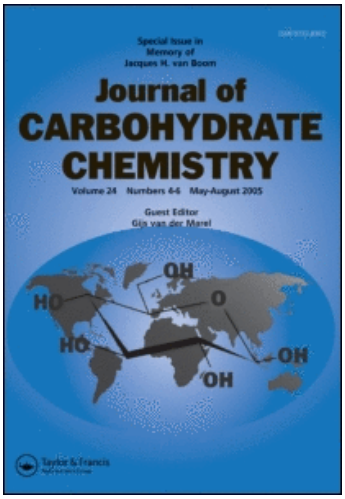


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# Structure Determination of Glycolipids Using Two Dimensional Proton NMR Spectroscopy: Globoside

T. A. W. Koerner Jr.[a]; J. N. Scarsdale[b]; J. H. Pxestegard[b]; R. K. Yu[c]
[a] Departaients of Laboratory Medicine, Yale University, New Haven, Connecticut [b] Departaients of Chemistry, Yale University, New Haven, Connecticut [c] Departaients of Neurology and Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut

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# STRUCTURE DETERMINATION OF GLYCOLIPIDS USING TWO DIMENSIONAL PROTON NMR SPECTROSCOPY: GLOBOSIDE

T. A. W. Koerner, Jr.,[1] J. N. Scarsdale,[2] J. H. Prestegard,[2] and R. K. Yu[3]*

Departments of Laboratory Medicine[1], Chemistry[2], Neurology[3] and Molecular Biophysics and Biochemistry[3], Yale University, New Haven, Connecticut, 06510

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## ABSTRACT

High resolution one and two dimensional proton nuclear magnetic resonance (2D-NMR) spectra of the glycosphingolipid globoside (the P blood group antigen) have been obtained at 500 MHz and 40-50 degrees C in dimethylsulfoxide-$d_6$-deuterium oxide (98:2 v/v). Analysis of a scalar coupling correlated spectrum (SECSY) allows assignment of resonances to residue types. Analysis of the oligosaccharide anomeric proton signals in the 3-6 ppm region of the 2D nuclear Overhauser effect (2D-NOE) spectrum of globoside reveals inter-residue couplings that allow identification of glycosidic linkage interactions and hence a primary structure. The diversity of linkage types in globoside demonstrates that oligosaccharide sequence determination via 2D-NMR spectroscopy may be considered a general approach, regardless of the type of anomeric linkage involved. Measurement of the intra-oligosaccharide and inter-oligosaccharide NOE-couplings allows an estimation of the glycosidic interproton distances for each glycosidic linkage. These distances are shown to be consistent with current views of preferred glycosidic bond conformations.



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## INTRODUCTION

Recently a combination of two dimensional NMR techniques which includes spin coupling correlated spectroscopy and cross relaxation correlated spectroscopy has been shown to be a non-destructive reliable method for the determination of oligosaccharide composition and sequence. The spin coupling correlation methods, often called coupling correlated spectroscopy (COSY) or spin echo correlated spectroscopy (SECSY), rely on through bond vicinal couplings between protons. They are very useful in assigning resonances belonging to the same sugar ring and in identifying residue type. The cross relaxation correlation methods, often called nuclear Overhauser effect spectroscopy (NOESY), rely on the same through-space dipolar relaxation mechanisms which give rise to nuclear Overhauser effects in one dimensional spectroscopy. They are very useful in establishing sequence and linkage sites. A number of examples of application of these and closely related experiments to oligosaccharides are now in the literature. Using one-dimensional NOE data, the oligosaccharide sequence of neutral glycosphingolipids, an acetylated disaccharide, and N-linked glycopeptides have been determined by Dabrowski *et al.*,[1] Berstein and Hall,[2] and Brisson and Carver,[3,4] respectively. Using two-dimensional NOE (2D-NOE) spectroscopy, we have determined the sequence and linkage sites of neutral glycosphingolipids and gangliosides.[5,6] When such sequence data are combined with composition data,[7] obtained through two-dimensional scalar coupling correlated NMR spectroscopy, the complete primary structure of an oligosaccharide may be obtained.[5,6]

Despite these successful applications, the methods as yet lack the demonstrated generality and ease of application that belong to more empirical methods of NMR structure analysis based on chemical shift correlations.[8] One question of generality relates to the fact that with the exception of some work by Brisson and Carver, studies have dealt with structures containing

only β-anomeric linkages. In this communication we test whether both α and β glycosidic linkages may be revealed through 2D-NOE spectroscopy, using the neutral glycosphingolipid globoside as a test case.

Globoside, a biomolecule interesting in its own right, was first proposed by Yamakawa and Suzuki[9] to have the tetraglycosyl ceramide structure depicted in FIG. 1, GalNac(β1-3)Gal(α1-4)Gal(β1-4)Glc(β1-1')ceramide, based on chemical and enzymatic degradation analyses. Its composition has recently been confirmed via one-dimensional[10,11] and two-dimensional proton NMR and carbon-13 NMR[12,13] spectroscopy. Globoside is the major glycosphingolipid present in the membrane of human erythrocytes from all but a small minority of individuals. Immunological studies by Marcus et al.[14,15] have shown that the P blood group substance is identical with globoside or the globoside oligosaccharide attached to other aglycones. A most intriguing finding of Kalenious et al.[19] is that a globoside-like substance present in the membrane of urinary tract epithelial cells acts as a receptor for strains of Escherichia coli responsible for pyelonephritis. Thus, individuals that are lacking membrane globoside and related structures appear to be protected against infection with pathogenic strains of E. coli.



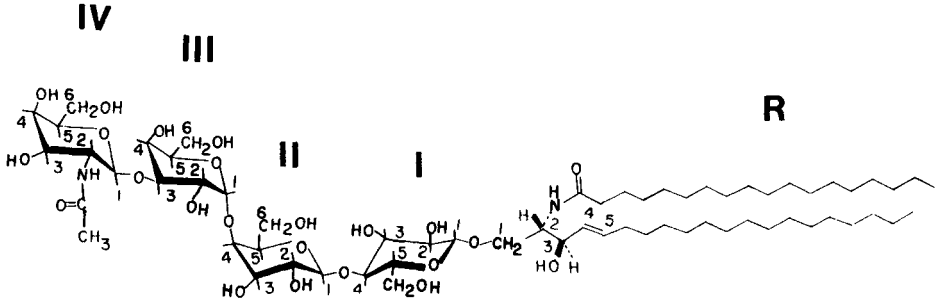


FIG. 1. Structure and nomenclature of globoside: GalNAc(β1-3)Gal(α1-4)Gal(β1-4)Glc(β1-1')ceramide.

Given the action of globoside as a receptor and determinant of antigenic activity, there is considerable interest in its tertiary as well as primary structure. In addition to providing sequence information, cross relaxation correlated spectra can provide information on interproton distances and thus place restrictions on allowed conformations. We will, therefore, also take this opportunity to discuss conformational implications of our 2D-NOE data.

## RESULTS AND DISCUSSION



The 1H NMR spectrum of globoside can, as can spectra of most glycolipids, be divided into a high field region containing largely lipid resonances, an intermediate field region containing oligosaccharide resonances, and a low field region containing olefinic resonances. The intermediate field region (3-5 ppm) is of most interest to us in this communication. A one dimensional as well as contour map of a 2D-SECSY spectrum of this region along with the olefinic region is presented in FIG. 2. Axes in the SECSY spectrum are normal chemical shift in the horizontal (F2) dimension and one half the difference in chemical shift in the vertical (F1) dimension.

A good representation of the one dimensional spectrum is found along the central horizontal ridge in the 2D-SECSY spectrum. Resonances on this ridge can be divided into subspectra representing each residue by successive construction of vertical lines from a central resonance to a cross peak, a 135 degree line to a diametrically opposed cross peak, and a vertical line back to a resonance on the central ridge. The central resonances so identified represent a spin coupled pair of protons. Repeating this procedure can in principle identify resonances from all protons on a single ring. The procedure is best begun at one of the anomeric resonances which are distinct doublets in a unique chemical shift region (4-5 ppm). The procedure identifying spin

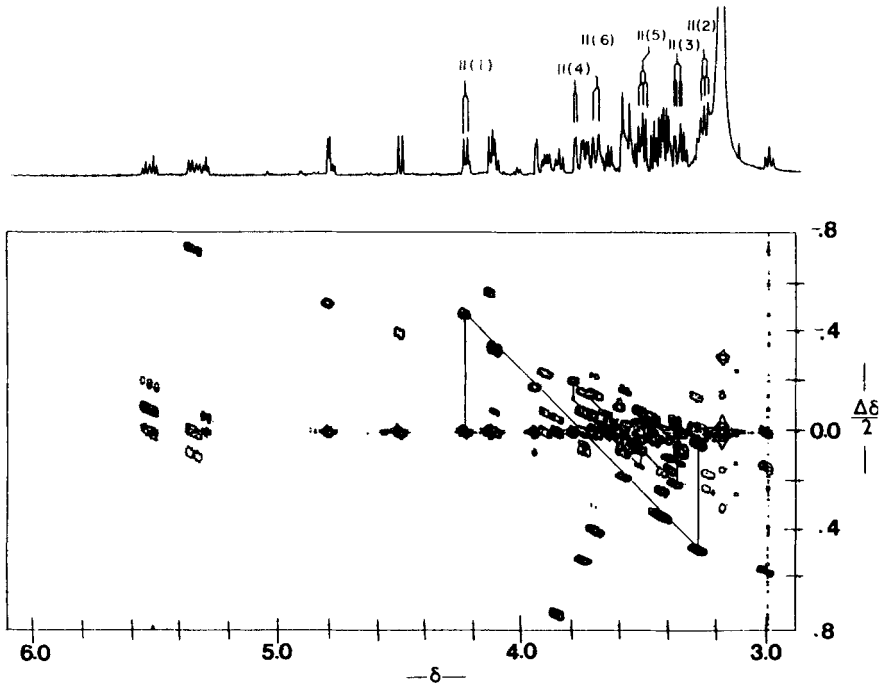


FIG. 2. Proton NMR spectra of globoside obtained at 500 MHz and 323K. A, 2D-SECSY spectrum showing construction for assignment of resonances in residue II. B, one dimensional spectrum showing resulting assignments.

coupled resonances in a single subspectrum is illustrated in FIG. 2 by the solid line. Connectivities through three or more protons can be followed for each ring. Resonances of a subspectrum are assigned to a residue type using the same chemical shift and scalar coupling correlations with model compounds used in assignment of one dimensional spectra. Confidence in assignments of subspectra resonances is, of course, greatly improved in that assignment relies not on the properties of one line or multiplet, but on the collective properties of the connected set. Any remaining resonances can usually be assigned by elimination in combination with characteristic shifts and multiplet structure.

Table 1. Proton Chemical Shifts of Globoside [a]

| Proton | Chemical Shift |
|---|---|
| IV(1) | 4.54 |
| IV(2) | 3.77 |
| IV(3) | 3.46 |
| IV(4) | 3.67 |
| IV(5) | 3.42 |
| IV(6A) | 3.60 |
| IV(6B) | 3.57 |
| III(1) | 4.82 |
| III(2) | 3.82 |
| III(3) | 3.65 |
| III(4) | 4.00 |
| III(5) | 4.15 |
| III(6) | 3.49 |
| II(1) | 4.24 |
| II(2) | 3.33 |
| II(3) | 3.43 |
| II(4) | 3.84 |
| II(5) | 3.58 |
| II(6) | 3.77 |
| I(1) | 4.14 |
| I(2) | 3.08 |
| I(3) | 3.34 |
| I(4) | 3.29 |
| I(5) | 3.29 |
| I(6a) | 3.63 |
| I(6b) | 3.76 |
| R(5) | 5.53 |
| R(4) | 5.36 |
| R(3) | 3.90 |
| R(2) | 3.82 |
| R(1a) | 3.49 |
| R(1b) | 3.88 |

a. 323 degrees K in 2% w/v $D_2O$ in DMSO. Chemical shifts are relative to internal TMS.[2] Proton labels are as indicated in FIG. 1.

Table I presents assignments based on constructions similar to that described above. Nomenclature follows that shown in the structure of FIG. 1 and chemical shifts are relative to internal TMS. All of the 1,2, and 3 proton resonances, all but one of the 4 proton resonances(I4), and one of the 5 proton resonances had been previously assigned by Dabrowski et al. using one dimensional spin decoupling methods.[10] Our assignments confirm as well as extend their assignments.

Shown in FIG. 3A is the contour plot of the 2D-NOE spectrum of globoside between 3 and 6 ppm. The presentation in terms of axes is the same as the SECSY experiment with chemical shift along the F2 axis and difference in chemical shift along the F1 axis. Cross peaks, however, now correspond to through-space dipolar relaxation connectivities. The connectivities originating in the anomeric region (4-5 ppm) are most clearly resolved and assigned. Inter-residue (glycosidic) as well as intra-residue NOE couplings are labeled below (FIG. 3A) and the one dimensional proton spectrum between 3 and 6 ppm is shown for reference in FIG. 3B. The NOE couplings of the anomeric protons of globoside are reported in Table 2.

The observation of interresidue couplings serves to establish the n-1 oligosaccharide residue and specific glycosidation site to which the anomeric proton of the nth residue is linked. As previously pointed out,[6,7] when such information is known for each residue (each anomeric proton) and combined with compositional data, the sequence of the oligosaccharide is obtained. Thus for globoside the following interresidue NOE couplings are observed (Table 1): I-1 -> R-1a, II-1 -> I-4, III-1 -> II-4, and IV-1 -> III-3. Combination of these sequence fragments yields the structure: GalNAc(β1-3)Gal(α1-4)Gal(β1-4)Glc(β1-1')ceramide, in agreement with that proposed by Yamakawa and Suzuki.[9]

Of particular importance is that one of the four interresidue NOE-couplings (III-1 -> II-4) is across an α-linkage and is of an intensity comparable to that of the three β-linkages.

It is not obvious that strong cross relaxation pathways should exist between anomeric and aglyconic protons in both β and α anomers. Because of the steep distance dependence of the dipolar cross relaxation mechanism ($1/r^6$), these protons, at opposite sides of the glycosidic linkage would have to be at similar short distances in both anomers for strong pathways to exist. Simple inversion of C-O and C-H bonds at the β anomeric center, to produce the α anomer, would dramatically increase the anomeric-aglyconic proton distance if compensating changes in

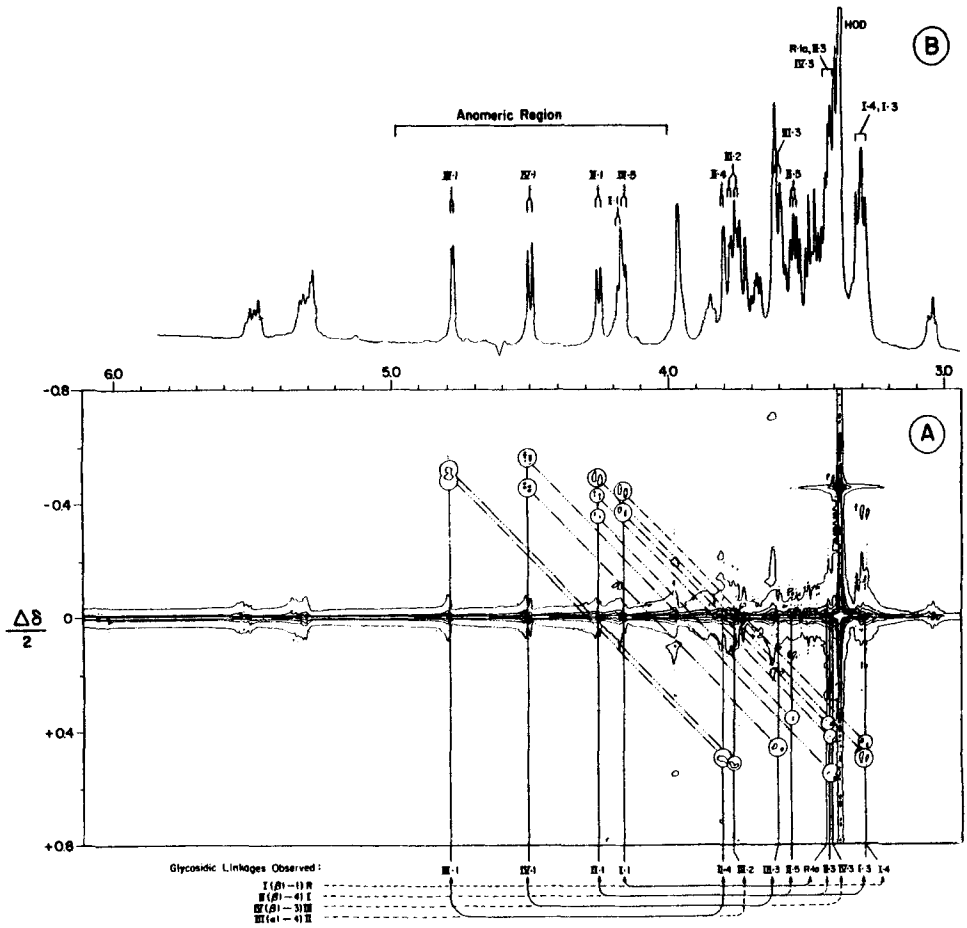


FIG. 3. Proton NMR spectra of globoside obtained at 500 MHz and 313K. A, 2D-NOE spectrum showing intra- and inter-residue NOE couplings of the anomeric protons of the oligosaccharide residues: I(-.-), II(-..-), III(-...-) and IV(-....-). Anomeric couplings are circled for emphasis. At the bottom inter-residue (glycosidic) couplings are labeled with solid lines and intra-residue couplings with dashed lines. B, one-dimensional spectrum with labeled anomeric resonances and the upfield region to which they are coupled.

Table 2. Observed NOE Couplings and Interprotonic Distances (r) Involving the Anomeric Protons of Globoside[a]

| Anomeric Signal | Upfield Signal | Interpretation | NOE Signal Integration[b] | Known[c] | r(A) Cal'd[d] |
|---|---|---|---|---|---|
| 4.788 | 3.800 | III-1 II-4 glycosidic | 1.4 | | 2.4 |
| | 3.761 | III-1 III-2 1,2-ea | 1.0 | 2.5 | |
| 4.502 | 3.597 | IV-1 III-3 glycosidic | 1.5 | | 2.4 |
| | 3.400 | IV-1 IV-3 1,3-aa | 1.3 | 2.5 | 2.4* |
| 4.253 | 3.554 | II-1 II-5 1.5-aa | 1.1 | 2.5 | |
| | 3.416 | II-1 II-3 1,3-aa | 0.8 | 2.5 | |
| | 3.289 | II-1 I-4 glycosidic | 1.6 | | 2.3 |
| 4.162 | 3.425 | I-1 R-1a glycosidic | 1.4 | | 2.4 |
| | 3.289 | I-1 I-5 1,5-aa | 1.3 | 2.5 | 2.4* |

* Uncertain because of overlap.

a. Obtained from 2D-NOE spectrum (3-6 ppm) at 500 MHz and 40°C in DMSO-$d_6$-$D_2O$ (98:2, v/v), FIG. 3.

b. Relative integrated areas of NOE-coupled signal measured from FIG. 2; error, +/- 10%.

c. Values based on x-ray crystallography measurements of monosaccharides (Ref. 18,19).

d. Calculated interprotonic distances as discussed in text; error, +/- 0.2Å.

glycosidic torsional angles did not occur. These compensating changes are known to occur for a wide variety of oligosaccharides and apparently do occur in globoside as well. The primary driving force underlying these changes has been termed the "exo-anomeric effect" and has been the subject of a number of theoretical and experimental studies.[14-16] A conclusion reached early by Lemieux and co-workers is that the virtual torsion angle defined by projection of anomeric carbon-hydrogen and aglyconic carbon-hydrogen bonds across the glycosidic bonds would normally be less than 30 degrees in both α and β linked oligosaccharides.[14] This leads to short trans-glycosidic proton-proton distances in a wide variety of linkages. To the extent that the exo-anomeric effect dominates conformational preferences, the use of trans-glycosidic NOEs as a method of oligosaccharide sequencing would be general. The present work supports the generality in that 2D-NOE trans-glycosidic connectivities have been demonstrated for a variety of different anomeric linkages, including α and β, as well as 1-3 and 1-4 linkages.

Another type of structural information that, in principle, can be obtained from the 2D-NOE spectrum of an oligosaccharide is the average interprotonic distance $\underline{r}$ across various glycosidic linkages. A rigorous treatment of NOEs in groups of dipolar coupled protons requires explicit consideration of all cross relaxation pathways and determination of a sufficient number of enhancements to yield a unique set of distances.[20] Where a sufficient number of determinations cannot be made, approximate treatments may suffice. For macromolecules, a treatment relying on the initial rates of NOE buildup, coupled with internal distance calibration via effects between a rigidly fixed pair of protons, has proven useful.[21,22] While the distance dependence in these experiments is straightforward ($1/r^6$), acquisition of a complete set of time dependent data can be very time consuming. We present here what amounts to a single point in a similar time dependent experiment carried out by two dimensional spectroscopy so that information on a large number of connectivities can be

simultaneously obtained. In the limit that the mixing time is sufficiently short, line shapes are the same for all resonances and similar correlation times characterize all dipolar interactions, the areas of cross peaks in the 2D experiment relate to one another as the ratio of the inverse sixth power of internuclear distances. We restrict our current presentation to data from one such 2D experiment. Quantitation via a more complete time dependent study will follow.

Ideally, to quantitate cross relaxation effects in two dimensional data sets, one would measure cross-peak volumes. The use of peak areas from cross-sectional plots is more convenient but, relies on the assumption of similar line shapes for the resonances of interest. Here, in order to estimate the anomeric NOE-couplings, cross-sectional plots through the intra-oligosaccharide anomeric signals at 4.788, 4.502 and 4.253 ppm were obtained. These cross-sectional plots are shown in FIG. 4. Integration of each NOE-coupled signal allows the relative areas of each to be measured. The resulting values of relative NOE-coupling are reported in Table 2.

In order to transform these relative NOE-coupling values into interproton distances, suitable distance calibrations must be chosen. With β-linked glucopyranoside and galactopyranoside rings, 1-3 and 1-5 proton pairs are expected to be rigidly held at a sufficiently short distance (2.5 Å) to provide such a calibration. In an α-linked galactopyranoside, the 1-2 pair at 2.5 Å, provides a suitable calibration.[23,24] We would expect to see NOE cross peaks from all of these pairs in addition to cross peaks related to interresidue interactions. Only one intra-ring cross peak can be identified for Gal-IV and Glc-I. Chemical shifts for protons 3 and 5 in these residues are expected to be very similar. SECSY experiments confirm actual overlap. These cross peaks are, therefore, assumed to result from two enhancements and one-half of the observed value is used in calculations. Interpretation of H-3,H-5 enhancements for Glc-I might be further questioned because of the overlap of H-3 and H-5

resonances with a resonance from the I-4 proton. Interpretation of cross peak intensities in strongly coupled spin systems is normally difficult. In the case of Glc-I, however, the exact overlap of protons 3,4, and 5, and similarity of distances from H-3 and H-5 to the anomeric proton allows a simple division by 1/2.

With these qualifications, suitable calibrations can be obtained and enhancements measured for anomeric -trans-glycosidic pairs can be converted to distances. Since enhancements were very similar for calibration distances in each ring, an average enhancement for a pair of protons at 2.5 Å was calculated. The sixth root of the ratio of this calibration enhancement to each individual enhancement was then multiplied by 2.5 to yield calculated distances which are presented in Table 2.

In general, the distances cited above represent averages over allowed conformers weighted by $1/r^6$. In the limit that one conformer dominates, interpretation in terms of a single conformer can be attempted. A single trans-glycosidic distance normally can be satisfied by several glycosidic bond conformations. A growing body of experimental measurements and theoretical calculations indicates that only a small number of $\phi$ and $\omega$ dihedral angles for glycosidic linkages are allowed ( $\phi = \pm 60$ degrees, $\omega = 0$ degrees).[16-18, 25-26] This is a manifestation of the exo-anomeric effect discussed above. The transglycoside distances calculated ($2.4 \pm .1$Å) are consistent with these glycosidic bond angles and strongly support the dominance of these conformers in DMSO solution.

Elimination of other possible conformers or more precise specification of $\phi$ and $\omega$ bond angles requires measurement of more than one inter-ring cross relaxation connectivity and is best done with a complete time development study. That additional connectivities can be measured is apparent in FIG 4. Peak c in a column containing resonance IV-1 can be assigned to a IV-1 -> III-4 connectivity. Combined with the IV-1 -> III-3 connectivity this gives the information needed to further refine structural

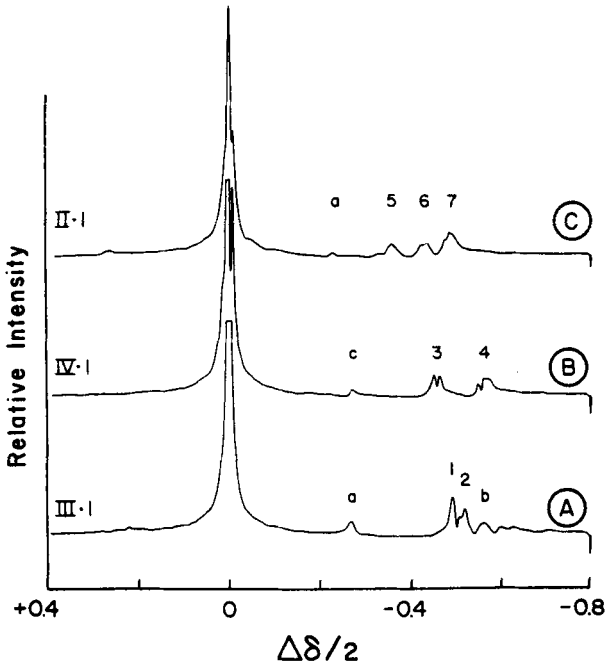


FIG. 4. Cross-sectional plots through the anomeric signals of FIG. 3 for quantitation of the relative intensity of NOE couplings. A, crossectional plot at 4.788 ppm (III-1): 1, III-1 - II-4 couple; 2, III-1 - III-2 couple. B, cross-sectional plot at 4.502 ppm (IV-1): 3, IV-1 - III-3 couple; 4, IV-1 - IV-3 couple. C, cross-sectional plot at 4.253 ppm (II-1): 5, II-1 - II-5 couple; 6, II-1 - II-3 couple; 7, II-1 - I-4 couple. a, and b, are artifacts. c, is tentatively assigned as the IV-1 - III-4 couple. The resonance at 0 ppm is in each case a result of direct excitation of the proton of interest (III-1, IV-1, or II-1).

parameters. Acquisition of more accurate cross relaxation data and acquisition of data which would allow investigation of conformation in water is underway.

## EXPERIMENTAL

Globoside (2 mg), obtained from human erythrocytes and kindly provided by Dr. S. Ando, Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan, was prepared for high-resolution proton NMR analysis in the solvent dimethyl sulfoxide-$d_6$-deuterium oxide (DMSO-$D_2O$) (98:2 v/v, 0.5 ml) as previously described.[6]

Proton spectra were obtained using a Bruker WM-500 spectrometer equipped with an Aspect 2000 computer operating in Fourier transform mode with quadrature detection. One dimensional spectra were typically acquired using a spectral width of 3000 Hz, a 2 sec cycle time, a 90 degree pulse of 11 μsec, and 128 to 256 scans. 2D spin coupling correlation spectroscopy was executed using two 90 degree pulses separated by a time 1/2 t1 and data were acquired during a period, t2, beginning after an additional time delay 1/2 t1 following the last 90 degree pulse. Acquisition of Spectra required 7 hr. This experiment, referred to as a SECSY experiment, contains the same basic information as a COSY experiment. The COSY experiment usually provides a higher signal to noise ratio because acquisition in the SECSY experiment is delayed by $t_1/2$, allowing further decay of the free induction decay. In cases where expected couplings occur over a small spectral region, however, the SECSY experiment allows contraction of the F1 frequency domain with substantial savings in the number of $t_1$ points that need to be acquired. In oligosaccharides this is the case and experiments provide comparable sensitivity.

Two dimensional, homonuclear nuclear Overhauser effect (2D-NOE) spectroscopy was executed with a sequence of three non-selective 90 degree pulses separated by t1/2 and a mixing delay, $\tau$. Mixing delay and t1 increments were 0.5 s and 1.25

msec, respectively. Phase cycling and a small random increment added to the mixing delay were used to suppress J-correlated peaks. The spectrum required a total of 88 scans in a 256 x 2048 data set, which took approximately 20 h to acquire.

The software used to transform the 2D-NOE spectra was obtained from Bruker Instruments, Billerica, MA. Zero filling and a window function of $\cos^2 \theta$ (phase shifted by $\pi/4$) were used in both dimensions. Processing and plotting required approximately 2 h each. The software used to transform the 2D SECSY spectrum was written by Dr. Dennis Hare, and run on a DEC VAX 11/750 computer. Zero filling in the F2 dimension and a window function of sin bell shifted by 0 degrees in both dimensions were used. Processing and plotting required a total of approximately 20 min.



## ACKNOWLEDGMENTS

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