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Carbohydrate Research 305 (1998) 65–72

CARBOHYDRATE RESEARCH

Structure of the capsular polysaccharide of *Clostridium perfringens* Hobbs 10 determined by NMR spectroscopy

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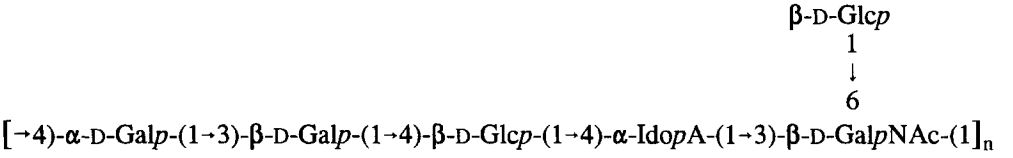
Received 9 July 1997; accepted 4 August 1997

Abstract

The complete primary structure of the type-specific capsular polysaccharide of *Clostridium perfringens* Hobbs 10 was determined. The polysaccharide was isolated from *C. perfringens* Hobbs 10 by cold-water extraction of whole, heavily encapsulated cells. The polysaccharide was purified, by ethanol precipitation, deproteination, selective precipitation with hexadecyltrimethylammonium bromide, ion-exchange chromatography and gel-filtration chromatography. The polysaccharide was comprised of D-glucose, D-galactose, *N*-acetylgalactosamine, and iduronic acid, in molar ratios of 2:2:1:1. Sequence and linkage assignments of the glycosyl residues were obtained by NMR spectroscopy, specifically by the combination of two-dimensional homonuclear DQF-COSY, TQF-COSY and TOCSY, heteronuclear {^{1}H,^{13}C} single-quantum coherence (HSQC) and heteronuclear multiple-bond correlation (HMBC) experiments. The capsular polysaccharide of *C. perfringens* Hobbs 10 is a polymer composed of a hexasaccharide repeating unit with the following structure:



This structure is novel among bacterial cell-surface polysaccharides, and it is only the second of many serotypically distinct capsular polysaccharides of *C. perfringens* to be described.
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Keywords: *Clostridium perfringens*; 2D NMR spectroscopy; DQF-COSY; TQF-COSY; TOCSY; HSQC; HMBC; Polysaccharide structure; Antigen

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PII S0008-6215(97)00280-2

1. Introduction

Clostridium perfringens is an histotoxic, anaerobic, spore-forming bacterium that is ubiquitously distributed in nature [1]. The bacterium is a known cause of human diseases such as clostridial myonecrosis (gas gangrene), food poisoning, and necrotizing enterocolitis in infants, and of animal diseases such as lamb dysentery, ovine enterotoxemia, and pulpy kidney disease in sheep [2]. *C. perfringens* is divided into five types (A through F) based on the production of extracellular toxins [3]. Most strains possess serologically and chemically distinct capsular polysaccharides that are the basis for subdividing *C. perfringens* by serotype [4,5].

The capsule is antiphagocytic, and it may be an essential virulence factor during disease [6,7]; but it is the toxins that are responsible for the severity of disease [8]. Although several hundred serological types of *C. perfringens* are known to exist, the capsular polysaccharides from only a few serotypes have been isolated, purified and their sugar compositions determined [9–12]. Only the primary structure of the serotype specific polysaccharide of *C. perfringens* Hobbs 5 has been determined to date [13].

Certain strains of *C. perfringens* that are associated with food-poisoning produced heat-resistant spores have been characterized by Arabic numerals and are designated as Hobbs serotypes [14,15]. The immunochemistry of the *C. perfringens* capsule has not attracted much attention in recent years in spite of its obvious antigenic significance. In view of this we decided to isolate capsular polysaccharide from *C. perfringens* serotype Hobbs 10 and determine its primary structure.

Chemical methods that were used initially to elucidate the molar composition of the *C. perfringens* Hobbs 10 polysaccharide showed glucose (Glc), galactose (Gal), *N*-acetylgalactosamine (GalNAc), and iduronic acid (IdoA) in the ratio of 2:2.5:1:0.5 [11]. No further information regarding the primary structure of this polysaccharide was available when we decided to explore the applicability of multidimensional NMR spectroscopy for its complete characterization.

Sequence and linkage assignments of the glycosyl residues were obtained by NMR spectroscopy, specifically by the combination of two-dimensional homonuclear DQF-COSY [16], TQF-COSY [17,18], and TOCSY [19], heteronuclear $\{^{1}H,^{13}C\}$ single-quantum coherence (HSQC) [20,21] and heteronuclear multiple-bond correlation (HMBC) [22,23] experiments.

2. Experimental

Isolation of the capsular polysaccharide of C.perfringens *Hobbs 10.*—The isolation and purification of the capsular type-specific polysaccharide of *C. perfringens Hobbs* 10 was described previously [11]. A portion (35 mg in 10 mL of deionized water) was treated by ultrasonic irradiation for 2 h. The sample was centrifuged (15,000 × g), dialyzed against deionized water, and finally recovered by lyophilization. This fraction of the Hobbs 10 polysaccharide was used for all subsequent NMR analyses. The apparent mol. wt. of the polysaccharide was determined by gel-filtration chromatography on a Sepharose CL-6B (Pharmacia Fine Chemicals) column (90 × 1.5 cm). The column was equilibrated with 0.01 M 2-amino-2-hydroxymethyl-1,3-propandiol (Tris)–0.1 M NaCl buffer (pH 7.2), and then it was calibrated with polysaccharide mol. wt. standards (Polymer Laboratories). The presence of carbohydrate was detected by the phenol–sulfuric acid method [24]. The polysaccharide (6.1 mg) in 1.0 mL of Tris buffer was applied to the column. The column was eluted at a flow rate of 26 mL h^{-1}. The polysaccharide eluted as a single polydisperse peak with an apparent mol. wt. of 98 kDa.

Absolute configuration.—The absolute configuration of the glycoses was determined by GLC-MS of the per-*O*-acetylated (+)-(*S*)-2-butyl glycosides [25,26]. Glc, Gal, and GalNAc had the D-configuration. In a previous study Glc and Gal were reactive with glucose oxidase and galactose oxidase [11]. We were not able to determine the absolute configuration of IdoA due to an insufficient amount of material available.

NMR analysis.—A portion of the Hobbs 10 polysaccharide (~ 20 mg) was repeatedly exchanged with deuterium oxide with intermediate lyophilization. The polysaccharide was dissolved in 0.7 mL deuterium oxide (99.96% D; Cambridge Isotope Laboratories) and transferred into a 5-mm NMR tube (Wilmad 535-PP). All NMR experiments were performed at 60 °C, except for the HMBC studies, which were done at 80 °C to improve the line shape of the spectra due to the reduction in viscosity. The 2D DQF-COSY and TOCSY experiments were done using a Varian UnityPlus 500 spectrometer equipped with a 5-mm

$^{1}H/^{19}F$ probe. The gradient-enhanced TQF-COSY, HSQC, and HMBC were performed on a Varian UnityPlus 600 spectrometer equipped with a gradient {^{1}H,X} probe. The proton spectral width was 3000 Hz for all experiments, and the carbon spectral width was 11,000 Hz. Typically, $2 \times 400 \times 2048$ data points were recorded for $^{1}H-^{1}H$, and $2 \times 512 \times 1024$ and $2 \times 256 \times 2048$ data points were recorded for the GHSQC experiment and for the GHMBC experiment, respectively. Quadrature detection in the t_1 dimension was achieved by the method of States et al. [27] in the DQF-COSY, TOCSY, and in the GHMBC experiments. The GTQF-COSY experiment was recorded in magnitude mode. *N*- and *P*-type spectra were recombined to give an absorption mode HSQC spectrum. The mixing time for the TOCSY was 80 ms (MLEV-17; flanked by two 2 ms trim pluses). The three gradient strengths used in the GTQF-COSY experiment were 8 G/cm with duration of 2.5 ms. The two gradient strengths used in the GHSQC experiment were 20 G/cm with duration times of 2 ms and 0.5 ms. Heteronuclear broad-band decoupling was achieved by GARP [28] pulse sequence. The delay for long-range couplings to evolve was set to 40 ms in the GHMBC experiment. The three gradient strengths were 16, 12, and −20 G/cm with duration of 1.5 ms. The data were processed off-line using the FELIX software package, version 95. Typically, a Lorentzian-to-Gaussian window function (lb = −0.5 and gb = 0.05) was applied in the t_2 dimension and a squared cosine-bell in the t_1 domain with multiplication of the first data point by 0.5. Zero-filling was applied in both dimensions. Chemical shifts were referenced to sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) at 0.0 ppm for ^{1}H and ^{13}C.

3. Results

NMR assignment of the ^{1}H *and* ^{13}C chemical shifts.—Chemical analysis of the Hobbs 10 polysaccharide showed that the polysaccharide was comprised of Glc, Gal, GalNac, and IdoA in the molar ratios of 2:2.5:1:0.5 [11]. The 1D ^{1}H NMR spectrum (Fig. 1) of the Hobbs 10 polysaccharide showed seven resonances of equal intensity in the anomeric region. This suggested that the polysaccharide may be composed of a heptasaccharide repeating unit. The seven resonances in the anomeric region were labeled A–G from left to right. The pair of signals labeled D and E, and F and G were not completely resolved.

A 2D TOCSY experiment was performed to reveal

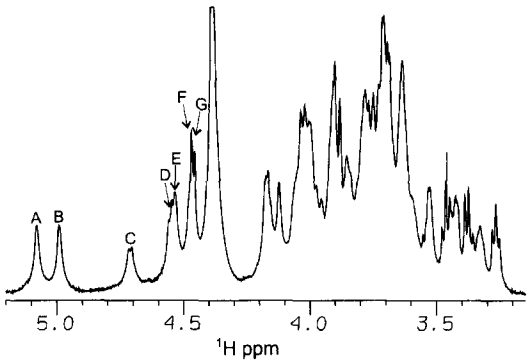


Fig. 1. One-dimensional ^{1}H NMR spectrum of the *C. perfringens* Hobbs 10 polysaccharide recorded at 600 MHz and 60 °C.

the ^{1}H coupling networks for each glycosyl residue. The traces for residues A, C, and F (Fig. 2b) displayed the characteristics of Hartman–Hahn transfer for galactopyranosyl (Gal*p*) residues. The traces for residues D and G showed the characteristics of glucopyranosyl (Glc*p*) residues. The very high intensity (long T_2) of the peaks for G suggested that this residue is more flexible than the rest of the polysaccharide molecule. This indicated that residue G was at a branch point in the polysaccharide. Residue B was assigned as IdoA since it showed Hartman–Hahn transfer from H-1 to H-5. The TOCSY peaks for B and E were not independent and, therefore, must be due to the same residue. The signal at 4.53 ppm noted as E in Fig. 1 is actually due to B-5, a non-anomeric signal of IdoA. At this point, we concluded that the polysaccharide was composed of a hexasaccharide repeating unit.

The assignment of individual protons for each monosaccharide residue was based on the 2D DQF-COSY spectrum (Fig. 2a) and on the 2D TOCSY spectrum (Fig. 2b). The anomeric protons identified in Fig. 1 provided a convenient starting point for tracing the spin network for the monosaccharide residues. For example, starting with H-1 of residue A at 5.08 ppm in the DQF-COSY spectrum (Fig. 2a) a cross peak at 5.08/3.72 ppm was observed; thus, the chemical shift of H-2 of residue A was 3.72 ppm. The chemical shift for H-3 of residue A was traced through its connectivity to H-2. The H-3/H-4 cross peak was not observed in the 2D DQF-COSY spectrum. However, since H-1, H-2, and H-3 of residue A were assigned, the remaining signal at 4.18 ppm in the 2D TOCSY spectrum was assigned to H-4 (Fig. 2b). The same procedure was used to assign the H-1 through H-4 in residues A, C, and F, H-1 through

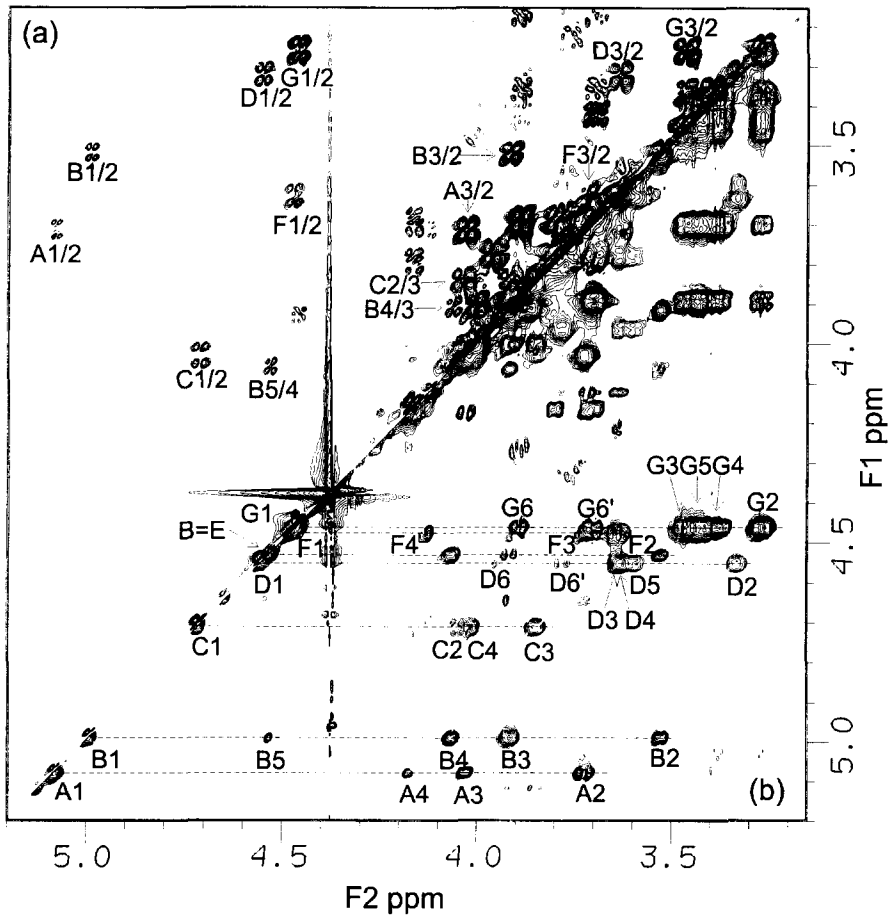


Fig. 2. (a) Two-dimensional DQF-COSY (above diagonal) and (b) two-dimensional TOCSY (below diagonal) spectra of the *C. perfringens* Hobbs 10 polysaccharide recorded at 500 MHz and 60 °C. The assignment for all the residues are marked.

H-5 in residue B, and H-1–H-6/H-6′ in residues D and G.

We were unable to be assign the H-5/H-6/H-6′ resonances of residues A, C, and F due to the small $^{3}J_{H4H5}$ (< 1.5 Hz) coupling constant of residues with the Gal*p* configuration and/or the extensive resonance overlap in the non-anomeric region using the same strategy. A TQF-COSY experiment was used to reveal the H-5/H-6/H-6′ connectivities for these residues. Four sets of H-5/H-6/H-6′ peaks were observed in the TQF-COSY spectrum (Fig. 3). The ^{1}H chemical shifts at 3.89, 3.70, and 3.42 ppm were due to H-6, H-6′, and H-5 of residue G (Glc*p*) as assigned previously. The remaining 3 sets in Fig. 3 belong to residues A, C, and F (Gal*p* configuration). The assignment of the three peaks in each set of H-5/H-6/H-6′ to H-5, H-6, and H-6′ was achieved by comparing the TQF-COSY spectrum with the HSQC spectrum as discussed later in this article.

We assigned many of the cross peaks in the HSQC spectrum starting with the unequivocal ^{1}H assignments (Fig. 4). The H-2 of residue C showed a cross peak at 54.01 ppm in the ^{13}C dimension typical of a 2-amino-2-deoxyhexose. Therefore, residue C was assigned as GalNAc and residues A and F were assigned as Gal. The assignment of most ^{13}C resonances was straightforward. The only ambiguities were the assignments of H-2/C-2 of residue F, H-3/C-3 and H-4/C-4 of residue D which were degenerate in the proton dimension (Fig. 4). These ambiguities were resolved by an HMBC experiment. The cross peak at 3.63/72.06 ppm in the HSQC spectrum (Fig. 4) was assigned to H-2/C-2 of residue F since multibond correlation between H-4 and C-2 (4.12/72.06) and H-3 and C-2 (3.71/72.06) was observed in the HMBC spectrum (Fig. 5). The cross peak at 3.96/81.30 ppm (H-6 of residue D) in the HMBC spectrum suggested that the cross peak at 3.63/81.30 ppm in the HSQC spectrum (Fig. 4) was due to H-4/C-4 of residue D; $^{3}J_{C-4,H-6}$ would give a cross peak whereas $^{4}J_{C-3,H-6}$ is too small to give a cross peak. The remaining peak at 3.63/76.57 ppm

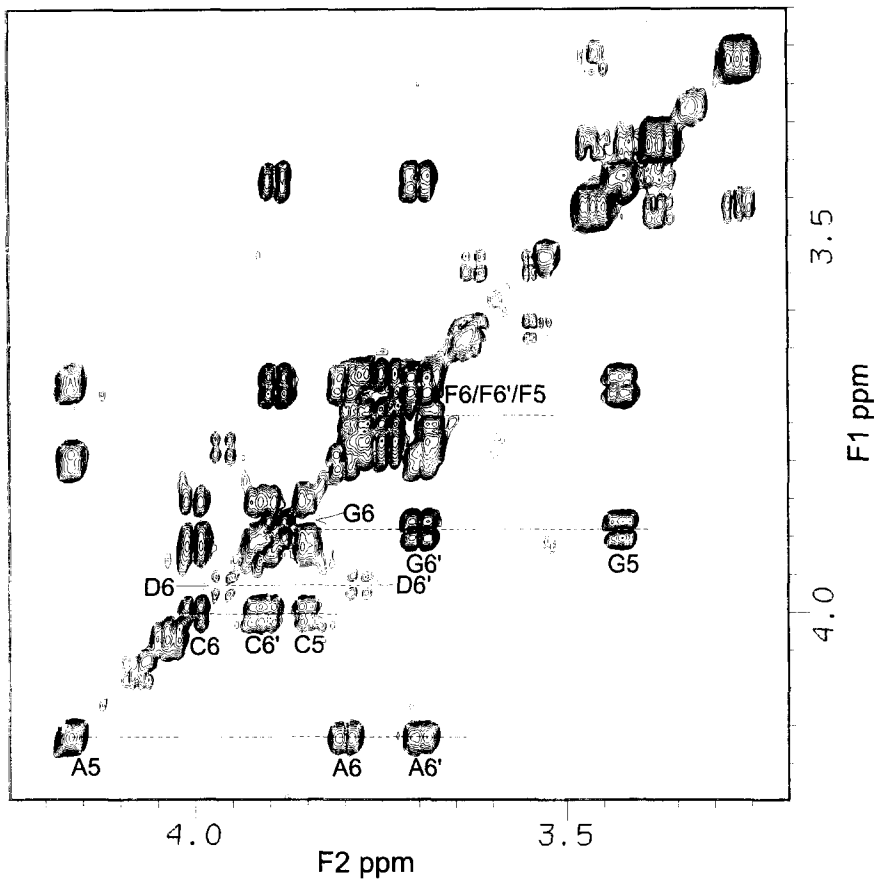


Fig. 3. Expansion of the two-dimensional TQF-COSY spectrum of the *C. perfringens* Hobbs 10 polysaccharide recorded at 600 MHz and 60 °C. The dashed horizontal lines show the H-5/H-6/H-6′ connectivities.

in the HSQC spectrum (Fig. 4) was assigned to H-3/C-3 of residue D.

In the typical C-6 region (60–65 ppm) in the HSQC spectrum, 4 pairs of cross peaks were observed. The other pair was located at 71.84 ppm. This suggested the presence of a (1 → 6) linkage in the polysaccharide. By comparing the chemical shifts of H-6/H-6′ obtained in the HSQC spectrum with the chemical shifts obtained in the TQF-COSY spectrum, we were able to identify the H-5s in residues A, C, and F that were not previously assigned. For example, there was a pair of cross peaks located at 3.80/63.10 ppm (H-6/C-6) and 3.70/63.10 ppm (H-6′/C-6) in the HSQC spectrum, and there were three peaks located at 4.16, 3.80 and 3.70 ppm in the TQF-COSY spectrum; thus, the peak at 4.16 was assigned to H-5. Based on the same reasoning, the peaks at 3.85 ppm in the 3.85/4.00/3.91 set, and 3.68 ppm in the 3.68/3.78/3.74 set were identified as H-5s.

At this stage, the only remaining ambiguity was in the correlation of H-5/H-6/H-6′ for residues A, C, and F. This could be done by tracing $^{2}J_{H-5,C-4}$ and/or $^{3}J_{C-4,H-6}$ couplings provided by the HMBC spectrum. The cross peaks at 4.16 (H-5)/79.58 (C-4 of A) ppm, 3.80 (H-6)/79.58 ppm, and 3.70 (H-6′)/79.58 ppm indicated that the set of H-5/H-6/H-6′ with chemical shifts of 4.16, 3.80, and 3.70 ppm belong to residue A. The observation of a cross peak at 3.78 (H-6)/67.54 (C-4 of F) shows that the set of H-5/H-6/H-6′ with chemical shifts of 3.68, 3.78, and 3.74 ppm belong to residue F. The remaining H-5/H-6/H-6′ set at 3.85, 4.00, and 3.91 ppm must belong to residue C. This completed the assignment of the ^{1}H and ^{13}C spectra of the polysaccharide. Table 1 contains the ^{1}H and ^{13}C chemical shifts for the capsular polysaccharide from *C. perfringens* Hobbs 10.

Configurations of the glycosidic linkages.—The values of the $^{1}J_{CH}$ coupling constants for the anomeric resonances were used to assign the configuration of each anomeric center. Values of ~ 160 Hz were associated with β-pyranosyl linkages, whereas values of ~ 170 Hz were associated with α-pyranosyl linkages [29]. The HMBC spectrum provided these $^{1}J_{CH}$ couplings. The value of 173 Hz for Gal (A), and

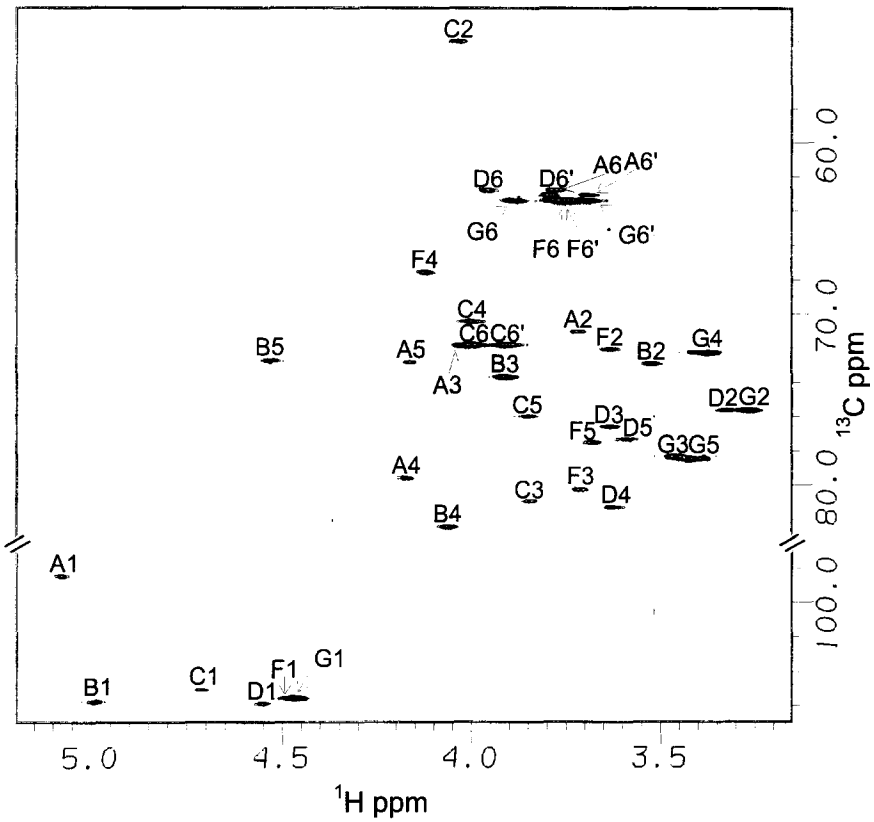


Fig. 4. HSQC spectrum of the *C. perfringens* Hobbs 10 polysaccharide recorded at 600 MHz and 60 °C. The resonances for all the residues are marked.

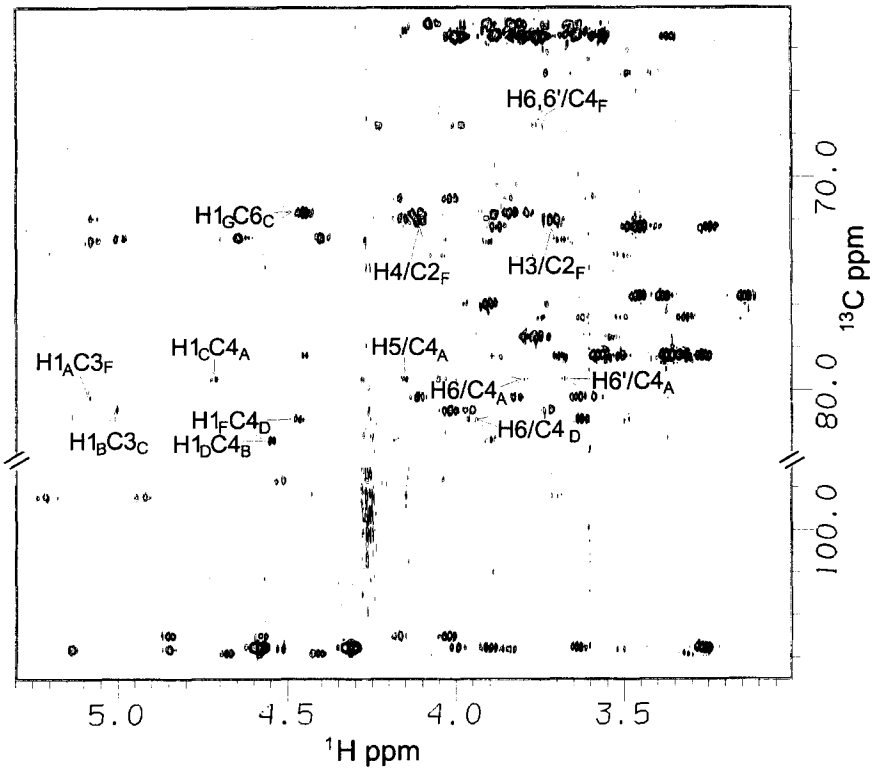


Fig. 5. {^{1}H,^{13}C} multiple-bond correlation (HMBC) spectrum of the *C. perfringens* Hobbs 10 polysaccharide recorded at 600 MHz and 80 °C. The cross peaks used for sequencing the polysaccharide and for correlating the H-1 through H-4 resonances to H-5/H-6/H-6′ resonances for Gal*p* residues are marked.

Table 1
^{1}H and ^{13}C NMR chemical shifts (ppm) of the *C. perfringens* Hobbs 10 capsular polysaccharide measured at 60 °C

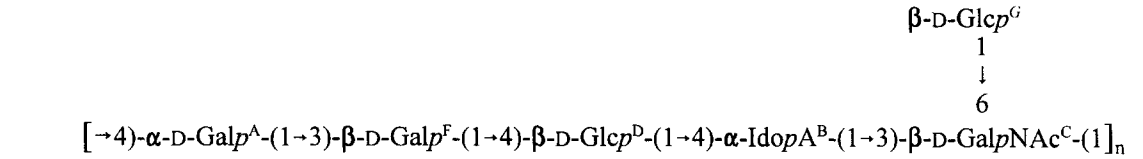
	Gal^{A}	$IdoA^{B}$	GalNac	Glc^{D}	Gal^{F}	Glc^{G}
H-1	5.08	4.99	4.71	4.55	4.48	4.46
H-2	3.72	3.52	4.03	3.33	3.63	3.27
H-3	4.03	3.91	3.85	3.63	3.72	3.46
H-4	4.18	4.06	4.00	3.63	4.12	3.37
H-5	4.16	4.53	3.85	3.59	3.68	3.42
H-6	3.80	–	4.00	3.96	3.78	3.89
H-6′	3.70	–	3.91	3.78	3.74	3.70
C-1	98.42	105.79	105.07	105.86	105.57	105.57
C-2	71.05	72.92	54.01	75.64	72.06	75.64
C-3	71.84	73.70	80.94	76.57	80.22	78.36
C-4	79.58	82.44	70.41	81.30	67.54	72.27
C-5	72.84	72.70	76.00	77.36	77.50	78.43
C-6	63.10	177	71.84	62.75	63.39	63.39

IdoA (B), lead to the assignment of the α configuration to these residues, whereas the value of 162–164 Hz for residues C, D, F, and G lead to the assignment of the β configuration to these residues. The assignment of the configuration of IdoA was predicated on the fact that the observed chemical shifts closely paralleled those observed in dermatan sulfate [30] and model systems [31].

Sequencing of the glycosyl residues.—The HMBC spectrum of the polysaccharide (Fig. 5) revealed long-range H-1 → C-x' and C-1 → H-x' connectivities for the six residues. The observation of a cross peak between H-1 of Gal (A) and C-3 of Gal (F) was evidence for assigning the linkage between Gal (A) and Gal (F) in the polysaccharide as Gal (A) (1 → 3) Gal (F). Similarly, the cross peaks H-1_F/C-4_D, H-1_D/C-4_B, H-1_B/C-3_C, and H-1_C/C-4_A indicate that Gal (F) is linked to Glc (D) through a (1 → 4)-linkage, Glc (D) is linked to IdoA (B) through a (1 → 4)-linkage, IdoA (B) is linked to GalNAc (C) through a (1 → 3)-linkage, and GalNAc (C) is linked to Gal (A) through a (1 → 4)-linkage. At this point, it is clear that the Gal (A), Gal (F), Glc (D), IdoA (B), and GalNAc (C) comprise the backbone of the Hobbs 10 polysaccharide. The observation of the cross peak between H-1 of Glc (G) and C-6 of GalNAc (C) indicates that Glc (G) is linked to the polysaccharide backbone at GalNAc (C) through a (1 → 6)-linkage. The down field shift of C-4 of Gal (A), C-4 of IdoA (B), C-3 of GalNAc (C), C-6 of GalNAc (C), C-4 of Glc (D), and C-3 of Gal (F) is consistent with the above assignment. This completed the determination of the primary structure of the Hobbs 10 polysaccharide.

4. Conclusions

The primary structure of the *C. perfringens* Hobbs 10 Capsular polysaccharide was determined to be as follows:



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