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Structure of an extracellular polysaccharide produced by *Lactobacillus rhamnosus* strain C83

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Abstract

The extracellular polysaccharide produced by *Lactobacillus rhamnosus* strain C83 was found to be composed of D-glucose and D-galactose in a molar ratio of 2:3. The primary structure of the polysaccharide was shown by sugar analysis, methylation analysis, FABMS, partial acid hydrolysis and nuclear magnetic resonance (NMR) spectroscopy to consist of a pentasaccharide repeating unit having the following structure:

→3)-α-D-Glc*p*-(1→2)-β-D-Gal*f*-(1→6)-α-D-Gal*p*-(1→6)-α-D-Glc*p*-(1→3)-β-D-Gal*f*-(1→

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1. Introduction

Lactic acid bacteria (LAB) are involved in the preparation of a large number of fermented food products derived from raw agricultural materials, such as milk, meat, vegetables and cereals. The dairy industry is the prime user of these bacteria [1].

The role of LAB in food fermentation processes is very diverse. Besides the production of lactate, as in many dairy products, these bacteria may also produce a large variety of other substances that contribute to the flavour, colour, texture and consistency of the final product. Although the effects of the different components on the properties of the food product are often not fully understood in chemical detail, it has been generally acknowledged that secreted extracellular polysaccharides play a key role in the rheological behaviour and the texture of fermented foods [2]. Furthermore, the popularity of '100% natural' food products without additives has increased, and it has been claimed that exopolysaccharides (EPSs) isolated from LAB cultures have antitumour activity [3].

In order to obtain insights into the relationship between the physicochemical properties and the three-dimensional structures of these polysaccharides, knowledge of the primary structures is a prerequisite. Since 1990, several structural studies of EPSs produced by different strains of LAB have been reported [4–12]. Galactose (Gal) and glucose (Glc) predomi-

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nate in these polysaccharides, although rhamnose and 2-acetamido-2-deoxy-D-glucose are sometimes observed. Moreover, all the EPSs characterised to date are thought to be branched.

This report describes the structural elucidation of the EPS produced by *Lactobacillus rhamnosus* strain C83 [13].

2. Results and discussion

Purification and sugar composition of the polysaccharide.—The crude polysaccharide (PS) was obtained as a 60% ethanol (v/v) precipitate from the culture supernatant solution of *L. rhamnosus* strain C83, as described by Gamar et al. [13]. Proteins were eliminated by filtration of the precipitate obtained after heating. The protein content of the purified material was less than 1%.

PS was subjected to total acid hydrolysis with trifluoroacetic acid (TFA). Quantitative analysis of neutral sugars by cation-exchange high performance liquid chromatography (HPLC) showed the presence of Gal and Glc in the ratio 3:2. Both sugars were shown to be D isomers by GLC–MS analysis of their trimethylsilylated (−)-2-butyl glycosides [14].

Methylation analysis.—The PS was methylated by the Hakomori method [15] and the derived alditol acetates analysed by GLC–MS. This methylation analysis showed that the polysaccharide is composed in approximately equimolar amounts of two- and three-linked Gal*f*; six-linked Gal*p*; and three- and six-linked Glc*p* residues, indicating a linear arrangement of the polysaccharide.

NMR spectroscopy.—The 500 MHz ^{1}H NMR spectrum of the PS (Fig. 1(A)) was recorded at 50 °C in D_2O. This spectrum contains five signals in the anomeric region suggesting a pentasaccharide-repeating unit. The five-monosaccharide units were arbitrarily labelled **A**–**E**, as indicated in the spectrum. Based on their small coupling constants, the anomeric signals at δ 5.17 (residue **A**, $^3J_{1,2} < 2$ Hz) and δ 5.05 (residue **B**, $^3J_{1,2} < 2$ Hz) were assigned to H-1 of the two β-Gal*f* groups [16]. The coupling constants of the anomeric signals at δ 4.93 (residue **C**, $^3J_{1,2}$ 2.9 Hz), δ 4.91 (residue **D**, $^3J_{1,2}$ 2.9 Hz) and δ 4.87 (residue **E**, $^3J_{1,2}$ 2.9 Hz) suggest the presence of three α-hexopyranosyl (Hex*p*) residues. The absence of signals between 1 and 3 ppm in the ^{1}H NMR spectrum indicated that no *O*-acetyl or *N*-acetyl groups were present in the PS.

The ^{13}C distortionless enhancement by polarisation transfer (DEPT) 135 NMR spectrum of the polysaccharide (Fig. 1(B)) is in agreement with the suggested pentasaccharide-repeating unit, since five signals are observed in the region of anomeric carbons. The two downfield signals at δ 110.12 and 107.67 were attributed to C-1 of the β-Gal*f* groups **A** and **B**. Based on their chemical shifts, the C-1 signals at δ 99.73, 99.99 and 100.73 were assigned to three α-Hex*p* residues (**C**, **D**, **E**). The signals at δ 68.28 and 68.68 were assigned to C-6 of six-substituted Hex*p* residues, the signals at δ 64.60 and 64.65 to C-6 of the two Gal*f* groups **A** and **B** and the signal at δ 62.44 to C-6 of six-unsubstituted Hex*p* unit.

The ^{31}P NMR spectrum of the polysaccharide contained no signals, indicating the absence of phosphorylated substituents.

Partial acid hydrolysis.—In order to elucidate the structure of the PS, oligosaccharides were produced by partial acid hydrolysis of the native polysaccharide. The high viscosity of the polysaccharide at high concentrations did not allow the use of 2D NMR spectroscopy. The mixture of oligosaccharides, obtained after partial acid hydrolysis of PS, was fractionated on Bio-Gel P2, yielding Fractions I–III. Fraction I contained only Gal and Glc.

Analysis of Fraction II.—According to its ^{1}H NMR spectrum, Fraction II is a trisaccharide.

In the high-mass region of the negative-ion mode fast atom bombardment (FAB) mass spectrum of II, a $[M-H]^-$ pseudomolecular ion at m/z 503 was detected, corresponding to a composition of Hex–Hex–Hex. Reduction and hydrolysis of II showed the presence of galactitol, Glc and Gal.

Five anomeric signals were observed in the 1D ^{1}H NMR spectrum of II (Fig. 2(A)); two of them at δ 4.50 and 5.17 were assigned to the β- and α-Gal*p* forms of the reducing end called **X** ($^3J_{1,2}$ 7.8 and 1.9 Hz) which is derived from either residue **A** or **B** of the PS. The two

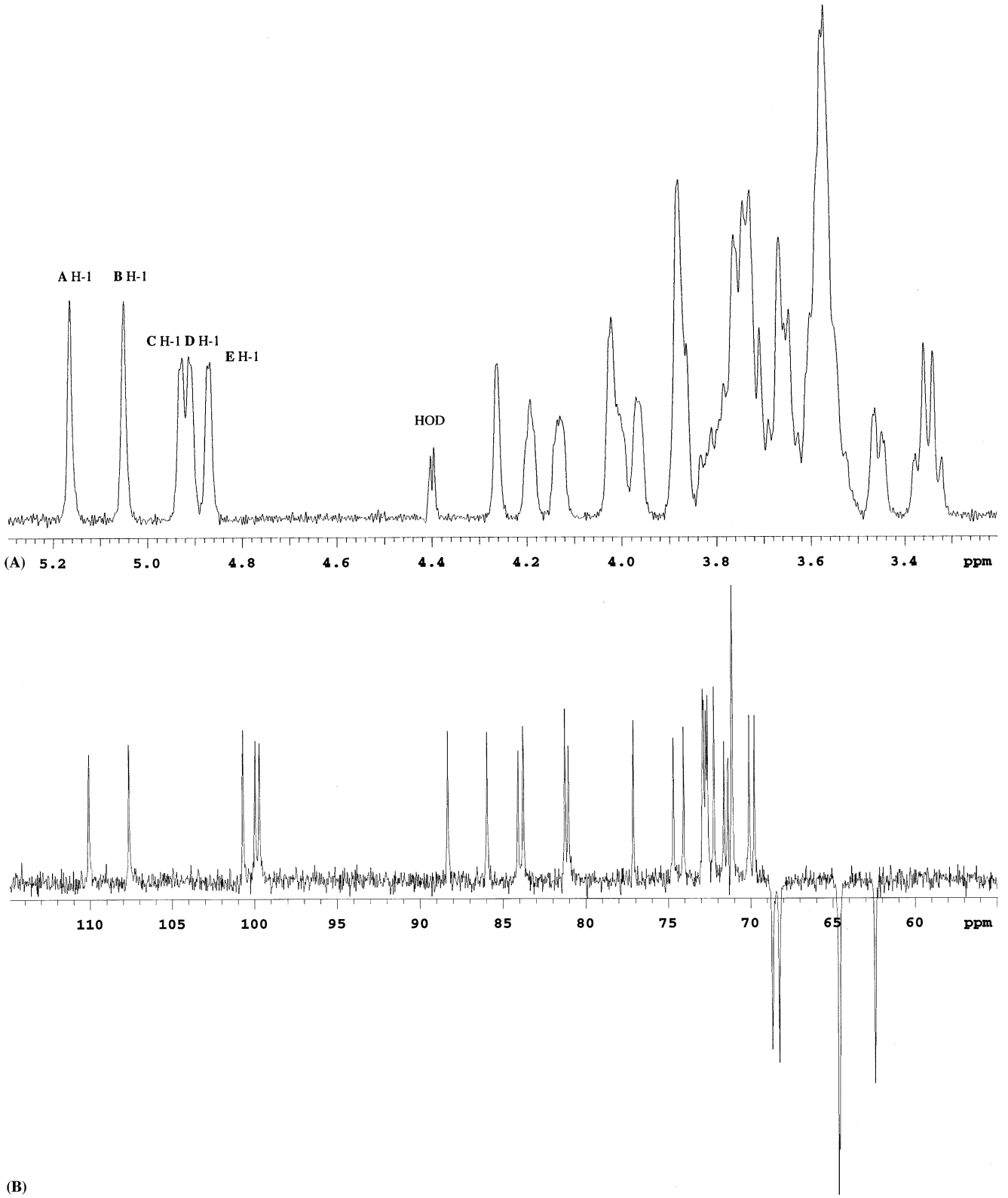

Fig. 1. (A) 500 MHz ^{1}H NMR spectrum of the crude polysaccharide in D_2O at 50 °C. (B) 75 MHz ^{13}C DEPT 135 NMR spectrum of the crude polysaccharide in D_2O at 50 °C.

Table 1
Chemical shift (δ, ppm) of the signals in the ^1H and ^{13}C spectra of Fraction II

Sugar residue		$^1H/^{13}C$						
		1	2	3	4	5	6	
→3)-Gal*p*	β	4.50	3.51	3.61	4.04	3.56	3.62	
X		98.08	72.90	79.52	66.77	76.49	62.74	
	α	5.17	3.84	3.84	4.10	3.93	3.66	
		93.94	68.38	75.87	67.22	71.95	62.56	
→6)-α-Glc*p*-(1→		5.00	3.49	3.68	3.41	4.02	3.84	3.64
Y		97.07	71.90	74.74	71.25	72.08	67.39	
		5.01	3.50	3.68	3.38	4.02	3.82	3.68
		96.53	71.90	74.72	71.35	72.08	67.63	
α-Gal*p*-(1→		4.87	3.71	3.77	3.88	3.84	3.62	
E		99.60	70.10	71.15	70.87	72.50	62.67	

signals at δ 5.00 and 5.01 ($^3J_{1,2}$ 3.9) were assigned to a α-Hex*p* residue called **Y,** which was influenced by the α–β equilibrium of residue **X** and which arises from residue **C** or **D** of PS. The signal at δ 4.87 ($^3J_{1,2}$ 3.2 Hz) was easily assigned to the α-Hex*p* residue **E**. The complete proton assignments for residues **X**, **Y** and **E**, presented in Table 1, are based on 1D total correlation spectroscopy (TOCSY) and correlation spectroscopy (COSY) experiments. The 1D TOCSY spectra of **X** H-1 at δ 4.50 and 5.17 showed only signals for **X** H-2,3,4. The signal for **X** H-5 could not be observed in the TOCSY spectra because of the small coupling constant $^3J_{4,5}$ for Gal*p* residues. The **X** H-5,6a resonances were assigned from the COSY spectrum, starting from **X** H-4 resonance. In the 1D TOCSY spectrum with selective excitation of the **Y** H-1 at δ 5.00 and 5.01 the complete series of signals for **Y** H-2,3,4,5,6a,6b were observed, indicating a Glc*p* residue. The 1D TOCSY spectra of **E** H-1 at δ 4.87 showed signals for **E** H-2,3,4 and resonances **E** H-5,6a were assigned with the COSY experiment, as for residue **X**, indicating a Gal*p* residue.

The $^{13}C-^1H$ correlation (XHCORR) spectrum [17] allowed the complete carbon assignments for residues **X**, **Y** and **E** to be obtained, as presented in Table 1. The ^{13}C DEPT 135 experiment (Fig. 2B) contained five signals in the anomeric region. The two signals at δ 93.94 and 98.08 were assigned to the α and β forms of the residue **X**, the two signals at δ 96.53 and 97.07 were attributed to residue **Y** and the signal at δ 99.60 to the residue **E**. The DEPT experiment showed that two ^{13}C signals from C-6 are present at δ 67.39 and 67.63, indicating a six-substituted sugar residue (**Y**), and the other three at δ 62.56, 62.67 and 62.74. The chemical shifts of C-3 for residue **X** (δ 79.52 for the β form and δ 75.87 for the α form) compared with those of an unsubstituted Gal*p* (δ 73.80 and 70.20, respectively) indicate that the residue **X** is a three-linked Gal*p* residue.

Hence, the structure of II is assigned as:

α-D-Gal*p*-(1→6)-α-D-Glc*p*-(1→3)-D-Gal*p*
E **Y** **X**

Analysis of Fraction III.—1D ^1H NMR analysis of Fraction III is consistent with the presence of a pentasaccharide.

In the high-mass region of the negative-ion mode FAB mass spectrum of III, a $[M-H]^-$ pseudomolecular ion at m/z 827 was detected, corresponding to a composition of Hex_5.

In the 1D ^1H NMR spectrum of III (Fig. 3(A)), seven anomeric signals are present. The two signals at δ 4.51 and 5.18 were assigned to residue **X** ($^3J_{1,2}$ 7.8 and 1.9 Hz), the two signals at δ 5.00 and 5.02 to residue **Y** ($^3J_{1,2}$ 3.8 Hz), and the signal at δ 4.87 to residue **E** ($^3J_{1,2}$ 3.4 Hz). These three residues are identical with those of the Fraction II (except for the substitution of residue **E**), which implied that the Fraction III had the same reducing end as Fraction II and was composed in part of these three residues. The signal at δ 4.93 ($^3J_{1,2}$ 3.8 Hz) was assigned to the α-Hex*p*

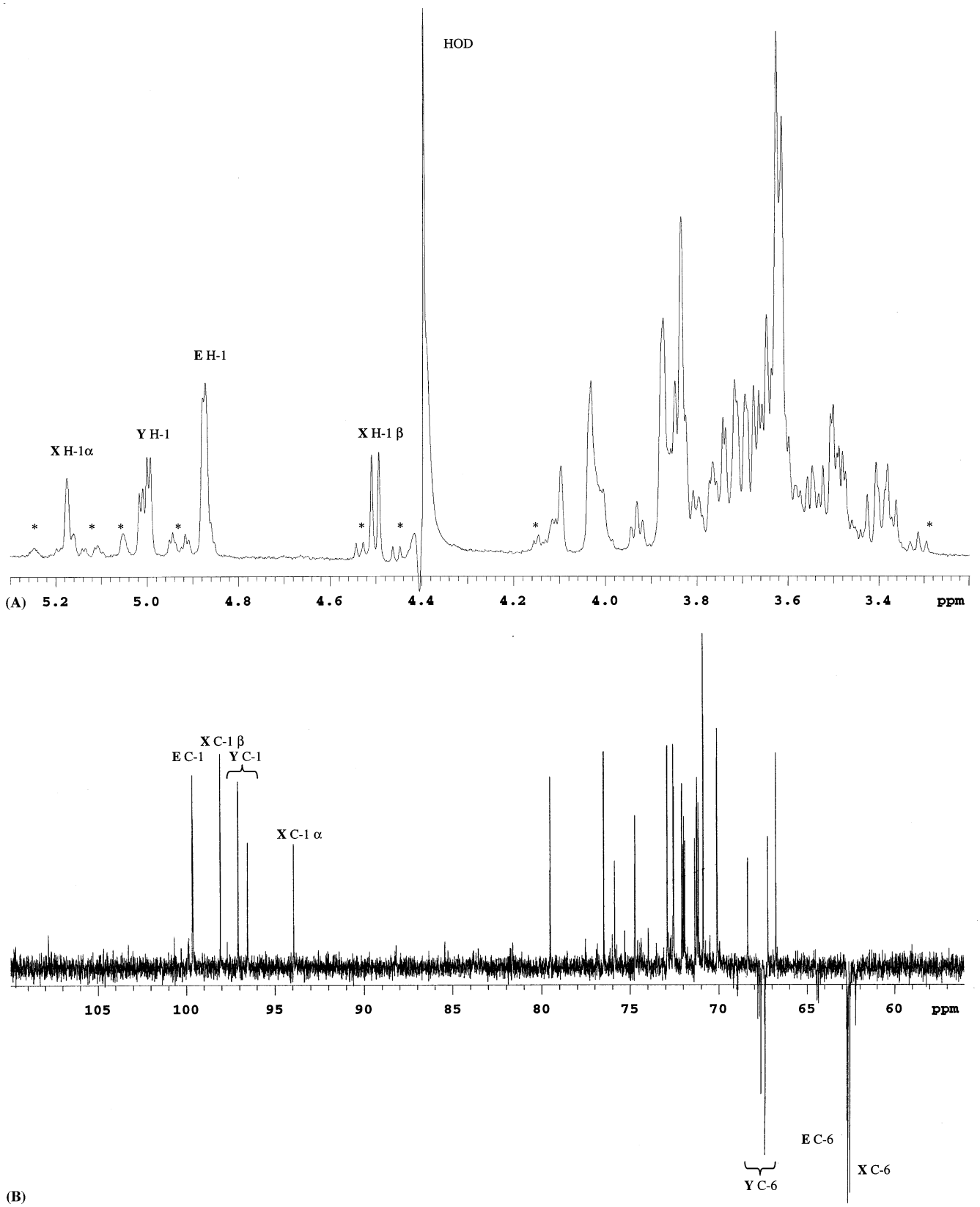

Fig. 2. (A) 500 MHz 1D ^{1}H NMR spectrum of Fraction II in D_2O at 50 °C (* impurities). (B) 75 MHz ^{13}C DEPT 135 NMR spectrum of Fraction II in D_2O at 50 °C.

Table 2
Chemical shifts (δ, ppm) of the signals in the ^1H and ^{13}C spectra of Fraction III

Sugar residue		$^1H/^{13}C$						
		1	2	3	4	5	6	
→3)-Gal*p*	β	4.51	3.50	3.61	4.04	3.53	3.62	
X		98.10	72.98	79.45	66.74	76.56	62.76	
	α	5.18	3.83	3.83	4.11	3.93	3.64	
		93.97	68.35	75.63	67.14	71.94	62.57	
→6)-α-Glc*p*-(1→		5.00	3.49	3.67	3.38	4.02	3.62	3.84
Y		97.06	71.90	74.77	71.34	72.06	67.33	
		5.02	3.50	3.68	3.38	4.02	3.68	3.82
		96.35	71.90	74.77	71.39	72.06	67.23	
→6)-α-Gal*p*-(1→		4.87	3.71	3.77	3.88	3.73	3.61	3.78
E		99.70	70.04	71.13	71.03	72.02	68.60	
→2)-β-Gal*f*-(1→		5.05	4.02	4.12	3.87	3.73	3.54	3.57
B		107.62	88.34	76.99	83.81	71.91	64.45	
α-Glc*p*-(1→		4.93	3.44	3.58	3.32	3.64	3.63	3.75
C		99.62	72.76	74.45	71.19	73.96	62.19	

residue **C** and the signal at δ 5.05 ($^3J_{1,2} < 2$ Hz) to the β-Gal*f* residue **B** because of their chemical shifts. The assignments of these two signals to H-1 of residues **C** and **B** implies that residues **X** and **Y** correspond to residues **A** and **D**, respectively, in the PS. It is important to note here that the chemical shifts of **D** H-1 are very different in the PS (δ 4.91) and in the Fractions II and III (δ 5.00 and 5.02 for Fraction III). This will be explained later. The complete proton assignments for residues **X**, **B**, **C**, **Y** and **E**, presented in Table 2, are based on 1D TOCSY and COSY experiments.

As for Fraction II, 1D TOCSY experiments of Fraction III showed most of the protons for residues **X**, **Y** and **E** and confirmed that **X** and **E** are Gal*p* residues and that residue **Y** is Glc*p*. In the 1D TOCSY spectra with selective excitation of **B** H-1 at δ 5.05, the complete series of signals for **B** H-2,3,4,5,6a,6b was observed, confirming the β-Gal*f* form. The 1D TOCSY spectra with excitation of **C** H-1 at δ 4.93 showed signals for **C** H-2,3,4,5,6a,6b, indicating a Glc*p* form.

The ^{13}C NMR spectrum (Fig. 3(B)) contained seven signals in the anomeric region. The signals at δ 93.97 and 98.10 were assigned to the α and β forms of residue **X**, the signals at δ 96.35 and 97.06 to residue **Y** and the signal at δ 99.70 to residue **E**, as in Fraction II. The downfield signal at δ 107.62 was attributed to C-1 of the Gal*f* group **B** and based on its chemical shift, the C-1 signal at δ 99.62 was assigned to a α-Glc*p* residue **C**. The resonances at δ 62.57, 62.76 and 62.19 were assigned to C-6 of six-unsubstituted Hex*p* residues (**X** and **C**), the signal at δ 64.45 to C-6 of the β-Gal*f* group **B** and the signals at δ 67.23, 67.33 and 68.60 to C-6 of six-substituted Hex*p* residues (**Y** and **E**).

The 2D $^{13}C-^1H$ HMQC spectrum of Fraction III allowed complete assignment of the ^{13}C NMR spectrum. The $^1J_{C\text{-}1,H\text{-}1}$ coupling constants observed in the HMQC experiment, without ^{13}C decoupling (Table 2), of 160.5 and 170 Hz for residue **X** confirm the assignments of their anomeric configuration as β and α, respectively. The $^1J_{C\text{-}1,H\text{-}1}$ coupling constants of 170 Hz for residues **C**, **Y** and **E** indicate an α-anomeric configuration and the coupling constant for residue **B** (174 Hz) confirms the β-Gal*f* form of this residue [16]. In the 2D $^{13}C-^1H$ heteronuclear multibond correlation (HMBC) spectrum of Fraction III (Fig. 4), intraresidual two- and three-bond $^{13}C-^1H$ couplings can be observed, as well as interresidual three-bond connectivities over the glycosidic linkages. The **Y**(1→3)**X** linkage was verified by a cross-peak between **Y** C-1 and **X** H-3 and the **E**(1→6)**Y** linkage was confirmed by cross-peaks between **E** C-1 and **Y** H-6a and **Y** H-6b. Furthermore, the two other linkages were determined from long-range couplings: the **B**(1→6)**E** linkage by cross-peaks between

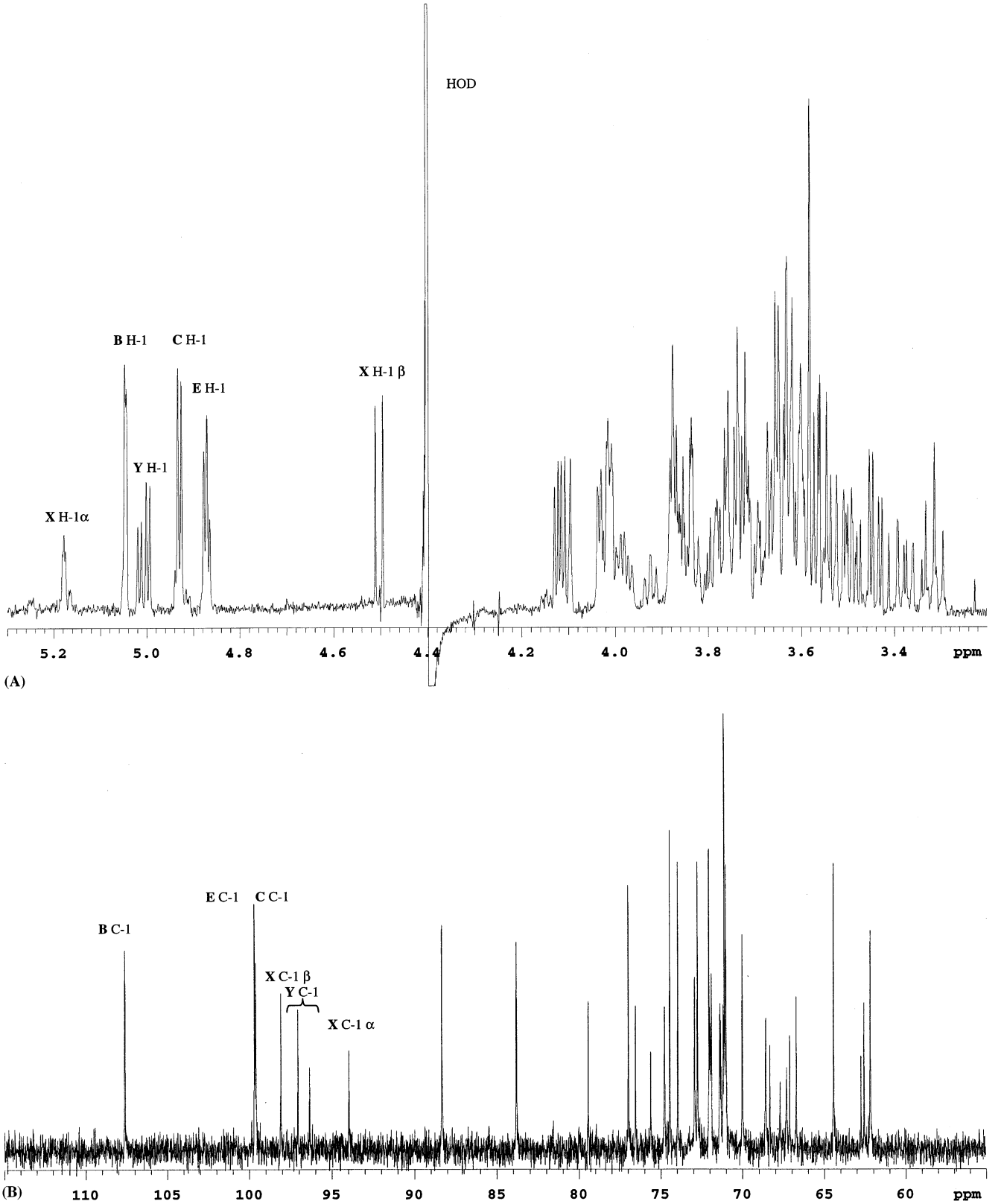

Fig. 3. (A) 500 MHz 1D ^{1}H NMR spectrum of Fraction III in D_2O at 50 °C. (B) 75 MHz ^{13}C NMR spectrum of Fraction III in D_2O at 50 °C.

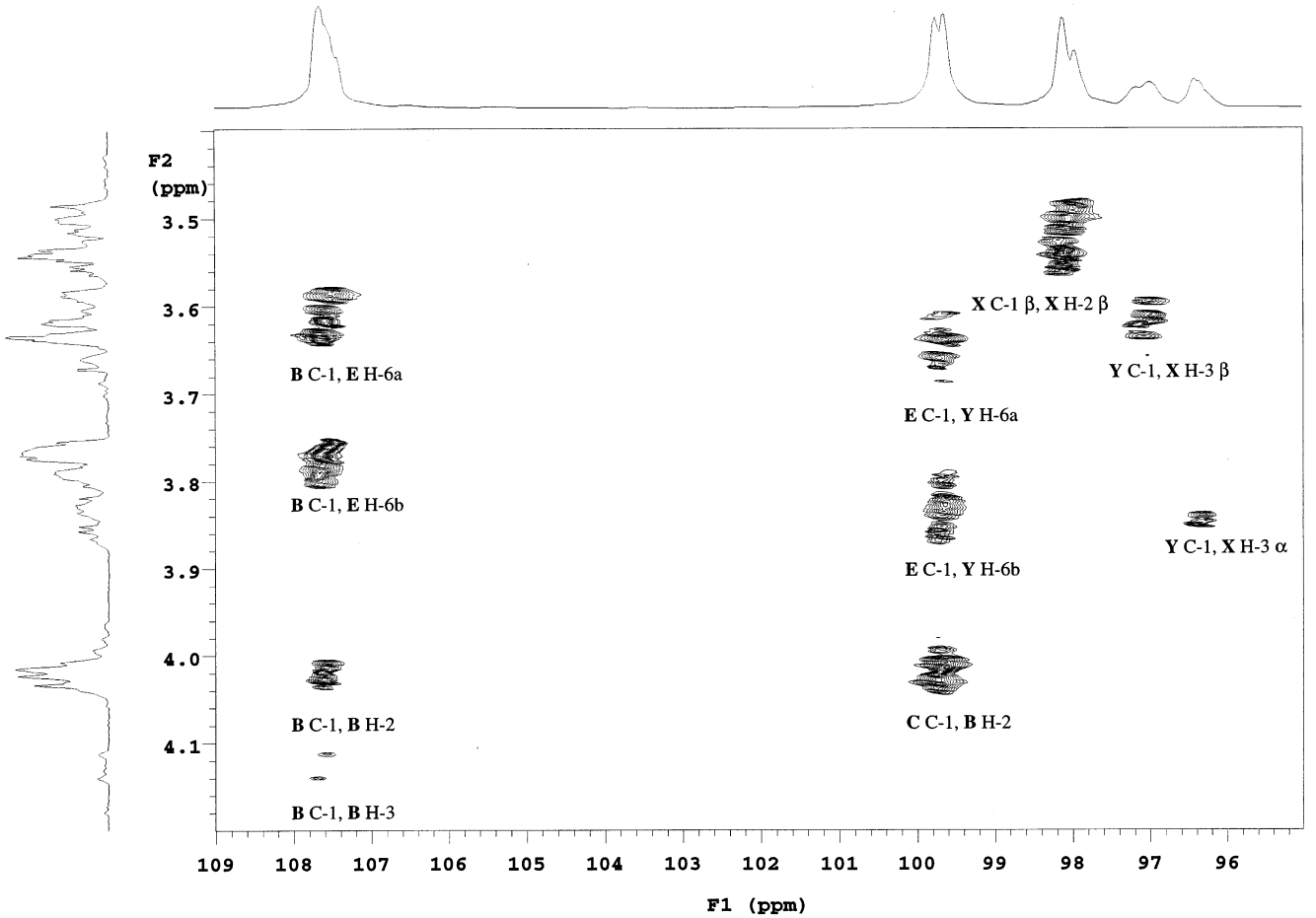

Fig. 4. 2D $^{13}C-^{1}H$ HMBC spectrum (mixing time 50 ms) of Fraction III in D_2O at 50 °C.

B C-1 and **E** H-6a and H-6b, and the **C**(1→2)**B** by a cross-peak between **C** C-1 and **B** H-2. Hence, the structure of III is assigned:

α-D-Glc*p*-(1→2)-β-D-Gal*f*-(1→6)
C **B**

-α-D-Gal*p*-(1→6)-α-D-Glc*p*-(1→3)-D-Gal*p*
E **Y** **X**

From the combined results of methylation analysis and the structure of Fraction III, the structure of the repeating unit of the polysaccharide could be determined. Methylation analysis of the PS showed the presence of three-linked Gal*f*, two-linked Gal*f*, three-linked Glc*p*, six-linked Glc*p* and six-linked Gal*p*. The change in the chemical shift of the anomeric proton resonance between residues **D** and **Y** is due to the change in ring form of residue **A** on hydrolysis, when residue **X** is formed. The linkage of residue **C** in PS seems to be an **A**(1→3)**C** linkage according to methylation analysis. So it is concluded that the neutral exopolysaccharide from *L. rhamnosus* strain C83 is composed of a pentasaccharide-repeating unit with the following structure:

→3)-α-D-Glc*p*-(1→2)-β-D-Gal*f*-(1→6)
C **B**

-α-D-Gal*p*-(1→6)-α-D-Glc*p*-(1→3)
E **D**

-β-D-Gal*f*-(1→
A

The composition of this PS is similar to these of many PSs produced by LAB since it is composed of Gal and Glc. Nevertheless, the presence of Gal*f* and the linear structure make it remarkable.

3. Experimental

Monosaccharide and methylation analyses.—The PS was hydrolysed in 4 M TFA at 100 °C for 2 h. The TFA was removed by successive coevaporations with H_2O. Quantitative HPLC of monosaccharides was performed on a Waters HPLC system with RI detection, using a CHO-682 cation exchange column (300 × 7.8 mm Interchim, France) eluted with water at 85 °C. For methylation analysis, the polysaccharide was permethylated according to the Hakomori method and the resulting per-*O*-methylated alditol acetates were analysed by GLC–MS.

Partial acid hydrolysis.—Native polysaccharide was hydrolysed in 1 M TFA for 30 min at 100 °C. After evaporation of TFA, oligosaccharide fractions were eluted from a Bio-Gel P2 column (2 m × 1.5 cm) with water at 60 °C and at a flow rate of 40 mL/h. The fractions were analysed by HPLC with a Interchrom CP5C18-25F apolar column (250 × 4.6 mm Interchim, France) eluted with water at room temperature with at a flow rate of 0.6 mL/min.

Gas–liquid chromatography and mass spectrometry.—GLC–MS analyses were carried out on a NERMAG R10-10C mass spectrometer (electron energy, 70 eV), coupled to a Delsi Instruments gas chromatograph equipped with a SP-2380 capillary column (30 m × 0.22 mm) using He as carrier gas. The oven conditions were: 180 °C for 5 min; 180–235 °C at 2 °C/min. The positive and negative-ion mode FAB mass spectra were recorded using the same mass spectrometer (accelerating voltage, 9 kV; matrix, glycerol).

Reduction and hydrolysis of Fraction II.—Fraction II (5 mg) was reduced with sodium borohydride in 1 mL of water for at least 4 h. After successive evaporations with 1% HCl–MeOH, hydrolysis was performed with 2 mL of 2 M TFA (100 °C, 3 h).

NMR experiments.—The samples were dissolved in D_2O. All the spectra were recorded at 323 K except the ^{31}P NMR spectrum. Proton-decoupled 75.470-MHz ^{13}C NMR spectra were recorded on a Bruker AC-300 NB spectrometer, equipped with a 5-mm $^{13}C/^{1}H$ dual probe. The 161.906-MHz 31P NMR spectrum was recorded on a Varian Unity 400 NB spectrometer, equipped with a 5-mm HX-ID-PFG probe, at a temperature of 65 °C. Chemical shifts are referenced to external $NaPO_2H_2$ (δ 0.00). Chemical shifts were given taking acetone as reference for ^{1}H NMR spectra (δ 2.04) and ^{13}C NMR spectra (δ 31.45) in D_2O at 357 K. The data were collected in 16k data sets and before Fourier transformation an exponential multiplication was applied. 1D ^{1}H and 2D NMR spectra for PS and fraction III were recorded, on a Varian Unity plus 500 spectrometer equipped with a 5-mm HX-ID-PFG probe (except the HMBC spectra). 1D ^{1}H NMR spectra were recorded with a spectral width of 1200 Hz in 64k complex data sets. Suppression of the HOD signal was achieved by presaturation for 1.5 s. The ^{13}C-^{1}H 2D HMQC experiment for Fraction III was carried out at a ^{1}H frequency of 499.837 MHz (125.694 MHz for ^{13}C) with a spectral width of 1247 Hz for t_2 and 10,000 Hz for t_1, using the standard pulse sequence. The time domain data sets were multiplied with a phase-shifted sine bell and, after Fourier transformation and zero-filling, data sets of 4k × 4k points were obtained.

The HMBC spectra were recorded on a Bruker AM-400 NB spectrometer using different mixing times; 50, 100 and 200 ms. 1D TOCSY spectra were recorded with mixing times of 5–89 ms. 2D NMR spectra of Fraction II (COSY and XHCORR) were recorded on a Varian Unity 400 NB spectrometer.

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