelia*, the scales on the cell body fuse extracellularly to form a cell wall, called theca [3–5].

Chemical analysis of individual scales and the theca showed the presence of mainly acidic polysaccharide material and only a low protein content was found (theca: 4% of dry weight [6,7], individual scales: 0–10% of dry weight [8]). In the cell wall, D-galacturonic acid (D-GalA) and the 2-keto-sugar acids 3-deoxy-*manno*-2-octulosonic acid (Kdo), 3-deoxy-5-*O*-methyl-*manno*-2-octulosonic acid (5OMeKdo), and 3-deoxy-*lyxo*-2-heptulosaric acid (Dha) occur as the dominant residues. In the theca of *Tetraselmis striata* Butcher, the relative amounts based on dry weight are GalA, 14%; Kdo, 42%; 5OMeKdo, 7%; and Dha, 11%. Other constituents comprise D-galactose (4%), D-gulose (2%), L-arabinose (1%), sulfate (6%), and Ca^{2+} (4%). To gain insight into the biosynthesis of this unusual type of cell wall, more details about its supramolecular structure are needed. The characterisation of an acidic trisaccharide and its sulfated analogue, obtained by partial hydrolysis of a theca polysaccharide fraction of *T. striata* Butcher, will be discussed.

* Corresponding author.

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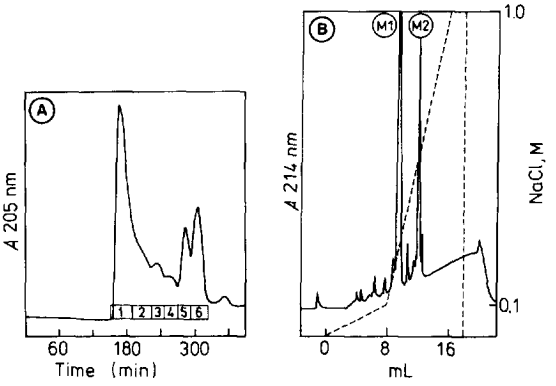

Fig. 1. Elution patterns of the partially hydrolysed aqueous-EDTA-solubilised polysaccharide preparation. **A**, Fractionation of the partial hydrolysate on Bio-Gel P-2; **B**, FPLC of Bio-Gel P-2 fraction 3 on Mono Q. For elution systems, see Experimental section.

Treatment of cell wall material of *T. striata* with aqueous EDTA yielded a soluble polysaccharide preparation (containing 2-keto-sugar acids) and an insoluble residue (containing no 2-keto-sugar acids). Gel permeation chromatography of the aqueous-EDTA-solubilised polysaccharide preparation on Superose 12 yielded several subfractions of different molecular mass, but monosaccharide analysis of these fractions demonstrated only small quantitative differences in monosaccharide composition. Therefore, oligosaccharides were hydrolytically generated (1% AcOH, 1 h, 100°C) by using the unfractionated solubilised polysaccharide preparation. The saccharide mixture obtained was fractionated on Bio-Gel P-2 (Fig. 1A), yielding mainly high molecular mass material (fraction 1), Kdo/5OMeKdo-containing material (fraction 5), and free Kdo (fraction 6). Subfractionation of fractions 2–5 on Mono Q gave rise to highly complex peak patterns. In the case of fraction 3, separation on Mono Q (Fig. 1B) gave two major fractions **M1** and **M2**, containing sufficient carbohydrate for analysis by NMR spectroscopy.

Monosaccharide analysis of fraction **M1** revealed the presence of GalA and Kdo in the molar ratio of 1.9:1.0. The 500-MHz ^{1}H NMR spectrum of **M1** at 300 K (Fig. 2A) showed, in the downfield region, two complex α-GalA H-1 patterns of doublets at δ 5.088 ($J_{1,2}$ 3.9 Hz)/5.080 ($J_{1,2}$ 3.9 Hz) and at δ 5.046 ($J_{1,2}$ 3.8 Hz)/5.028/5.024/5.017 ($J_{1,2}$ 3.7 Hz), respectively. In the high-field region, a complex patern of multiplets for Kdo H-3 is observed (α-pyranose, H-3*eq* δ 1.865, H-3*ax* δ 1.980; α-furanose, H-3a δ 2.296, H-3b δ 2.361; β-furanose, H-3a δ 2.064, H-3b δ 2.567), comparable to the H-3 pattern of free Kdo as ammonium salt (α-pyranose, H-3*eq* δ 1.878, H-3*ax* δ 1.978; β-pyranose, H-3*eq* δ 2.386, H-3*ax* δ 1.751; α-furanose, H-3a δ 2.296, H-3b δ 2.366; β-furanose, H-3a δ 2.069, H-3b δ 2.585) (see also Refs. [9–11]). ^{1}H NMR analysis of $NaBH_4$-reduced **M1** demonstrated the conversion of the complex α-GalA H-1 patterns into single doublets at δ 5.096 ($J_{1,2}$ 3.8 Hz) and 5.035 ($J_{1,2}$ 3.7 Hz), indicating a trisaccharide structure with Kdo at the reducing end. It is evident that the complex GalA H-1 patterns in **M1** are caused by the presence of all four ring forms of Kdo.

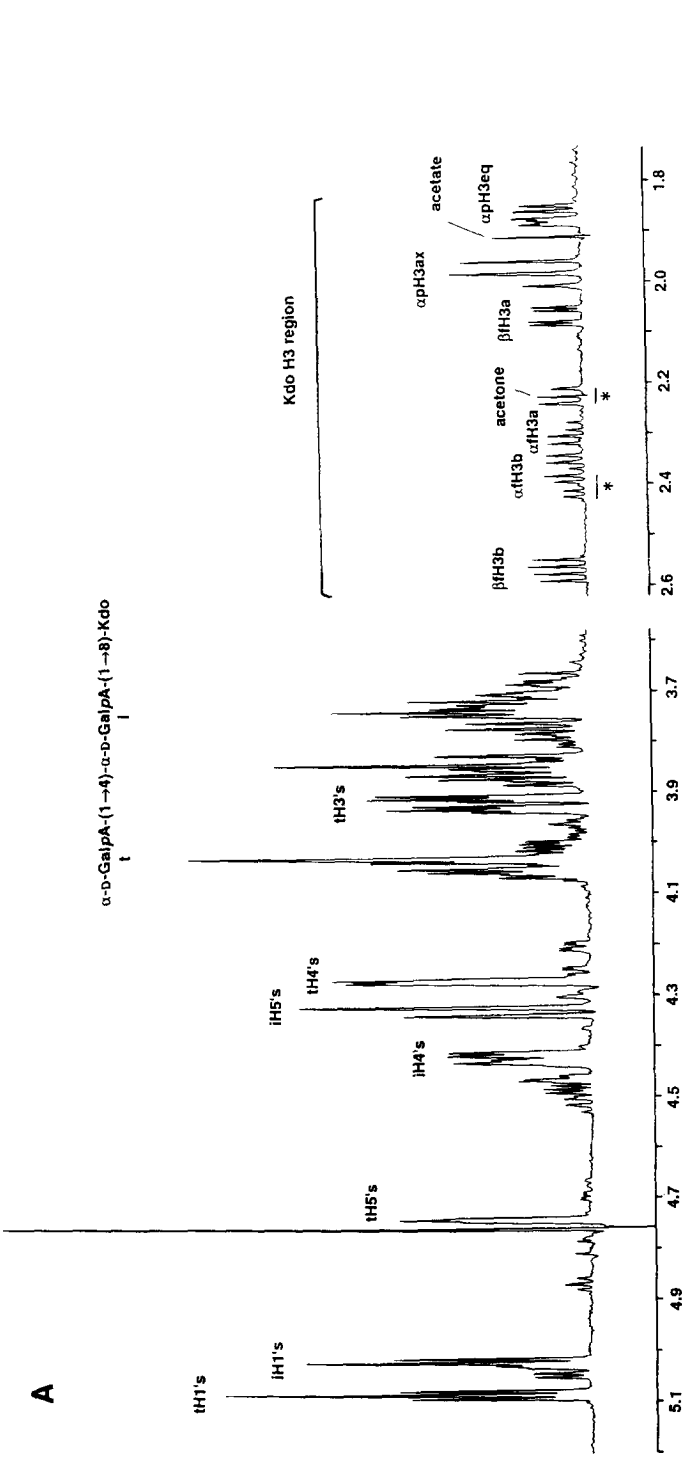

Fig. 2. 500-MHz ^{1}H NMR spectra of **A**, fraction **M1** and **B**, fraction **M2** in D_2O. In the spectrum of **M1**, the coupled signals marked with an asterisk at δ 2.402 (doublet of doublets) and 2.223 (doublet) could not be explained so far.

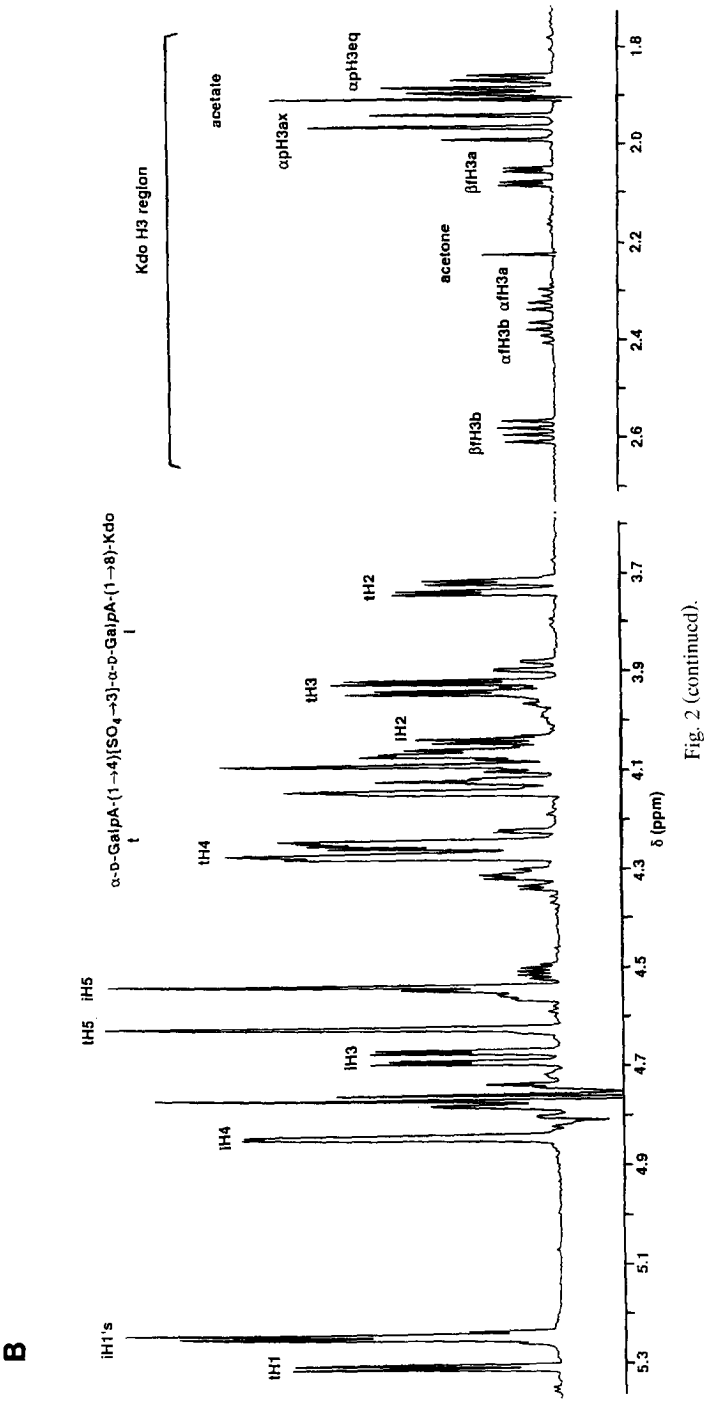

Fig. 2 (continued).

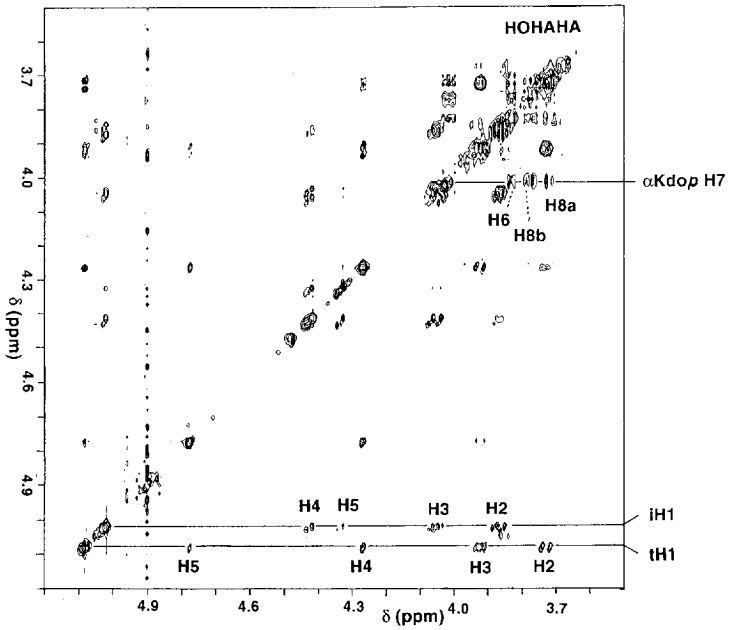

Fig. 3. Partial 2D HOHAHA spectrum of fraction **M1**.

For a more complete interpretation of the ^{1}H NMR spectrum of **M1**, 2D DQF ^{1}H–^{1}H COSY, 2D HOHAHA (Fig. 3), and 2D ROESY experiments were carried out at 288 K and the ^{1}H NMR data are presented in Table 1. H-1 of the Gal*p*A residues occur at δ 5.08 and 5.02 in the HOHAHA spectrum and for each unit the total scalar coupled network was assigned making use of the COSY data. The GalpA H-1 pattern at δ 5.08 represents the terminal Gal*p*A residue (α-tGal*p*A), whereas the Gal*p*A H-1 pattern at δ 5.02 reflects the internal Gal*p*A residue (α-iGal*p*A), as supported by a strong ROE between α-tGal*p*A H-1 and α-iGal*p*A H-4 in the ROESY spectrum. The presence of this connectivity, in combination with the absence of an ROE effect between α-tGal*p*A H-1 and α-iGal*p*A H-3, suggests an α-Gal*p*A-(1 → 4)-α-Gal*p*A element. Since it had been observed that an α-GalNAc-(1 → 3)-Gal linkage gives rise to a strong NOE between H-1 of α-GalNAc and H-4 of Gal [12–14], this ROE alone is not sufficient evidence for the assignment of the 1 → 4 linkage. However, the chemical shift values of α-tGal*p*A H-4 and α-iGal*p*A H-4 in **M1** match those reported for the non-reducing and reducing Gal*p*A H-4 resonances in the reference compound α-Gal*p*A-(1 → 4)-Gal*p*A [15]. With regard to the reducing Kdo unit, Table 1 only includes the ^{1}H NMR data of the major α Kdo*p* residue. On the H-3*eq* track at δ 1.86 of α-Kdo*p* in the HOHAHA spectrum, cross-peaks were observed for H-3*ax* and H-4, and on the H-7 track at δ 4.05 cross-peaks for H-6, H-8a, and H-8b. The ROE between α-iGal*p*A H-1 and α-Kdo*p* H-8a in the ROESY spectrum is in accordance with the occurrence of an α-Gal*p*A-(1 → 8)-α-Kdo*p* element.

Table 1
^{1}H NMR data of the *T. striata* cell wall-derived oligosaccharides **M1** and **M2**, together with those of reference compounds α-Kdo*p* and α-D-Gal*p*A-(1 → 4)-α-D-Gal*p*A [15]

Residue	Chemical shift in			
	M1 [a]	**M2** [b]	α-Kdo*p*	α-D-Gal*p*A-(1 → 4)-α-D-Gal*p*A
α-Gal*p*A-(1 → (α-t-Gal*p*A)				
H-1	5.08 [c]	5.308		5.088
H-2	3.73	3.726		3.721
H-3	3.92 [c]	3.929		3.914
H-4	4.27 [c]	4.273		4.274
H-5	4.78 [c,d]	4.621		4.735
→ 4)-α-Gal*p*A[-(1 → (α-i-Gal*p*A)				
H-1	5.02 [c]	5.248		5.310
H-2	3.86	4.049		3.830
H-3	4.05	4.680		3.995
H-4	4.42 [c]	4.842		4.420
H-5	4.33 [c]	4.536		4.417
[→ 8)-]α-Kdo*p*				
H-3*ax*	1.97 [c]	1.965	1.978	
H-3*eq*	1.86 [c]	1.878	1.878	
H-4	4.04	n.d.	4.049	
H-5	n.d [e]	n.d.	4.008	
H-6	3.83	n.d	3.804	
H-7	4.05	n.d.	3.880	
H-8a	3.70	n.d.	3.804	
H-8b	3.78	n.d.	3.622	

[a] Data from 2D HOHAHA spectrum at 288 K.
[b] Recorded at 300 K.
[c] For 300 K, see 1D spectrum and text.
[d] δ 4.74 at 300 K, may also be influenced by a slight shift in pD.
[e] n.d., Not determined.

Additional evidence for the type of linkages in **M1** was obtained from its ^{13}C NMR spectrum. In Table 2 the ^{13}C NMR data of **M1** are summarised, together with those of the reference compounds α-Kdo*p*, α-L-Rha*p*-(1 → 3)-α-L-Rha*p*-(1 → 8)-α-Kdo*p* [16], and α-Gal*p*A-(1 → 4)-Gal*p*A [15]. Comparison of the chemical shift values for α-Kdo*p* in free Kdo and in **M1** shows a downfield shift for C-8 ($\Delta\delta$ +6.2 ppm) and an upfield shift for C-7 ($\Delta\delta$ −1.0 ppm), in agreement with a C-8 substituted α-Kdo*p* unit (compare also with α-L-Rha*p*-(1 → 3)-α-L-Rha*p*-(1 → 8)-α-Kdo*p* [16]). The presence of an α-Gal*p*A-(1 → 4)-α-Gal*p*A-(1 → element follows from a comparison of the chemical shifts of **M1** and reference α-Gal*p*A-(1 → 4)-α-Gal*p*A [15]. In conclusion, the structure of **M1** is:

α-D-Gal*p*A-(1 → 4)-α-D-Gal*p*A-(1 → 8)-Kdo

Monosaccharide analysis of fraction **M2** gave rise to the same molar ratio for GalA and Kdo (2:1) as **M1**. However, the 500-MHz ^{1}H NMR spectra of **M2** and **M1** are different, and it is proposed that **M2** is the following sulfated analogue of **M1**, in accordance with the occurrence of sulfate in the native preparation.

Table 2
^{13}C NMR data of *T. striata* cell wall-derived oligosaccharide **M1**, together with those of the reference compounds α-Kdo*p*, α-L-Rha*p*-(1 → 3)-α-L-Rha*p*-(1 → 8)-α-Kdo*p* [16], and α-D-Gal*p*A-(1 → 4)-α-D-Gal*p*A [15]

Residue	Chemical shift in			
	M1	α-Kdo*p*	→ 8)-α-Kdo*p*	α-D-Gal*p*A-(1 → 4)-α-D-Gal*p*A
α-Gal*p*A-(1 → (α-t-Gal*p*A)				
C-1	99.1			99.1
C-2	68.2			68.1
C-3	69.5			69.1
C-4	70.7			70.5
C-2	72.7			72.1
→ 4)-α-Gal*p*A[-(1 → (α-i-Gal*p*A)				
C-1	98.1			91.0
C-2	67.8			67.9
C-3	69.0			68.6
C-4	78.1			78.1
C-5	70.6			70.4
[→ 8)-]α-Kdo*p*				
C-1	n.d. [a]	n.d.	176.4	
C-2	n.d.	96.3	96.2	
C-3	n.d.	33.5	33.5	
C-4	65.9	66.1	66.1	
C-5	66.4	66.5	66.5	
C-6	70.9	71.0	70.7	
C-7	68.1	69.1	68.0	
C-8	69.1	62.9	68.9	

[a] n.d., Not determined.

α-D-Gal*p*A-(1 → 4)[SO_4 → 3]-α-D-Gal*p*A-(1 → 8)-Kdo

In the anomeric region the ^1H NMR spectrum at 300 K of **M2** (Fig. 2B) showed an α-Gal*p*A H-1 signal at δ 5.308 ($J_{1,2}$ 4.0 Hz) and a complex α-Gal*p*A H-1 pattern of doublets at δ 5.256/5.248 ($J_{1,2}$ 3.7 Hz)/5.240, respectively. In the Kdo H-3 region a complex pattern of multiplets (α-pyranose, H-3*eq* δ 1.878, H-3*ax* δ 1.965; α-furanose, H-3a δ 2.318, H-3b δ 2.385; β-furanose, H-3a δ 2.067, H-3b δ 2.585) was observed. The ^1H NMR spectrum of $NaBH_4$-reduced **M2** gave rise to two doublets at δ 5.216 ($J_{1,2}$ 3.7 Hz) and 5.203 ($J_{1,2}$ 3.9 Hz), respectively. 2D HOHAHA spectroscopy at 288 K of reduced **M2** afforded a complete assignment of the protons of both Gal*p*A residues and, based on these data, the H-atoms of the GalA residues in the major anomer of **M2** were assigned (Table 1). Comparison of the ^1H NMR data of **M1** and **M2** showed large differences in chemical shift for several protons of the two Gal*p*A residues, in particular those of α-iGalpA. Especially, a high downfield shift was observed for α-iGal*p*A H-3 ($\Delta\delta$ +0.625 ppm), supporting the presence of a sulfate group at C-3 of α-iGal*p*A. Similar shift increments were observed when comparing the ^1H NMR data of α-Gal*p*-OMe and α-Gal*p*-OMe 3-sulfate: H-3 $\Delta\delta$ +0.67 ppm; H-2, $\Delta\delta$ +0.16 ppm; H-4, $\Delta\delta$ +0.36 ppm [17].

1. Experimental

Cell wall polysaccharide preparation.—Cell culturing of algae and isolation of cell wall material from *T. striata* Butcher was performed as described [6]. The cell wall material was resuspended in 10 mM EDTA (pH 8.0) at a concentration of 5 mg/mL, and incubated overnight at 56°C. After removal of insoluble material by centrifugation (10 min, 12 000 *g*), the supernatant solution was desalted on a column (40 × 2.6 cm) of Bio-Gel P-4 (Bio-Rad, 200–400 mesh), using water as eluent, and lyophilised.

Isolation of **M1** *and* **M2**.—The aqueous-EDTA-solubilised polysaccharide preparation (120 mg) was treated with aq 1% (v/v) AcOH (4 mL) for 1 h at 100°C. After neutralisation with M NaOH and removal of insoluble material by low speed centrifugation, the supernatant solution was applied to a column (40 × 2.2 cm) of Bio-Gel P-2 (Bio-Rad, 200–400 mesh), equilibrated, and eluted with 100 mM NH_4HCO_3. The flow rate was 15 mL/h, the eluate was monitored at 206 nm, and fractions of 2.5 mL were collected. Carbohydrate-positive fractions (phenol–H_2SO_4 spot test) were lyophilised at least three times.

Medium-pressure anion-exchange chromatography of Bio-Gel P-2 fraction 3 was performed on a Pharmacia FPLC system equipped with a Mono Q HR 5/5 column. After collecting the neutral fraction, elutions were carried out using a gradient from 0–0.1 M NaCl in water (8 mL), followed by a steeper gradient from 0.1–1.0 M NaCl in water (8 mL), and UV-detection at 214 nm. Collected fractions were desalted on a column (27 × 1.6 cm) of Bio-Gel P-2.

Monosaccharide analysis.—Samples (0.1–0.5 mg) were subjected to methanolysis (1.0 M methanolic HCl) for 24 h at 85°C and, after trimethylsilylation, analysed by GLC on an SE-30 fused-silica capillary column (25 m × 0.32 mm, Pierce) using a Varian 3700 gas chromatograph [18].

NMR spectroscopy.—Samples were treated twice with D_2O (99.9 atom% D, MSD Isotopes) with intermediate lyophilisation, and finally dissolved in D_2O (99.96 atom% D, MSD Isotopes) at pD 7.

Resolution-enhanced 500-MHz ^1H NMR spectra were recorded on a Bruker AM-500 spectrometer (Bijvoet Center, Department of NMR Spectroscopy) at a probe temperature of 300 K. ^1H NMR chemical shifts are expressed in ppm relative to internal acetone (δ 2.225). 2D DQF COSY, HOHAHA, and ROESY spectra were recorded at 500 MHz at a probe temperature of 288 K. The HOD signal was suppressed by pre-saturation during 1.0 s. In the HOHAHA experiments a spin-lock time of 100 ms was applied at a field strength of 10.2 kHz, corresponding to a 90° pulse-width of 24.6 μs. The spectral width was 2000 Hz in each dimension and 512 FIDs with 4K data points were recorded. In the ROESY experiments a continuous spin-lock pulse of 150 ms was applied at a field strength of 2.4 kHz, corresponding to a 90° pulse-width of 104.5 ms. The carrier-frequency was set at the left side of the spectrum at 5.45 ppm. The spectral width was 5000 Hz in each dimension, and 496 FIDs with 4K data points were recorded.

Natural-abundance proton-decoupled ^{13}C NMR spectroscopy was performed on a Bruker AC-300 spectrometer (Department of Organic Chemistry, Utrecht University) equipped with a 10-mm broad-band probe-head at a probe temperature of 300 K at 75 MHz. Chemical shifts are expressed in ppm relative to internal acetone (δ 31.55).

Acknowledgements

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