Sillerud, L. O., Prestegard, J. H., Yu, R. K., & Schafer, D. E. (1978) *Biochemistry 17*, 2619–2628.
Sillerud, L. O., Yu, R. K., & Schafer, D. E. (1982) *Biochemistry 21*, 1260–1271.
Svennerholm, L. (1980) *Adv. Exp. Med. Biol. 125*, 533–544.
Vliegenthart, J. F. G. (1980) *Adv. Exp. Med. Biol. 125*, 77–91.
Wagner, G., Kumar, A., & Wüthrich, K. (1981) *Eur. J. Biochem. 114*, 375–384.
Yamada, A., Dabrowski, J., Hanfland, P., & Eggē, H. (1980) *Biochim. Biophys. Acta 618*, 473–479.
Yu, R. K., & Sillerud, L. O. (1982) *Adv. Exp. Med. Biol. 152*, 41–46.

High-Resolution Proton NMR Studies of Gangliosides. 2. Use of Two-Dimensional Nuclear Overhauser Effect Spectroscopy and Sialylation Shifts for Determination of Oligosaccharide Sequence and Linkage Sites†

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ABSTRACT: Homonuclear two-dimensional proton nuclear Overhauser effect (2-D-NOE) spectra have been obtained for asialo-G_{M2} (**4**) and gangliosides G_{M2} (**8**) and G_{M1} (**9**) at 500 MHz and 40 °C in Me_2SO-d_6–D_2O (98:2 v/v). The anomeric protons of each oligosaccharide residue of **4**, **8**, and **9** are observed to NOE couple via intraresidue 1,3- and 1,5-diaxial interactions and interresidue interactions across the glycosidic linkages. The former couplings are used to confirm the H-3 and H-5 assignments for each residue. From the latter couplings the *sequence* and glycosidic linkage sites of all oligosaccharide residues, except the sialic acid residues, are revealed. Sialic acid attachment sites are determined for the monosialogangliosides (**6**–**9**) through consideration of sialylation-induced glycosidation shifts. Combination of the above sequence and linkage-site data with the monosaccharide composition, anomeric configurations, and characterization of the aglycon (information obtained via two-dimensional spin-echo *J*-correlated spectroscopy or 2-D-SECSY) allows complete assignment of monosialoganglioside primary structure, independent of other methods of structural analysis. Compared to conventional chemical and enzymatic methods, high-resolution two-dimensional proton nuclear magnetic resonance spectroscopy has the advantages of speed, sensitivity, and sample preservation.

Previously, we have shown that the monosaccharide composition, anomeric configurations, and aglycon structure of gangliosides can be determined through homonuclear proton two-dimensional spin-echo *J*-correlated NMR[1] spectroscopy or 2-D-SECSY (Koerner et al., 1983). Through this 2-D NMR method, all information is obtained that is necessary to determine the complete primary structure of a ganglioside, except the sequence and linkage sites of the oligosaccharide residues. In theory, the potential proximity of the glycosidic-linkage protons of the core oligosaccharide residues should allow its sequence and glycosidation sites to be determined, if through-space couplings could be systematically measured. In fact, Ernst, Wüthrich, and co-workers (Jeener et al., 1979; Kumar et al., 1980a,b; Macura et al., 1981) have shown in studies of polypeptides that through-space couplings of protons can be systematically measured by another homonuclear proton NMR method, namely, two-dimensional nuclear Overhauser effect spectroscopy (2-D-NOE). Recently, Dabrowski et al. (1981) and Berstein & Hall (1982) have used 1-D NOE data to determine the sequence and linkage sites of neutral glycosphingolipids and an acetylated disaccharide, respectively. We have reported the use of 2-D-NOE data to determine the sequence and linkage sites of a ceramide trisaccharide (**4**; Prestegard et al., 1982).

In this paper we show how the *sequence* and linkage sites of the core oligosaccharide of gangliosides may be determined by 2-D-NOE and how their sialic acid residue linkage sites may be deduced from consideration of sialylation shifts. When this information is combined with composition data, generated through 2-D-SECSY, the complete primary structure of monosialogangliosides may be elucidated, nondestructively and independent of other methods of analysis. This study is an extension of our use of two-dimensional proton NMR methods for the complete analysis of oligosaccharide primary structure (Prestegard et al., 1982) and high-resolution proton NMR studies of gangliosides (Koerner et al., 1982). All numbering, symbols, and structures used in this paper are the same as those previously used [Figure 1 in Koerner et al. (1983)].

Materials and Methods

Asialo-G_{M2} (**4**) and gangliosides G_{M2} (**8**) and G_{M1} (**9**) were isolated from human brain and prepared for proton NMR

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[1] Abbreviations: 2-D-NOE, two-dimensional nuclear Overhauser effect; 2-D-SECSY, two-dimensional spin-echo *J*-correlated spectroscopy; Me_2SO-d_6, dimethyl-d_6 sulfoxide; D_2O, deuterium oxide; G_{M1}, G_{M2}, G_{M3}, G_{M4}, G_{A1}, and G_{A2}, symbols for ganglioside structures [as shown in Figure 1 of Koerner et al. (1983)]; I, II, III, IV, R, and A, ganglioside residue structures; NMR, nuclear magnetic resonance. All monosaccharides are assumed to be of the D configuration.

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analysis (Bruker WM-500) as previously described (Koerner et al., 1983). Two-dimensional nuclear Overhauser effect (2-D-NOE) spectroscopy, which establishes connectivities due to dipole–dipole cross relaxation and depends on through-space proximity of coupled protons, was executed with a sequence of three nonselective 90° pulses. The first two pulses are separated by $(1/2)t_1$ (evolution period) and used to selectively invert magnetization and frequency label the various magnetization components by perturbing magnetization to a degree dependent on frequency offset. After a time ND (mixing time) during which cross relaxation leads to exchange of magnetization between nearby protons, a third 90° pulse is applied followed by an additional delay of $(1/2)t_1$. The signal (S) at the end of this delay is stored as a function of the second time domain, t_2. A two-dimensional Fourier transformation of the data matrix $S(t_1,t_2)$ then produces the frequency domain spectrum that is plotted. Mixing delay and pulse times were 0.5 s and 11 μs, respectively. Zero filling and a window function of $\cos^2 \theta$ (phase shifted by $\pi/4$) were used in both dimensions. Phase cycling and a small random increment added to the mixing delay were used to suppress *J*-correlated peaks. Except for small displacements due to *J* coupling, the central horizontal region of the 2-D-NOE spectrum corresponds to a normal one-dimensional spectrum, and off-axis peaks occur at a vertical position corresponding to 0.5 the chemical shift distance to an NOE-coupled resonance. Thus, sequential construction of vertical, 135° diagonal, and vertical lines identifies NOE-coupled resonances (Figure 1). The software used to obtain the 2-D-NOE spectra was obtained from Bruker Instruments, Billerica, MA (Manual for the Aspect 2000, FTNMR2D program, version 810515, developed by A. D. Bain, G. Baliman, and H. C. Jost). Each spectrum required a total of 88 scans in a 256 × 2048 data set ($t_1 \times t_2$), which took approximately 20 h to acquire. Processing and plotting required approximately 2 h.

Results

2-D-NOE spectra were obtained for the complete oligosaccharide residue ring region (3–5 ppm) of asialo-G_{M2} (**4**) and gangliosides G_{M2} (**8**) and G_{M1} (**9**). The spectrum of G_{M2} is shown in Figure 1 as an example. Through construction of vertical, 135° diagonal, and vertical lines, NOE-coupled resonances are identified in each spectrum as illustrated in Figure 1. NOE couplings were observed between anomeric (H-1) and nonanomeric resonances and between pairs of nonanomeric resonances. The latter are valuable in confirmation of assignments and residue characterizations, previously made through 2-D-SECSY (Koerner et al., 1983); however, since they add no new structural information, we will not describe them in detail. Only anomeric to nonanomeric interactions will be considered, since they are essential for the establishment of ganglioside primary structure.

All NOE couplings involving anomeric resonances of **4**, **8**, and **9** are shown in Table I. The identity of coupled protons was established by comparison of their chemical shifts with those assigned through 2-D-SECSY (Koerner et al., 1983). Each anomeric resonance is observed to be coupled upfield, both to inter- and intraresidue protons. The latter are seen to result from 1,3- and 1,5-diaxial interactions and are expected from previous studies of derivatized monosaccharides (Hall, 1981). The observation of these intraresidue couplings confirms the assignments for H-3 and H-5 of each residue.

Far more interesting are the observed *interresidue* couplings, which are seen to be interactions across glycosidic linkages. Observation of this type of coupling establishes the *n*-1 oligosaccharide residue and specific glycosidation site to which the anomeric proton of the *n*th residue is linked. When such information is known for each residue (each anomeric proton) and combined, the *sequence* of the core oligosaccharide is obtained. For example, ganglioside G_{M2} (**8**) and asialo-G_{M2} manifest the following interresidue NOE couplings (Table I): I-1→R-1a, II-1→I-4, and III-1→II-4. If one overlaps these sequence fragments, the sequence of the core oligosaccharide of **8** and **4** is III(1→4)II(1→4)I(1→1')R. To our knowledge, this is the first application of this nondestructive method for sequencing to the core oligosaccharides of gangliosides.

Table I: Observed NOE Couplings Involving the Anomeric Protons of Asialo-G_{M2} (4) and Gangliosides G_{M2} (8) and G_{M1} (9)[a]

compd	chemical shifts (ppm) of coupled proton pairs (error ±0.005 ppm): anomeric signal	upfield signal	$\lvert\Delta\delta/2\rvert$[b]	interpretation[c]
4	4.467	3.804	0.338	III-1→II-4 (glycosidic)
		3.444	0.516	III-1→III-3 (1,3-aa)
		3.338	0.563	III-1→III-5 (1,5-aa)
	4.231	3.505	0.361	II-1→II-3 (1,3-aa)
		3.476	0.380	II-1→II-5 (1,5-aa)
		3.285	0.474	II-1→I-4 (glycosidic)
	4.169	3.438	0.373	I-1→R-1a (glycosidic)
		3.338	0.419	I-1→I-3 (1,3-aa)
		3.294	0.443	I-1→I-5 (1,5-aa)
8	4.800	3.932	0.443	III-1→II-4 (glycosidic)
		3.638	0.583	III-1→III-5 (1,5-aa)
	4.276	3.749	0.268	II-1→II-3 (1,3-aa)
		3.484	0.402	II-1→II-5 (1,5-aa)
		3.280	0.501	II-1→I-4 (glycosidic)
	4.147	3.437	0.361	I-1→R-1a (glycosidic)
		3.287	0.437	I-1→I-5 (1,5-aa)
9	4.869	3.945	0.463	III-1→II-4 (glycosidic)
	4.277	3.748	0.265	II-1→II-3 (1,3-aa)
		3.476	0.396	II-1→II-5 (1,5-aa)
		3.261	0.501	II-1→I-4 (glycosidic)
	4.221	3.463	0.373	IV-1→III-3 (glycosidic)
		3.364	0.425	IV-1→IV-5 (1,5-aa)
		3.300	0.472	IV-1→IV-3 (1,3-aa)
	4.149	3.421	0.361	I-1→R-1a (glycosidic)
		3.273	0.437	I-1→I-5 (1,5-aa)

[a] Obtained from 2-D-NOE spectrum (3–5 ppm) at 500 MHz and 40 °C in Me_2SO-d_6-D_2O (98:2 v/v). [b] Absolute value of the ordinate-axis displacement; values are positive for the upfield signal and negative for the anomeric signal. [c] Signals are assigned by comparison of chemical shifts with those previously assigned via 2-D-SECSY (Koerner et al., 1983). Couplings are either across the glycosidic linkages or through 1,3-diaxial (1,3-aa) or 1,5-diaxial (1,5-aa) interactions.

The magnitude of the anomeric NOE couplings varies in amplitude as can be noted by observing the contour densities in Figure 1. Given the single mixing delay, the absolute magnitudes of these couplings are not highly significant. However, it is noteworthy that the interresidue couplings are smaller than the intraresidue ones. This suggests that the trans-glycosidic pairs of protons are close in space. If quantitative experiments[2] confirm this observation, the range of possible secondary structures for the oligosaccharide residues of gangliosides and their derivatives would be very limited.

Discussion

In order to determine the complete primary structure of a monosialoganglioside, one must establish (1) its composition, i.e., the number, constitution and configuration of its oligo-

[2] T. A. W. Koerner, J. H. Prestegard, P. C. Demou, and R. K. Yu, unpublished data.

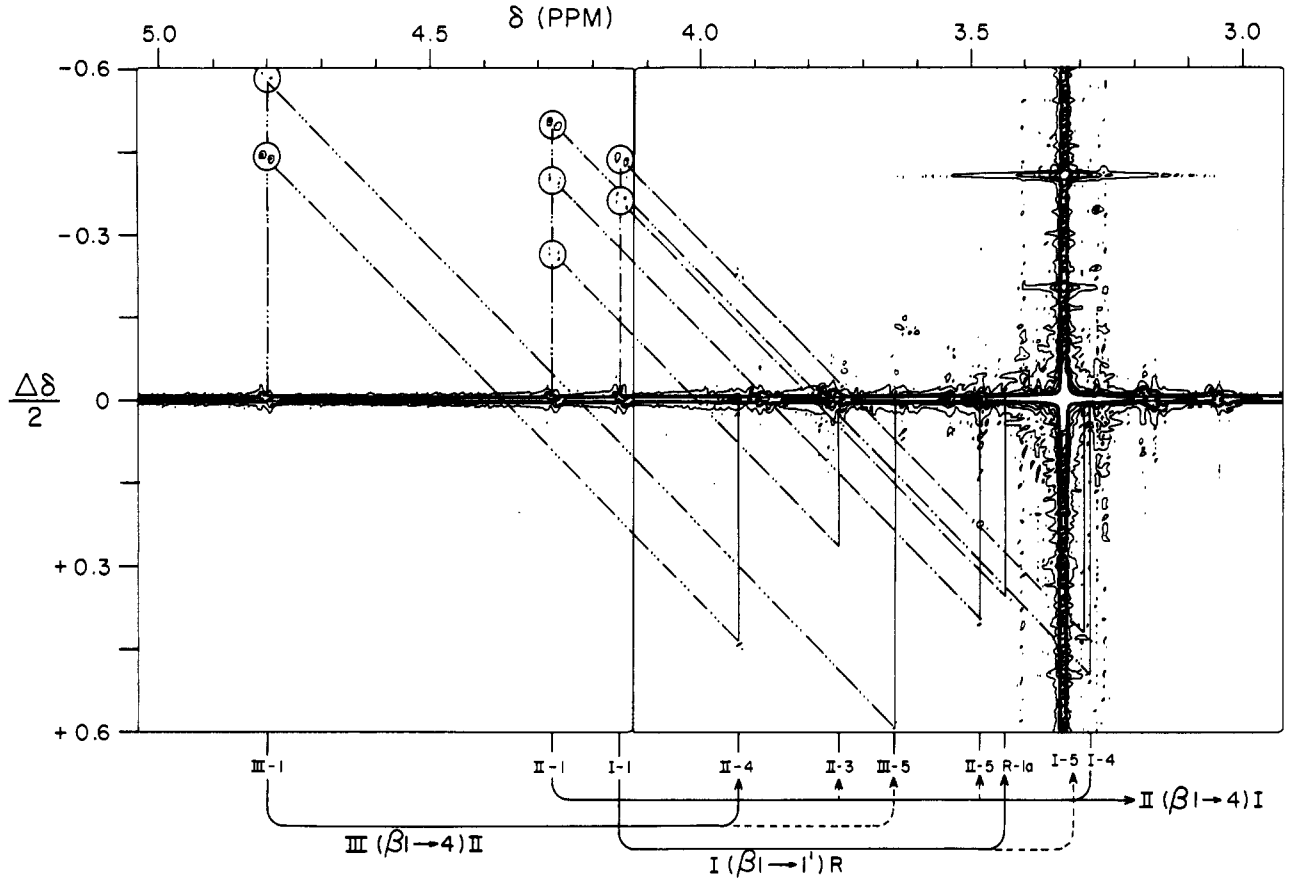

FIGURE 1: Two-dimensional homonuclear proton NOE spectrum of the oligosaccharide ring proton region (3–5 ppm) of ganglioside G_{M2} (**8**), obtained at 500 MHz and 40 °C. NOE couplings are labeled for the anomeric protons of the following oligosaccharide residues: I, β-glucopyranosyl (–·–); II, β-galactopyranosyl (–··–); III, 2-acetamido-2-deoxy-β-galactopyranosyl (–···–). Anomeric couplings are circled for emphasis. At the *bottom*, *interresidue* couplings are labeled with solid lines and *intraresidue* couplings with dashed lines. The glycosidic linkages that are indicated by interresidue proton pairs are noted.

Table II: Sialylation-Induced Chemical Shift Differences (Δ_γ^A) for Gangliosides (**6–9**)

	proton no.														
example	I-2	I-3	I-4	I-6b	II-2	II-3	II-4	II-6b	III-3	III-4	III-6b	IV-2	IV-3	IV-4	IV-6b
6–2					−0.04	*+0.63*	+0.03	−0.02							
7–3	+0.01	+0.03	−0.01	−0.00	−0.09	*+0.66*	+0.08	+0.02							
8–4	+0.00	−0.01	−0.01	−0.00	−0.09	*+0.23*	+0.13	+0.01	−0.13	−0.09	−0.02				
9–5	+0.00	+0.01	−0.01	−0.01	−0.10	*+0.24*	+0.14	+0.02	−0.18	−0.08	−0.03	+0.05	+0.07	+0.00	+0.03

saccharide residues, and the structure of its aglycon, (2) the sequence and linkage sites of its core oligosaccharide, and (3) the linkage site of its sialic acid residue. This structural information has traditionally been obtained by application of a combination of such procedures as compositional analysis by gas–liquid chromatography, permethylation studies, Smith degradation, mass spectrometry, partial acid or enzyme hydrolysis, optical rotation measurement, etc. (Ledeen & Yu, 1982). These procedures are time-consuming, taking many weeks of effort, and frequently require elaborate derivatization of the intact molecules, as well as access to many different instruments and mastery of many physical and chemical methods. With high-field, 2-D proton NMR, all three of the above types of data may be obtained nondestructively from a 1–2-mg sample, independent of other methods of structural analysis. The procedure for proton NMR analysis of gangliosides and related glycolipids is summarized and discussed in the following four steps.

Step One. The high-field 1-D- and 2-D-SECSY spectra of an unknown ganglioside or derivative are obtained between 0 and 10 and 3 and 5 ppm, respectively. Subspectra are extracted and a complete set of chemical shift and coupling constant data are generated; then, residue assignments are made and the *composition* is revealed through integration of well-resolved resonances.

Step Two. The high-field 2-D-NOE spectrum between 3 and 5 ppm is obtained, anomeric NOE couplings are revealed, and *core oligosaccharide sequence* and linkage sites are assigned.

Step Three. Combination of the compositional data (step one) and the sequence and linkage-site data (step two) determines the complete core oligosaccharide primary structure.

Step Four. Calculation of sialylation-induced glycosidation shifts reveals the site of sialic acid residue attachment.

Step one has been discussed in detail previously (Koerner et al., 1983) and step two has been demonstrated under Results and in Table I. Steps three and four will be discussed with **8** as an example. From 2-D-SECSY data of **8** (step one), it is known that one αNeuAc (A) residue is present, the aglycon is ceramide (R), and the core oligosaccharide is composed of three residues: I, β-glucopyranoside; II, β-galactopyranoside; III, 2-acetamido-2-deoxy-β-galactopyranoside. From its 2-D-NOE spectrum (Figure 1, Table I), it is known that the sequence and linkage sites of the core oligosaccharide of **8** are

III(1→4)II(1→4)I(1→1)R (step two). Thus the complete structure must be 2-acetamido-2-deoxygalactopyranosyl-(β1→4)galactopyranosyl(β1→4)glucopyranosyl(β1→1)ceramide.

It should be noted that oligosaccharides composed of aldopyranoside residues, such as the core oligosaccharide of gangliosides, are well suited for sequencing via 2-D proton NMR since they contain a continuously coupled series of protons from one end of the molecule to the other, when through-space (NOE) and through-bond (scalar) couplings are considered. Furthermore, this series of protons is regularly interspersed with very characteristic and well-resolved protons from each residue (the anomeric or H-1 protons). It is also important to point out that α-anomeric protons NOE couple across glycosidic linkages as strongly and as regularly as do β-anomeric protons.[2]

In step four the calculation of a sialylation shift (Δ_γ^A) is made for the proton resonance at each possible sialylation site, using the chemical shifts of these protons and those of the corresponding proton of the asialoganglioside core oligosaccharide (Koerner et al., 1983). In the case of **8**, the core oligosaccharide is that of **4**, as revealed in step three. Thus Δ_γ^A are calculated **8** minus **4** for protons I-2, -3, -4, -6a, and -6b, II-2, -3, -4, -6a, and -6b, and III-3, -4, -6a, and -6b (Table II). Only for II-3 is Δ_γ^A greater than 0.20 ppm, indicating A is attached at II-3 and only one sialylation site is present.

Other Δ_γ^A noted in Table II are due to long-range glycosidation shifts in **8**. Since only one αNeuAc is present in **8** (step one), the sialic acid structure must be NeuAc(α2→3)III. Combining this information with the structure deduced for the core oligosaccharide, it is concluded that **8** is NeuAc(α2→3)[GalNAc(β1→4)]Gal(β1→4)Glc(β1→1)ceramide [Figure 1 in Koerner et al. (1983)]. This structure is in agreement with that assigned through chemical and enzymatic methods (Kuhn & Wiegandt, 1964; Ledeen & Salsman, 1965).

Analysis of the 2-D-NOE spectrum and Δ_γ^A shifts of ganglioside G_{M1} (**9**) in the same manner as illustrated above for **8** resulted in the deduction of a unique and correct structure [Figure 1 in Koerner et al. (1983)], in agreement with that in the literature (Ledeen & Yu, 1982). The sialylation-induced glycosidation shift (Δ_γ^A) data generated in this analysis are shown in Table II. Also shown in Table II are Δ_γ^A calculations that were made for two other monosialogangliosides, G_{M4} (**6**) and G_{M3} (**7**), and their asialo derivatives **2** and **3**, respectively, for which data were available (Koerner et al., 1983). In both of these examples as well, the correct sialylation site was predicted. It should be noted for future studies that for gangliosides that have core oligosaccharide structures that differ from the ganglio type, e.g., lacto or globo type, there are also data available (Dabrowski et al., 1980)[2] to carry out a Δ_γ^A calculation. For determination of the structures of disialo-, trisialo-, and polysialogangliosides, Δ_γ^A data for NeuAc(α2→8)NeuAc linkages will be needed, which are currently being obtained.[2]

Finally, it should be noted that, though sequencing via 2-D-NOE is rapid when compared with classical methods, this 2-D-NMR method does require large investments of spectrometer time. Thus its proper usage is in the context of an overall protocol. In our experience 2-D experiments are more efficient when five or more data sets need to be acquired. For the largest molecule considered here, ganglioside G_{M1} (**9**), one would have anticipated Overhauser effects involving the four anomeric resonances to be particularly useful in sequencing. However, we observed five additional connectivities in the 2-D set that provided assignment confirmation and unexpected structural data, which justified the 2-D approach.

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References

Bernstein, M. A., & Hall, L. D. (1982) *J. Am. Chem. Soc. 104*, 5553–5555.

Dabrowski, J., Hanfland, P., & Egge, H. (1980) *Biochemistry 19*, 5652–5658.

Dabrowski, J., Hanfland, P., Egge, H., & Dabrowski, U. (1981) *Arch. Biochem. Biophys. 210*, 405–411.

Hall, L. D. (1981) in *The Carbohydrates* (Pigman, W., Horton, D., & Wander, J. D., Eds.) Vol. IB, pp 1299–1326, Academic Press, New York.

Jeener, J., Meier, B. H., Bachmann, P., & Ernst, R. R. (1979) *J. Chem. Phys. 71*, 4546–4553.

Koerner, T. A. W., Prestegard, J. H., & Yu, R. K. (1982) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 41*, 1170.

Koerner, T. A. W., Prestegard, J. H., Demou, P. C., & Yu, R. K. (1983) *Biochemistry* (preceding paper in this issue).

Kuhn, R., & Wiegandt, H. (1964) *Z. Naturforsh., B 19B*, 256–266.

Kumar, A., Ernst, R. R., & Wüthrich, K. (1980a) *Biochem. Biophys. Res. Commun. 95*, 1–6.

Kumar, A., Wagner, G., Ernst, R. R., & Wüthrich, K. (1980b) *Biochem. Biophys. Res. Commun. 96*, 1156–1163.

Ledeen, R., & Salsman, K. (1965) *Biochemistry 4*, 2225–2233.

Ledeen, R., & Yu, R. K. (1982) *Methods Enzymol. 83*, 139–190.

Macura, S., Huang, Y., Suter, D., & Ernst, R. R. (1981) *J. Magn. Reson. 43*, 259–281.

Prestegard, J. H., Koerner, T. A. W., Demou, P. C., & Yu, R. K. (1982) *J. Am. Chem. Soc. 104*, 4993–4995.