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The teichoic acid (C-polysaccharide) synthesized by *Streptococcus pneumoniae* serotype 5 has a specific structure

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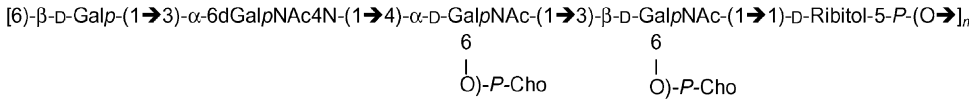
Abstract—The teichoic acid synthesized by *Streptococcus pneumoniae* serotype 5, also known as pneumococcal common antigen (C-polysaccharide), was purified. On the basis of compositional analysis, HPAEC-PAD analysis, MALDI-TOF mass spectrometry and NMR spectroscopy, made on the native polysaccharide and on the dephosphorylated repeating unit, the following structure is proposed:



This C-polysaccharide (C-PS), differs from those previously described by the replacement of Glc by Gal in its repeating unit structure.

Keywords: *Streptococcus pneumoniae*; Serotype 5; Teichoic acid; C-polysaccharide; Pneumococcal capsular polysaccharides; Pneumococcal vaccine

1. Introduction

Streptococcus pneumoniae is a major cause of morbidity and mortality worldwide. Pneumococci account for a large number of diseases such as bacteremia, pneumonia, meningitis and otitis.[1]

Capsular polysaccharides separately purified from the outer capsules of 23 pneumococcal strains, used in various combinations, have proven effective as vaccines against pneumococcal infection in older children and adults.[2] The pneumococcal teichoic acid, an antigen common to all pneumococci also known as C-polysaccharide (C-PS), is a cell wall component composed of repeating tetrasaccharides (Fig. 1) joined by ribitol phosphate diester bonds.[3–6] In addition, C-PS can be mono- or disubstituted with phosphocholine.[7] As C-PS is linked to the cell wall peptidoglycan,[8] capsular polysaccharides used in the preparation of pneumococcal

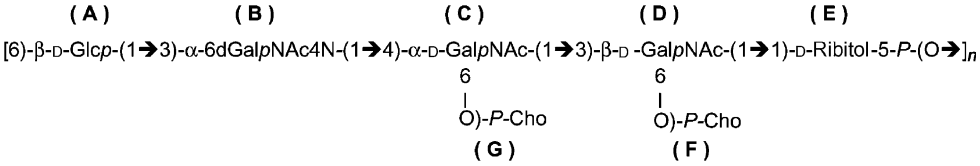


Figure 1. Structure of the previously determined repeating unit of C-PS. C-PS with two phosphocholine residues is represented.

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polysaccharide-based vaccines may contain some amounts of C-PS.

In the present study, we isolated the C-PS synthesized by pneumococcal types 1, 5, 6, 7F and 14. Structural studies on the type 5 C-PS (C-PS5) revealed the presence of Gal in its repeat unit whereas the C-PS associated with other serotypes contained Glc in their repeat units as previously described.[3–7]

2. Results and discussion

2.1. HPAEC-PAD and MALDI-MS analysis of the oligosaccharides generated by HF treatment

Previous studies[9,10] have shown that oligosaccharides are generated after hydrofluoric acid (HF) treatment (48%, 4 °C, 48 h) of C-PS from *S. pneumoniae*. HPAEC-PAD analysis of the HF treated C-PS5, from Aventis Pasteur and from ATCC, revealed a peak that eluted at about 3.5 min (Fig. 2B and C), whereas the C-PS from strain CSR SCS2 produced a peak corresponding to this oligosaccharide eluting at about 4.5 min (Fig. 2A). A peak eluting at 4.5 min was also detected after HF treatment of 22 of the 23 capsular polysaccharides present in the polysaccharide vaccines (data not shown), indicating a different composition for C-PS5.

The oligosaccharides present in the HF hydrolyzates were characterized by MALDI-TOFMS analysis. One quasi-molecular ion at *m/z* 795.3 corresponding to the $[M+Na]^+$ ion of an oligosaccharide composed of one hexose, one 6dGal*p*NAc4N, and two HexNAc residues was detected in all cases (data not shown). As dephosphorylation with 48% HF of C-PS results in cleavage of the labile β-GalNAc bond to ribitol,[9,10] these oligosaccharides contain no Ribitol in their repeat units.

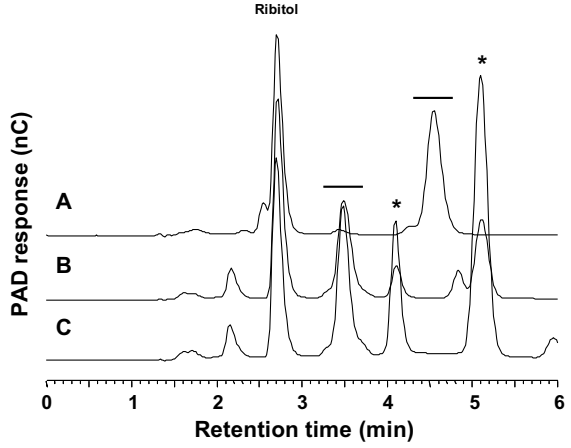


Figure 2. HPAEC-PAD analysis of HF treated C-PS. (A) C-PS from strain CRS SCS2; (B) C-PS associated with pneumococcal type 5 from Aventis Pasteur; (C) C-PS associated with pneumococcal type 5 from ATCC. Fractions indicated by horizontal bars were collected and desalted. The peaks marked with an asterisk are those originating from HF treatment of type 5 capsular polysaccharide.

2.2. Semi-preparative HPAEC-PAD purification of the oligosaccharides generated by HF hydrolysis

The oligosaccharides generated by HF treatment of C-PS were purified using a semi-preparative CarboPac PA-1 column. The fractions (see Fig. 2) corresponding to the generated oligosaccharides were collected, desalted and freeze dried. By compositional analysis, each of these oligosaccharides was shown to contain GalN, but in the C-PS5, Gal was present instead of Glc (data not shown). 6dGal*p*NAc4N, a component of all C-PS, was not detected as it is most likely destroyed under the acid hydrolysis conditions used for the compositional analysis.[5,6,11,12]

2.3. C-PS from pneumococcal type 5 contains Gal instead of Glc in its repeat unit

^{1}H NMR analysis performed on purified oligosaccharides (Fig. 3) confirmed the presence of Gal in C-PS5. Purified C-PS oligosaccharides from strain CSR SCS2 (Fig. 3A) and from C-PS5 (Fig. 3B) were analyzed by ^{1}H NMR spectroscopy. Assignments were made by TOCSY experiments in conjunction with previously published NMR data for C-PS oligosaccharide fragments.[3,4] Signals for α-anomeric protons are observed, in both cases, at δ 4.96 ($J_{1,2}$ 4.4 Hz, residue B), and δ 5.15 ($J_{1,2}$ 3.9 Hz, residue C) and for a β-anomeric proton at δ 4.60 ($J_{1,2}$ 8.1 Hz, residue A). D H-1 and B H-5 overlap at δ ~4.60. The main difference observed was for the hexosyl residue. For C-PS oligosaccharides from strain CSR SCS2 (Fig. 3A), Glc can be distinguished by the presence of its H-2 at δ 3.30 ($J_{2,3}$ 8.3 Hz, residue A), and by the $^3J_{H,H}$ values of ~9 Hz for its H-3, H-4, and H-5 protons (δ 3.48, 3.40, 3.44, respectively). These data confirmed the structure of C-PS oligosaccharides from strain CSR SCS2 to be β-D-Glc*p*-(1→3)-α-6dGal*p*NAc4N-(1→4)-α-D-Gal*p*NAc-(1→3)-β-D-GalNAc in agreement with previous results.[3–6] For C-PS5 (Fig. 3B), and in conjunction with HPAEC-PAD compositional analysis, residue A was assigned to β-Gal based on its H-2 at δ 3.53 ($J_{2,3}$ 8.9 Hz) and by the $J_{3,4}$ value of 3.6 Hz.

Oligosaccharides of C-PS from strain CSR SCS2 (Fig. 4A) and C-PS5 (Fig. 4B) were treated with β-galactosidase and then subsequent MALDI-TOFMS analysis performed on the enzyme hydrolyzates.

A quasi-molecular ion at *m/z* 795.3 was observed for the C-PS from strain CSR SCS2 (Fig. 4A) while a quasi-molecular ion at *m/z* 633.3 was observed for C-PS5 indicating the loss of a β-Gal residue from the C-PS5 oligosaccharide (Fig. 4B). This demonstrates the presence of a nonreducing terminal β-(1→3 or 6)-linked Gal on the C-PS5 oligosaccharide.

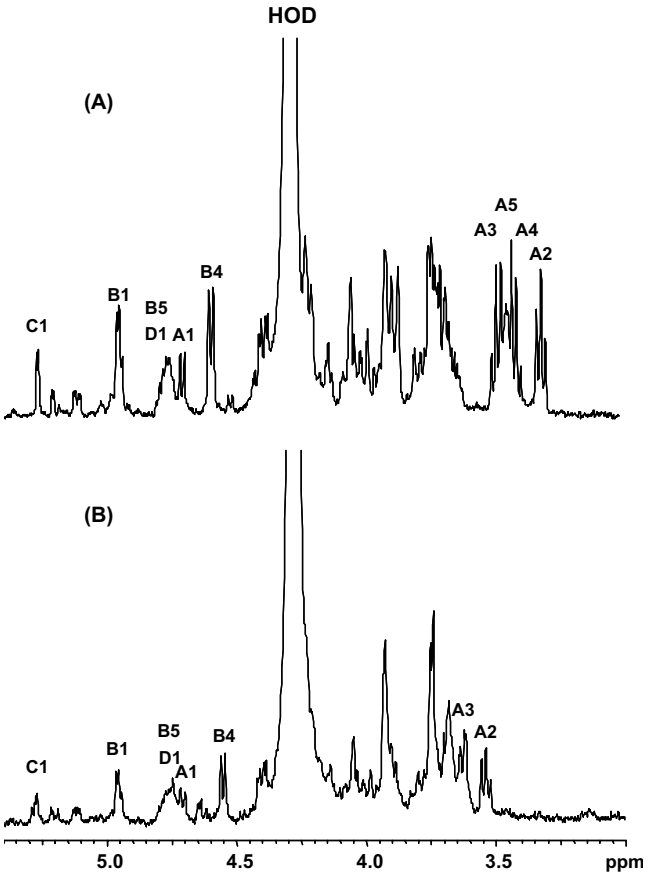


Figure 3. ^{1}H NMR spectrum of the HPAEC purified oligosaccharides generated by HF treatment of (A) C-PS from strain CSR SCS2; (B) C-PS5. Nonassigned signals of the products generated by the base-catalyzed degradation of reducing glycoses can be detected in the anomeric region.

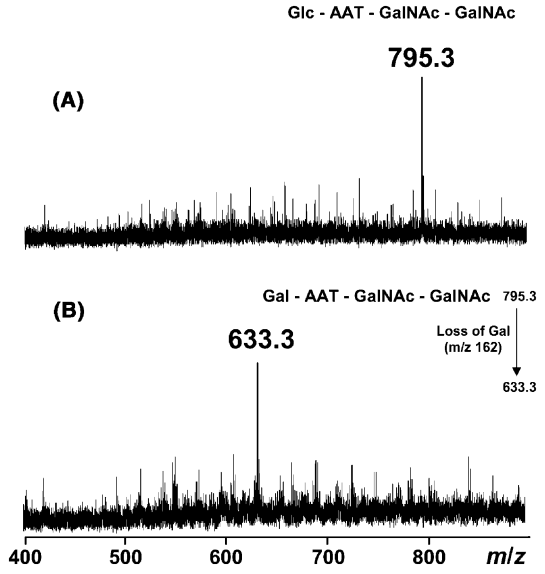


Figure 4. MALDI-TOF mass spectra of the products obtained after β-galactosidase hydrolysis of the oligosaccharides generated by HF treatment of (A) C-PS from strain CSR SCS2; and (B) C-PS5.

2.4. Structure of the purified C-PS5

Structure determination of C-PS5 was carried out on purified native C-PS5 (see Section 3 for details). The assignment of proton signals of this C-PS5 was carried out using 2D COSY and TOCSY experiments (Table 1). The sequence of the sugars has been established by 2D NOESY experiment, which showed the inter residue correlations between the *trans*-glycosidic protons: β-Gal H-1 (residue A), α-6dGal*p*NAc4N H-3 (residue B) at δ 4.60/4.40; α-6dGal*p*NAc4N H-1 (residue B), α-GalNAc H-4 (residue C) at δ 4.96/4.11; α-GalNAc H-1 (residue C), β-GalNAc H-3 (residue D) at δ 5.15/3.85; and β-GalNAc H-1 (residue D), ribitol H-1 (residue E) at δ 4.61/3.96. All these data revealed the sequence of residues in the C-PS5 to be β-D-Gal*p*-(1→3)-α-6dGal*p*NAc4N-(1→4)-α-D-Gal*p*NAc-(1→3)-β-D-GalNAc-(1→1)-Ribitol.

The assignment of carbon signals was carried out using ^{1}H–^{13}C HSQC experiment (Table 2).

The ^{31}P NMR spectrum showed the presence of three resonances at δ −0.79, 0.30 and 1.06 for the C-PS5 from Aventis Pasteur and from ATCC (Fig. 5C and D). This corresponds to the phosphate groups from the two *P*-Cho substituents and from the *P*-Ribitol residue, respectively. The change in the ^{31}P chemical shift of the *P*-Ribitol residue from δ 1.06 to δ 1.30 (δ of *P*-Ribitol in Glc containing C-PS) was observed irrespective of the degree of *P*-Cho substitution (either mono-substituted (Fig. 5A) or disubstituted (Fig. 5B)). ^{31}P NMR analysis performed on purified C-PS from type 1, 6B, 7F and 14 revealed that these Glc containing C-PS′ are also

Table 1. ^{1}H NMR data (δ ppm) of C-PS5

Sugar residue	H-1	H-2	H-3	H-4	H-5	H-6
→6)-β-Gal-(1→ (A)	4.60	3.54	3.67	4.28	3.98	3.99
→3)-α-6dGal*p*NAc4N-(1→ (B)	4.96	4.22	4.40	3.97	4.78	1.24
→4)-α-GalNAc-(1→ (C)	5.15	4.31	3.92	4.10	4.02	4.02
→3)-β-GalNAc-(1→ (D)	4.61	4.10	3.85	4.17	4.05	4.05
→1)-Ribitol-5-*P*-(O→ (E)	3.96	3.77[a]	3.90[a]	ND	3.96, 4.05	
Cho-*P*-(O→ (F)	4.33	3.68	3.23			
Cho-*P*-(O→ (G)	4.28	3.67	3.23			

ND, not determined.
[a] Assignment could be interchanged.

Table 2. ^{13}C NMR data (δ ppm) of C-PS5

Sugar residue	C-1	C-2	C-3	C-4	C-5	C-6
→6)-β-Gal-(1→ (A)	104.8	71	72.9	68.0	70.9	68.6
→3)-α-6dGal*p*NAc4N-(1→ (B)	98.6	48.8	75.2	55.2	63.6	15.9
→4)-α-GalNAc-(1→ (C)	93.8	49.9	67.2	77.1	70.9	64.8
→3)-β-GalNAc-(1→ (D)	101.7	51.1	75.1	63.7	71.0	64.9
→1)-Ribitol-5-*P*-(O→ (E)	70.9	72.1[a]	71.0[a]	ND	67.0	
Cho-*P*-(O→ (F)	59.9	66.5	54.5			
Cho-*P*-(O→ (G)	59.8	66.5	54.5			

ND, not determined.
[a] Assignment could be interchanged.

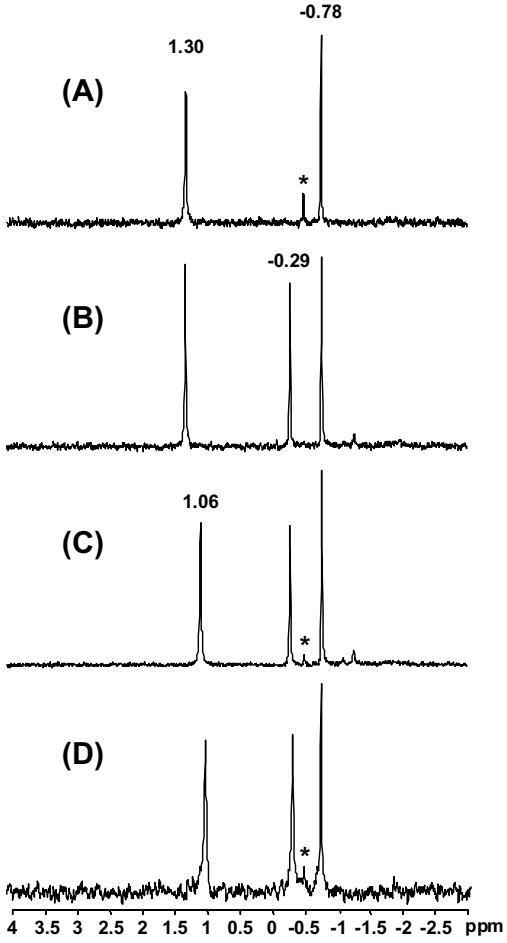


Figure 5. ^{31}P NMR spectrum of (A) C-PS from strain CSR SCS2; (B) C-PS from type 14; (C) C-PS5 from Aventis Pasteur; and (D) C-PS5 from ATCC. The peak marked with an asterisk is originating from terminal repeat units.[16]

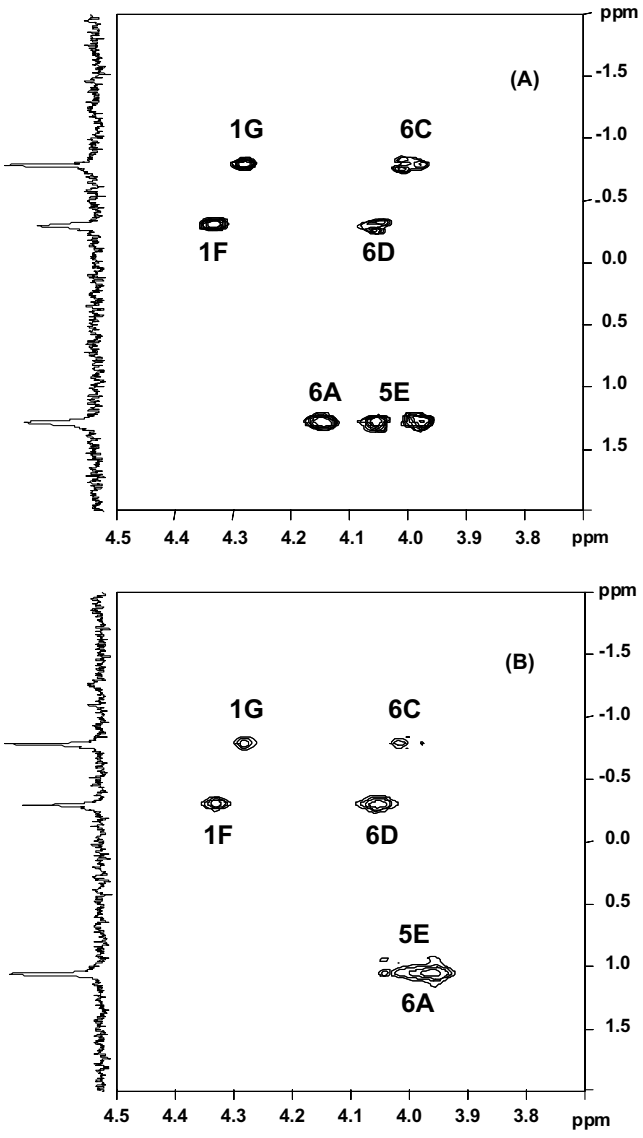


Figure 6. The ^{1}H–^{31}P HMQC spectrum of (A) C-PS from strains 14; and (B) C-PS5.

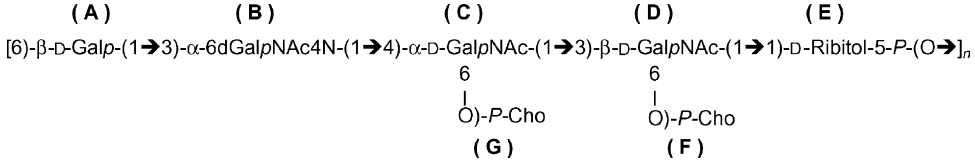
disubstituted with *P*-Cho (Fig. 5B for C-PS from type 14 and data not shown for C-PS from others types).

For all C-PS′ disubstituted with *P*-Cho, the ^{1}H–^{31}P HMQC analysis, consistent with previous assignments,[3,4,7] showed cross-peaks for the ^{31}P signals of the *P*-Cho at δ −0.79 (residue G) with α-GalNAc H-6 (residue C) at δ 4.03 and with Cho H-1 at δ 4.29; and of the *P*-Cho at δ −0.30 (residue F) with β-GalNAc H-6 (residue D) at δ 4.06 and with Cho H-1 at δ 4.34 (Fig. 6A and B).

The ^{31}P signal of the *P*-Ribitol at δ 1.30 from the Glc containing C-PS (Fig. 6A) showed cross-peaks with β-Glc H-6 (residue A) at δ 4.16 and with Ribitol (residue E) H-5a, H-5b at δ 3.99 and 4.06, respectively.

However, in conjunction with the proton assignment for the C-PS5, the ^{31}P signal of the *P*-Ribitol at δ 1.06 (Fig. 6B) showed cross-peaks with β-Gal H-6 (residue A) at δ 3.99 and with Ribitol (residue E) H-5a, H-5b at δ 3.96 and 4.05, respectively.

Therefore, on the basis of the data obtained, it is concluded that the C-PS synthesized by *S. pneumoniae* serotype 5 has the following structure and is different from previously described C-PS's synthesized by the 22 other pneumococcal vaccine strains, by the replacement of Glc by Gal in its repeat unit structure:



3. Experimental

3.1. Materials

Purified pneumococcal capsular polysaccharides and polysaccharide crude extracts were obtained from Aventis Pasteur Manufacturing Division, Marcy l'Etoile, France. Another purified type 5 pneumococcal capsular polysaccharide was obtained from American Type Culture Collection (ATCC, Manassas, VA). A purified C-polysaccharide, prepared from the noncapsulated pneumococcal strain CSR SCS2 clone was obtained from Statens Serum Institut (Copenhagen, Denmark).

Recombinant β-galactosidase from *Xanthomonas manihotis* (GALase II) was purchased from Glyko, Oxfordshire, UK.

3.2. Preparation of the C-polysaccharides

The type 5 C-PS was purified from pneumococcal type 5 polysaccharide crude extracts by a gel filtration chromatography on a Sepharose CL-4B column. Fractions were collected, assayed for phosphate[13] and uronic acid.[14] Phosphate-containing fractions were pooled and lyophilized.

Purification of the C-PS from pneumococcal types 1, 6B, 7F and 14 was achieved by anion-exchange chromatography on Q-sepharose. The purified capsular polysaccharides of these four types were dissolved in 25 mM Tris solution (pH 8.3) and the elution was performed using the same buffer and a 0–0.3 M NaCl gradient. Fractions were collected and phosphorus containing fractions were pooled, dialyzed and lyophilized.

3.3. Sugar analysis

The C-PS were hydrolyzed with 2 M trifluoroacetic acid for 2 h at 120 °C. The sugars were identified by HPAEC-PAD.[12,15]

3.4. HF treatment

The C-PS were treated with aq HF (48% by mass) in a concentration of 5 g/mL at 4 °C for 48 h in a polypropylene microfuge tube. HF was removed by drying under a stream of nitrogen.

3.5. High-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD)

HPAEC-PAD was performed with a Dionex DX-500 system with a PA-1 analytical (4 × 250 mm) column (Dionex Corp, Sunnyvale, CA). Analyses of the oligosaccharides generated by HF treatment were carried out by isocratic elution at a flow rate of 1 mL/min with 75 mM NaOH as the eluent. Automatic sample injection was accomplished using a spectra system AS3500 autosampler (Spectra-Physics, San Jose, CA). Components in the eluent were detected by PAD using a gold electrode and an Ag/AgCl reference electrode. The quadruple potential waveform was applied. Data were collected using the DIONEX PEAKNET software.

Purification of these oligosaccharides generated by HF was done in a similar way on a semi-preparative CarboPac PA-1 column (9 × 250 mm) at a flow rate of 2 mL/min. Fractions were collected manually (see Fig. 2), desalted (chromatography on an AG 50W-X8 column), and then freeze dried.

3.6. β-Galactosidase treatment of purified oligosaccharides

Oligosaccharides (1 μg) generated by HF treatment of C-PS were dissolved in 20 μL of 50 mM sodium phosphate buffer, pH 5.0, containing 10 mU of β-galactosidase and incubated at 37 °C overnight. Resulting oligosaccharides were analyzed by MALDI-TOFMS.

3.7. Mass spectrometry

MALDI-TOFMS was performed with a Bruker Biflex III apparatus (Bruker Daltonics, Wissembourg, France) in the positive ion mode using 2,5-dihydroxybenzoic acid matrix. Solutions of oligosaccharides (10–50 μg/mL) were diluted 1:1 (v/v) with the matrix solution, and 1 μL of the resulting solution was deposited onto a stainless target, and allowed to dry at room temperature. Positive ion mass spectra were acquired in the reflectron mode with pulse ion extraction. Mass assignments were based on an external calibration of the instrument.

3.8. NMR spectroscopy

NMR spectra were obtained on a Bruker Avance 500 spectrometer running XWIN-NMR 2.6 software and using 5 and 3 mm NMR probes. Spectra were measured for solutions in D_2O at pD 7.4 using phosphoric acid (2%) as an external standard for ^{31}P NMR (δ 0.00 ppm), DSS as an external standard for ^{1}H NMR (δ 0.00 ppm) and acetone for ^{13}C NMR (δ 30.02 ppm). One-dimensional TOCSY, two-dimensional homonuclear DFQ-COSY, TOCSY and NOESY experiments and two-dimensional heteronuclear HMQC (^{1}H–^{31}P and ^{1}H–^{13}C) were performed using standard Bruker pulse programs. NMR analysis on oligosaccharides and native C-PS were recorded at 313 and 303 K, respectively. TOCSY and NOESY experiments were performed in the phase-sensitive mode using mixing times of 120 and 350 ms, respectively.

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