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Structural elucidation of the capsular polysaccharide isolated from *Kaistella flava*

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ABSTRACT

The Gram-negative bacterium under study belongs to the genus *Kaistella*. It was isolated from a soil sample of the Haian Island in China, and it produces a lipophilic polysaccharide characterised by a branched hexasaccharide repeating unit, counting four 6-deoxy-α-L-mannose (Rha) residues, one 2-acetamido-2-deoxy-β-D-glucose (GlcNAc) and a 2-acetamido-2,6-dideoxy-β-D-galactose (FucNAc) unit.

The structure of the repeating unit, assigned through 2D-NMR spectroscopy, is herein reported for the first time:

[3)-β-D-FucNAc-(1→3)-α-L-Rha-(1→2)-α-L-Rha-(1→3)-α-L-Rha-(1→2)-α-L-Rha-(1→]$_n$
2
β-D-GlcNAc┘

1. Introduction

Kaistella flava, strain YIM 47657, is a Gram-negative bacterium isolated from a soil sample collected in the secondary forest of the Wuzhi Mountain in the Haian Island in China. It is taxonomically related to *Kaistella koreensis* with only 95.41% similarity of its 16S rRNA sequence. It is a possible new species and, as all the members of the genus *Kaistella*, it is placed into the *Chryseobacterium–Bergeyella–Riemerella* branch of the *Flavobacteriaceae* family.[1] *Kaistella flava* grows aerobically, is Gram-negative, appears as non-sporulating rods and forms circular, entire, low-convex, smooth, opaque and yellow colonies.

In the context of the chemotaxonomical classification of this bacterium, we have started a study of the cell wall components and of the capsular and/or excreted polysaccharides.

Exopolysaccharides and capsular polysaccharides are essential components of the biofilm matrix, providing the framework into which microbial cells are inserted. They are synthesised by microbial cells and vary greatly in their chemical and physico-chemical properties. Some are neutral macromolecules, but the majority presents a polyanionic nature due to the presence of either uronic acids (D-glucuronic acid being very common, although D-galacturonic and D-mannuronic acids are also found) and/or carboxylate-bearing substituents. Inorganic residues, such as phosphate or rarely sulfate, may also confer polyanionic status. A very few others may even be polycationic, as exemplified by the adhesive polymer obtained from strains of *Staphylococcus epidermidis* strains associated with biofilms.[2] In general, exopolysaccharides are very important for their rheological and biological properties, and are widely used in many fields such as in biotechnology and pharmacy; therefore, the study of the primary structure of EPS is of pivotal importance.

Within this frame, the repeating unit of a polysaccharide component isolated from cells of *Kaistella flava* was determined.

2. Results and discussion

2.1. Isolation of PCP, W and P fraction and chemical analysis

Freeze-dried cells were extracted with a mixture of phenol/chloroform/petroleum ether[3] and the remaining pellet was treated according to the hot water/phenol extraction method,[4] the work up of each phase (Scheme 1) led to the following samples: **PCP** (from the first extraction protocol), **W** and **P** (from the second method). The three fractions were screened by SDS–PAGE electrophoresis, and the occurrence of R-type LPS was ascertained in the **PCP** fraction (Fig. 1, lane B), although minor amounts were visible in both the water and phenol phases (figure not shown).

PCP compositional analysis revealed the presence of D-glucose and D-mannose as principal components and D-glucuronic acid,

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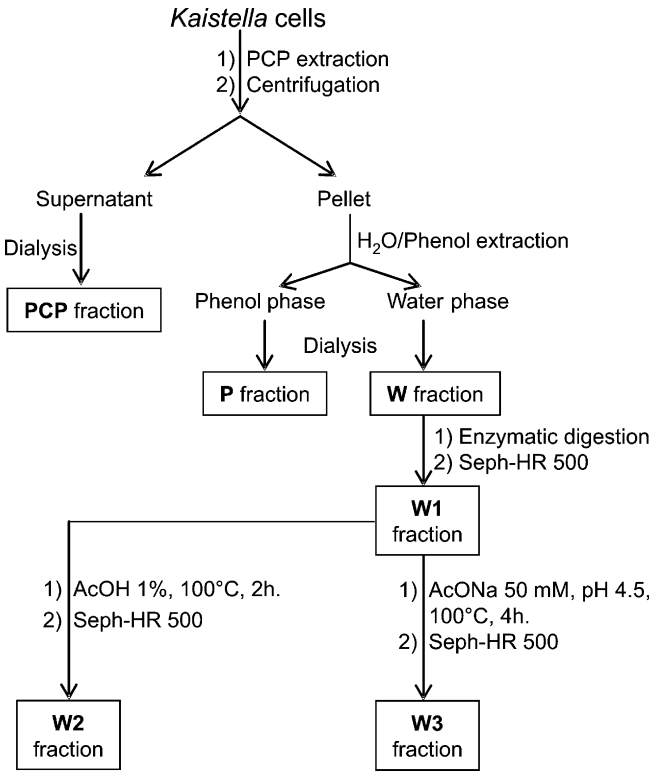

Scheme 1. Purification strategy used to isolate **PCP**, **P**, **W**, **W1**, **W2** and **W3** fractions from the dry cells of *Kaistella* strain YIM 47657.

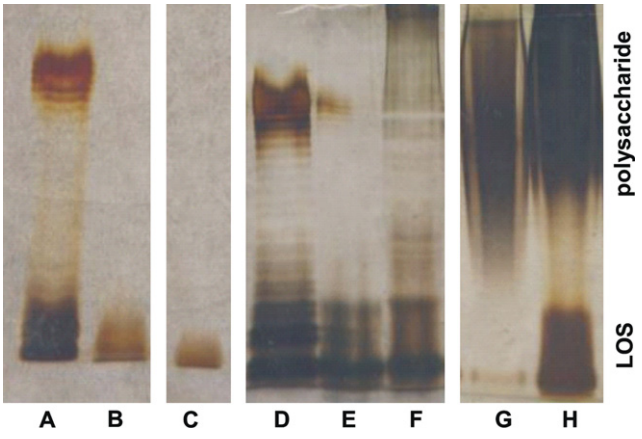

Figure 1. 15% SDS–PAGE electrophoresis analysis of polysaccharide fractions from *Kaistella*, lanes A–C are visualised according to the silver-staining protocol, lanes D–H are visualised applying Alcian blue prior to the standard-staining procedure. (A) and (D): reference, *E. coli* O:55 (4 μg), (B) and (E): **PCP** 4 μg; (C) and (F): **W1** 4 μg; (G): **W2** 8 μg; (H): **W3** 8 μg.

D-glucosamine and Kdo as minor ones, and the following fatty acids (*i*- stands for *iso*, *a*- for *anteiso* and *n*- for *normal*): *i*- and *a*-C14:0, *i*- and *a*-C15:0, *i*-C16:0 and *n*-C16:0, *i*- *a*- and *n*-C15:0-3OH, C18:1, C18:2, C16:0-3OH, C17:0-3OH confirmed the lipid pattern described for the genus *Kaistella*.[1] Differently, **W** and **P** fractions contained large amounts of L-rhamnose (Rha), D-glucose, D-glucosamine (GlcN) and 2-amino-2,6-dideoxy-D-galactose (FucN), whilst Kdo, Man and GlcA could be found in trace amount. These data suggested that these two samples contained small proportions of LOS together with an abundant polysaccharide component, not detected with the classical silver-staining protocol of the SDS–PAGE.[5]

2.2. Isolation of W1, W2 and W3 fractions and SDS–PAGE analysis with Alcian blue

In order to separate the polysaccharide from the LOS component, analyses were focused on **W** fraction (see Scheme 1) that was sequentially treated with RNAse, DNAse and protease to eliminate nucleic acids and proteins, dialysed and successively chromatographed on a Sephacryl HR-S500 (profile not shown).

The resulting polysaccharide component coeluted together with the LOS as a broad peak in the void volume of the column (fraction **W1**), whereas nucleic acid and protein fragments were eluted afterwards, together with a glucose-containing component that was not further studied.

Methylation analysis of **W1** led to the identification of a terminal unit of GlcN, 2-, 3- and 2,3-substituted rhamnose units, and a 3-substituted FucN. Peaks related to the LOS fraction were not detected, and a trace of 4-substituted glucose was probably related to the contaminant glucan.

LOS elution in the void volume of the column was dictated from the formation of the high molecular weight micelle complexes and, in order to purify the polysaccharide component from these aggregates, LOS was degraded cleaving the lipid A from the rest of the molecule by acid hydrolysis. Applying this procedure, the insoluble lipid A was recovered by centrifugation and the core oligosaccharides, unable to aggregate without the lipophilic moiety, were expected in the low molecular weight region of the chromatographic profile.

The **W1** fraction was hydrolysed, and two different protocols were selected in order to detect any possible artefact on the polysaccharide component (i.e., depolymerisation or loss of labile substituents): AcOH 1% at 100 °C for 2 h, and AcONa 50 mM pH 4.5 at 100 °C for 4 h, obtaining, after chromatographic purification, **W2** and **W3** fractions, respectively (Scheme 1).

In each case, precipitation of the Lipid A was observed as expected, and the supernatant was purified in the same chromatographic conditions used for **W1**: the elution profile showed one main peak in the void volume of the column, named **W2** or **W3** depending on the hydrolysis conditions used (AcOH 1% or AcONa 50 mM, respectively); oligosaccharides related to the LOS were not detected probably due to their low abundance.

Electrophoretic analysis was performed by fixing the gel with Alcian blue prior to silver staining.[6] As a result **W1**, **W2** and **W3**, but not **PCP** fraction (Fig. 1, lanes F, G, H and E, respectively) showed a new high molecular weight band pattern; in the case of **W1**, these bands were located almost at the start of the separating gel (Fig. 1, lane F) and were not visible when the gel was stained without fixative (Fig. 1, lane C).

Staining pattern of fraction **W2** (Fig. 1, lane G) showed that the hydrolysis process was complete (LOS was absent), and the polysaccharide bands were located in the high molecular weight area. In contrast with **W2**, the polysaccharide component in **W3** sample (Fig. 1, lane H) was less depolymerised and the presence of LOS bands in the lower part of the gel suggested the partial hydrolysis of this last component; the **W3** proton spectrum (not shown) was identical to that of **W2** (Fig. 2), ruling out the occurrence of acid labile substituents.

On the basis of the above results, it appeared that this bacterium produces a polysaccharide, co-extracted with the LOS in the water phase. This polymer could be detected in the **W1** (or **W2** or **W3**) fraction only applying a fixative prior to the classical silver staining, as reported for many polysaccharides.[7] Unexpectedly, Alcian blue acted as a fixative and staining enhancer for a neutral polysaccharide, whereas its use is usually considered specific for anionic carbohydrates.

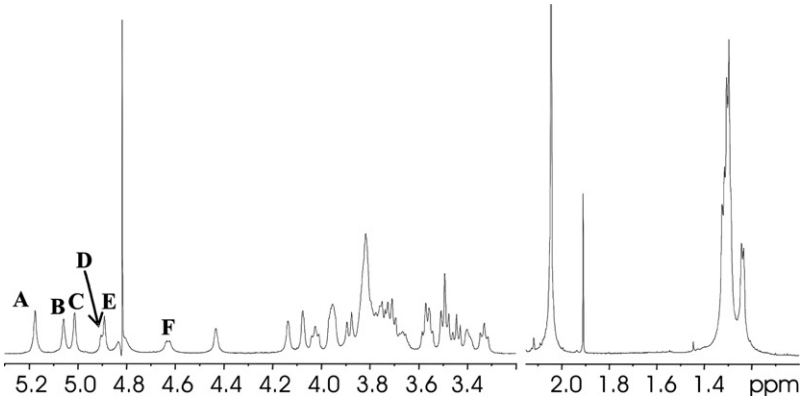

Figure 2. (D_2O, 500 MHz, 25 °C) ^1H NMR spectrum of **W2** sample. The six anomeric protons in the low field region are labelled with capital letters (**A**–**F**) and the signal at 2.04 ppm belongs to two *N*-acetyl groups.

2.3. NMR analysis of the capsular polysaccharide

The primary structure of the polymer was obtained by spectroscopic analysis of the **W2** fraction.

The ^1H NMR spectrum (Fig. 2) showed six anomeric protons (labelled with letters from **A** to **F** according to their decreasing chemical shift values), one singlet at 2.04 ppm equivalent to two *N*-acetyl signals, and several overlapped doublets at high fields, due to the methyl groups of the FucN and the Rha units.

Rhamnose spin systems, labelled as **A**, **B**, **C** and **E**, were easily recognised on the basis of the coupling constant information in the DQ-COSY spectrum, in particular from the diagnostic shape of the H-3/H-2 cross peak that displayed a small active coupling dictated by the $^3J_{H2,H3}$ value and a passive but large one with proton H-4.

Their anomeric configuration was α on the basis of their C-5 chemical shift (70.5, 70.8, 70.4 and 70.5, respectively) and the low field displacement of C-2 of both **A** and **C** residues with respect to the typical value (71 ppm) proved the glycosylation at that position, whereas **E** was substituted at O-3 and **B** at O-2 and O-3 positions (Table 1).[8]

The fifth anomeric signal, **D**, was classified as a terminal β-GlcNAc: the *gluco* stereochemistry was explained by the strong scalar correlations in both the COSY and TOCSY spectra, the β configuration was established on the basis of the $^3J_{H1,H2}$ coupling constant detectable on the COSY spectrum (8.4 Hz) and by the chemical shift value of its C-5 (77.1 ppm).[8] The carbon chemical shift of the ring carbons pointed out to the occurrence of a carbon-bearing nitrogen in position C-2, N-acetylated as proven by the H-2 deshielding chemical shift and by the presence of *N*-acetyl signals in the proton spectrum. The other carbon signals had similar values to those reported for the non-substituted glycoside,[8] confirming the occurrence of a terminal 2-acetamidodeoxyglucose unit.

The last anomeric signal **F** was attributed to an O-3 substituted β-FucNAc residue. The HSQC correlation between the H-2 and a nitrogen-bearing carbon confirmed the location of an N-acetylated amino group at C-2, the $^3J_{H1,H2}$ coupling constant (8.1 Hz) indicated that the β configuration at the anomeric carbon and the absence of the scalar correlation between H-4 and H-5 proved the *galacto* stereochemistry of the residue. The chemical shift values for H-4 and H-5 were established on the basis of the NOE contacts between H-3/H-4, H-3/H-5 and H-4/H-5.

The following strong NOE contacts (Fig. 3) contribute to build the sequence of the backbone repeating unit: H-1 of **B** and H-2 of **A**, H-1 of **A** and H-3 of **E**, H-1 of **E** and H-2 of **C**, H-1 of **C** with H-3 of **F** and H-1 of **F** with H-3 of **B**; this pentasaccharide sequence presented a single monosaccharide branch due to **D** residue located at O-2 of **B**, as inferred from the NOE between H-1 of **D** and H-2 of **B**.

NOE correlations were in agreement with gHMBC data.

Summarising NMR data, the repeating unit of the polysaccharide from *Kaistella flava*, is made up of six pyranosidic sugars, the linear backbone is constituted by five 6-deoxyglucose residues, one fucosamine and four rhamnose units, whereas glucosamine is terminal and stands as appendage of one of the rhamnose residues, as depicted in Figure 4.

Table 1
^1H (plain) and ^{13}C NMR (italic) chemical shifts of the constituents of the repeating unit (D_2O, 500 MHz, 25 °C)

H/C	1	2	3	4	5	6
2)-α-L-Rha-(1→	5.17	4.08	3.95	3.49	3.83	1.31
A	*102.1*	*79.0*	*71.0*	*73.4*	*70.5*	*18.0*
2,3)-α-L-Rha-(1→	5.06	4.43	3.79	3.33	3.67	1.24
B	*102.7*	*78.4*	*81.9*	*72.0*	*70.8*	*18.0*
2)-α-L-Rha-(1→	5.01	3.82	3.96	3.49	3.83	1.31
C	*102.1*	*80.5*	*71.0*	*73.4*	*70.4*	*18.0*
β-D-GlcNAc-(1→	4.90	3.71	3.57	3.45	3.41	3.74–3.89
D	*103.5*	*56.8*	*74.9*	*71.0*	*77.0*	*61.8*
3)-α-L-Rha-(1→	4.89	4.14	3.82	3.56	3.77	1.29
E	*103.5*	*71.0*	*80.5*	*72.9*	*70.5*	*18.0*
3)-β-D-FucNAc-(1→	4.64	4.03	3.72	3.82	3.80	1.30
F	*104.1*	*53.1*	*80.7*	*71.5*	*71.7*	*18.0*

Chemical shifts are expressed in δ relative to internal acetone.

2.4. Conclusions

The genus *Kaistella* was described for the first time quite recently[1] and no data are available concerning the composition or the structure of its glycosidic components, information of importance in the context of the chemiotaxonomical description of this genus.

Kaistella Y47567 is a new member of this genus and it is a soil isolate; in the course of this work, it was found that it produces a branched polysaccharide characterised by a hexasaccharidic repeating unit (Fig. 4) built up of one fucosamine residue joined to a rhamnose tetrasaccharide, in which the first one is branched with a terminal glucosamine unit.

The structure of this polysaccharide and the kind of monosaccharides employed in its construction confers to the bacterial membranes a rather neutral if not lipophilic character, a feature shared from the capsule or the lipopolysaccharides of many other bacteria living in the same habitat, like most of the members of the *Rhizobiaceae* family,[9] or other genera as *Azospirillum*,[10] *Xanthomonas* or the phytopathogenic *Pseudomonas* and *Burkholderia* species.[11]

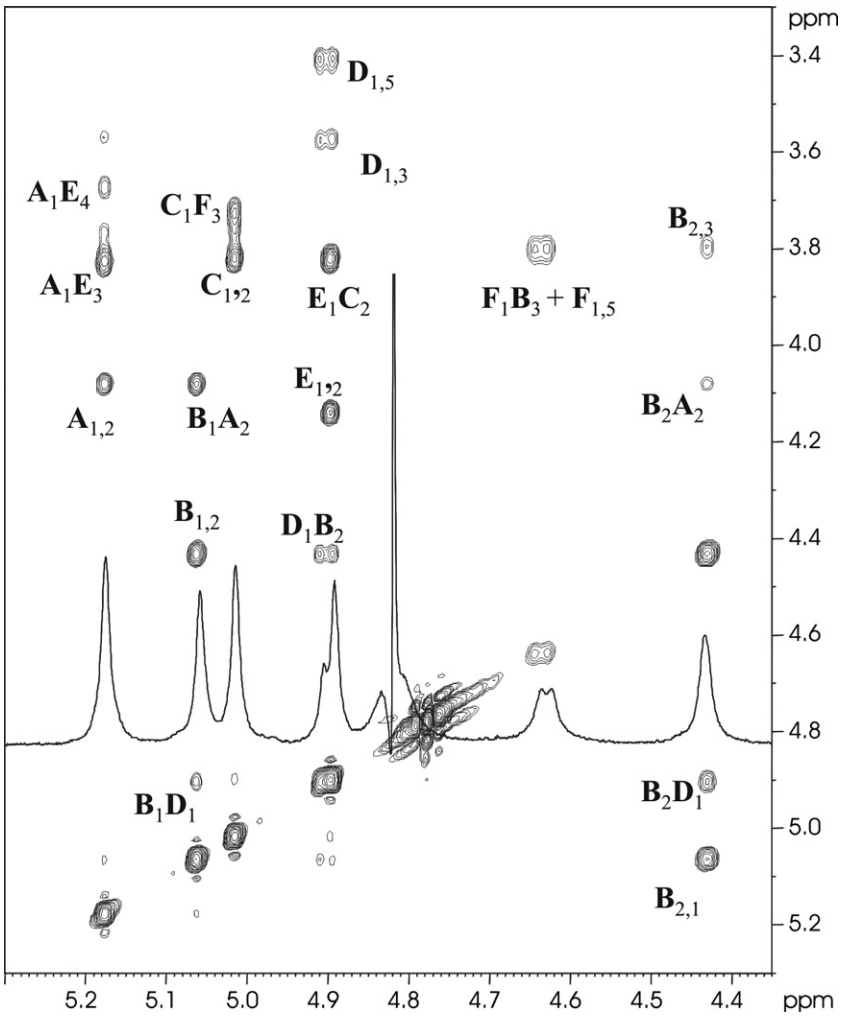

Figure 3. (D_2O, 500 MHz, 25 °C) expansion of the anomeric region of **W2** NOESY spectrum. The principal NOE contacts are reported.

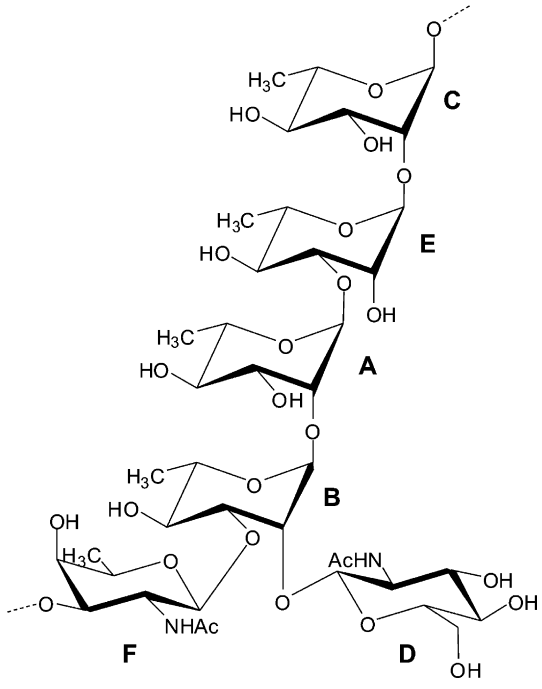

Figure 4. Structure of the branched hexasaccharide repeating unit.

The role of this polysaccharide in the environmental adaptation process of this bacterium is still unknown, and further work is needed to address this point.

3. Experimental

3.1. *Kaistella flava* bacterial cultivation and chemical composition analysis

Bacterial growth,[1] GC–MS analysis of monosaccharide and lipid derivatives were performed as reported.[12]

The absolute configuration of GlcN was inferred analysing its 2-butyl derivatives,[13] whereas that of Rha and FucN was determined by analysis of the 2-octyl derivates according to the procedure of Leontein et al.,[14] L-fucosamine from *Pseudomonas cychorii* O-chain was used as standard.[15]

Methylation analysis was performed according to Sandford and Conrad procedure,[16] the polysaccharide was methylated with iodomethane (CH_3I) in DMSO and anhydrous dimsyl anion, hydrolysed with TFA 2 M (100 °C, 2 h), carbonyl-reduced with $NaBD_4$ (rt, 18 h), and acetylated and analysed by GC–MS.

All GC–MS analyses were performed with an Agilent 5973 instrument, using a SPB-5 capillary column (Supelco, 30 m × 0.25 i.d. flow rate 0.8 ml/min, He as carrier gas), mass spectra were recorded in EI (70 eV) and an ionising current of 0.2 mA. The

temperature programme used was: 150 °C for 5 min, 150→280 °C at 3 °C/min, 300 °C for 5 min.

3.2. Isolation and purification of the polysaccharide component (Scheme 1)

Dried cells (1.6 g) were extracted with 8:5:2 petroleum ether–$CHCl_3$–phenol.[2] The suspension was centrifuged, the solvents were removed from the supernatant by diminished pressure evaporation, the resulting solution was dialysed against distilled water (cut off 12–14 kDa) and freeze-dried yielding to **PCP** fraction (72 mg). The pellet obtained from the previous centrifugation was treated according to the phenol–water method,[3] both water and phenol phases were dialysed against distilled water and freeze-dried, yielding to fractions **W** (100 mg) and **P** (147 mg), respectively.

PCP, **W** and **P** fractions were screened by discontinuous SDS–PAGE[17] using a miniprotean gel system from Bio-Rad and a 15% separating gel, samples were run at constant voltage (150 V) and stained with the silver-staining protocol directly[5] or using Alcian blue as fixative first.[6]

Fraction **W** (80 mg) was digested with RNAse (Sigma, cod. R5503), DNAse (Sigma, cod. DN25) and protease (Sigma, cod. P5147), and purified by SEC on a Sephacryl HR-S500 (Amersham Bioscience) using NH_4HCO_3 50 mM as eluent (60 cm × 1.5 cm, 14 mL/h), the eluate was monitored by an on-line refractive index, the first peak appeared in the void volume of the column and it was found to contain the purified polysaccharide (fraction **W1**, 23 mg), the second, retained, peak contained nucleic acid and protein fragments, and a glucan that was not further studied.

Ten milligrams of **W1** were treated with 1% AcOH (1 mL, 2 h, 100 °C), the precipitate (lipid A) was removed by centrifugation, the supernatant was lyophilised and fractionated by SEC on a Sephacryl HR-S500 column as above, polysaccharide fraction **W2** (7 mg) was recovered in the void volume.

Similar results were obtained using milder hydrolysis conditions, namely sodium acetate 50 mM, pH 4.5, 100 °C, 4 h (fraction **W3**).

3.3. NMR spectroscopy

NMR experiments were carried out at 25 °C with Varian Inova 500 of Consortium INCA (L488/92, Cluster 11) equipped with a z-gradient, inverse probe. Chemical shifts are expressed in δ relative to internal acetone (^1H at 2.225 ppm, ^{13}C at 31.45 ppm). Two-dimensional spectra (DQ-COSY, TOCSY, NOESY, gHSQC and gHMBC) were measured using standard Varian software.

For the homonuclear experiment, 512 FIDs of 2048 complex data points were collected, with 40 scans per FID. The spectral width was set to 10 ppm and the frequency carrier was placed at the residual HOD peak. For the HSQC and HMBC spectra, 256 FIDs of 2048 complex points were acquired with 50 scans per FID, the GARP sequence was used for ^{13}C decoupling during acquisition. Conversion of the Varian data, processing and analysis were performed with Bruker TOPSPIN 1.3 program.

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