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A simple method to quantitatively measure polypeptide $J_{H^N H^\alpha}$ coupling constants from TOCSY or NOESY spectra*

Yunjun Wang, Alex M. Nip and David S. Wishart**

Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, AB, Canada T6G 2N8

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Summary

A simple linear relationship between the $J_{H^N H^\alpha}$ coupling constant and the linewidth ($\Delta\nu_{1/2}$) of in-phase NMR peaks has been identified. This relationship permits the rapid and accurate determination of polypeptide $J_{H^N H^\alpha}$ coupling constants from a simple inspection of amide cross peaks in homonuclear ^{1}H TOCSY or ^{1}H NOESY spectra. By using the appropriate set of processing parameters we show that $J_{H^N H^\alpha} = 0.5(\Delta\nu_{1/2}) - MW/5000 + 1.8$ for TOCSY spectra and $J_{H^N H^\alpha} = 0.6(\Delta\nu_{1/2}) - MW/5000 - 0.9$ for NOESY spectra, where $\Delta\nu_{1/2}$ is the half-height linewidth in Hz and MW is the molecular weight of the protein in Da. The simplicity of this relationship, combined with the ease with which $\Delta\nu_{1/2}$ measurements can be made, means that $J_{H^N H^\alpha}$ coupling constants can now be rapidly determined (up to 100 measurements in less than 30 min) without the need for any complex curve-fitting algorithms. Tests on 11 different polypeptides involving more than 650 separate $J_{H^N H^\alpha}$ measurements have shown that this method yields coupling constants with an rmsd error (relative to X-ray data) of less than 0.9 Hz. Furthermore, the correlation coefficient between the predicted NMR coupling constants and those derived from high-resolution X-ray crystal structures is typically better than 0.89. These simple linear relationships have been found to be valid for peptides as small as 1 kDa to proteins as large as 20 kDa. Despite the method's simplicity, these results are comparable to the accuracy and precision of the best techniques published to date.

Introduction

Coupling constant measurements are playing an increasingly important role in the determination and refinement of peptide and protein structures (Bax et al., 1994). Their rise in importance has had much to do with the recent introduction of innovative experimental and analytical methods which have facilitated the extraction of these otherwise difficult-to-measure parameters. A variety of ingenious curve-fitting techniques are now available for determining $J_{H^N H^\alpha}$ coupling constants from homonuclear DQF-COSY spectra (Pardi et al., 1984; Kim and Prestegard, 1989; Smith et al., 1991), NOESY spectra (Szyperski et al., 1992) or a combination of DQF-COSY and NOESY spectra (Ludvigsen et al., 1991). Each of these methods is based on computationally intensive procedures which attempt to fit theoretical curves to anti-phase or in-phase doublets.

With the widespread use of isotopic labels in biological NMR, a number of elegant experimental methods have also been developed to measure $J_{H^N H^\alpha}$ values from ^{1}H-^{15}N heteronuclear experiments. These include the HMQC-J experiment (Kay and Bax, 1990), the J-modulated [^{15}N, ^{1}H] COSY experiment (Billeter et al., 1992), the HNCA E.COSY experiment (Weisemann et al., 1994) and the HNHA experiment (Vuister and Bax, 1993). By combining spectral data from these experiments with experiment-specific computer curve-fitting routines (Kay and Bax, 1990; Goodgame and Greer, 1993) or peak integration

*This paper is dedicated to the memory of Casey Wishart (1986–1997).
**To whom correspondence should be addressed.
Abbreviations: COSY, correlation spectroscopy; $\Delta\nu_{1/2}$, linewidth at half-height; DQF-COSY, double quantum filtered correlation spectroscopy; DSS, 2,2-dimethyl-2-silapentane-5-sulfonic acid; GS, gramicidin S; kDa, kilodaltons; MW, molecular weight; NOESY, nuclear Overhauser effect spectroscopy; rmsd, root-mean-square deviation; TOCSY, total correlation spectroscopy.
Supplementary material available from the authors: tables listing the J_{lw} and J_{xray} values as well as relevant statistics for all of the peptides and proteins described in this study.

routines (Billeter et al., 1992; Vuister and Bax, 1993), it is possible to extract relatively accurate $J_{H^N H^\alpha}$ coupling constants. Furthermore, the use of isotopically labeled proteins, with their inherently greater chemical shift dispersion, now permits the measurement of J-coupling values of much larger proteins (up to 18 kDa – Billeter et al., 1992).

Despite the arsenal of experimental and computational techniques available to quantitatively determine $J_{H^N H^\alpha}$ coupling constants, it is still quite rare to see $J_{H^N H^\alpha}$ coupling constants reported in the literature. Indeed, while there are more than 200 000 chemical shifts of peptides and proteins deposited in the BioMagResBank (Seavey et al., 1991) there are still fewer than 1300 $J_{H^N H^\alpha}$ coupling constants reported in the literature (D.S. Wishart, unpublished observations). The fact that quantitative (as opposed to qualitative) coupling constant measurements often require special hardware (gradients, three or four channels) or special computer programs, or the implementation of a complex pulse sequence, or the use of isotopically labeled material, or the collection and reprocessing of multiple data sets, or the complete reassignment of 'yet another spectrum' suggests that the experimental and practical barriers to measuring coupling constants are still quite significant. Even in the simplest situation (analyzing a homonuclear DQF-COSY) one is still confronted with the onerous task of recollecting and reassigning a spectrum which has notoriously poor signal-to-noise. Even if the signal is sufficiently good (which it rarely is), it is still necessary to use a computer program to perform a four-parameter fit over a very bumpy hypersurface. However, an apparently successful computer fit is no guarantee of correctness, as spectral noise can often lead even the best curve-fitting program into a false local minimum.

Given these concerns and given our own frustrations at unsuccessfully implementing previously published techniques, we decided to investigate the possibility of developing a robust method for measuring $J_{H^N H^\alpha}$ coupling constants that would be (i) simple (something that could be done in one's head or with a hand-held calculator); (ii) quick (allowing $J_{H^N H^\alpha}$ determinations to be made in seconds); (iii) accurate (having an rmsd of less than 1.0 Hz); (iv) easy to learn; (v) independent of any requirement for specific isotopic labels; (vi) applicable to conventional, easily obtained spectra (TOCSY, NOESY, COSY, etc.); (vii) applicable to NMR spectra with inherently high signal-to-noise (in-phase as opposed to anti-phase peaks); (viii) applicable to both small peptides and large proteins; (ix) generalizable to both homonuclear and heteronuclear experiments; (x) independent of any requirement for specialized hardware; (xi) independent of the spectrometer make, size or type; and (xii) extensively tested and verified. We believe we have developed such a method. It is based on the simple observation that $J_{H^N H^\alpha}$ coupling constants are linearly related to the linewidth at half-height ($\Delta\nu_{1/2}$) of in-phase TOCSY and NOESY amide cross peaks. This method is described, in detail, below.

Materials and Methods

Sample preparation and data collection

The peptides and proteins used in this study were selected on the basis of their availability, cost and the requirement that each peptide or protein had to have a high-resolution (<2.0 Å) X-ray crystal structure deposited in the Protein Databank (PDB) (Bernstein et al., 1977). Using these criteria a total of 11 compounds was selected, including gramicidin S (GS) (five synthetic analogs), interleukin 8 (human), ubiquitin (bovine), thioredoxin (T4 phage), thioredoxin (*E. coli*), lysozyme (chicken) and ribonuclease A (bovine). Samples of ribonuclease A, lysozyme and ubiquitin were obtained as crystallized powders from Sigma. Samples of T4 and *E. coli* thioredoxin were prepared and purified as previously described (Wishart, 1991; Wishart et al., 1993). GS analogs (small cyclic peptides with well-defined β-sheet structure) were synthesized and purified using previously published methods (Wishart et al., 1996). Five analogs were used: peptide #1 (sequence: $[PVKLF]_2$), peptide #2 (sequence: $[PVKLH]_2$), peptide #3 (sequence: $[PVKLN]_2$), peptide #4 (sequence: $[PVKLY]_2$ and peptide #5 (sequence: [GKLYPVKLYP]).

For the NMR analysis, all polypeptide samples used for this study were dissolved in 500 µl 80% H_2O/20% D_2O yielding a typical concentration of 1–3 mM and an uncorrected pH reading between 4.0 and 5.0. Each sample was referenced to internal DSS (Wishart et al., 1995) with the temperature being maintained at 25±0.1 °C. The TOCSY spectrum of GS peptide #1 was collected on a Varian Unity 300 MHz spectrometer. The TOCSY and NOESY spectra of interleukin 8 were collected on a Varian Unity 600 MHz spectrometer. All other data were collected on

TABLE 1
LISTING OF HIGH-RESOLUTION X-RAY STRUCTURES USED IN CALCULATING J_{xray} VALUES

Protein	Accession	Resolution (Å)	R factor	Reference
Thioredoxin (*E. coli*)	2TRX	1.68	0.165	Katti et al. (1990)
Ubiquitin (bovine)	1UBI	1.80	0.165	Vijay-Kumar et al. (1987)
Ribonuclease A (bovine)	8RAT	1.50	0.158	Tilton et al. (1992)
Thioredoxin (T4 phage)	1AAZ	2.00	0.210	H. Eklund et al. (unpublished)
Lysozyme (chicken)	193L	1.33	0.184	Young et al. (1994)
Interleukin 8 (human)	xIL8	1.50	0.180	Baldwin et al. (1991)

an extensively modified Varian VXR 500 MHz spectrometer equipped with a 5 mm inverse detection probe. One-dimensional ^{1}H data were acquired with a ^{1}H sweepwidth of 6000 Hz and an acquisition time of 3.0 s. The residual HDO signal was suppressed by presaturation. TOCSY (Braunschweiler and Ernst, 1983; Bax and Davis, 1985) spectra were collected using 256 t_1 increments and spectral widths of 6000 Hz in both dimensions. Acquisition times were set to 0.171 s, relaxation delays were 2.5 s and spin-lock (MLEV-17) mixing times were 50 ms. The spin-lock pulse width (90°) was 35.4 μs and the trim pulses were set to 0.5 ms. Data were zero-filled to produce a matrix of $4K \times 4K$ complex points and processed using a shifted sine-bell weighting function (see details below) followed by baseline correction. Quadrature detection was achieved using the method of States et al. (1982). ^{1}H NOESY (Jeener et al., 1979; Kumar et al., 1980) data were collected essentially identically to the TOCSY data, with mixing times ranging from 150 to 300 ms (depending on the size of the molecule).

Assignments of all spectra were based on previously published chemical shift values (with suitable corrections for reference standards, pH and temperature). In particular, the ^{1}H assignments for the GS analogs were based on those of Wishart et al. (1996), the ^{1}H assignments for *E. coli* thioredoxin were based on the tabulation by LeMaster and Richards (1988), the ^{1}H assignments for ubiquitin were based on those of Weber et al. (1987), the ^{1}H assignments for ribonuclease A were based on published shifts from Robertson et al. (1989), the ^{1}H assignments for hen lysozyme were based on values reported by Redfield and Dobson (1988), the ^{1}H assignments for interleukin 8 were from the report by Clore et al. (1989) and the T4 thioredoxin shifts were based on unpublished work from our laboratory (Y. Wang et al., manuscript in preparation). On average, more than 75% of each protein's amide-alpha resonances (excluding glycines) were included in our analysis. Practical considerations of time and effort in addition to difficulties associated with ambiguous assignments, interference from the H_2O peak and problems associated with resonance overlap generally prevented complete utilization of all resonances.

In determining the coupling constants for each polypeptide, we made use of the following Brookhaven PDB entries: *E. coli* thioredoxin (2TRX), ubiquitin (1UBI), ribonuclease A (8RAT), T4 thioredoxin (1AAZ), hen lysozyme (193L) and interleukin 8 (xIL8). Table 1 provides further details concerning the resolution and R factors for each of these crystal structures.

$J_{H^NH^\alpha}$ coupling constants for each of the above crystal structures were determined from the reported backbone ϕ angles using the following equation: $J = 5.9 \cos^2\theta - 1.3 \cos\theta + 2.2$, where $\theta = |\phi - 60°|$. This equation, which differs slightly from previously published versions of the Karplus equation (Pardi et al., 1984; Vuister and Bax, 1993; Wang and Bax, 1996), was derived from an analysis of nearly 1000 coupling constants reported by various sources for 15 different proteins (D.S. Wishart, manuscript in preparation). The use of other widely used Karplus parameters led to only minor differences in the overall performance of this method. Note that, in contrast to the protein samples, $J_{H^NH^\alpha}$ coupling constants for the GS analogs were obtained by direct measurement of the 1D ^{1}H NMR spectra.

Calculation of $J_{H^NH^\alpha}$ coupling constants from $\Delta\nu_{1/2}$

We have attempted to develop a protocol that should allow the determination of $J_{H^NH^\alpha}$ coupling constants independent of specific spectrometer characteristics. However, it is important to precisely follow the procedures outlined below to ensure reproducibility and accuracy:

(1) After shimming the sample to get optimum lineshape and linewidth characteristics, collect a ^{1}H TOCSY or NOESY spectrum of the protein of interest. For optimal results, TOCSY spectra must be collected with an MLEV-17 mixing scheme with 0.5–2 ms trim pulses (Bax and Davis, 1985). For all spectra, acquisition times should be set such that the resolution (prior to zero-filling) in the F2 dimension is better than 6.0 Hz/pt.

(2) Assign the spectrum (using previously determined assignments or assign using conventional NOE-directed techniques).

(3) Zero-fill in both dimensions to produce a $4K \times 4K$ spectrum, perform a baseline correction and apply a squared sine-bell weighting function in the form of

$$\sin^2[\pi(t - sbs)/2sb] \qquad (1)$$

For Varian spectrometers running VNMR software (v. 5.1 or higher),

$$|sb| = 0.100,\ sbs = -0.066 \quad \text{for the F2 dimension}$$

where sb and sbs are given in seconds and sb must be toggled to a negative value (–0.100) to indicate that the weighting function is squared. Processing along the F1 dimension is somewhat less important although we generally choose values equal to 1/3 of those used for the F2 dimension (sb1 = –0.033, sbs1 = –0.022). For other kinds of spectral processing software, the sine-bell weighting function (Eq. 1) can be cast into several different forms. According to the Bruker convention, the sine-bell function is expressed as

$$\sin^2[\{(\pi - \phi)/T\}t + \phi] \qquad (2)$$

where the parameters T and ϕ are related to sb and sbs as $T = 2sb + sbs$ and $\phi = \pi\, sbs/2sb$. In NMRPipe (Delaglio et al., 1995), the sine-bell function is written as

$$\sin^2\{\pi[off + sw\ t\ (end - off)/(tsize - 1)]\} \qquad (3)$$

where tsize is the total number of points in the time domain, sw is the sweepwidth and the parameters off and end can be expressed in terms of tsize, sw, sb and sbs as

off = –sbs/2sb and end = [(tsize–1) - sbs sw]/2sb sw. Finally, for FELIX, the sine-bell function is given as

$$\sin^2\{\pi[(1 - \text{wshift}/180)(\text{t sw}/\text{wsize}) + \text{wshift}/180]\} \quad (4)$$

where sw is the sweepwidth and the phase shift wshift and window size wsize can be expressed as wshift = –90 sbs/sb and wsize = 2sb sw(1 – wshift/180), where wsize is rounded off to the nearest integer. Using the above relationships, one can easily transform between different spectral processing systems.

(4) In order to exclude the effects of passive α–β coupling constants, select F2 traces from the *upper* diagonal only and determine the linewidth at half-height ($\Delta\nu_{1/2}$) for the central portion of each assigned ^{1}HN-^{1}Hα cross peak (note that for NOESY spectra, any cross peak connected to a NH resonance can be used – not just the $^1H^N$-$1H^\alpha$ cross peak). Consistent selection of the centermost region of each cross peak can greatly reduce the experimental or measurement error associated with this technique. In Varian spectrometers the command 'dres' automatically determines $\Delta\nu_{1/2}$ for any given trace or any given 2D peak using a simple 'half-width at half-height' algorithm (least-squares curve-fitting is not used). In Bruker spectrometers, $\Delta\nu_{1/2}$ determination takes slightly more effort.

Protocol #1

From the measured linewidth at half-height ($\Delta\nu_{1/2}$) substitute this value into one of the following two equations to determine the $J_{H^NH^\alpha}$ coupling constant (in Hz).

$$J_{H^NH^\alpha} = 0.5(\Delta\nu_{1/2}) - \text{MW}/5000 + 1.8 \quad \text{for TOCSY data} \quad (5)$$

$$J_{H^NH^\alpha} = 0.6(\Delta\nu_{1/2}) - \text{MW}/5000 - 0.9 \quad \text{for NOESY data} \quad (6)$$

where $\Delta\nu_{1/2}$ is the half-height linewidth (in Hz) of a given ^{1}HN-^{1}Hα (for TOCSY) or ^{1}HN-^{1}Hx (for NOESY) cross peak and MW is the molecular weight of the protein in Da. Both equations work well in practically all situations. However, care must be taken in using the correct molecular weight (i.e., is the polypeptide of interest a monomer or a dimer at NMR concentrations?) and in making sure that the temperature of the sample is between 20 and 35 °C. Under certain circumstances, the situation can be complicated by the presence of inherently broad linewidths, poor shimming, paramagnetic contaminants or the use of unusually high (> 40 °C) or low (< 20 °C) temperatures. In situations where 'unusual' temperatures are used, we have found that the following linewidth correction can be employed: $\Delta\nu_{1/2} = \Delta\nu_{1/2}(\text{obs}) - 0.04(\text{T} - 25)$, where T is the sample temperature (in °C) and $\Delta\nu_{1/2}$(obs) is the observed linewidth (in Hz) at the given temperature. Because experimental conditions can occasionally lead to the above-mentioned complications, we elaborate on two alternative procedures that could serve as independent checks to Eqs. 5 and 6 while at the same time eliminating the problems associated with intrinsic linewidth, temperature, sample or spectrometer differences.

Protocol #2

In situations where there is some uncertainty about the MW, it would be useful to rely on an internal linewidth standard. By identifying a resonance belonging to the protein or peptide of interest that is not affected by conformational (i.e., dihedral angle) variations, it should be possible to use its $\Delta\nu_{1/2}$ as a benchmark or reference from which to calibrate the linewidths of those resonances, such as the ^{1}HN-^{1}Hα peaks, which are affected by conformational variations. Interestingly, the doublets belonging to the 2,6 and 3,5 aromatic protons of tyrosine (~7.1 ppm) serve as ideal, easily identified, internal linewidth references. By determining the average linewidth for all assigned tyrosine cross peaks in a TOCSY spectrum (averaging both the upper and lower diagonal signals), it is possible to use this number ($\Delta\nu_{1/2}$(Tyr)) instead of the apparent molecular weight to calculate $J_{H^NH^\alpha}$. In this case, the two best-fit equations become

$$J_{H^NH^\alpha} = 0.5(\Delta\nu_{1/2}) + 6.6 - 0.5(\Delta\nu_{1/2}(\text{Tyr})) \quad \text{for TOCSY data} \quad (7)$$

$$J_{H^NH^\alpha} = 0.6(\Delta\nu_{1/2}) + 4.0 - 0.5(\Delta\nu_{1/2}(\text{Tyr})) \quad \text{for NOESY data} \quad (8)$$

where $\Delta\nu_{1/2}$ is the half-height linewidth (in Hz) of a given amide cross peak and $\Delta\nu_{1/2}$(Tyr) is the average half-height linewidth (in Hz) for all tyrosine cross peaks in the peptide or protein of interest (as determined from TOCSY spectra). Note that the intrinsic linewidths for tyrosine resonances in NOESY spectra tend to vary significantly; consequently, TOCSY data must be used to determine $\Delta\nu_{1/2}$(Tyr).

Protocol #3

A third, albeit less refined, approach may also be used to determine the linewidth correction factor. This is based on the observation that the narrowest amide cross peak, whether in a TOCSY or a NOESY spectrum, invariably has a $J_{H^NH^\alpha}$ coupling constant of close to 4.0 Hz. This phenomenon was observed for all 11 protein samples tested in this study. Furthermore, given the distribution of ϕ angles in solved protein structures from the Brookhaven PDB (Bernstein et al., 1977), this observation should be generally valid for just about any structured peptide or protein one is likely to encounter (even molecules with mostly β-sheet structure). Consequently, we have found that $J_{H^NH^\alpha}$ can be determined quite accurately using either one of the following equations:

$$J_{H^NH^\alpha} = 0.5(\Delta\nu_{1/2}) - 0.5(\Delta\nu_{1/2}(\text{min})) + 4.0 \quad \text{for TOCSY data} \quad (9)$$

$$J_{H^NH^\alpha} = 0.6(\Delta\nu_{1/2}) - 0.6(\Delta\nu_{1/2}(\text{min})) + 4.0 \quad \text{for NOESY data} \quad (10)$$

where $\Delta\nu_{1/2}$ is the half-height linewidth (in Hz) of a given ^{1}HN-^{1}Hα (for TOCSY) or ^{1}HN-^{1}Hx (for NOESY) cross peak and $\Delta\nu_{1/2}$(min) is the half-height linewidth (in Hz) of

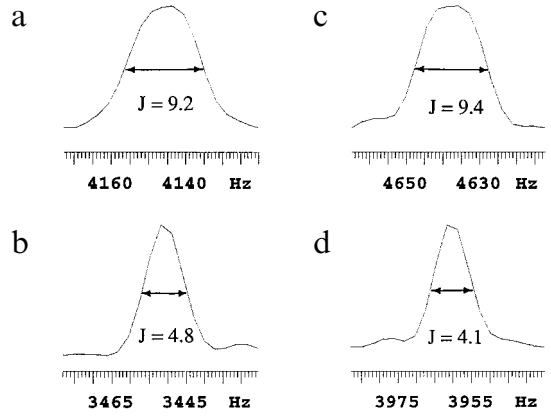

Fig. 1. Four examples of traces taken through the F2 dimension of $^1H^N$-$^1H^\alpha$ and $^1H^N$-$^1H^x$ cross peaks from TOCSY and NOESY spectra. Illustrated in (a) and (b) are TOCSY traces of amide cross peaks from ribonuclease A (Asn[103]: $\Delta\nu_{1/2} = 21.1$ Hz; Ser[90]: $\Delta\nu_{1/2} = 11.7$ Hz). Illustrated in (c) and (d) are NOESY traces of amide cross peaks from ubiquitin (Val[5]: $\Delta\nu_{1/2} = 19.9$ Hz; His[58]: $\Delta\nu_{1/2} = 11.2$ Hz). The $J_{H^NH^\alpha}$ value (in Hz) as determined from high-resolution X-ray data is indicated in each figure. Note that broad peaks are associated with large coupling constants while narrow peaks are associated with small coupling constants.

the *narrowest* amide cross peak in the spectrum. A small disadvantage to this approach is that one cannot determine the coupling constants until after all of the resonance linewidths have been measured and the narrowest line identified. Furthermore, one must exercise caution in applying this protocol to the measurement of unstructured peptides or denatured proteins. In these situations the narrowest amide cross peak would likely correspond to a coupling constant of 6 or 7 Hz instead of 4.0 Hz.

Results

Figure 1 illustrates four examples of traces taken through the F2 dimension of $^1H^N$-$^1H^\alpha$ and $^1H^N$-$^1H^x$ cross peaks from (a) TOCSY and (b) NOESY spectra. The $J_{H^NH^\alpha}$ value as determined from high-resolution X-ray data is indicated in each figure. From these four examples it is quite clear that there is a consistent, quantitative relationship between the width of the resonance and the observed $J_{H^NH^\alpha}$ coupling constant. This can be further verified if we plot the relationship between $\Delta\nu_{1/2}$ and the $J_{H^NH^\alpha}$ coupling constant as derived from X-ray data. In Fig. 2 we show the linear relationship that exists between $\Delta\nu_{1/2}$ and $J_{H^NH^\alpha}$ for a much larger data set including all measurable resonances from (a) *E. coli* thioredoxin and (b) bovine ubiquitin as determined from ^{1}H TOCSY and ^{1}H NOESY spectra, respectively. An excellent fit is obtained for both examples with correlation coefficients (r) of 0.94 for *E. coli* thioredoxin and 0.91 for ubiquitin. The strong correlation between $\Delta\nu_{1/2}$ and $J_{H^NH^\alpha}$ and the clear linear relationship observed for these and other examples suggested that a simple equation of the form

$$J_{HN_H\alpha} = m\,\Delta\nu_{1/2} + B \qquad (11)$$

(where m is the slope, B is the y-intercept and $\Delta\nu_{1/2}$ is the half-height linewidth) could be developed to predict coupling constants from $\Delta\nu_{1/2}$ measurements from ^{1}H TOCSY and NOESY spectra.

Extensive curve-fitting combined with various combinations of processing parameters allowed us to identify a common slope (m = 0.5) to all of these J versus $\Delta\nu_{1/2}$ plots for TOCSY spectra and a slope (m = 0.6) for NOESY spectra. Further comparisons revealed a clear and consistent relationship between the y-intercept (B) and the molecular weight of the peptide or protein. This relationship between the 'best-fit' y-intercept (for TOCSY data) and the molecular weight of the peptide is plotted in Fig. 3. Note that interleukin 8 forms a well-defined dimer (MW = 16.2 kDa), while T4 thioredoxin (MW = 20.1 kDa) shows strong evidence of forming a nonspecific dimer at the concentrations used in this study. The remaining compounds are known to be monomeric. Also plotted in Fig. 3 is the relationship between the best-fit y-intercept and the half-height linewidth of the aromatic protons of each compound's tyrosine resonances ($\Delta\nu_{1/2}$(Tyr)) as well as the

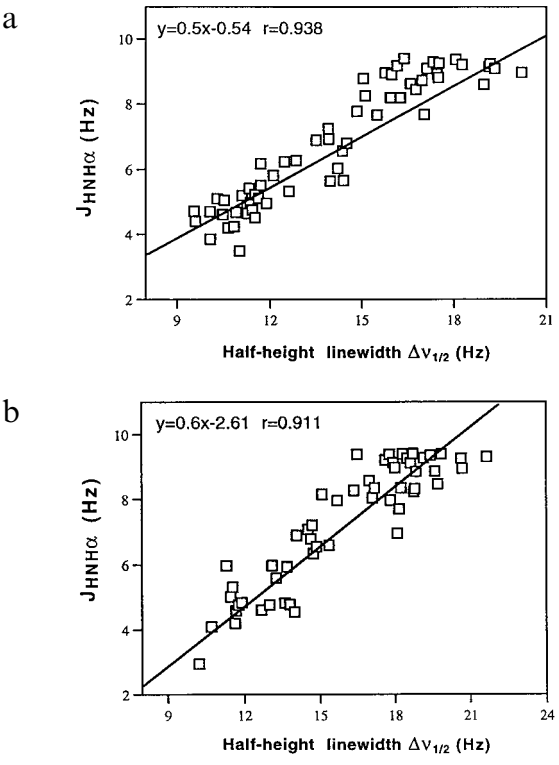

Fig. 2. Relationship between $\Delta\nu_{1/2}$ and $J_{H^NH^\alpha}$ for (a) *E. coli* thioredoxin and (b) bovine ubiquitin as determined from ^{1}H TOCSY and ^{1}H NOESY spectra, respectively. The equation for the best-fit line derived from protocol #1 and the correlation coefficient (r) are shown in the top left corner of each graph. Note that the superimposed line is a best-fit line for all of the data (650 points) and all of the proteins (11) and so, for any given protein, there may be slight systematic deviations at certain extrema.

relationship between the y-intercept and the half-height linewidth of the narrowest line ($\Delta\nu_{1/2}$(min)). Excellent fits are obtained for all three of these plots (r = 0.96, 0.91 and 0.97, respectively), indicating that Eqs. 4–9 are essentially equally valid and equally accurate.

Using these equations, we were able to predict the $J_{H^NH^\alpha}$ coupling constant for a total of 11 peptides and proteins. The correlation between these predicted coupling constants (designated as J_{lw} – since they were derived from linewidth measurements) and the coupling constants derived from the corresponding high-resolution X-ray structures (designated as J_{xray}) is shown in Tables 2 and 3. These two tables summarize the results of more than 650 coupling constant measurements (383 from TOCSY data; 279 from NOESY data) made using this simple technique. Both tables clearly show the excellent agreement obtained for both large (20 kDa) and small (1 kDa) polypeptides and that this agreement is consistently good from experiment to experiment and protein to protein. Overall, for the 11 polypeptides tested, TOCSY data yielded an average correlation coefficient of 0.89 and an rmsd from J_{xray} of 0.86 Hz while NOESY data yielded an average correlation coefficient of 0.90 and an rmsd from J_{xray} of 0.85 Hz.

Discussion

The methods described in this manuscript offer a unique approach to quantitatively measuring J-coupling constants. Almost every method previously published depends on the measurement of peak-to-peak separation (Pardi et al., 1984; Kim and Prestegard, 1989; Ludvigsen et al., 1991; Szyperski et al., 1992) combined with detailed computer-aided curve-fitting to determine J-coupling constants. In our approach, no attempt is made to measure peak-to-peak separations. Rather, the half-height linewidth ($\Delta\nu_{1/2}$) of in-phase peaks is used as a simple proxy for peak-to-peak separation. This avoids the difficulties and frailties of earlier methods because linewidths are less affected by noise, peak intensity and digital resolution than are peak positions. This is underlined by the fact that our spectra could be analyzed at a digital resolution (after zero-filling) of 3.0 Hz/pt while most DQF-COSY analyses, for instance, require a digital resolution of 0.5 Hz/pt or better.

While a relationship between $\Delta\nu_{1/2}$ and $J_{H^NH^\alpha}$ was not unexpected, what was particularly surprising was that this relationship was so linear and that the same general equation (J = mx + B) could be applied to such a range of differently sized molecules. Simulations, using the same weighting functions as employed in this paper, on spectral doublets having a variety of linewidths, revealed that the relationship between $\Delta\nu_{1/2}$ and $J_{H^NH^\alpha}$ is only approximately linear (see Fig. 4). Furthermore, these simulations predict that the slope of the curve (a parabola) should increase with increasing molecular weight (or decreasing T_2). Given the gradual nature of the predicted curve, we

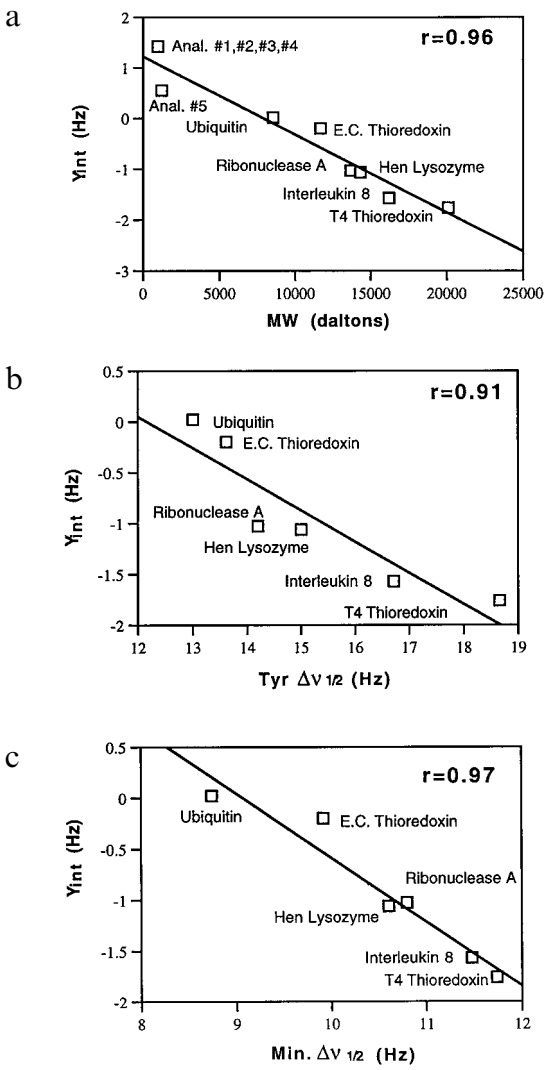

Fig. 3. Relationship between the best-fit y-intercept (for TOCSY data) and (a) the molecular weight of the peptide, (b) the tyrosine half-height linewidth, and (c) the linewidth of the narrowest amide resonance. The correlation coefficient (r) for each line is given in the top right corner of each graph.

believe the scatter associated with our plots is probably too great to distinguish between a straight line and a parabola. Consequently, a linear approximation, as we have employed in this paper, is entirely adequate to quantitatively predict J-coupling constants. Interestingly, we were unable to detect a trend in our experimental data which reiterated the predicted relationship between the slope and the molecular weight. This difference between experimental and theoretical results may have to await further investigation. Nevertheless, the apparent independence between the slope and molecular weight (or T_2) certainly makes protocols we have described much easier to use and far easier to remember.

Because this method makes use of linewidth measurements, and because linewidths are sensitive to T_2's, correlation times, temperature, molecular weights, sample

TABLE 2
SUMMARY OF RESULTS OBTAINED USING LINEWIDTH ANALYSIS (PROTOCOL #1) FOR DETERMINATION OF $J_{H^N H^\alpha}$ COUPLING CONSTANTS (TOCSY DATA ONLY)

Protein	MW (Da)	No. of points	r (J_{xray} vs. J_{lw})	Rmsd (J_{xray} vs. J_{lw}) (Hz)
T4 thioredoxin	20 100	47	0.88	0.88
Interleukin 8	16 200	47	0.91	0.88
Lysozyme	14 300	87	0.85	0.94
Ribonuclease A	13 700	69	0.86	0.98
E. coli thioredoxin	11 700	62	0.94	0.72
Ubiquitin	8600	48	0.91	0.81
GS peptide #1–#4	1200	16	0.96	0.74
GS peptide #5	1200	7	0.96	0.50

conditions and shimming, it is important that appropriate correction factors be identified and properly used. We have described three independent methods for determining these correction factors. One is based on molecular weight (protocol #1), another is based on an internal linewidth standard (protocol #2) and the third is based on the linewidth of the narrowest amide cross peak (protocol #3). Protocol #1 is perhaps the simplest to use and is our preferred choice in deriving coupling constants from TOCSY or NOESY data. Protocol #2 is perhaps the most rigorous and least sensitive to sample or spectrometer variations. Its only disadvantage is that it requires a few additional linewidth measurements for calibration purposes. We recommend that protocol #3 be used only if there are no tyrosines in the molecule or if one wishes to perform an independent check of protocols #1 or #2. The option of choosing any one of three independent methods for calculating $J_{H^N H^\alpha}$ coupling constants certainly provides a level of redundancy and robustness not often found in many other NMR measurements.

In assessing the accuracy of this method we were careful to select a subset of polypeptides spanning a significant size range (1–20 kDa) for which there were readily available high-resolution X-ray structures. It has been noted by others (Pardi et al., 1984; Smith et al., 1991; Garrett et al., 1994) that higher resolution X-ray structures invariably yield better agreement with measured J-coupling constants than lower resolution X-ray structures. Furthermore, X-ray structures (no matter what resolution) consistently show better agreement between measured J-coupling constants than structures generated through NMR distance geometry methods (Garrett et al., 1994). Indeed, our own investigations on almost 1000 $J_{H^N H^\alpha}$ coupling constants extracted from the literature (D.S. Wishart, manuscript in preparation) show that the standard deviation between measured $J_{H^N H^\alpha}$ values and those derived from NMR-generated structures is typically greater than 1.69 Hz, while the standard deviation between measured $J_{H^N H^\alpha}$ values and those derived from X-ray structures is less than 0.96 Hz. This difference is quite substantial and certainly justifies our reliance on X-ray dihedral angles rather than on NMR-derived dihedral angles to assess the accuracy of our method.

Nevertheless, differences between one X-ray crystal form and another (say tetragonal versus triclinic) or between one structure at high resolution and another at marginally lower resolution typically introduce a mean error (averaged over the length of the polypeptide) of $\pm 10°$ for the ϕ dihedral angle. This suggests that the best possible agreement between crystal-structure-derived J-coupling constants and a 'perfect' method for determining coupling constants directly from NMR data would lead to a 'best-case' rmsd of 0.54 Hz and a correlation coefficient of slightly less than 0.98 (Wang and Bax, 1996). A more reasonable set of values, which would account for the inherent differences between solution and crystal structures, would likely add a further 0.50 Hz to the uncertainty in $J_{H^N H^\alpha}$, thereby giving a predicted correlation coefficient of 0.93. With these theoretical limits in mind, we compared our results to both these predictions and to the results from other popular methods for quantitatively measuring $J_{H^N H^\alpha}$ coupling constants.

In total, more than 650 $J_{H^N H^\alpha}$ coupling constants were evaluated from our data set. This is roughly equal to 1/2 of

TABLE 3
SUMMARY OF RESULTS OBTAINED USING LINEWIDTH ANALYSIS (PROTOCOL #1) FOR DETERMINATION OF $J_{H^N H^\alpha}$ COUPLING CONSTANTS (NOESY DATA ONLY)

Protein	MW (Da)	No. of points	r (J_{xray} vs. J_{lw})	Rmsd (J_{xray} vs. J_{lw}) (Hz)
Interleukin 8	16 200	46	0.89	0.93
Lysozyme	14 300	80	0.88	0.86
Ribonuclease A	13 700	46	0.92	0.85
E. coli thioredoxin	11 700	53	0.91	0.83
Ubiquitin	8600	54	0.91	0.85

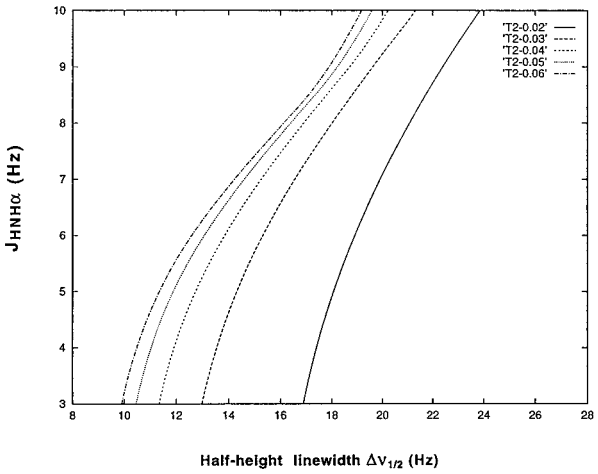

Fig. 4. Computer simulation of the dependence of the $J_{H^NH^\alpha}$ coupling constant on the half-height linewidth ($\Delta\nu_{1/2}$) using six different T_2 values. Note that this family of curves is slightly parabolic.

all (quantitative) $J_{H^NH^\alpha}$ coupling constants reported over the past 20 years. On average, our experimentally measured J_{lw} values agreed with derived J_{xray} values with a correlation coefficient (r) of 0.89 and an rmsd error of 0.86 Hz. This compares very favorably to the results obtained by Kim and Prestegard (1989) for their DQF-COSY method where r = 0.74 and rmsd = 1.95 Hz for acyl carrier protein. It also compares favorably with the results reported by Smith et al. (1991) wherein their method yielded r = 0.96 and rmsd = 0.93 Hz for DQF-COSY data collected on hen lysozyme. Similarly, Ludvigsen et al. (1991) obtained an r value of 0.90 and an rmsd of 1.16 Hz for their NOESY/DQ-COSY method as applied to the CI-2 inhibitor. The HMQC-J approach (Kay et al., 1989) when applied to staphylococcal nuclease yielded an r value of 0.89 and an rmsd of 1.01 Hz. The HNHA method of Vuister and Bax (1993) as similarly applied to staphylococcal nuclease yielded an r value of 0.91 and an rmsd of 0.76 Hz.

With the possible exception of the method of Kim and Prestegard (1989), nearly all of the methods (including ours) achieve a level of agreement that is reasonably close to optimal (rmsd = 0.54 Hz, r = 0.96) and essentially identical to expected (rmsd = 1.04 Hz, r = 0.93). Overall, the average correlation coefficient for the four best methods was r = 0.92 and the rms deviation was 0.97 Hz. While the average correlation coefficient for the four best methods is slightly higher than ours (0.92 versus 0.89), it is important to note that our calculations were performed on a substantially larger (5–15 times larger) sample and a significantly more diverse set of polypeptides (in both size and structure) than any of the other methods. Had any of these previously published methods been applied to a comparable number of data points (>650) or to proteins as large (>20 kDa) as those analyzed here, we expect the results would have been somewhat different. In this regard, the work of Garrett et al. (1994) is of particular note. These workers recently re-evaluated the HNHA experiment using a much larger data set (264 points versus 96 points) and found that the rms deviation for this more representative sample increased to 1.42 Hz (from 0.76 Hz) and the correlation coefficient fell from 0.91 to 0.78.

To summarize, we have described a novel method that allows $J_{H^NH^\alpha}$ coupling constants to be rapidly determined from simple linewidth measurements of ^{1}H TOCSY or ^{1}H NOESY spectra. This method makes use of the linear relationship between $J_{H^NH^\alpha}$ and $\Delta\nu_{1/2}$ of appropriately processed NMR spectra. We believe this new approach offers several significant advantages over most other published techniques.

First, it is simple enough that coupling constants can be determined almost by inspection and without the need of a computer program or any kind of complex curve-fitting routine. Indeed, only a quick calculation based on the measured linewidth of amide ^{1}H traces is needed to obtain an accurate $J_{H^NH^\alpha}$ coupling constant.

Second, the method is very quick. Once the assignment process has been completed, we have generally found it to be possible to manually determine the $J_{H^NH^\alpha}$ coupling constants of a 100-residue protein in less than 30 min. This has allowed one of us (Y.W.) to measure more than 650 $J_{H^NH^\alpha}$ coupling constants – nearly 1/2 of the total of all $J_{H^NH^\alpha}$ values ever reported over the last 20 years – in less than a few hours (this total, however, does not include the considerable time required to prepare the samples, collect the spectra and assign all of the proteins used in this study).

Third, the method is accurate. As described previously, this new approach has an rmsd of less than 0.9 Hz and a correlation coefficient of 0.89 when compared to X-ray-derived coupling constants. This equals or betters the performance or accuracy of nearly every other method published to date.

Fourth, the method is applicable to conventional, easily obtained, high signal-to-noise NMR experiments. In particular, we have shown that this method works well for both TOCSY and NOESY data. These are robust NMR experiments which can be routinely collected on almost any modern high-field spectrometer. Further, TOCSY and NOESY spectra have signal-to-noise ratios that are minimally 16 times higher than conventional DQF-COSY spectra, thereby allowing higher quality J-coupling data to be collected at a much faster rate than DQF-COSY's.

Fifth, the method is applicable to both small peptides and large proteins. In particular, we have been able to obtain accurate coupling constants for proteins as large as 20.1 kDa (T4 thioredoxin) using only homonuclear TOCSY data. Previously, the largest protein or protein complex for which quantitative coupling constants have been reported was 18 kDa (Billeter et al., 1992) and this complex required the use of heteronuclear spectroscopy. We have every reason to believe that our technique could be applied to molecules significantly larger than 20 kDa.

Sixth, the method is independent of special requirements in terms of isotopic labeling (we are currently developing approaches to apply this method to ^{15}N-labeled material), spectrometer hardware (third and fourth channels are not needed, nor are pulsed-field gradients) and spectrometer software (special pulse sequences and special deconvolution programs are not required). This generalizability makes this method highly portable and easily implemented – even by the most naive NMR spectroscopist.

Seventh, the method has been thoroughly tested. Having measured and verified more than 650 coupling constants from 11 different polypeptides (from 1 to 20 kDa) collected at three different field strengths (300, 500 and 600 MHz) over a range of temperatures (20–35 °C), we believe this approach to coupling constant measurement is among the most thoroughly tested methods yet presented.

While there are many positive aspects to this simple approach to $J_{H^N H^\alpha}$ coupling constant determination, it is still important to remember that it is not without some flaws. Certainly there may arise circumstances where protein linewidths, for whatever reason, may become sufficiently large (perhaps >30 Hz) such that the general linear relationship (Eq. 11) does not hold. Similarly, for very small peptides (<10 residues), it is often possible to measure the peak-to-peak separation of in-phase TOCSY or NOESY doublets without having to measure their linewidths. In these extreme situations it is neither practical nor particularly useful to apply linewidth analysis. It is also important to note that even under ideal circumstances, it is possible to introduce a systematic error (up to 0.5 Hz) in coupling constant measurements through an incorrect determination of the 'y-intercept' or correction factor. Care, therefore, must be taken to ensure that this correction factor is consistent with what is known about the molecule (i.e., is it a dimer or a monomer?) and that it yields a range of $J_{H^N H^\alpha}$ values typical for proteins (between 3 and 10 Hz).

Still another limitation with this approach to $J_{H^N H^\alpha}$ measurement is the fact that it requires TOCSY or NOESY spectra to be collected and processed in a very precise manner. Obviously computer-based curve-fitting programs are more flexible and do not typically constrain the user to follow special spectral collection and processing conditions. Nevertheless, we have found that our approach is somewhat more flexible than what might be expected. In this regard, we investigated whether measured linewidths were sensitive to different levels of digital resolution (both before and after zero-filling). Using three sets of ubiquitin TOCSY data with digital resolutions ranging from 6 to 1.5 Hz/pt (before and after zero-filling), we found that measured linewidths for all three spectra were essentially identical with an average rms difference of less than 0.2 Hz and a correlation coefficient of 0.997. Consequently, the coupling constants predicted from these three data sets were essentially identical. This indicates that spectral resolution (so long as the intrinsic linewidth is not a result of poor digitization) does not seem to adversely affect the lineshapes, the linewidths or the predicted coupling constants. In addition to this work on digital resolution, we investigated how the use of different kinds of signal processing functions (Gaussian, shifted Gaussian, shifted-sinebell, etc.) on TOCSY and NOESY data might affect the correlation between $\Delta\nu_{1/2}$ and $J_{H^N H^\alpha}$. While the numbers for the slope and intercept do change, the linear relationship between linewidth and $J_{H^N H^\alpha}$ appears to hold for all processing parameters so far tested.

Because the method we have described is fundamentally based on linewidth measurements and because linewidths are sensitive to segmental motions, fast and slow exchange, polymer 'end' effects, spectral overlap and decoupler distortion, it is important to be aware of the complications that these phenomena can cause. For instance, if the protein has a very flexible head or tail, or if it contains a mobile 'hinge' region, then it is likely that the linewidths for these segments will be somewhat different from the rest of the protein. Because the peptides and proteins we selected were all stable, well-structured, single domain molecules, we did not encounter this problem. However, we did observe amide resonances at the C-termini of two proteins that were somewhat narrower than other resonances. Similarly, we found at least one occurrence where the linewidth of a particular resonance was nearly twice as broad (40 Hz!) as the next widest resonance. Whether this was due to exchange broadening, decoupler distortion or spectral overlap is not clear, but the presence of such an outlier is usually sufficiently obvious that it can be dealt with appropriately.

One unexpected result from this work was the observation that the linewidths from TOCSY cross peaks are generally narrower than NOESY cross peaks. This observation led to the development of separate equations relating $J_{H^N H^\alpha}$ coupling constants to measured TOCSY and NOESY linewidths. To understand why this difference was observed, it is important to remember that TOCSY cross peaks, unlike NOESY cross peaks, have a mixture of both in-phase and anti-phase components (Bax and Davis, 1985). These anti-phase components, if not completely removed, will cause slight phase distortions in TOCSY lineshapes (this distortion is most obvious for glycines) which will lead to changes (i.e., narrowing) in the apparent linewidth of an unresolved doublet. The only way to remove these anti-phase components is to employ z-filtering (Sørensen et al., 1984). As shown by Subramanian and Bax (1987), the use of z-filters in a 1D TOCSY experiment can yield spectra that are sufficiently free from distortion to permit very accurate peak-to-peak measurements of well-resolved multiplets. To investigate this issue further, z-filtered TOCSY spectra employing a DIPSI mixing sequence were collected for a mid-sized protein (ubiquitin) and compared to both 'unfiltered'

TOCSY and conventional NOESY spectra of the same molecule. The results indicate that the use of a z-filtered TOCSY experiment led to spectra with linewidths very similar to those measured from our NOESY spectra. This suggests that if one were to employ z-filtered TOCSY experiments (instead of the unfiltered ones employed in this study), in all likelihood, only a single set of equations would be needed to extract coupling constants from either NOESY or TOCSY spectra. This result underlines the dependence that this particular technique has on the spectral collection conditions and serves to emphasize the importance of adhering to the collection and processing conditions described in the Materials and Methods section.

Conclusions

As this simple method of $J_{H^NH^\alpha}$ coupling constant determination is applied to other biomolecular systems, we expect further refinements and improvements will be possible. We are also hopeful that this very general concept of linewidth measurement, as opposed to peak-to-peak measurement, will find applications beyond the determination of protein $J_{H^NH^\alpha}$ coupling constants through homonuclear ^1H spectroscopy. Currently we are working on methods to quantitatively measure $J_{H^NH^\alpha}$ coupling constants using heteronuclear techniques. We are also working to develop methods for measuring other vicinal and geminal coupling constants ($J_{\alpha\beta}$) using a combination of both homonuclear and heteronuclear spectroscopy. We believe that this and other related work will make quantitative coupling constant measurements far simpler and far easier to use in analyzing the conformation of peptides, proteins, carbohydrates and other biomolecules through NMR.

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