ELSEVIER

Carbohydrate Research 277 (1995) 1–9

CARBOHYDRATE RESEARCH

Structural elucidation of the novel type VII group B *Streptococcus* capsular polysaccharide by high resolution NMR spectroscopy ☆

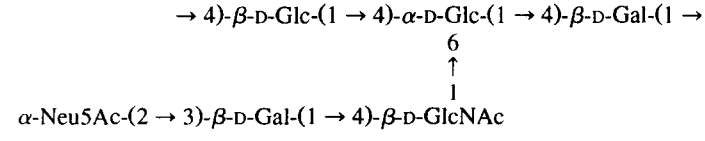
Grigorij Kogan [a,1], Jean-Robert Brisson [a], Dennis L. Kasper [b], Christina von Hunolstein [c], Graziella Orefici [c], Harold J. Jennings [a,*]

[a] *Institute for Biological Sciences, National Research Council of Canada, Ottawa, Ontario K1A 0R6, Canada*
[b] *Channing Laboratories, Harvard Medical School, Boston, MA 02115, USA*
[c] *Laboratory of Bacteriology and Medical Mycology, Istituto Superiore di Sanità, 00161 Rome, Italy*

Received 17 January 1995; accepted 20 April 1995

Abstract

The type VII capsular polysaccharide isolated from the newly discovered group B *Streptococcus* (GBS) strain contains D-glucose, D-galactose, *N*-acetyl-D-glucosamine and *N*-acetylneuraminic acid in the molar ratio 2:2:1:1. High-resolution one- and two-dimensional (1D and 2D) ^{1}H and ^{13}C NMR spectroscopy of the native and desialylated polysaccharides showed the type VII GBS capsular polysaccharide to contain the following branched hexasaccharide repeating unit:



Despite extensive structural similarity with the previously described GBS polysaccharides, the type VII polysaccharide showed no cross-reaction with the heterologous antisera.

Keywords: Group B *Streptococcus*; Type VII capsular polysaccharide; NMR spectroscopy

☆ This is National Research Council of Canada publication no. 394 90.

[1] Permanent address: Institute of Chemistry, Slovak Academy of Sciences, Bratislava, 84238 Slovakia.

* Corresponding author.

SSDI 0008-6215(95)00195-6

1. Introduction

Group B *Streptococcus* (GBS) is a major cause of neonatal sepsis and meningitis [1–3]. GBS are classified into serotypes on the basis of their type-specific capsular polysaccharides. Along with the four strains that are most commonly isolated from the clinical cases (serotypes Ia, Ib, II, and III [2]), several additional GBS serotypes IV, V and VI have been isolated recently and the structures and immunochemical properties of their capsular antigens were elucidated [4–6]. We now report the structural and immunochemical characterization of the most recently isolated type VII GBS capsular polysaccharide. Like all other type-specific GBS polysaccharides, it contains terminal sialic acid which plays a crucial role in defining the conformation of the GBS polysaccharide antigens.

2. Results and discussion

Isolation and characterization of the type VII GBS polysaccharide.—The capsular polysaccharide was isolated from the bacterial mass using a modification of a previously described procedure [7]. The polysaccharide was purified by elution from a DEAE-Sephacel column by a linear gradient of NaCl in Tris buffer, and the fractions that were reactive against the type-specific rabbit antiserum were pooled. The average molecular weight of the capsular polysaccharide was determined to be 186 000 Da using FPLC on a Superose 6 column calibrated with dextran standards. UV spectrophotometry of a 1 mg mL^{-1} solution of the purified polysaccharide revealed a single major absorbance peak at 206 nm with the minor absorbances at 260 and 280 nm, indicative of minimal contamination by nucleic acids or protein. Protein content estimated by the method of Lowry et al. [8] was 2%. Monosaccharide composition was determined by pulsed amperometric potentiometry and confirmed by the GLC of alditol acetates. The data showed that the type VII GBS polysaccharide consisted of glucose, galactose, 2-amino-2-deoxyglucose (GlcN) and neuraminic acid (Neu) in the molar ratio 2:2:1:1. The amino groups of the two latter sugars were shown to carry acetyl substituents by means of ^{1}H and ^{13}C NMR analyses. Content of sialic acid, estimated using thiobarbituric acid assay according to Warren [9], was 25% by weight, which is in agreement with the results of the monosaccharide analysis.

In a double immunodiffusion assay [10], type VII polysaccharide formed a single precipitin line with rabbit antiserum raised to the whole bacteria. No cross-reaction was observed between the type VII polysaccharide and rabbit antisera raised to types Ia, Ib, II and III GBS group-specific polysaccharide-tetanus toxoid conjugates or types IV, V and VI GBS whole bacteria, and conversely no cross-reaction was observed between the antiserum raised against type VII GBS whole bacteria and GBS type-specific polysaccharides of other serotypes.

NMR analysis.—The anomeric region of the ^{1}H NMR spectrum of type VII GBS polysaccharide contained, along with the other signals, a broad unresolved peak integrating to three protons resulting from the partial overlap of three of the five anomeric proton signals. Since in the ^{1}H NMR spectrum of the desialylated polysaccharide these

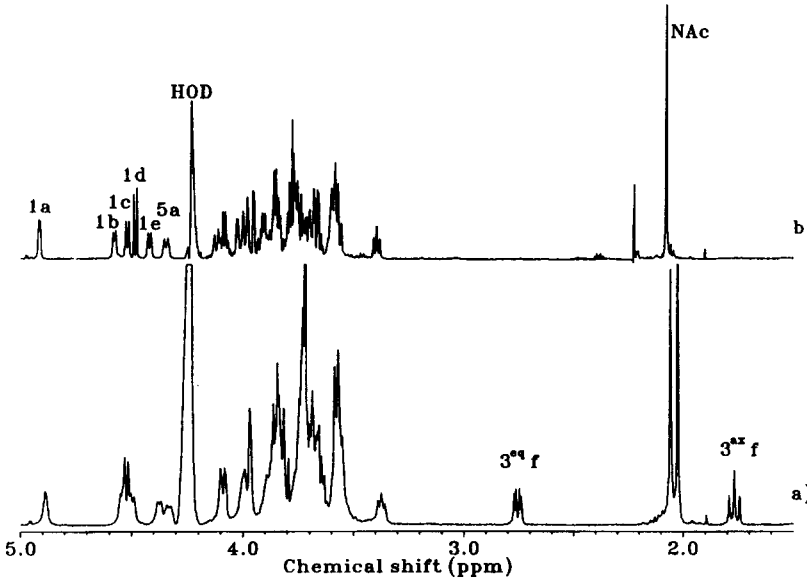


Fig. 1. 600 MHz ^{1}H NMR spectra (345 K) of the native (a) and desialylated type VII GBS polysaccharides (b).

signals were resolved and the spectrum was further simplified due to the smaller number of the protons, the complete assignment of the signals and the structure elucidation was carried out primarily on the desialylated type VII GBS polysaccharide. Fig. 1 shows the ^{1}H NMR spectra of the native (a) and desialylated (b) type VII GBS polysaccharides (600 MHz, 345 K).

In accordance with the results of the monosaccharide composition analysis, the ^{1}H NMR spectrum of the desialylated type VII GBS polysaccharide (ds-PS) contained five anomeric proton signals for *gluco/galacto* configurations, indicating that it was built of pentasaccharide repeating units. One signal (4.92 ppm, $^3J_{H1,H2}$ 4.2 Hz) was attributed to an α-glycosidic configuration, whereas the remaining four signals at 4.58, 4.52, 4.48 and 4.42 ppm showed $^3J_{H1,H2}$ 7.8 Hz and thus belonged to β-anomeric protons. According to the sequence of the anomeric signals from the lower field, the corresponding monosaccharide units were designated as units **a**, **b**, **c**, **d** and **e**.

The specific shape of the anomeric signal at 4.58 ppm (unit **b**) indicated strong coupling between H-2 and H-3 of this unit. This phenomenon is often characteristic of 4-substituted β-D-GlcNAc [11,12]. Due to β-glycosylation, substitution at position 4 causes downfield displacement of the chemical shift of H-3. This shift results in strong coupling with H-2, and in the formation of the specific pseudo-multiplet shape of the H-1 signal due to virtual coupling [12]. The triplet at 3.39 ppm was characteristic of H-2 of the β-D-glucosyl unit (**e**) and the presence of *N*-acetyl-D-glucosamine was indicated by the acetamido methyl group signal at 2.08 ppm. Assignments of the signals in the ^{1}H NMR spectrum were obtained by means of 2D COSY and TOCSY, as well as NOESY techniques that confirmed the α-anomeric configuration of unit **a** (α-D-glucose) and

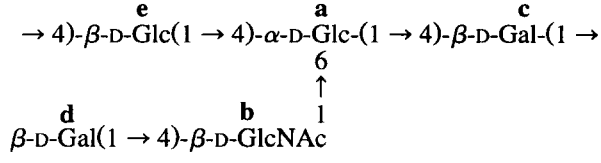
Table 1
Assignments of the ^{1}H resonances for the type VII GBS polysaccharide (PS), desialylated core polysaccharide (ds-PS) [a] and corresponding data for monosaccharides (mono) [14,15]

Unit	H-1	H-2	H-3	H-4	H-5	H-6	NAc
a PS	4.88	3.58	3.84	3.53	4.37	4.10,3.90	
ds-PS	4.92	3.59	3.86	3.57	4.34	4.12,3.90	
mono	5.23	3.54	3.72	3.42	3.84	3.84,3.76	
b PS	4.53	3.77	3.73	3.73	3.58	4.00,3.84	2.08
ds-PS	4.58	3.76	3.76	3.71	3.59	3.99,3.84	2.08
mono	4.72	3.65	3.56	3.46	3.46	3.91,3.75	2.06
c PS	4.51	3.57	3.76	4.00	3.78	3.91,3.84	
ds-PS	4.52	3.58	3.76	4.02	3.79	3.92,3.84	
mono	4.53	3.45	3.59	3.89	3.65	3.72,3.64	
d PS	4.55	3.57	4.11	3.96	3.71	3.74,3.73	
ds-PS	4.48	3.56	3.67	3.95	3.73	3.79,3.78	
mono	4.53	3.45	3.59	3.89	3.65	3.72,3.64	
e PS	4.36	3.37	3.65	3.73	3.60	3.85,3.84	
ds-PS	4.42	3.39	3.67	3.69	3.60	3.86,3.84	
mono	4.64	3.25	3.50	3.42	3.46	3.90,3.72	
f PS			1.80,2.78	3.69	3.85	3.64	2.03
mono			1.80,2.77	3.69	3.85	3.63	2.03
	H-7	H-8	H-9				
f PS	3.59	3.89	3.87,3.64				
mono	3.60	3.87	3.86,3.64				

[a] Chemical shift values were taken from 2D spectra (±0.01 ppm error), except for isolated resonances.

β-anomeric configuration of the other units. The assignments of the ^{1}H NMR signals of the ds-PS are shown in Table 1. Interestingly, the H-5 signal of unit **a** (H-5**a**) was located at an unusually low field (4.34 ppm) and had the shape of a doublet of two almost symmetrical triplets. The $^3J_{H5,H6}$ and $^3J_{H5,H6'}$ values were both very small (3 ± 0.5 Hz), which implied that both H-6**a** and H-6′**a** were preferentially in the *gauche* conformation relative to H-5**a**. Using the modified Karplus equation proposed by Haasnoot et al. [13], the fractional populations of the rotamers for the C-5–C-6 linkage were calculated, and the relative population of the *gauche–gauche* rotamer for the $-CH_2O$-group in unit **a** was found to be ca. 80%.

The sequence of the monosaccharides in the repeating unit of ds-PS was established from the 2D NOESY spectrum, cross-sections of which are shown in Fig. 2. The observed interglycosidic connectivities were consistent with the following structure for the repeating unit of the desialylated type VII GBS polysaccharide:



As can be seen, substitution of unit **a** in positions 4 and 6 accounted for both the unusually low-field location of the H-5**a** signal and also for the preferentially *gauche–*

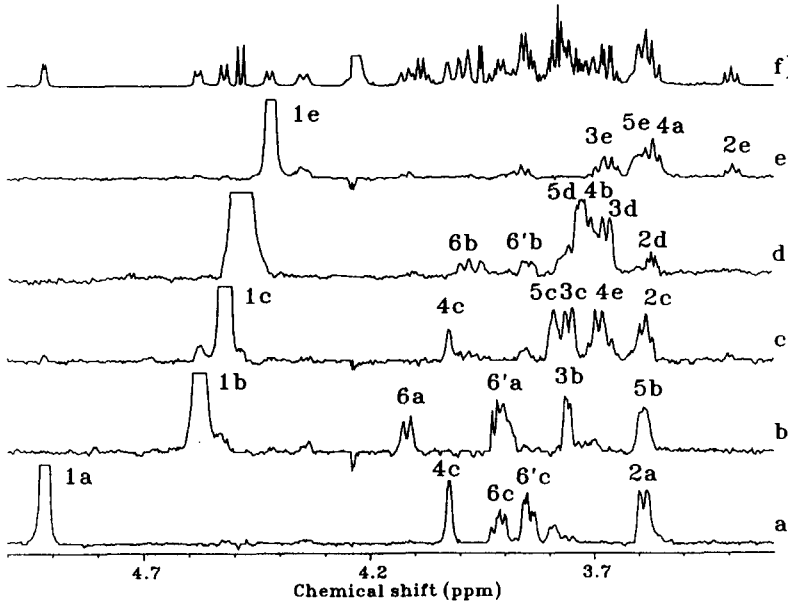


Fig. 2. NOE spectrum of the ds-PS showing the NOEs for the anomeric protons of residues **a**–**e**. The ^{1}H NMR spectrum is shown on the top (**f**).

gauche conformation of the C-5–C-6 bond. Attachment of unit **d** to position 4 of unit **b** was confirmed by interglycosidic NOEs from H-1**d** to H-4**b**, as well as from H-1**d** to H-6**b** and H-6′**b** (Fig. 2, trace **d**). Such NOEs to H-6 are often observed in four-substituted sugar units and were also present in unit **a** (Fig. 2, trace **a**). Unit **b** being 4-substituted *N*-acetyl-β-D-glucosamine was corroborated also by the unusual shape of its anomeric proton signal, and finally, the β-D-galactosyl unit (**d**) was shown to be terminal. Assignment of the ^{13}C NMR signals of the ds-PS was performed using HMQC techniques and the results are presented in Table 2. Observed displacements of the chemical shifts of C-4**a**, C-6**a**, C-4**b**, C-4**c**, and C-4**e** in comparison with the corresponding values for the free monosaccharides [14] confirmed the assignments for the positions of glycosidic substitution in the respective units, which were established previously by the NOESY experiment.

The ^{1}H NMR spectrum of the native type VII GBS capsular polysaccharide contained, in addition to the signals observed in the spectrum of ds-PS, inter alia, signals at 1.80 and 2.78 ppm due to the axial and equatorial H-3 protons of sialic acid [15,16], as well as the signal at 2.03 ppm integrating to three protons due to its acetamido methyl group. In agreement with these observations, the ^{13}C NMR spectrum of the polysaccharide (Fig. 3) contained additional characteristic signals at 174.70, 100.66, 63.44, and 40.49 ppm corresponding to C-1, C-2, C-9, and C-3 of sialic acid, as well as signals at 175.85 and 22.88 ppm arising from its acetamido group.

As mentioned above, sialylation caused displacement of some signals in the ^{1}H NMR spectrum when compared with the spectrum of the ds-PS. This displacement resulted in

Table 2
Assignments of the ^{13}C NMR resonances for the type VII GBS polysaccharide (PS), desialylated core polysaccharide (ds-PS) [a], and corresponding data for monosaccharides (mono) [14,16]

Unit	C-1	C-2	C-3	C-4	C-5	C-6	NAc
a PS	100.46	72.14	72.14	80.24	70.00	67.68	
ds-PS	101.4	72.2	72.0	80.2	70.2	67.9	
mono	92.99	72.47	73.78	70.71	72.37	61.84	
b PS	101.77	55.96	73.13	79.16	75.46	60.88	23.48,175.06
ds-PS	101.7	55.9	73.1	79.8	75.6	61.1	23.4,175.0
mono	95.85	57.86	74.81	71.06	76.82	61.85	23.10,175.49
c PS	104.17	71.48	72.60	77.80	76.00	60.88	
ds-PS	103.8	71.9	73.5	69.4	76.1	61.7	
mono	97.37	72.96	73.78	69.69	75.93	61.64	
d PS	103.45	70.22	76.32	68.33	76.00	61.86	
ds-PS	103.8	71.9	73.5	69.4	76.1	61.7	
mono	97.37	72.96	73.78	69.69	75.93	61.84	
e PS	103.86	73.73	74.95	79.16	75.46	60.88	
ds-PS	103.4	73.8	75.1	79.7	75.6	61.1	
mono	96.84	75.20	76.76	70.71	76.76	61.84	
f PS	174.70	100.66	40.49	69.20	52.54	73.73	22.88,175.85
mono	174.6	101.9	41.3	69.5	53.2	73.8	23.3,176.3
	C-7	C-8	C-9				
f PS	68.96	72.59	63.44				
mono	69.5	72.9	63.9				

[a] Data for PS were obtained from the 1D ^{13}C NMR spectrum (± 0.01 ppm error). Data for ds-PS were taken from the HMQC spectrum (± 0.1 ppm error).

significant overlap of the anomeric as well as ring resonances, making their assignment more difficult. However, knowledge of the chemical shifts of the desialylated polysaccharide permitted a complete assignment of the ^{1}H and ^{13}C NMR signals of the native polysaccharide to be made using the HMQC technique. In cases where similar values of the ^{1}H chemical shifts (as in H-2**c** and H-2**d**) were encountered, assignment was made by taking β-glycosylation effects into consideration, as well as the sharper appearance of the side-chain units signals in comparison with those of the backbone units. The data

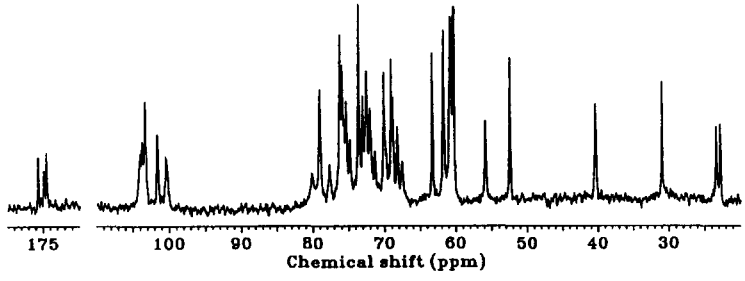
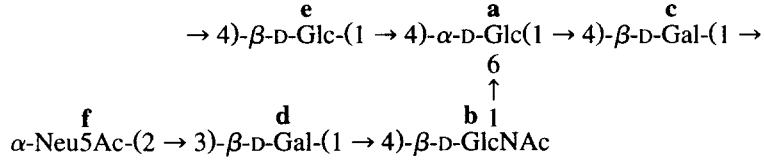


Fig. 3. 125 MHz ^{13}C NMR spectrum (300 K) of the type VII GBS polysaccharide.

obtained are listed in the Tables 1 and 2, together with the respective data for the ds-PS and for the monosaccharides [14–16]. Comparison of the respective data for the native and desialylated polysaccharides shows that sialylation caused significant low-field shifts for the H-3**d** and C-3**d** signals (from 3.67 to 4.11, and from 73.53 to 76.32, respectively). This behavior implied glycosidic substitution at position 3 of the β-D-galactosyl unit **d** by sialic acid [15,16]. Such a pattern of sialylation is common for all previously described type-specific GBS polysaccharides [3–6]. Thus, the structure of the repeating unit of the type VII GBS capsular polysaccharide may be presented as follows:



Besides glycosylation effects on the galactosyl unit **d**, sialylation caused marked upfield displacements of the ^{13}C chemical shifts of the C-3 signal of unit **a** (0.71 ppm) and of the C-3 signal of the backbone galactosyl unit **c** (1.14 ppm). These effects may be due to the steric proximity of the sialic acid to certain backbone sugar units and/or to different conformations of the desialylated and native polysaccharides.

Immunochemical characterization.—Despite structural similarities common to all other GBS type-specific polysaccharides, especially with the type VI GBS polysaccharide, where the only difference is that the trisaccharide side-chain is linked to position 6 of the backbone β-D-galactose instead of α-D-glucose [4], the type VII polysaccharide is immunologically distinct. This behavior was demonstrated by the absence of cross-reaction with antisera raised against all other GBS polysaccharides. This immunological uniqueness, despite such striking structural resemblance, implies that the antigenic specificity could be controlled by spatial, conformational organization of the polysaccharide antigen, rather than by its primary chemical structure alone. Such conformational control is believed to be attributed to the presence of terminal sialic acid, which although not immunodominant in the classical sense due to its ubiquitous presence in animal and human tissues, is crucial for the epitopic expression of the polysaccharide antigens [17].

3. Experimental

Bacteria and cultivation.—Type VII Group B *Streptococcus* strain 7271 was received from Dr P. Ferrieri (Departments of Laboratory Medicine and Pathology and Pediatrics, University of Minnesota, Medical School, Minneapolis, MN) on a chocolate slant agar. A 500-mL log-phase culture was used to inoculate 16 L of a dialysate (10 000 NMWL membranes, Pellicon Cassette System, Millipore Corp., Bedford, MA) of Columbia broth (Difco) containing 80 g glucose per L. Cultivation was carried out in a 20 L fermenter (LSL BioLafitte, France) for 18 h at 37°C with 0.2% antifoam 204 (Sigma), continuous stirring at 200 rpm, aeration at 10 L min^{-1} and pH maintained at 7.1. Bacterial cells were recovered by microfiltration (0.45 μm Durapore membrane, Prostack System, Millipore Corp., Bedford, MA) and suspended in 20 mM potassium

phosphate buffer, pH 7.0, containing 20% (w/v) sucrose and 1 mM $MgCl_2$ and subsequently treated with 50 000 U of mutanolysin (Sigma).

Isolation of the capsular polysaccharide and preparation of ds-PS.—After incubation at 37°C for 18 h, the supernatant was recovered by centrifugation at 10 000 × *g* for 20 min at 4°C. The liquid was then brought into 0.5 M NaOH and kept at 50°C for 48 h. Afterwards the preparation was neutralized with 12 M HCl and clarified by centrifugation at 10 000 × *g* for 20 min. The supernatant was dialyzed and concentrated (10 000 NMWL membranes, Minitan System, Millipore), then freeze-dried. The preparation was re-*N*-acetylated by treatment with 4% acetic anhydride in 5% sodium bicarbonate at room temperature for 2 h, then neutralized, dialyzed and concentrated using the Minitan system, and lyophilized overnight. The crude preparation was treated with 20 mg DNase (Worthington), 50 mg RNase A (Worthington) and 10 μL RNase T_1 (Sigma) at 37°C for 2 h, followed by treatment with 25 mg Pronase (Calbiochem) for 2 h at 37°C, dialyzed, concentrated and lyophilized.

The preparation was resuspended in a minimum volume of 10 mM Tris buffer, pH 7.2 and applied to a DEAE-Sephacel (Pharmacia) column (2.6 × 30 cm) equilibrated with the same Tris buffer. After being washed with 500 mL of buffer, the material bound to the column was eluted with a linear gradient of 0 0.22 M NaCl in Tris buffer. Fractions were tested by radial immunodiffusion against type-specific rabbit antiserum and reactive fractions were pooled. The pool was dialyzed and concentrated by Minitan system, then lyophilized.

Desialylated core polysaccharide (ds-PS) was prepared by 1% acetic acid hydrolysis of the native polysaccharide for 1 h at 80°C, followed by dialysis and lyophilization.

Analytical procedures.—Protein content was determined by the method of Lowry et al. [8]. Sialic acid was determined using thiobarbituric acid assay according to Warren [9]. Monosaccharide composition was assessed by pulsed amperometric potentiometry (Dionex, Sunnyvale, CA) as well as using the alditol acetates procedure as previously described [6]. Determination of the absolute configurations of the glycoses was performed by the modified method of Leontein et al. [18] as described previously [6]. The molecular size of the native polysaccharide was determined using FPLC using a refractive index detector on an analytical Superose 6 column (Pharmacia).

Immunologic assays.—Double diffusion in 1% agarose with 1% polyethylene glycol in phosphate-buffered saline was performed as described by Ouchterlony [10].

Preparation of antisera.—GBS types Ia, Ib, II and III specific antisera were prepared by immunizing New Zealand white rabbits with type-specific polysaccharide-tetanus toxoid conjugate vaccines [19–21]. Formalin-killed GBS cells of serotypes IV, V, VI and VII were used as whole cell immunogens for the preparation of rabbit antisera by the method of Lancefield et al. [22].

NMR spectroscopy.—All NMR experiments were performed on Bruker AMX 600 and AMX 500 spectrometers using a 5 mm broadband probe with the ^1H coil nearest to the sample. ^1H and 2D experiments with the ds-PS were performed at 345 K, while its ^{13}C spectrum was recorded at 300 K. The differences in the ^{13}C chemical shift values observed between spectra measured at 300 and 345 K were negligible. All NMR experiments with the native polysaccharide were performed at 300 K. Native polysaccharide (5mg) or ds-PS (1 mg) was dissolved in 0.5 mL of 10 mM phosphate buffer in

D_2O, pH 7.0 (measured in H_2O prior to lyophilization). Acetone was used as an internal standard with the CH_3 resonance set at 31.07 ppm for ^{13}C spectra and 2.225 ppm for ^1H spectra. The experiments were carried out without sample spinning. Two dimensional homo- and hetero-correlated experiments (COSY, TOCSY, NOESY, HMQC) were carried out as previously described [23]. For NOESY, a mixing time of 400 ms was used. For increased signal to noise, the NOE spectrum of H-**1d** was obtained from a 1D NOESY.

References

[1] C.J. Baker and M.S. Edwards, *Ann. N.Y. Acad. Sci.*, 459 (1988) 193–202.
[2] P. Ferrieri, *Rev. Infect. Dis.*, 12 (Suppl. 4) (1990) s394–s400.
[3] H.J. Jennings, *Curr. Top. Microbiol. Immunol.*, 150 (1990) 97–127.
[4] J.L. DiFabio, F. Michon, J.-R. Brisson, H.J. Jennings, M.R. Wessels, V.-J. Benedí, and D.L. Kasper, *Can. J. Chem.*, 67 (1989) 877–882.
[5] M.R. Wessels, J.L. DiFabio, V.-J. Benedí, D.L. Kasper, F. Michon, J.-R. Brisson, J. Jelínková, and H.J. Jennings, *J. Biol. Chem.*, 266 (1991) 6714–6719.
[6] G. Kogan, D. Uhrín, J.-R. Brisson, L.C. Paoletti, D.L. Kasper, C. von Hunolstein, G. Orefici, and H.J. Jennings, *J. Carbohydr. Chem.*, 13 (1994) 1071–1078.
[7] M.R. Wessels, V.-J. Benedí, H.J. Jennings, F. Michon, J.L. DiFabio, and D.L. Kasper, *Infect. Immun.*, 57 (1989) 1089–1094.
[8] O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, 193 (1951) 265–275.
[9] L. Warren, *J. Biol. Chem.*, 234 (1959) 1971–1975.
[10] O. Ouchterlony, *Prog. Allergy*, 6 (1962) 30–154.
[11] R.C. Bruch and M.D. Bruch, *J. Biol. Chem.*, 257 (1982) 3409–3413.
[12] J.-R. Brisson and J.P. Carver, *J. Biol. Chem.*, 257 (1982) 11207–11209.
[13] C.A.G. Haasnoot, F.A.A.M. de Leeuw, and C. Altona, *Tetrahedron*, 36 (1980) 2783–2792.
[14] P.-E. Jannson, L. Kenne, and G. Widmalm, *Carbohydr. Res.*, 188 (1989) 169–191.
[15] L. Poppe, J. Dabrowski, C.-W. von der Lieth, M. Numata, and T. Ogawa, *Eur. J. Biochem.*, 180 (1989) 337–342.
[16] J.F.G. Vliegenhart, L. Dorland, H. van Halbeek, and J. Haverkamp, in R. Schauer (Ed.) *Sialic Acids. Chemistry, Metabolism and Function*, Springer Verlag, Wien, 1982, pp 127–172.
[17] H.J. Jennings, E. Katzenellenbogen, C. Lugowski, F. Michon, R. Roy, and D.L. Kasper, *Pure Appl. Chem.*, 56 (1984) 893–905.
[18] K. Leontein, B. Lindberg, and J. Lönngren, *Carbohydr. Res.*, 62 (1978) 359–362.
[19] L.C. Paoletti, M.R. Wessels, F. Michon, J.L. DiFabio, H.J. Jennings, and D.L. Kasper, *Infect. Immun.*, 60 (1992) 4009–4014.
[20] M.R. Wessels, L.C. Paoletti, D.L. Kasper, J.L. DiFabio, F. Michon, K. Holme, and H.J. Jennings, *J. Clin. Invest.*, 86 (1990) 1428–1433.
[21] M.R. Wessels, L.C. Paoletti, A.K. Rodewald, F. Michon, J.L. DiFabio, H.J. Jennings, and D.L. Kasper, *Infect. Immun.*, 61 (1993) 4760–4766.
[22] R.C. Lancefield, M. McCarty, and W.N. Everly, *J. Exp. Med.*, 142 (1975) 165–179.
[23] H. Baumann, A.O. Tzianabos, J.-R. Brisson, D.L. Kasper, and H.J. Jennings, *Biochemistry*, 31 (1992) 4081–4089.