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# THE DETERMINATION OF THE STRUCTURE OF BLOOD GROUP OLIGOSACCHARIDES FROM FULLY ASSIGNED $^1$H-N.M.R. SPECTRA FOR SOLUTIONS IN NON-AQUEOUS SOLVENTS*

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ABSTRACT

The fully assigned $^1$H-n.m.r. spectra of a blood group A tetrasaccharide and of a blood group H hexasaccharide in dimethyl sulfoxide and in pyridine by use of two-dimensional COSY and homonuclear Hartmann–Hann coherence transfer methods are reported. The $^1$H-n.m.r. spectra of both of these compounds in deuterium oxide had been previously assigned. Since the relative proton chemical shifts in the three solvents are quite different, resonances which overlap or are strongly coupled for one solvent may be well resolved for another, thus providing an extension of the method of complete proton assignments for determination of the structure of complex oligosaccharides. Although the rotational correlation times ($\tau_c$) of these oligosaccharides are similar to the reciprocal of the spectrometer frequency, either negative or positive n.O.e. values were measurable for both oligosacharides in all three solvents in one-dimensional difference spectroscopy by taking advantage of the dependence of $\tau_c$ on the solvent viscosity and, thus, on sample temperature. Whereas n.O.e. depend strongly on temperature and solvent viscosity, the ratios of the effects between protons on the same pyranoside ring and those on different rings were observed to be similar, suggesting that the oligosaccharide conformations are not strongly dependent on solvent or temperature.

## INTRODUCTION

Nearly ten years ago, Vliegenthart and assoc. introduced the use of $^1$H-n.m.r. spectroscopy as a method for determination of the complex oligosaccharide structures of glycopeptides (for reviews, see Vliegenthart *et al.*[1,2]). The method introduced by this group, which has been adopted for structural studies of glycopeptides, oligosaccharides, and oligosaccharide alditols by many laboratories, involves assignment of "structural reporter group resonances" which are single

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isolated signals, such as those of anomeric protons in the $^1$H-n.m.r. spectra for deuterium oxide solution. More recently, Dabrowski and assoc.[3,4] have extended the utility of this method to the determination of the complex oligosaccharide structures of glycolipids by the use of dimethyl sulfoxide as a solvent in which these amphiphilic molecules are soluble in disaggregated form. This group as well as others[5] have suggested, as an extension of the structural reporter group method, a complete proton assignment in which the coupled spins of each glycosidic ring are identified by spin correlation either by one-dimensional spin difference decoupling or by a two-dimensional method such as COSY. Given the complete proton assignments for a pyranoside ring, its stereochemistry can be deduced from the proton coupling constants by examination of the multiplet structure of each peak, thus providing identification of each residue and its anomeric configuration[6]. The linkage of each residue to its neighbors is determined by the nuclear Overhauser enhancements either by 1D difference method or by NOESY. Although it has not always been made clear in publications on the topic, these advanced techniques requiring a complete proton assignment for a complex oligosaccharide can be severely hampered by cases of overlapping resonances, or more seriously by cases of strong coupling. This latter effect is serious for oligosaccharides in deuterium oxide where the chemical shifts of the methine protons are quite similar and many resonances are crowded into the region between $\delta$ 3.5 and 4.0. Although the coupling constants, and thus the pyranoside stereochemistry, can be extracted by spin simulation in cases of intermediate or moderately strong coupling, they cannot be determined if the chemical shift differences are less than the coupling constants.

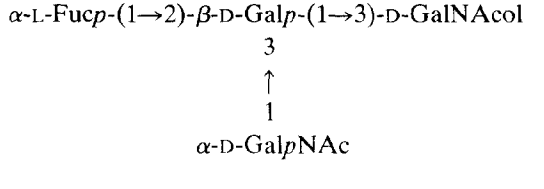
In spite of the technical problems involved, a number of complete proton assignments of complex oligosaccharide structures have appeared both for glycolipids in dimethyl sulfoxide and for related oligosaccharides in deuterium oxide. A survey of the results of these complete proton assignments reveals that the chemical shifts of the protons in similar oligosaccharide structures for dimethyl sulfoxide and deuterium oxide solutions are not identical, and furthermore that the discrepancies between the chemical shifts of similar oligosaccharides measured for dimethyl sulfoxide and deuterium oxide solutions do not result simply from a difference in the chemical shift referencing; real differences in the relative proton chemical shifts exist[7]. The difference in relative chemical shift suggests, but does not prove, that there may be a substantial difference between the conformations of the oligosaccharides in the two solvents, dimethyl sulfoxide and deuterium oxide. We have attempted to address this question directly by measuring nuclear Overhauser effects, for the non-aqueous solvents, dimethyl sulfoxide and pyridine, of some blood group oligosaccharides which have been previously studied in deuterium oxide solutions. In order to interpret the n.O.e. data, it has been necessary to make complete proton assignments for the oligosaccharides in these non-aqueous solvents.

We found that not only do the chemical shifts of the same blood group oligosaccharide in dimethyl sulfoxide and deuterium oxide differ substantially, but the

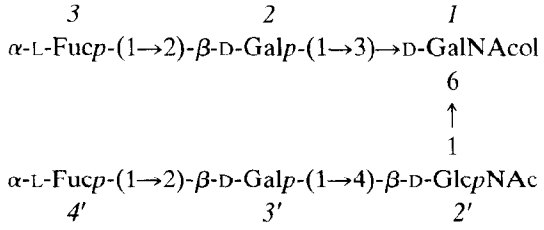
chemical shifts for the pyridine solution differ even more remarkably. Resonances, which overlap severly or which are strongly coupled for the deuterium oxide solution, may exhibit well separated resonances for these other solvents, thus considerably extending the potential of the method of complete proton assignments for determination of the complete structure of a complex oligosaccharide. Since the n.O.e. measurements for non-aqueous solvents imply that the oligosaccharide conformations are similar to those for a deuterium oxide solution, the added chemical shift dispersion obtained by this method may also be valuable for conformational studies of complex oligosaccharides[8].

## EXPERIMENTAL

*Materials.* — The blood-group A-tetrasaccharide (**1**) and the H-hexasaccharide (**2**) alditols were isolated from ovarian cyst mucin glycoproteins as described previously[6,9]. For studies of solutions in $D_2O$, the samples were treated




**1**




**2**

three times with $D_2O$, followed by lyophilization, and the final solutions were prepared by dissolving the samples in high purity (99.96 atom% D) $D_2O$ (Merck, Sharp, and Dohme Co.). Before dissolving the blood group A-tetrasaccharide **1** in $Me_2SO$, the sample was treaed with $D_2O$, followed by lyophilization, and the final solution was prepared by dissolving in ($^2H_3$)$Me_2SO$ (0.4 mL, 99.96 atom% D; Merck, Sharp, and Dohme Co). For studies of pyridine solutions, both samples were first lyophilized after exchange with $D_2O$, followed by a second cycle of lyophilization after exchange with ($^2H_5$)pyridine. The final solutions were prepared by dissolving the samples in ($^2H_5$)pyridine (0.4 mL, 99.5 atom% D). The spectra of **1**

were recorded with 4% $D_2O$ and that of **2** with 2.5% $D_2O$. For the solutions in $D_2O$, the reference was the signal of internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS), acetone being a secondary internal reference ($\delta$ 2.225 downfield from the signal of DSS). Internal $Me_4Si$ was the reference for the two non-aqueous solvents.

*Methods.* — The $^1$H-n.m.r. spectra were recorded with a 300-MHz Nicolet NT-300 NMR spectrometer, equipped with a 293C pulse programmer. The scalar-coupled peaks were assigned by COSY[10], 1D-HOHAHA (Homonuclear Hartman–Hahn coherence transfer)[11], and phase-sensitive 2D-HOHAHA[12,13] methods. The COSY spectra were recorded in the magnitude mode using the $90°$–$t_1$–$90°$-Acq pulse sequence with the appropriate phase cycling to obtain N-type peaks. A constant delay of 15 ms was used before and after the mixing pulse to reduce the antiphase character of the cross-peak multiplets[14]. Prior to the Fourier transformation, the data were apodized by a sine-bell function in each dimension. The final data matrix consisted of 512 × 512 real data points in the two dimensions. The 1D-HOHAHA spectrum was recorded by the pulse sequence[11]; $180°_{sel}$–$90°$–$(SL_y$-$60°_{-y}$–$300°_y$–$SL_{-y}$–$60°_y$–$300°_{-y})_n$–Acq[11], where $SL_y$ and $SL_{-y}$ denote spin lock along the y and $-$y axes, respectively, and the $180°_{sel}$ is a selective-inversion pulse. A 3-ms pulse was used for the spin-lock field and the number of cycles $n$ was chosen to be 15 with a total propagation time of 90 ms for the spin magnetization. The experiment was carried out in the low power r.f. mode with an ENI 411LA power amplifier to amplify the transmitter power. For spectral widths of ±750.0 Hz (maximum offset of a resonance of interest from the transmitter <750 Hz), r.f. power levels of 1-2 watts proved quite adequate to effectively spin-lock the resonances. The 90°-pulse widths were between 40 and 30 $\mu$s depending on the power levels used.

The scalar-coupled network was obtained by the 2D-HOHAHA method with MLEV-17 mixing[13] using the pulse sequence, $90°_\phi$–$SL_x$–$(MLEV\text{-}17)_n$–$SL_x$–$Acq_{\psi\prime}$, where $n$, the number of times the MLEV-17 sequence is cycled, determines the duration of spin propagation. Two sets of 1K × 256 data points were acquired in adjacent blocks of memory and the data processed by the method of States *et al.*[15] to obtain pure absorptive spectra. Prior to Fourier transformation, a Gaussian line broadening of 2 Hz was used in the two dimensions and, in the $t_1$ dimension, FID data were zero-filled to 512 points to obtain the final data matrix of 512 × 512 real points. The sweep width was ±722.5 Hz with a digital resolution of 2.82 Hz. In the low power transmitter mode, 2 watts of r.f. power was used (90° pulse, 30 $\mu$s). The 2D data sets were transferred *via* a high speed parallel data link to a DEC VAX computer for processing with the FTNMR program of Dennis Hare (Infinity Systems, Seattle, Washington).

1D Nuclear Overhauser effects were measured in the steady state by the difference method where the on-resonance irradiated spectrum was subtracted from the one in which the irradiation frequency was off-resonance shifted. A 3-s irradiation pulse was used for saturating the resonance. Quantitative n.O.e. values were obtained by integration where the intensity of the saturated resonance was set

to a single proton (with appropriate scaling for incomplete saturation). For isolated resonances, the integrated intensities were verified by scaled substraction of the irradiated spectrum from the off-resonance shifted spectrum.

## RESULTS

In the COSY spectrum of the blood group A-tetrasaccharide alditol **1** in dimethyl sulfoxide ($Me_2SO$) (Fig. 1), the signals of H-1$\alpha$ of Fuc and GalNAc are at $\delta$ 5.090 and 4.956, respectively, and that of H-1$\beta$ of Gal is at $\delta$ 4.412. The chemical shift dispersion of ~1.1 p.p.m. for the rest of the methine protons permitted a complete assignment of the ring protons of the four residues, including the alditol residue. A comparison of the chemical shifts for the solution in $Me_2SO$ given in Table I with these for the solution[6] in $D_2O$ showed an upfield shift of all resonances by amounts varying from 0.10 to 0.30 p.p.m. The different relative shifts resolved the overlapping resonances of Gal H-4 and GalNAc H-2 [$\Delta\delta$ 0.025 ($D_2O$) and 0.2 p.p.m. ($Me_2SO$)] as well as that of Gal H-2 and GalNAc H-3 [$\Delta\delta$ 0.03 ($D_2O$) and 0.1 p.p.m. ($Me_2SO$)]. These chemical-shift dispersion proved to be very useful for n.O.e. measurements, since both resonances in a given pair showed n.O.e. simultaneously to the anomeric protons of GalNAc and Fuc, respectively. Despite the chemical-shift differences outlined above, there are similarities between spectra for solutions in $D_2O$ and $Me_2SO$. For example, the signal for Fuc H-1 is downfield of that for GalNAc H-1 and has a line shape that is distorted by the strong coupling of H-2 and H-3, as is the case for the solution in $D_2O$. All the nonanomeric proton resonances were upfield of that of H-1$\beta$ (Gal H-1). The two geminal H-6 of Gal and GalNAcol, as well as H-1 of GalNAcol remained strongly coupled in the two solvents.

The COSY of tetrasaccharide **1** in pyridine is shown in Fig. 2. The scalar-coupled networks for Fuc, GalNAc, and GalNAcol were readily obtained in the COSY spectrum. However, for Gal the close proximity of the H-3 and H-4 resonances precluded assignment of that for H-4, and this resonance was assigned by the 1D-HOHAHA technique. The chemical-shifts of tetrasaccharide **1** in pyridine are given in Table I. In comparison to the resonances for the solution in $Me_2SO$ (Table I), there is a deshielding of all the ring protons of which the $\alpha$-GalNAc resonances showed the largest down-field shift by as much as 1.2 p.p.m. As a result, the signal of GalNAc H-1 was downfield of that for Fuc H-1, in contrast to the observations for the solution in $D_2O$ (ref. 6) as well as in $Me_2SO$. Contrary to the rule in carbohydrate n.m.r. spectroscopy that the anomeric proton resonances are usually at a lower field than the nonanomeric signals, the resonance assigned to GalNAc H-2 was downfield of that of Gal H-1$\beta$. Gal H-5 and the two geminal H-6 protons form an AMX pattern unlike the case in the other two solvents where they form a tightly coupled system.

For the blood group H hexasaccharide **2** in pyridine, the spin-coupled resonances were assigned by COSY and the 2D-HOHAHA method. In the 2D-

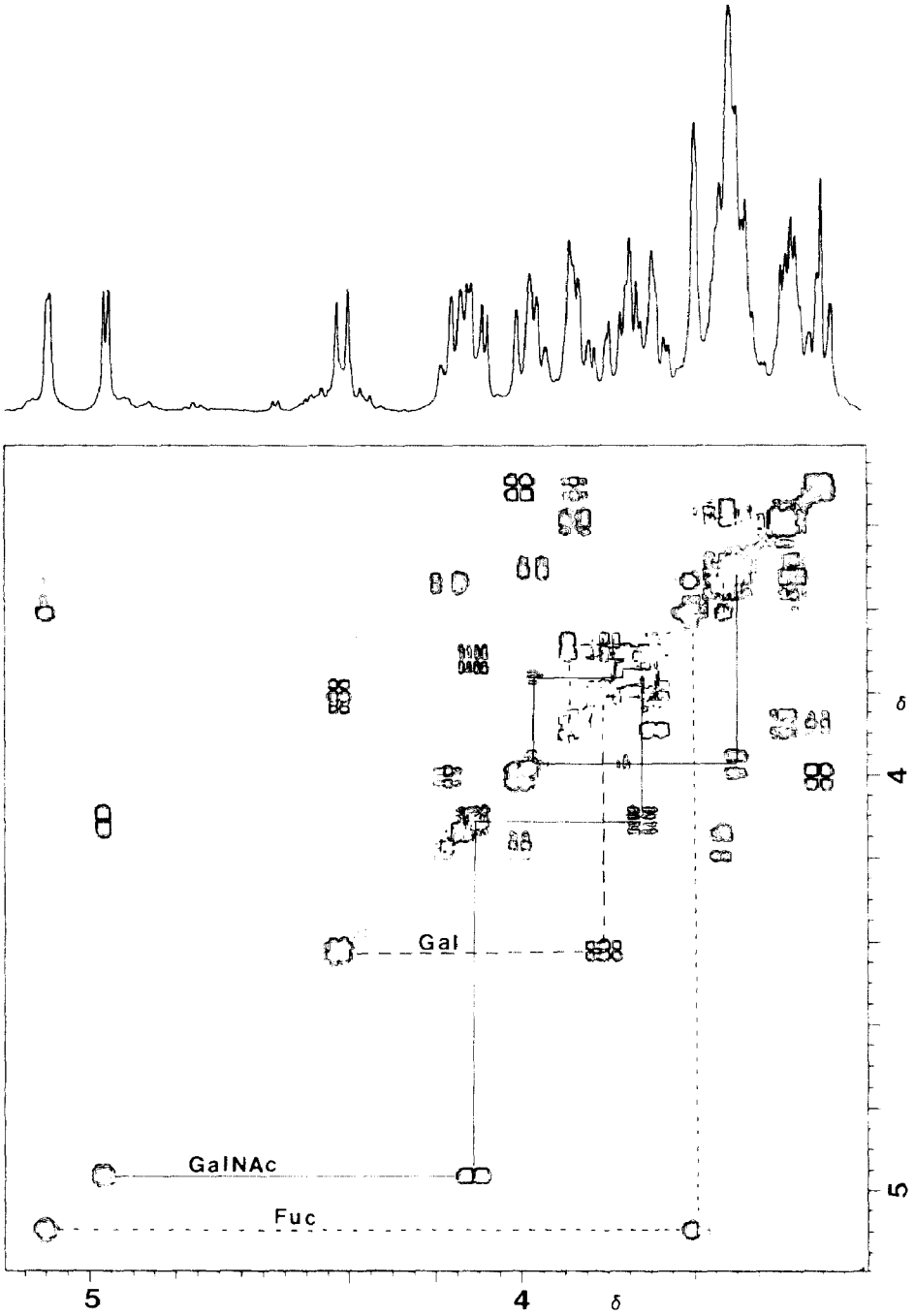



Fig. 1. 300-MHz Partial $^1$H-n.m.r. COSY spectrum of the blood group A tetrasaccharide alditol (1) in $Me_2SO$ at 24°. The full spectrum consists of 512 × 512 real data points over a spectral width of ±680.27 Hz (digital resolution 2.65 Hz).

TABLE I

300-MHz, $^1$H-N.M.R. CHEMICAL SHIFTS ($\delta$)[a] OF THE BLOOD GROUP A TETRASACCHARIDE ALDITOL (**1**) AND THE BLOOD GROUP H-HEXASACCHARIDE ALDITOL (**2**) AT 24°

| *Residue* | *H-1* | *H-2* | *H-3* | *H-4* | *H-5* | *H-6a,b* |
|---|---|---|---|---|---|---|
| *Compound* **1** *in* ($^2H_3$)$Me_2SO$ | | | | | | |
| Fuc | 5.090 | 3.60 | 3.60 | 3.540 | 4.150 | 1.105 |
| Gal | 4.412 | 3.810 | 3.682 | 3.895 | 3.350 | 3.52 |
| GalNAc | 4.956 | 4.110 | 3.723 | 3.755 | 3.965 | 3.50 |
| GalNAcol | 3.54 | 4.166 | 3.995 | 3.310 | 3.875 | 3.39 |
| *Compound* **1** *in* ($^2H_5$)*pyridine* | | | | | | |
| Fuc | 5.711 | 4.513 | 4.600 | 4.200 | 4.556 | 1.521 |
| Gal | 5.221 | 4.675 | 4.500 | 4.53 | 3.914 | 4.020, 4.451 |
| GalNAc | 5.982 | 5.300 | 4.891 | 4.796 | 4.955 | 4.500 |
| GalNAcol | 4.262 | 5.245 | 5.100 | 4.265 | 4.955 | 4.305 |
| *Compound* **2** *in* ($^2H_5$)*pyridine* | | | | | | |
| Fuc **3** | 5.591 | 4.617 | 4.510 | 4.173 | 4.447 | 1.598 |
| Fuc **4'** | 5.863 | 4.63 | 4.63 | 4.280 | 4.690 | 1.541 |
| Gal **2** | 5.161 | 4.501 | 4.180 | 4.294 | 4.045 | 4.197, 4.551 |
| Gal **3'** | 5.108 | 4.560 | 4.190 | 4.412 | 4.05 | 4.33 |
| GlcNAc **2'** | 4.968 | 4.584 | 4.30 | 4.31 | 3.84 | 4.42 |
| GalNAcol **1** | 4.27 | 5.240 | 5.060 | 4.009 | 4.989 | 4.121, 4.439 |

[a]Chemical shifts are with reference to the internal signal of $Me_4Si$. Although the spin connectivities were established by COSY, wherever possible, accurate chemical shifts (±0.003 p.p.m.) were obtained either by 1D-spin difference decoupling or 1D-HOHAHA experiments. For strongly coupled resonances, however, accuracy is ±0.01 p.p.m.

HOHAHA spectrum (see Fig. 3), cross-peaks occurred between resonances which are directly J-coupled, as in COSY, as well as those which are remotely coupled within the same spin system. If spins *A* and *M* are scalar coupled, during the mixing period, magnetization propagates from proton A to M at a rate determined by $J_{AM}$. If *M* is coupled to the proton X, the magnetization of *A* is relayed to X (even if $J_{AX} = 0$) during the MLEV-17 mixing period *via* the spin *M*. For sufficiently long mixing-times, magnetization propagates from one proton to all the other protons which are within the scalar-coupling network. This technique is analogous to the TOCSY experiment[16]. In Fig. 3, for each diagonal peak, the scalar-coupled resonances in the spin system were obtained by examining the sections either along the *F1* or *F2* axis (see, *e.g.*, Fig. 4). Thus, the two fucosyl $\alpha$-anomeric proton resonances showed cross-peaks to H-3 and H-4, as did the two $\beta$-anomeric signals of Gal. The connectivity of H-1 to H-5, and further to H-6, was not observed owing to the small scalar coupling between H-4 and H-5 ($J_{4,5} \sim 1.0$ Hz) for both Fuc and Gal. For GlcNAc, for which $J_{4,5}$ is larger (9.6 Hz), cross peaks were observed for all resonances from H-1 to H-6. Owing to the strong coupling of H-4 and H-5, as well as of the two H-6's, not all cross peaks were at distinct frequencies. In Fig. 4 are shown cross-sections along the *F1* axis for the two fucosyl and the GlcNAc

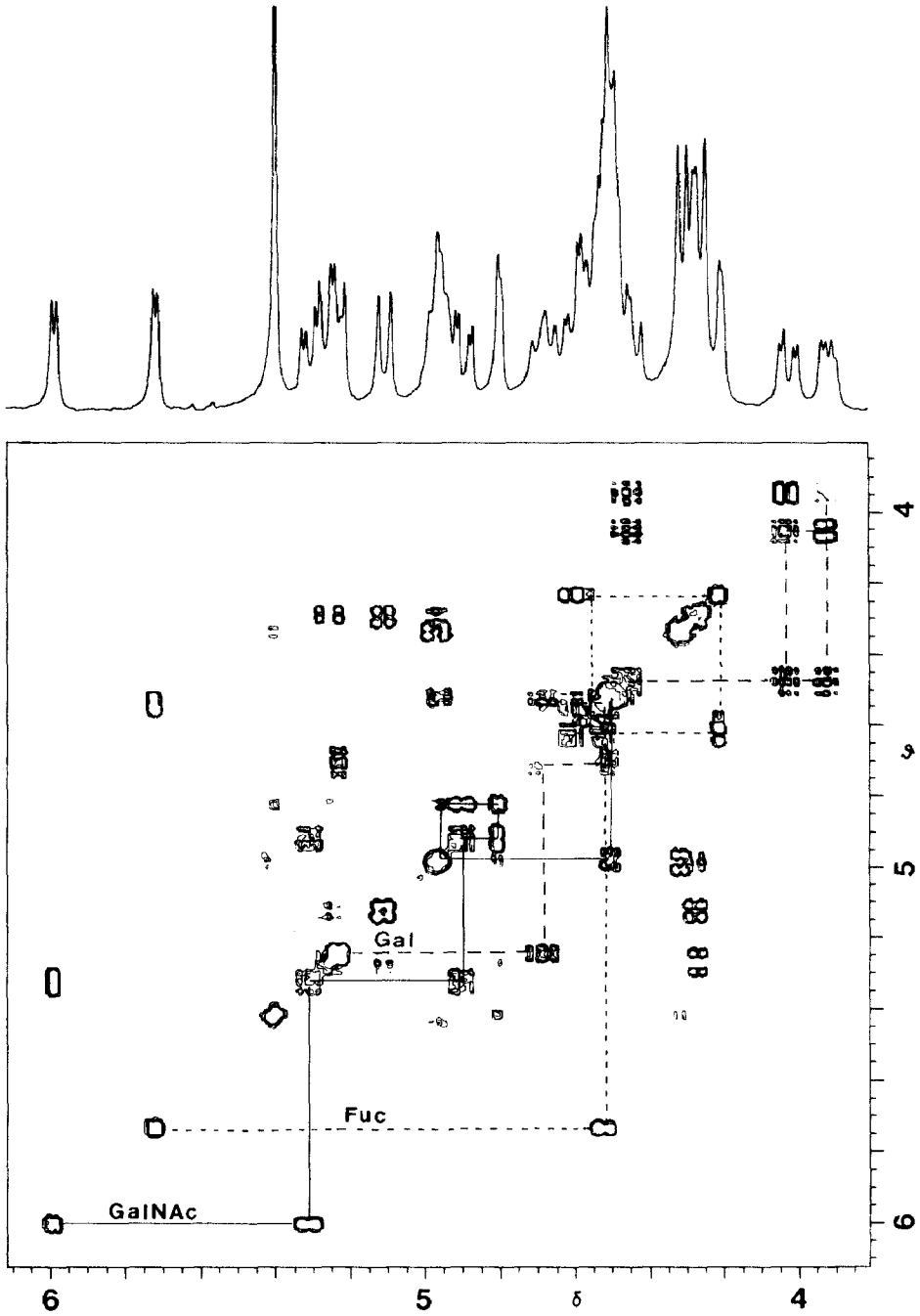



Fig. 2. 300-MHz Partial $^1$H-n.m.r. COSY spectrum of the blood group A-tetrasaccharide alditol (**1**) in pyridine +4% $D_2O$ at 24°. The full spectrum consists of 512 × 512 real data points with a sweep width of ±750.5 Hz (digital resolution 2.92 Hz).

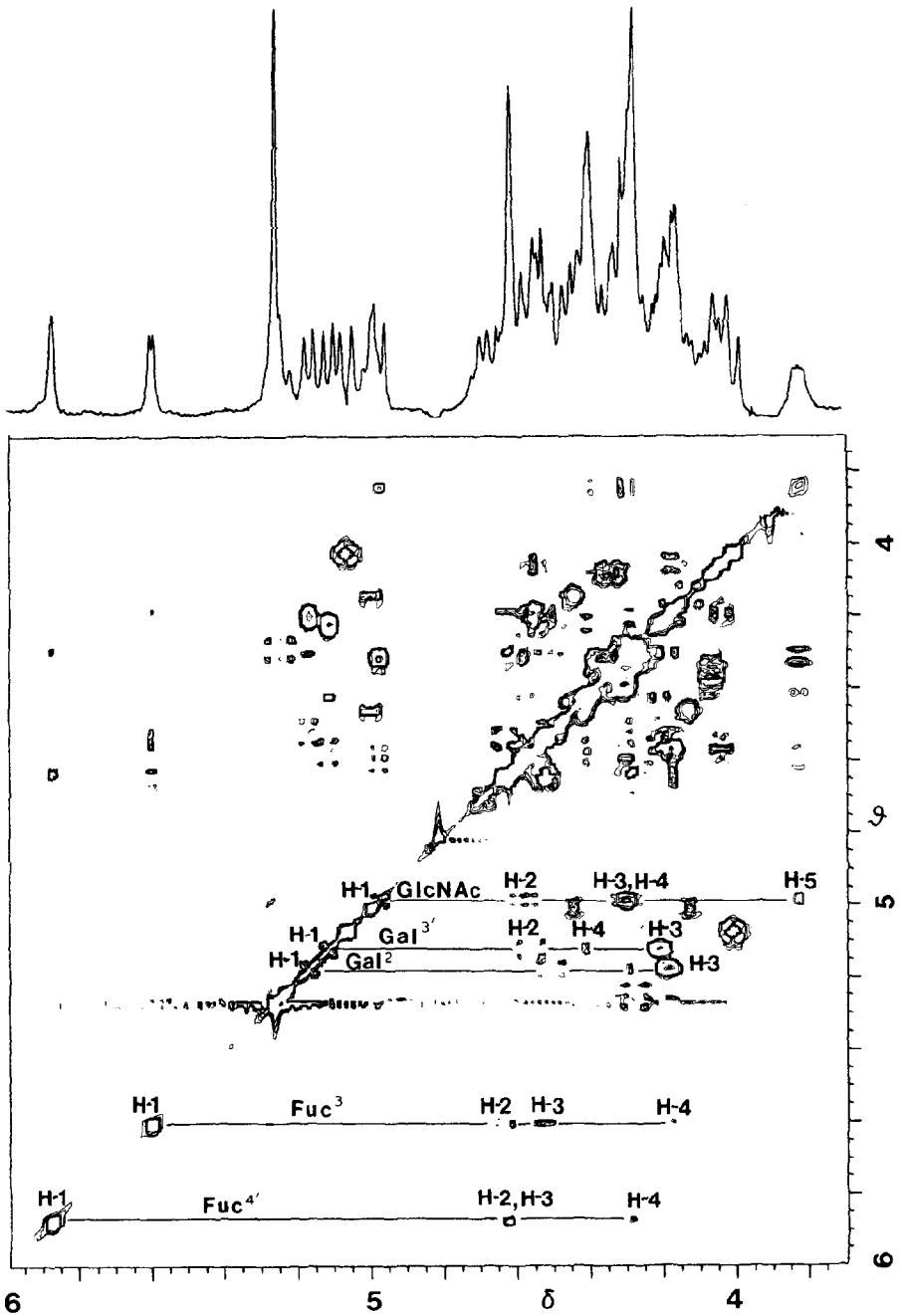



Fig. 3. 300-MHz Partial $^1$H-n.m.r. 2D-HOHAHA spectrum of the blood group H-hexasaccharide alditol (**2**) in pyridine +2.5% $D_2O$ at 24°. The total spin-propagation time including two 2.5 ms spin-lock pulses at the beginning and end of the [MLEV-17] sequence is 55 ms. The full data matrix consists of 512 × 512 real points after zero-filling along $t_1$ (digital resolution 2.9 Hz/point).

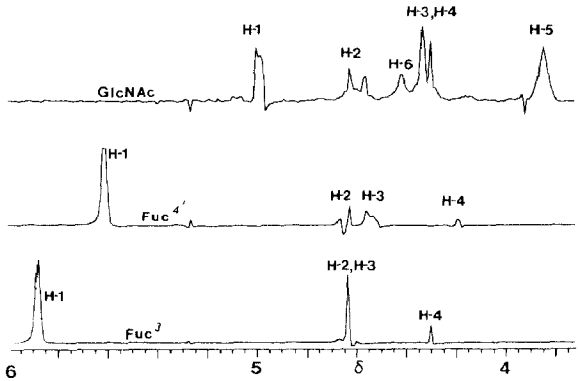



Fig. 4. Cross-sections along the *F2* axis in the 2D-HOHAHA spectrum of Fig. 3: (a) Section along GlcNAc H-1; (b) section along Fuc$^{4'}$ H-1; and (c) section along Fuc$^{3}$ H-1. Note that in (a) connectives involving spins H-1···H-6 are observed.

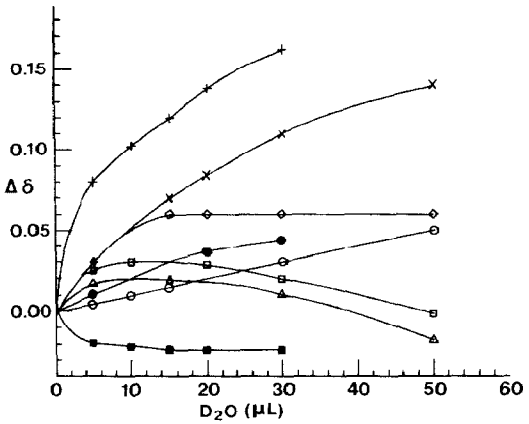



Fig. 5. Plot of $\Delta\delta$ (relative shifts) *vs.* added $D_2O$ for **1** in pyridine: (+) Gal H-3; (×) N-Acetamido methyl; (◇) GalNAc H-3; (●) Gal H-1; (○) Fuc H-1; (□) GalNAc H-1; (△) GalNAc H-2; and (■) Fuc H-3.

anomeric protons. These cross sections are analogous to the 1D-HOHAHA spectra in their information content and have a very similar appearance to 1D HOHAHA difference spectra. The line shapes are slightly distorted from pure absorptive in both the cross-sections of the phased 2D spectra[12], as well as in the 1D difference spectra[11]. The chemical shifts of **2** in pyridine are given in Table I.

A comparison of the chemical shifts of hexasaccharide **2** in $D_2O$ (ref. 17) and pyridine showed a deshielding of all the resonances by differing amounts in the range of 0.30–0.50 p.p.m. Despite the chemical shift differences, certain characteristic features remain in the two spectra. For example, the signal of Fuc$^{4'}$ H-1 is downfield from that of Fuc$^{3}$ H-1 and showed the distorted line shape resulting from the strong coupling of Fuc H-2 and H-3. Also, GlcNAc H-3 and H-4 showed a strong coupling which arose from substitution of GlcNAc at O-4. In both

solvents, the chemical equivalence of the geminal $H_2$-6 of GalNAcol was removed by the substitution at O-6. A notable difference, however, was the appearance of an AMX pattern for Gal[2] H-5 and the $H_2$-6, as in the spectra of **1**. On the other hand, the signals for GlcNAc H-5 and H-6, which form an AMX subsystem in $D_2O$, lost the first-order spectral nature because of strong coupling of the geminal $H_2$-6.

The chemical shifts of tetrasaccharide **1** in pyridine were very sensitive to the water content of the solution. A plot (Fig. 5) showing the chemical shifts as a function of added $D_2O$ from 0 to 12.5% by volume showed varying downfield shifts for most of the resonances with deshieldings of 0.02–0.18 p.p.m. For Fuc H-3, an upfield shift with increasing hydration was observed, whereas the signals for GalNAc H-1 and H-2 showed a downfield shift initially (by 0.02 p.p.m.) followed by a reversal in the direction of the shift beyond a 4% $D_2O$ content. Advantage was taken of this dependence of chemical shifts on added water by adding small amounts of water to the pyridine solution in order to resolve overlapping signals of Gal H-1, GalNAc H-2, and GalNAcol H-3 (see Fig. 2). In contrast to the effect of water on the chemical shifts for the solution in pyridine, the chemical-shift perturbations resulting from the addition of water (in the range of 0–12.5% of $D_2O$ by volume) to a $Me_2SO$ solution were much less significant. The chemical-shift changes were a maximum for the amide methyl resonances (0.02 p.p.m.).

For both spectra of **1** and **2**, the change of solvents led to large chemical-shift perturbations. These chemical-shift differences arise from the different effective magnetic fields experienced by the nuclei due to different local magnetic susceptibilities in the solvent media. Additionally, chemical-shift differences could also result from conformational changes that affect the magnetic environment around a given nucleus. However, unlike chemical shifts, n.O.e. is not sensitive to bulk magnetic susceptibility of the solvent and depends only on the geometry and rotational correlation-time of the molecule. Therefore, any change in the relative magnitudes of n.O.e. values offers direct evidence for a solvent-mediated conformational transition. For tetrasaccharide **1**, the solvent dependence of chemical shifts offered an advantage as some of the overlaping resonances that show n.O.e. to the same saturated proton were resolved because of different relative displacements.

For a solution in $D_2O$, the observed n.O.e. values of the tetrasaccharide **1** at 24° were positive, and the largest effects of the order of 10–12%. On saturation of Fuc H-1, 11% n.O.e. was observed at the intraring Fuc H-2, and a 10% effect at Gal H-2. There were two other smaller n.O.e.'s, one at GalNAc H-3 (~3%) and the other at GalNAc H-5 (2%). These small effects were outside the experimental error limits (±1%), the source of which have been discussed elsewhere[18]. The n.O.e.'s between the two nonneighboring residues were especially useful for model building, as they restricted the permissible values of two pairs of glycosidic torsion angles. On saturation of GalNAc H-1, the intraring n.O.e. at GalNAc H-2 was

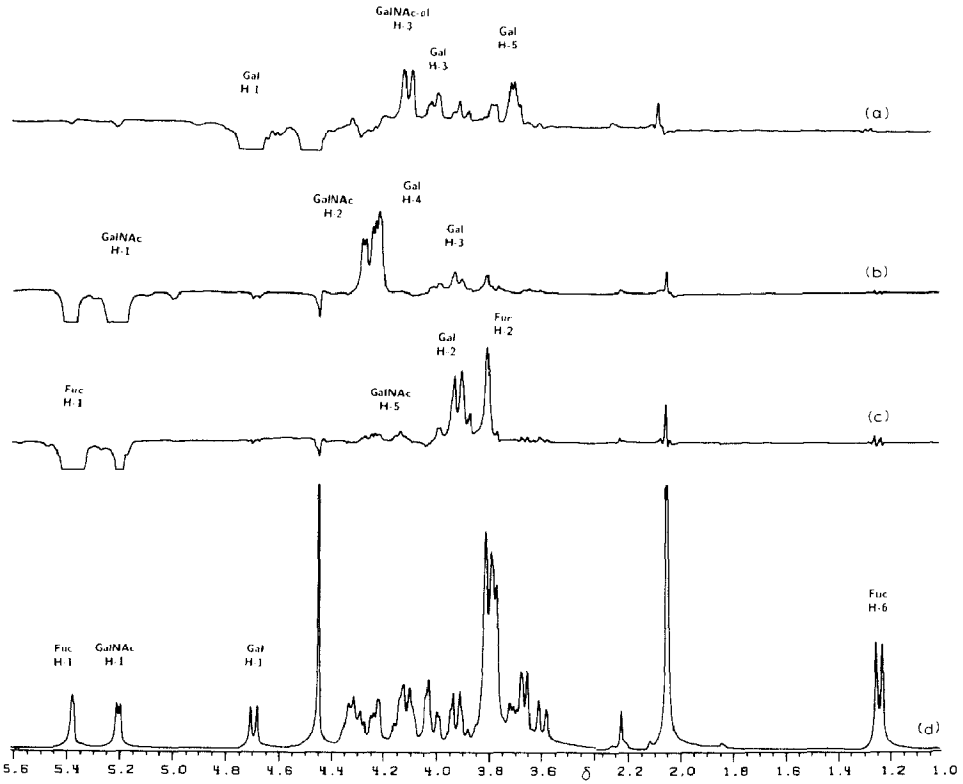



Fig. 6. 300-MHz 1D-n.O.e. difference spectra of tetrasaccharide **1** in $D_2O$ at 60°: (a) Saturation of Gal H-1, (b) saturation of GalNAc H-1, (c) saturation of Fuc H-1, and (d) normal spectrum.

10%, and 8% at Gal H-4. Although the GalNAc group is (1→3)-linked to the Gal residue, the magnitude of the effect at H-3 (3%) was far less than at Gal H-4. These effects are in contrast to the general observation that the interresidue n.O.e. is predominantly at the proton across the glycosidic linkage. The conformational implications of this effect have been studied by Bush *et al.*[18], and such effects have also been observed for the blood group A trisaccharide[19] and B-active glycolipids[4], where the larger n.O.e. is at the equatorial H-4 of the (1→3)-substituted Gal residue. At 60°, larger positive n.O.e.'s were observed because of decreased rotational-correlation time resulting from temperature dependence of viscosity as well as loss of bound water[17]. Otherwise, the observed effects at both room temperature and 60° were essentially similar. The observed n.O.e. values for tetrasaccharide **1** at 60° are shown in Fig. 6.

For the tetrasaccharide **1** in $Me_2SO$ solution, negative n.O.e. values were observed at room temperature, as result of the slower tumbling of the molecule in the relatively viscous solvent (Fig. 7). A comparison of the observed n.O.e. for solutions in $D_2O$ and $Me_2SO$ showed that the ratios of the interring-to-intraring effects in the two solvents were not only quite similar, but the non-nearest neighbor

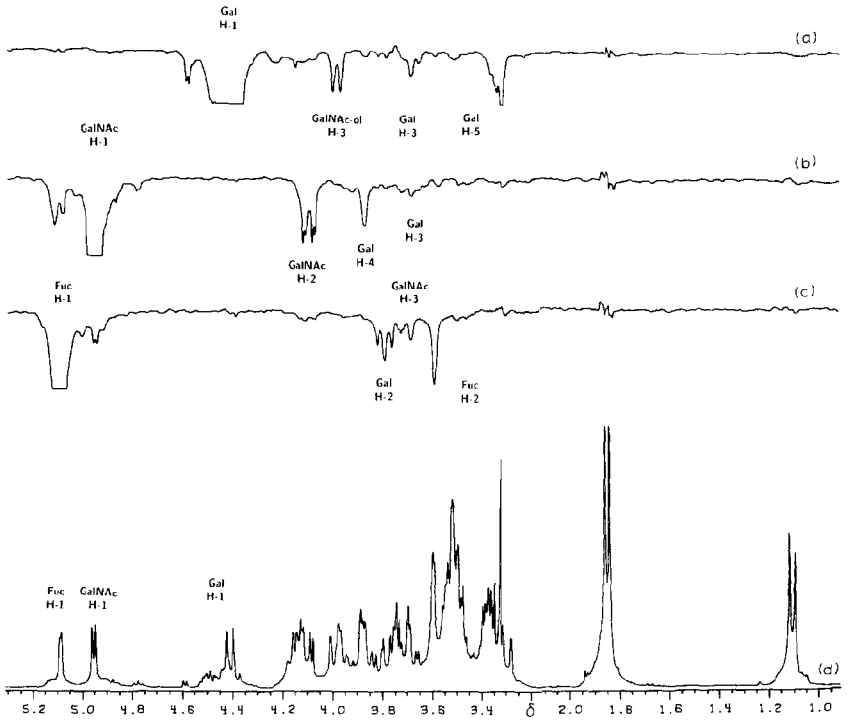



Fig. 7. 300-MHz 1D-n.O.e. difference spectra of tetrasaccharide **1** in $Me_2SO$ at 20°: (a) Saturation of Gal H-1, (b) saturation of GalNAc H-1, (c) saturation of Fuc H-1, and (d) normal spectrum.

effects between Fuc H-1, and GalNAc H-3 and H-5 were also present. Measurements of n.O.e. for this oligosaccharide in $Me_2SO$ were also carried out at 60° (Fig. 8). In contrast to the data obtained at room temperature, the observed n.O.e. values were positive at higher temperature. Comparison of Figs. 7 and 8 showed that the n.O.e.'s are qualitatively very similar, except for the change of sign. On saturation of Fuc H-1, GalNAc H-3 (3%) and H-5 (1%) showed small effects, as at 15°, and also the ratio of interresidue to intraresidue was nearly equal at the two temperatures for both linkages. The observation of small n.O.e.'s at GalNAc H-3 and H-5, at both low and high temperatures, implied a substantial degree of conformational rigidity, as these effects occur among protons whose interproton distances are sensitive to variation of any of the four glycosidic torsional angles[8].

For tetrasaccharide **1** in pyridine, the room temperature n.O.e.'s were very small, as the rotational correlation times were $\sim 1/\omega$, the reciprocal of the spectrometer frequency. Hence, n.O.e. experiments were done at 60° to take advantage of the strong dependence of the rotational correlation time on temperature[17] (Fig. 9). For saturation of Fuc H-1, the predominant n.O.e.'s were at Fuc H-2 (8%) and Gal H-2 (11%). The smaller effects at GalNAc H-3, and H-5, observed for the solutions in both $D_2O$ and $Me_2SO$, were absent for the solution in pyridine. However, for the

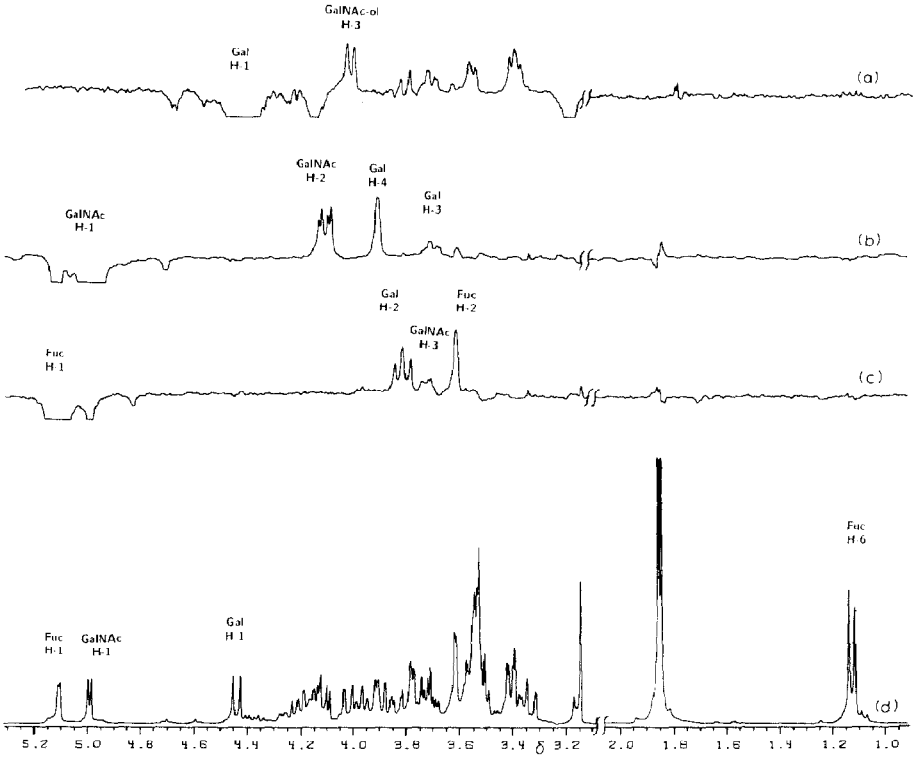



Fig. 8. 300-MHz 1D-n.O.e. difference spectra of tetrasaccharide **1** in $Me_2SO$ at 60°: (a) Saturation of Gal H-1, (b) saturation of GalNAc H-1, (c) saturation of Fuc H-1, and (d) normal spectrum.

GalNAc-(1→3)-Gal linkage, the larger of the two interresidue n.O.e.'s was observed at Gal H-4 (9%), as compared with 2% at H-3, indicating that the conformation around this linkage was still similar to that observed for the solutions in $D_2O$ and $Me_2SO$. In order to examine the effect of water content in pyridine on the conformation of **1**, n.O.e. measurements done for a 4% water content were compared to those measured for dry pyridine at 60°. Under dry conditions, the observed values of 10% at GalNAc H-2, 7% at Gal H-4, and 4% at GalNAc H-3 on saturation of GalNAc H-1; and 13% at Gal H-2 and 9% at Fuc H-2 on saturation of Fuc H-1, were in good agreement with those measured for the solution containing 4% $D_2O$.

At room temperature, negative n.O.e.'s were observed for hexasaccharide **2** in pyridine. In order to obtain larger n.O.e. effects, measurements were done at 15°. On saturation of $Fuc^{4'}$ H-1, the intraring Fuc H-2 (−10%) and the interring Gal H-2 (−8%) showed comparable effects, whereas saturation of $Fuc^{3}$ H-1 caused a slightly larger interring n.O.e. at Gal H-2 (−18%) than the intraring effect at Fuc H-2 (−13%). These observations were similar to those made for the solution[17] in $D_2O$. Additional n.O.e.'s observed included a −16% n.O.e. at GlcNAc H-4 on saturation of $Gal^{3'}$ H-1 and several intraring effects in the two Gal and the GlcNAc

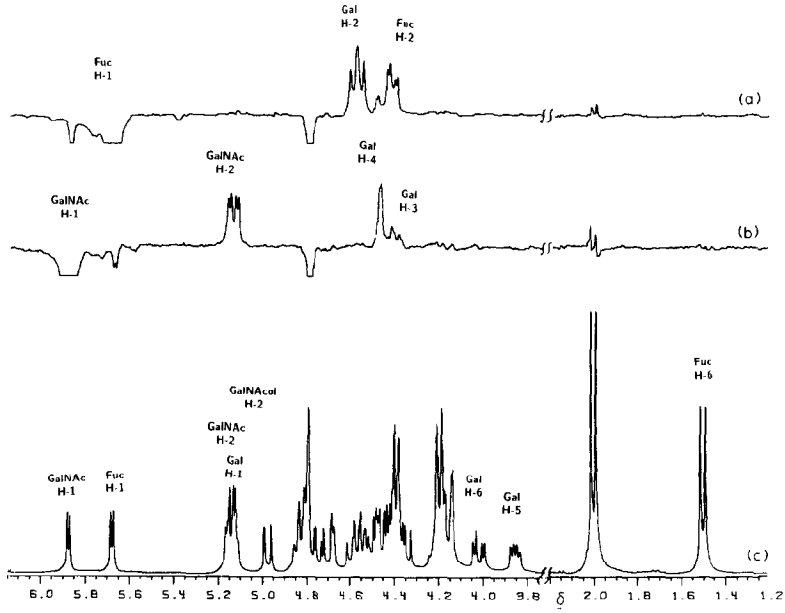



Fig. 9. 300-MHz 1D-n.O.e. difference spectra of tetrasaccharide **1** in pyridine at 60°: (a) Saturation of Fuc H-1, (b) saturation of GalNAc H-1, and (c) normal spectrum.

residues. For both solutions in $D_2O$ and pyridine, the interresidue n.O.e.'s were only to the next nearest neighbor, unlike for tetrasaccharide **1**. The similarities in the observed n.O.e.'s for both solutions in $D_2O$ and pyridine indicated no significant change in the molecular conformation as a result of change in molecular environment[8].

## DISCUSSION

With the help of modern high-field n.m.r. spectrometers, it is possible to elucidate carbohydrate primary structures, even without the help of structural reporter groups and chemical-shift analogies to closely related oligosaccharide structures, provided that the sample quantities are sufficient to obtain complete proton assignment. For pyranosides, the coupling constants give an unambiguous identification of the axial and equatorial protons, and hence of the sugar molecular geometry. Unambiguous distinction between neutral and acetamido sugars, such as Gal and GalNAc, is possible by assignment of the amide proton resonances of the latter residue by decoupling to the assigned H-2 signals[6]. Measurement of n.O.e. between protons of adjacent residues provides unambiguous evidence for identity of residues that are linked, although identification of the linkage position requires careful consideration of both the n.O.e. and of the chemical shifts of the protons of the sugar residue linked at a nonanomeric oxygen atom.

In the present study, complete proton assignment was done by $^1$H-n.m.r. at

300 MHz using some of the more recent methods. Although COSY provides most of the direct *J*-connectivities, resonance overlaps and strong coupling lead to ambiguities in obtaining the complete scalar-coupled network. Also, cross-peaks between resonances having similar chemical shifts are generally not well resolved from the broad diagonal peaks, precluding assignment of those resonances. In the 2D-HOHAHA method, which is especially useful for complete assignments of protons of carbohydrates that contain chains of mutually coupled spins, both the aforementioned problems are eliminated as a single cross section along the *F1* or *F2* axis for a given resonance provides the entire *J*-connectivity within the spin network. This is illustrated in Fig. 4 which shows cross sections for the anomeric resonances of the GlcNAc and the two Fuc residues of the blood group H hexasaccharide **2**.

Several points must be considered in comparing the relative sensitivity of the COSY and HOHAHA methods. As a result of the partial decay of magnetization during the spin-locking interval, there is a loss of sensitivity in 2D HOHAHA, as compared to COSY, and the intensity is spread among more crosspeaks in the former method. On the other hand, the absorptive character of the phased HOHAHA spectrum permits the use of either an exponential or a Gaussian apodization function, which does not severely degrade the signal-to-noise ratio as does the sine bell apodization commonly used in the magnitude COSY data processing[15]. Although the 2D-HOHAHA experiment is absorptive, there is anti-phase dispersive character in the spin multiplets which causes distortion of the multiplet structure.

In cases where only a few spin connectivities are to be obtained, 1D-HOHAHA serves as a good alternative to the 2D-method, owing to substantial reduction in the experimental time. This method has the advantage that one can assign resonances in a sample that is not in quantity sufficient for 2D experiments. In our observations on the oligosaccharides, one can derive exactly the same information either by use of a 1D- or 2D-HOHAHA experiment. However, 1D-HOHAHA is suitable only for cases in which one of the resonances of a spin system is well isolated, as the selectivity of the inversion pulse is generally not less than ±20 Hz. Similar to the case of the cross section from the 2D-HOHAHA spectra shown in Fig. 4, the spin-coupled resonances exhibit a distorted multiplet shape due to partial dispersive component in the line shape.

The use of n.O.e. for either structural correlations or for conformational studies requires that the effect be measured. Since the rotational correlation times of tetra- to hexa-saccharides in $D_2O$ are generally of the order of the reciprocal of the spectrometer frequency (300 to 500 MHz) at room temperature, the observed n.O.e.'s are quite small. An approach to overcoming the problem of unfavorable rotational-correlation time for observing n.O.e. is the transient n.O.e. method in the rotating frame (CAMELSPIN) suggested by Bothner-By *et al.*[20]. In this method, the n.O.e. is always positive and the magnitude increases with increasing rotational-correlation time of the molecule. However, we have shown here and in

earlier work that it is generaly possible to observe ordinary n.O.e.'s for oligosaccharides in $D_2O$ by changing the temperature, and in the present work we have shown that this approach is effective in two non-aqueous solvents. Although 1D difference spectroscopy for measurement of n.O.e. is more difficult and requires better instrument stability than NOESY, it is generally more suitable for experiments on modest-sized oligosaccharides whose rotational correlation times are close to the spectrometer frequency. The rotational correlation times of these oligosaccharides are determined by the solvent viscosity, which in turn depends on the temperature. The ratios of the n.O.e. are similar for the different solvents, so we concluded that the conformation is not strongly dependent on the solvent and that explanations other than conformational differences must be sought to explain the differences observed in chemical shifts.

The chemical-shift perturbations observed for the two non-aqueous solvents result from the different magnetic susceptibilities of the solvent media. In pyridine, such local perturbations result from diamagnetic anisotropic effects due to the $\pi$-ring system. These cause either shielding or deshielding depending on the direction of the plane of the ring to the vector joining the proton and the center of the ring, as well as the distance of the proton from the ring[21]. The variation of the chemical shifts on adding $D_2O$ to pyridine (shown in Fig. 5) resulted from changes in hydrogen bonding as well as local magnetic susceptibilities within the pyridine–water system. Inasmuch as the chemical shift changes result from intermolecular interactions, a conformational interpretation of these effects becomes impossible. On the other hand, the observed n.O.e.'s provide a basis for conformational-model building, as these effects depend only on the molecular geometry. Details of the calculations using the steady state n.O.e.'s to derive molecular models consistent with the experimental data are presented elsewhere[8].

Our results for the blood group A and H oligosaccharides **1** and **2** showed that the perturbation of solvent on proton chemical shifts is quite large, especially in the case of pyridine as solvent. The utility of this effect in structural studies of complex oligosaccharides is that protons, the chemical shifts of which are the same in $D_2O$ solution, may be separately observed in one of the alternative solvents discussed herein. The data of Table I showed that the resonances of the two Gal and GalNAc H-6 protons are distinct for a pyridine solution, whereas these resonances are generally strongly coupled for a $D_2O$ solution. The three solvents, $D_2O$, $Me_2SO$, and pyridine, have different macroscopic and hydrogen-bonding properties, which have different influences on the molecular interaction with the solvent. As a result of the magnetic anisotropy of the aromatic ring, pyridine causes the largest chemical-shift dispersion among the three solvents. Despite this advantage, the value of pyridine as solvent to establish structural reporter groups for determination of the structure of complex oligosaccharides is limited by the strong chemical-shift dependence on the water content. Especially for higher mol.-wt. oligosaccharides, small amounts of water might be essential for adequate solubility, which would require the exact specification of the water content for comparison of chemical shifts.

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