

2D NMR ANALYSIS OF GROUP B CAPSULAR POLYSACCHARIDE OF *N. MENINGITIDIS*: COMPLETE ASSIGNMENT OF ^1H -NMR SPECTRUM OF B POLYSACCHARIDE OF STRAIN 6275 *

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Summary: Group B capsular polysaccharide of *N. meningitidis* was analyzed by 2D NMR spectroscopy. The ^1H -NMR spectrum of the polymer was completely assigned by 2D homonuclear (COSY and HOHAHA) and heteronuclear (^1H , ^{13}C) NMR experiments. © 1988 Academic Press, Inc.

Capsular polysaccharide of *Neisseria meningitidis* B is an α -2,8 linked sialic acid homopolymer (Fig. 1) (1). This sialic acid polymer elicits very low levels of antibodies when injected in man (2). The poor immunogenicity of the polymer has been postulated to be due to its structural similarity to host tissues (3,4). Jennings *et al.* has carried out immunochemical analysis of α -2,8 linked sialic acid oligomers and suggested that the polymer might have a conformational epitope within the molecule (5). Therefore, characterization of conformations of the B polysaccharide is very important in the understanding of the immunogenicity of the polymer. Conformational analysis of B and C polysaccharides has been carried out by Lindon *et al.* (6) by NMR and modelling and a difference of the segmental motion of the exocyclic carbon chain has been reported. However, the overall conformations of the B polymer are not yet clear. Our approach to this problem is the use of 2D NOE (Nuclear Overhauser Effect) NMR technique to determine intramolecular geometry of the polymer. For this analysis, a complete assignment of the ^1H NMR spectrum of the polymer is necessary. The present

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Abbreviations: COSY, Homonuclear J-correlation NMR spectroscopy; HOHAHA, Homonuclear Hartmann-Hahn NMR spectroscopy; TSP, 3-trimethyl[2,2,3,3- $^2\text{H}_4$]propionate.

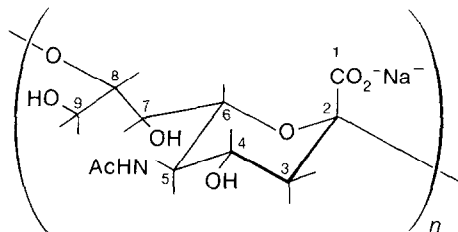


Fig. 1. Structure of group B polysaccharide of *Neisseria meningitidis*.

paper describes the complete assignment of the ^1H -NMR spectrum of B polysaccharide using 2D homo- and heteronuclear NMR experiments.

Materials and Methods

Materials. Strain 6275 of *N. meningitidis* was obtained from the culture collection of the Walter Reed Army Institute of Research (Washington, D.C.). The polysaccharide was isolated and purified according to the literature procedure (7).

NMR measurements. All NMR spectra were obtained (for ^1H at 500 MHz and for ^{13}C at 125 MHz) on a GE/Nicolet GN500 spectrometer equipped with an Oxford Instruments magnet and a Nicolet 1280 computer. A 5-mm proton probe and a 10-mm $^1\text{H}/^{13}\text{C}$ dual probe were used. The running temperature was 25 °C. For ^1H -NMR, the polysaccharide (10 mg) was lyophilized several times from D_2O solution and finally dissolved in 99.99% D_2O (0.4 ml). ^1H Chemical shifts are relative to the chemical shift of HOD which was carefully referenced to sodium 3-trimethyl[2,2,3,3- $^2\text{H}_4$]propionate (TSP) as a function of temperature. For ^{13}C -NMR, the polysaccharide (100 mg) was lyophilized as described above and finally dissolved in 99.99% D_2O (2 ml). ^{13}C chemical shifts are relative to acetonitrile (CH_3 : 1.56 ppm) that was used as an internal standard.

The 2D homonuclear J-correlation (COSY) (8) spectrum was acquired as 512 x 2K data points with a spectral width of ± 2000 Hz. The data matrix was processed with a phase-shifted sine-bell in the t_1 and t_2 dimensions and was zero filled in the t_1 dimension. Homonuclear Hartmann-Hahn (HOHAHA) (9) spectra were obtained as 512 x 4K data points using three different mixing times; 30, 70, and 100 ms. The spectral width was ± 1700 Hz, and the number of acquisitions n was 32. The data matrix was processed with zero filing and a phase-shifted sine-bell in the t_1 and t_2 dimensions to give 1K x 1K real data points. The 2D heteronuclear (^1H and ^{13}C) chemical shift correlation spectrum (10) was obtained as 512 x 4K data points. The ^1H spectral width was ± 2500 Hz and the ^{13}C spectral width was ± 12000 Hz. The data matrix was processed with a phase-shifted sine-bell in the t_1 and t_2 dimensions and was zero filled in the t_1 dimension.

Results and Discussion

In order to overcome lower spectral resolution due to a short T_2 time, we tried to perform NMR experiments at higher temperatures. However, degradation took place even at 37 °C, in particular during acquisition of 2D NMR spectra. This made the assignment of protons very difficult. Therefore, we performed our experiments at 25 °C, at which temperature the polymer was stable for several days.

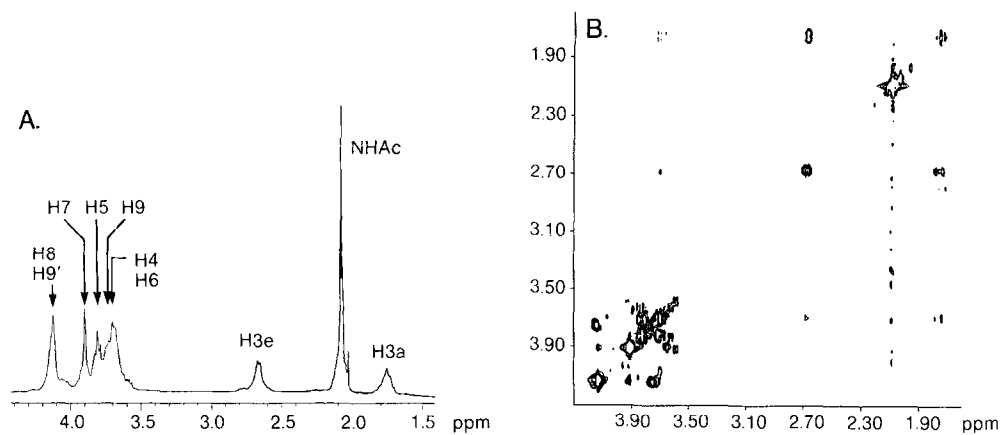


Fig. 2. 500-MHz ^1H -NMR(A) and COSY(B) spectra of the 6275 B polysaccharide at 25 °C.

Fig. 2 shows the ^1H -NMR spectrum (A) and COSY (B) of B polysaccharide of strain 6275 of *N. meningitidis* at 25°C. The C-3 methine protons, H-3a and H-3e, were located at a higher field (11) and assigned to be 1.76 and 2.66 ppm, respectively. From the COSY spectrum, the peak centered at 3.69 ppm was easily assigned to be H-4 as it showed cross peaks with H-3a (1.75 ppm) and H-3e (2.67 ppm). Similarly, the peak at 3.81 ppm was assigned to be H-5. The integration of the 1D spectrum indicated that there were two protons present in each peak centered at 3.69 and 4.12 ppm. In addition, there were two sets of interactions of directly coupled protons: a proton at 3.90 ppm and a proton at 4.12 ppm, and a proton at 3.74 ppm and a proton at 4.12 ppm. The intensity of the latter cross peak suggested that this interaction was due to the geminal protons, H-9 and H-9'. The proton at 3.90 ppm was assigned to be H-7 or H-8. If the proton centered at 3.90 ppm were H-6, it should give a cross peak due to the interaction with H-5 (3.81 ppm) as H-5 and H-6 are trans-axially oriented (Fig. 1). Further identification of the proton could not be made as the absence of the cross peak between H-8 and H-9 can be expected due to a cancellation when the $J_{8,9}$ value is small. The proton (at 3.64 ppm) that overlapped with H-4 was suggested to be H-6. The absence of the cross peak between H-6 and H-7 can be expected for the same reason as described above. Comparison of the 1D spectrum (A) with the COSY spectrum (B) also showed that there exist small populations of smaller molecular-weight B polymer. The cross peaks due to the interactions amongst the protons (3.61, 3.65, 3.67, and 3.89 ppm) and the protons that overlapped with H-5 (3.81 ppm) were found to be derived from them. These peaks became more pronounced because of their long T_2 times than those due to the interactions between the C3 methine protons and H-4 of the major higher molecular-weight B poly-

mer. These peaks became more dominant in the sample that was hydrolyzed at a higher temperature (data not shown).

To identify H-6, H-7, and H-8, we carried out 2D homonuclear Hartmann-Hahn (HOHAHA) experiments. Fig. 3 shows the HOHAHA spectra of the polymer at different mixing times (B: 30 ms; C: 70 ms; D: 100 ms). Bond connectivities became much clearer by HOHAHA experiments, and the assignments of H-9 and H-9' were confirmed. Directly coupled interactions were more pronounced at 30ms (Fig. 3B); cross peaks between H-4 (3.69 ppm) and H-5 (3.81 ppm), and between H-9 (3.74 ppm) and H-9' (4.12 ppm). As expected, the intensities of the two cross peaks due to the two sets of geminal protons at C-3 and C-9 decreased as the mixing time increased. At the same time, the intensity of a new cross peak between a proton at 3.90 ppm and H-9 (3.74 ppm) increased with the increase of the mixing time. This showed that the proton was H-7; if the proton were H-8, the intensity of the observed cross peak should decrease with the increase of the mixing time. The assignment of H-7 was also supported by the increase of the cross peak due to the H-7-H-9' interaction. H-

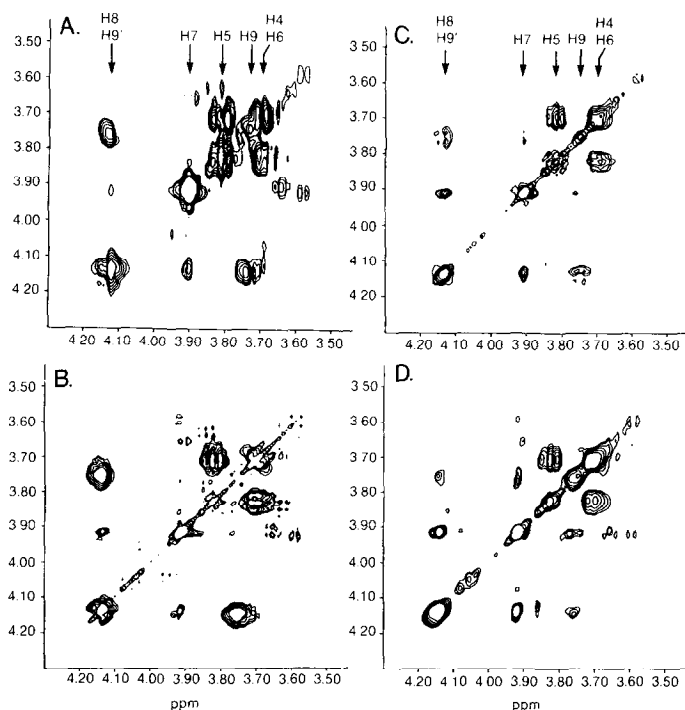


Fig. 3. 500-MHz COSY and HOHAHA spectra (4.4-3.5 ppm) of the 6275 polymer at 25°C: (A) the COSY spectrum; (B) HOHAHA spectrum at 30ms; (C) HOHAHA at 70 ms; (D) HOHAHA spectrum at 100 ms. HOHAHA spectra (B-D) are shown at the same contour level.

6 was assigned to be at 3.69 ppm. The absence of the directly coupled interaction between H-6 and H-7 in the HOHAHA spectra was due to the small $J_{6,7}$ value.

In order to confirm the above assignment, we carried out 2D ^1H - ^{13}C chemical shift correlation NMR analysis of the same polymer. Fig. 4 shows the spectrum and ^{13}C chemical shifts are given in Table 1. The presence of the two protons (H-4 and H-6) at 3.69 ppm was confirmed by the heteronuclear 2D experiment, and the proton chemical shift of H-4 and H-6 was found to be identical. The C9 carbon showed two cross peaks due to its interactions with H9 and H9' protons. The identity of H-8 was also confirmed, and the chemical shift difference of the two protons was only 0.01 ppm. Although the HOHAHA spectroscopy is a useful technique to determine bond connectivities, the complete assignment of the ^1H -spectrum was only possible by using the 2D homo- and heteronuclear experiments because of the overlapping of the two sets of protons.

While preparing this manuscript, Michon *et al.* published a paper and reported NMR assignments of colominic acid and its oligosaccharides (12). Although the data for the α -2,8-

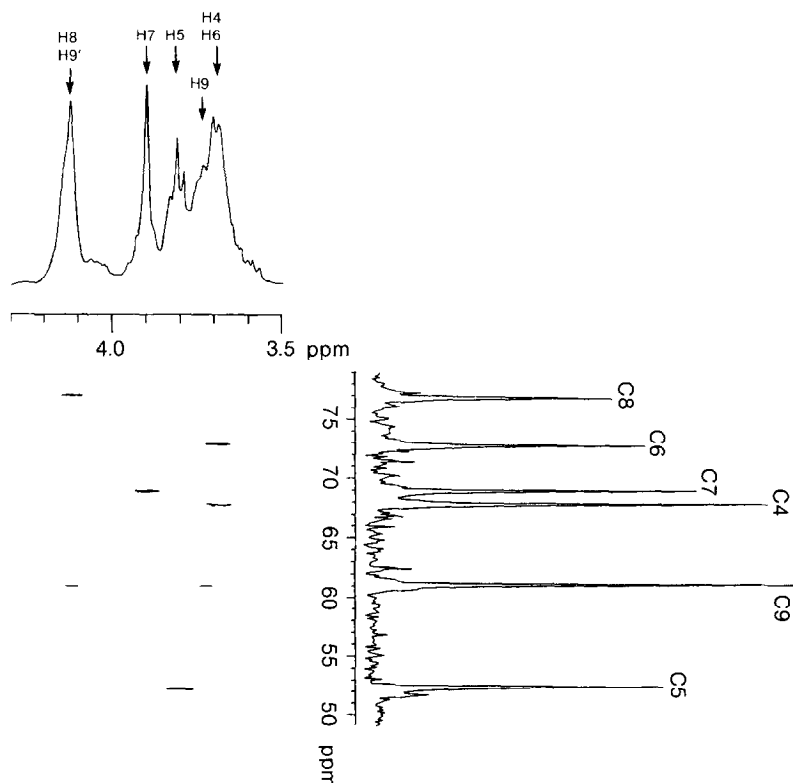


Fig. 4. Part of 2D heteronuclear ($^1\text{H}/^{13}\text{C}$) chemical shift correlation spectrum of the 6275 B polysaccharide at 500/125 MHz.

Table 1. ^1H and ^{13}C chemical shifts* of group B polysaccharide of *N. meningitidis* at 25 °C

H-3a	H-3c	H-4	H-5	H-6	H-7	H-8	H-9a	H-9b	NHAc
1.76	2.66	3.69	3.81	3.69	3.90	4.11	3.74	4.12	2.09

C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9	NHAc	C=O
101.79	40.53	69.01	53.29	73.94	70.03	78.24	62.08	23.29	174.04 175.81

*: The HOD signal was used as a chemical shift reference 4.78 ppm downfield from TSP. Acetonitrile (CH_3 : 1.56 ppm) was used as an internal standard for the measurement of ^{13}C chemical shifts.

linked trisaccharide were shown, the evidence for the spectral assignment of the colominic acid was not presented. Although they stated that the previous assignments of C-4 and C-7 made by Bhattacharjee *et al.* (1) should have been reversed, their recent assignments of the two carbons were not reversed. The present study gives the complete assignment of the ^1H -NMR spectrum of group B polysaccharide of *N. meningitidis*. Further conformational analysis of the 6275 polymer by 2D NOE NMR will be given in detail in a separate paper.

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