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A new 2D NMR method for measurement of J_{HH} coupling constants

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SUMMARY

A new 2D NMR pulse sequence for E.COSY-type measurement of J_{HH} coupling constants is introduced. It exploits a heteronuclear spin, e.g., ^{13}C, for displacement in the ω_1 frequency dimension via a large heteronuclear J coupling. The experiment is demonstrated by application to a heptapeptide at the natural abundance ^{13}C level. It is suitable, for example, for measurement of $^3J_{HH}$ and $^4J_{HH}$ coupling constants in peptides and proteins.

Although the first protein structure determinations by NMR did not rely on information about torsional angles from scalar spin–spin coupling constants, it is nowadays inconceivable that accurate protein NMR structures could be determined without rigorous compilation of J coupling constants. Practically all multidimensional NMR techniques for measurement of J couplings generate so-called E.COSY-type patterns (Griesinger et al., 1985,1986,1987) by which small Js can be conveniently determined when they are associated with larger Js that exceed the linewidth. In ^{13}C, ^{15}N-labelled proteins such large couplings are provided by ^{13}C-^1H and ^{15}N-^1H one-bond interactions.

The common multidimensional techniques for determination of J_{HH} coupling constants in proteins rely (apart from the original E.COSY technique) on multiply heterolabelled molecules (Montelione and Wagner, 1989; Olsen et al., 1993). However, whereas multiple labelling is a prerequisite for NMR structure determination of larger proteins, techniques requiring only single hetero spin labels are of interest in many other contexts. Such techniques would be relevant, for example, for medium- and small-sized proteins with low enrichment or possibly even at the natural abundance level.

In this communication we present a 2D technique of this type, namely an experiment for determination of J_{HH} coupling constants requiring only one heteronuclear spin isotope per mole-

cule. As an illustration we use the heptapeptide Gly^{23}-Phe^{24}-Phe^{25}-Tyr^{26}-Thr^{27}-Pro^{28}-Lys^{29}, that corresponds to the fragment of residues 23–29 in the B-chain of insulin. The heteronuclear spin isotope used is ^{13}C at the natural abundance level.

Before describing in detail the new experiment, it is appropriate to regard it in the context of alternative methods for measurement of J_{HH} coupling constants which do also not rely on a high level of heterolabelling (Kim and Prestegard, 1989; Titman and Keeler, 1990; Ludvigsen et al., 1991; Willker and Leibfritz, 1992). All these methods in their original form depend on cross peaks generated by the J_{HH} couplings to be determined. Consequently, there is a lower limit on the ratio J_{HH} : linewidth beneath which these methods fail.

For the first three alternative techniques quoted above, this is a matter of sensitivity as well as accuracy, although satisfactory results have been reported for ratios J : linewidth down to 1 : 2. Nevertheless, these techniques obviously have a sensitivity edge over other methods that rely on ^{13}C or ^{15}N at the natural abundance level.

The method of Willker and Leibfritz requires one heteronuclear spin per molecule, but in contrast to the three above, here only the sensitivity and not the accuracy depends on the active J_{HH} coupling (of course neglecting the inherent dependence of accuracy on signal-to-noise ratio). This is due to generation of convenient E.COSY cross-peak patterns. However, the latter method is limited to measurement of J_{HH} coupling constants where at least one of the protons is part of an XH group (e.g. CH, *not* CH_2 and CH_3).

Furthermore, our method and that of Willker and Leibfritz can be employed for high-accuracy measurement of four-bond J_{HH} coupling constants, which may serve as additional structural constraints as discussed recently in the context of homonuclear relayed E.COSY (Schmidt et al., submitted for publication). For this purpose, the techniques of Kim and Prestegard, Ludvigsen et al., and Titman and Keeler cannot be employed because of an in general devastating ratio $^4J_{HH}$: linewidth.

Our method differs from that of Willker and Leibfritz by entirely removing the J_{HH} coupling constant to be determined not only from the accuracy but also from the sensitivity limitation. In addition, there is no limitation on the X-spin multiplicity (e.g. CH, CH_2, CH_3), apart from the fact that normally only sums of two coupling constants can be determined for CH_2 groups. As far as sensitivity is concerned, the technique of Willker and Leibfritz is favoured by normally larger active coupling constants. This is to some degree compensated by the fact that our pulse sequence is considerably simpler. In the method of Willker and Leibfritz, the J_{HH} dependence can be removed from sensitivity by replacing the TOCSY element by a NOESY or ROESY element. This results in sensitivity dependence on the cross-relaxation rate, which can be advantageous, in particular for macromolecules.

In summary, we consider our method the method of choice as a general technique for determination of $^3J_{HH}$ and $^4J_{HH}$ coupling constants in medium-sized proteins, where the sensitivity penalty for having to rely on ^{13}C and ^{15}N has been remedied by an appropriate (e.g. 20–30%) level of nonspecific labelling. Incomplete heterolabelling has the advantages of lower costs and simpler multiplets in the hetero dimensions.

The new experiment shown in Fig. 1 is a selective version of a recently introduced pulse sequence XLOC (Sørensen et al., 1993). The selective ^{1}H refocusing π pulse has a dramatic effect on sensitivity and peak shapes.

When high resolution is desired in the ω_1 dimension, the nonselective XLOC experiment cannot

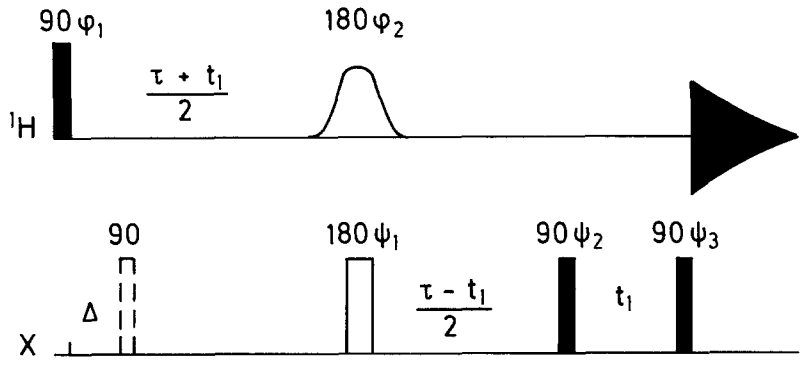

Fig. 1. Pulse sequence XLOC (X-nucleus for long-range couplings) for determination of ^{1}H-^{1}H J coupling constants. The basic phase cycle consists of $\psi_1 = 4(x,-x)$; $\psi_2 = 2(x,-x)$; $\psi_3 = (x,-x,-x,x)$, and acq. = (x,−x) while TPPI is applied to ψ_2. The numbers in front of ψ_1 and ψ_2 indicate the number of consecutive transients that are acquired with each phase before advancing to the next step in the phase cycle. In addition, the proton pulses may be phase cycled according to Sørensen et al. (1993). The dashed X-spin 90° pulse represents an optional (first-order) low-pass J filter (Kogler et al., 1983). Another option is to replace the ^{1}H 90° pulse by an equivalent selective one. This could also be considered for one of the X-spin 90° pulses surrounding t_1. Note that the effective evolution time for the long-range $^nJ_{XH}$ couplings is $\tau-\Delta$.

provide 2D pure absorption peak shapes because of an inherent asymmetry about $\omega_1 = 0$. This is caused by the different couplings of zero- and double-quantum coherences to passive spins; it manifests itself even though the proton chemical-shift part of the multiple-quantum coherences is suppressed during the evolution period. On top of this problem, the evolution of the ^{1}H-^{1}H couplings during $\tau + t_1$ leads to tilted peaks, as in homonuclear J spectroscopy.

In contrast, a truly selective ^{1}H refocusing π pulse ensures pure 2D absorption peak shapes and suppresses evolution of ^{1}H-^{1}H couplings prior to detection. This can be understood from two facts. Firstly, ignoring the X-part of the pulse sequence, the central selective π pulse reverses the evolution of the couplings by inversion of the active spin, leading to complete rephasing at the point of the echo. Secondly, the ^{1}H-^{1}H (in contrast to the ^{1}H-X) coupling part of the Hamiltonian commutes with the pulse sequence element, exciting and reconverting the heteronuclear multiple-quantum coherences.

In short, the basic idea of the XLOC experiment is an excitation of heteronuclear XH two-spin coherence (Sørensen et al., 1983) via heteronuclear long-range couplings as in HMBC (Bax and Summers, 1986). This coherence evolves in t_1 under the one-bond $^1J_{XH}$ coupling to the passive proton(s) attached to X, while J_{HH} couplings and ^{1}H chemical shifts are refocused during the period $\tau + t_1$. Because the spin state of the proton(s) attached to X is not perturbed between evolution and detection, convenient E.COSY-type multiplet patterns result. The displacement vectors have the components $^1J_{XH}$ and $^{n+1}J_{HH}$ in the ω_1 and ω_2 dimensions, respectively. Hence, measurement of $^{n+1}J_{HH}$ coupling constants is made possible by large heteronuclear $^1J_{XH}$ couplings. The sensitivity of the experiment is determined primarily by the T_2 of protons and by the magnitude of $^nJ_{XH}$ coupling constants responsible for excitation of heteronuclear two-spin coherences through a sinusoidal amplitude factor. The pure absorption peak shapes hinge on the ^{1}H π pulse being selective for the active ^{1}H spins, which is not always possible for side-chain protons but can be achieved easily for amide protons.

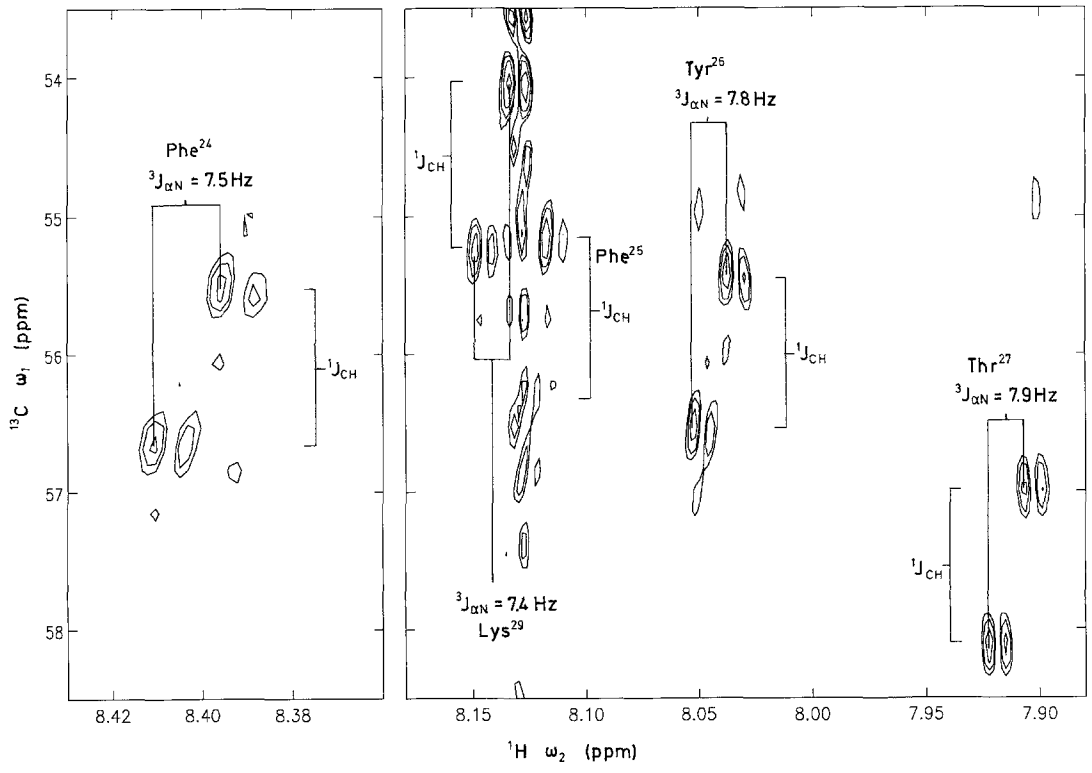

Fig. 2. Excerpts from an XLOC spectrum of the heptapeptide Gly^{23}-Phe^{24}-Phe^{25}-Tyr^{26}-Thr^{27}-Pro^{28}-Lys^{29} (9.8 mM in 90% H_2O/10% D_2O) recorded at 305 K and pH 3 (meter reading) on a Bruker AM 500 NMR spectrometer. An 8-ms REBURP (Geen and Freeman, 1991) pulse at 8.13 ppm was used for refocusing, while τ = 40 ms and t_1^{max} = 12.4 ms, t_2^{max} = 0.274 s. Water suppression was accomplished by a DANTE (Morris and Freeman, 1978) pulse train during a 1.125-s prescan delay. Four dummy scans were used and 896 scans were accumulated for each t_1 value. The real data matrix of 248 × 4096 points was apodized with a sine-square shifted 80°, combined with an exponential multiplication corresponding to −100 Hz in t_1 and a sine-bell in t_2, prior to zero-filling up to 1024 × 16384 points and 2D Fourier transformation. The ^{13}C frequencies were confirmed by assignment of the ^{13}C spectrum (not shown).

Figure 2 shows an example of correlations between amide protons and intraresidue α-carbons. The individual multiplet patterns consist of two doublets, in antiphase in ω_2 with respect to $^2J(H^NC^\alpha)$ and separated by a displacement vector $J_{H\alpha}$ = ($^1J(C^\alpha H^\alpha)$, $^3J(H^NH^\alpha)$). Hence, $^3J(H^NH^\alpha)$ can be conveniently extracted from the displacement in the ω_2 dimension. We consider this one of the most important applications of the XLOC pulse sequence.

As mentioned above, pure 2D absorption peak shapes can only be expected when the ^1H refocusing pulse is selective for the active ^1H spins. Nevertheless, even when such selectivity is impossible, the experiment still works, as will be shown in the examples to follow.

Figure 3a shows XLOC correlations between β-protons and α-carbons in the aromatic residues Phe^{24}, Phe^{25} and Tyr^{26} of the heptapeptide. For orientation and comparison, Fig. 3b shows an excerpt from a ^1H TOCSY (Braunschweiler and Ernst, 1983; Bax and Davis, 1985) spectrum displaying correlations with the same β-protons in ω_2 and the intraresidue amide protons in ω_1. From the 2D XLOC spectrum, three J_{HH} coupling constants can be extracted easily as shown by

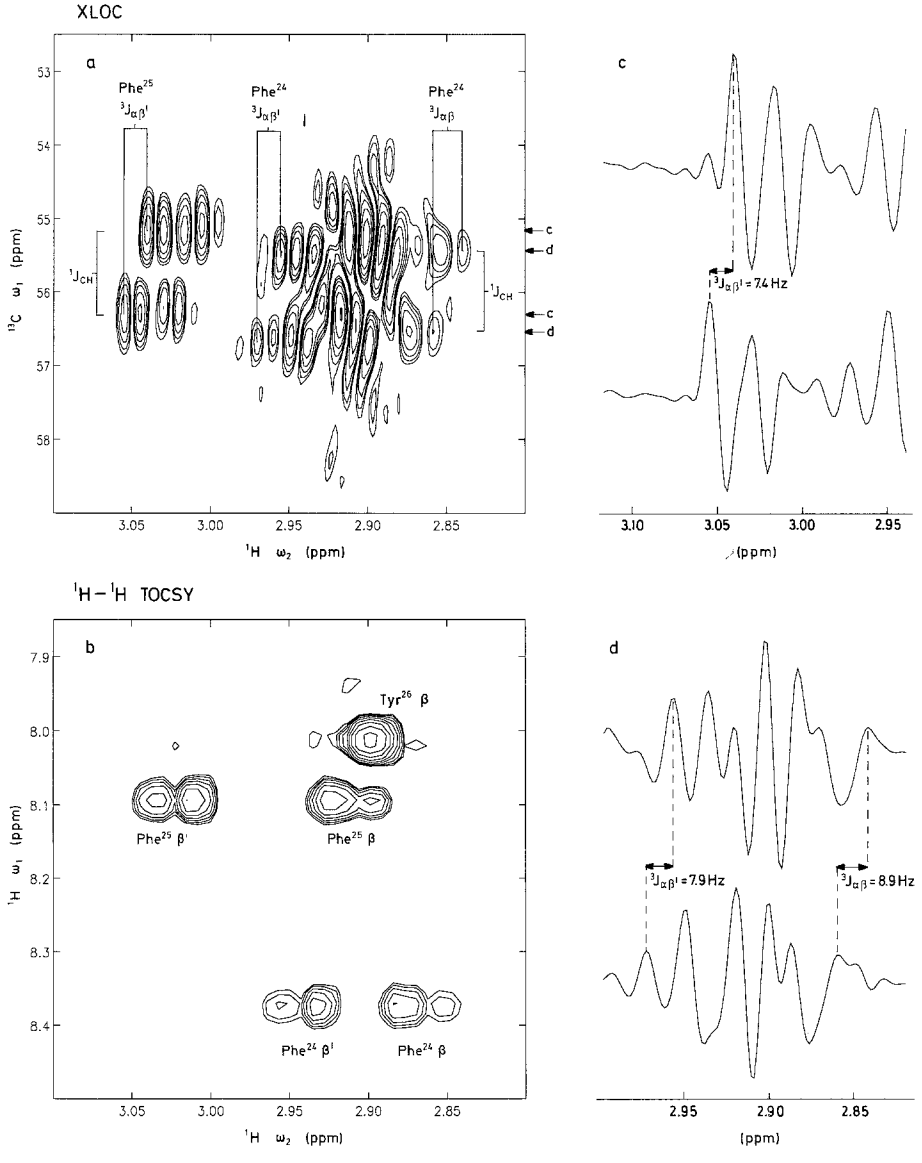

Fig. 3. (a) Excerpts from an XLOC spectrum of the heptapeptide Gly^{23}-Phe^{24}-Phe^{25}-Tyr^{26}-Thr^{27}-Pro^{28}-Lys^{29} (8.5 mM in D_2O), recorded at 310 K and pD 3.7 (meter reading) on a Bruker AM 500 NMR spectrometer. A 6-ms REBURP (Geen and Freeman, 1991) pulse at 2.17 ppm was used for refocusing, while τ = 50 ms and t_1^{max} = 12.2 ms, t_2^{max} = 0.274 s. Water suppression was accomplished by a DANTE (Morris and Freeman, 1978) pulse train during a 0.75-s prescan delay. Four dummy scans were used and 1088 scans were accumulated for each t_1 value. The real data matrix of 245 × 2985 points was apodized with a sine-square shifted 70° in t_1 and a sine-bell in t_2, prior to zero-filling up to 1024 × 16384 points and 2D Fourier transformation. (b) Excerpts from a TOCSY spectrum recorded at 310 K, showing correlations between amide protons and the same β-protons as in (a). (c) Two 1D spectra resulting from the coaddition of eight rows around each of the two rows indicated by the arrows marked 'c' in (a). (d) Two 1D spectra resulting from the coaddition of seven rows around each of the two rows indicated by the arrows marked 'd' in (a). An estimate for the uncertainty of the measured J coupling constants can be obtained by measuring the relative displacement for all four pairs of multiplet components in Phe^{25}. The result is an average of 7.3 Hz with a standard deviation of 0.3 Hz.

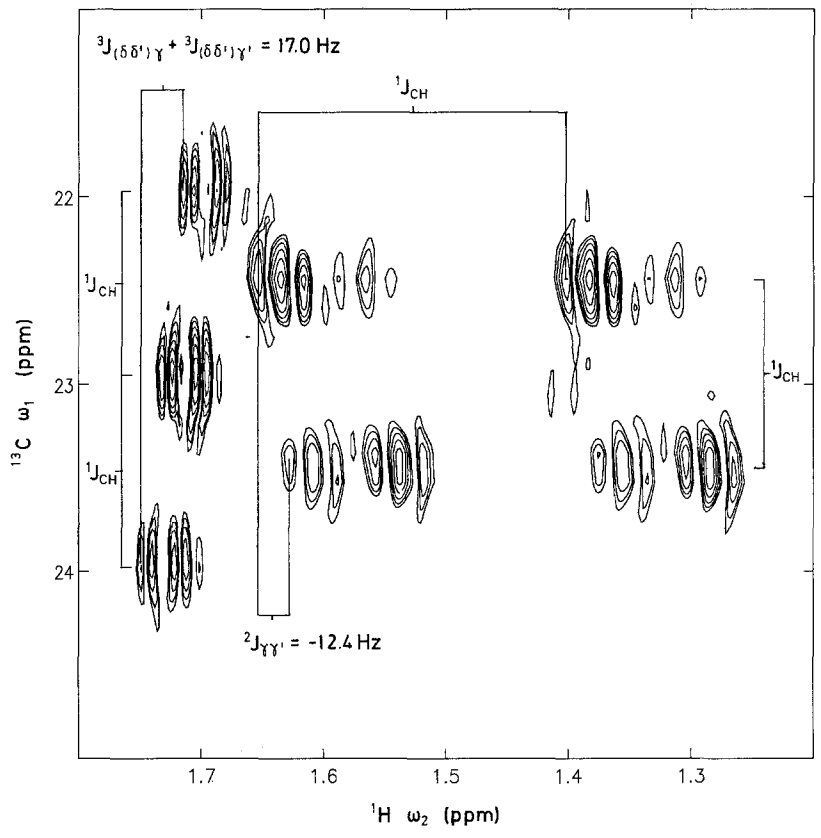

Fig. 4. Excerpts from an XLOC spectrum of lysine (0.2 M in D_2O), recorded at 300 K and pD 6.4 (meter reading) on a Bruker AM 500 NMR spectrometer. A 5-ms REBURP (Geen and Freeman, 1991) pulse at 1.9 ppm was used for refocusing, while $\tau = 40$ ms and $t_1^{max} = 25.6$ ms, $t_2^{max} = 0.274$ s. Water suppression was accomplished by a DANTE (Morris and Freeman, 1978) pulse train during a 0.75-s prescan delay. Four dummy scans were used and 384 scans were accumulated for each t_1 value. The real data matrix of 512×2985 points was apodized with a sine-square shifted 70° in t_1 and a sine-bell in t_2, prior to zero-filling up to 1024×16384 points and 2D Fourier transformation. In analogy to the error estimation procedure explained in the caption to Fig. 3, we obtain for the two indicated splittings 16.8 and −12.6 Hz with standard deviations of 0.3 and 0.4 Hz, respectively.

the traces in Figs. 3c and d. In order to resolve the overlap in Fig. 3a, one could consider extending the experiment by an additional dimension.

Figure 4 demonstrates two types of XLOC CH_2 correlations on a sample of lysine in D_2O. The triplet to the left is a correlation between the δ-protons and the γ-carbon from which normally only the sum of the J_{HH} coupling constant to the γ-protons can be extracted (from the outer components of the triplet). The remaining multiplet pattern in Fig. 4 represents two one-bond correlations of the γ- and γ'-protons with the γ-carbon. The H-C two-spin coherences are split in ω_1 by the $^1J_{CH}$ coupling constant to the other (passive) γ- or γ'-proton, resulting in doublets, i.e. displacement vectors $\mathbf{J}_{H^\gamma} = (^1J(C^\gamma H^\gamma), {}^2J(H^\gamma H^{\gamma'}))$ and $\mathbf{J}_{H^{\gamma'}} = (^1J(C^\gamma H^{\gamma'}), {}^2J(H^\gamma H^{\gamma'}))$. Note the opposite tilt of the multiplets resulting from the negative sign of the geminal coupling constant.

In conclusion, we have introduced a new method for measurement of ^1H-^1H J coupling con-

stants, exploiting a heteronuclear spin. The new technique, XLOC, addresses the shortcomings of homonuclear E.COSY when the linewidth exceeds the values of the relevant J_{HH} coupling constants. The technique should find widespread application to small- and medium-sized proteins, in particular for measurement of $^3J_{HH}$ and $^4J_{HH}$ coupling constants.

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