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Distance evaluation via heteronuclear SQC-NOESY experiments

Henriette Molinari^a, Gennaro Esposito^b, Roberto Consonni^c, Monica Pegna^d and Lucia Zetta^{c,*}

^a*Dipartimento di Chimica Organica e Industriale, Università di Milano, Via Golgi 19, 20133 Milano, Italy*

^b*Università degli Studi di Udine, Istituto di Biologia, Via Gervasutta 48, 33100 Udine, Italy*

^c*Laboratorio NMR, Istituto di Chimica delle Macromolecole del CNR, Via Ampere 56, 20131 Milano, Italy*

^d*ITALFARMACO c/o Laboratorio NMR, Istituto Chimica delle Macromolecole del CNR, Via Ampere 56, 20131 Milano, Italy*

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SUMMARY

A method for the quantitative determination of interproton distances from ¹H NOE relayed heteronuclear correlation is presented. Model compounds are investigated. Accurate distances are obtained if all the factors affecting such distance measurements, such as the local mobility and the presence of strong coupling in heteronuclear systems, are properly taken into account.

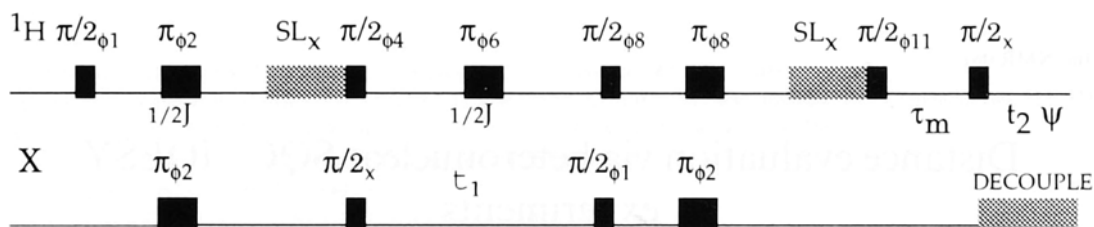
INTRODUCTION

We report a detailed analysis of the ¹H NOESY relayed heteronuclear correlation via single-quantum coherence (SQC), SQC-NOESY (Bax et al., 1990), with the aim of (i) testing the viability of this experimental approach to evaluate interproton distances, and (ii) exploring the effect of the presence of chemical exchange and strong coupling in heteronuclear systems.

THEORETICAL CONSIDERATIONS

The pulse sequence employed, along with the designed phase cycle, is depicted in Scheme 1. The heteronuclear multiple-quantum (HMQC) version of this experiment was first proposed by Shon

* To whom correspondence should be addressed.



Scheme 1. ϕ_1 and ϕ_4 are cycled independently in steps of 180° , along the x and y axis, respectively. ϕ_2 is cycled independently as $+x$, $-x$. The three pulses on the ^1H nucleus, before the mixing time, are cycled together in steps of 90° , independently of the rest of the cycle, as ϕ_8 : $+x, +y, -x, -y$ and ϕ_{11} : $y, -x, -y, x$. The phase of $\pi(^1\text{H})$, in the middle of t_1 , ϕ_6 is cycled independently in steps of 180° , as is ϕ_7 , the phase of the last $\pi/2(\text{X})$ pulse, giving a total of 128 step cycles. The receiver is cycled as $[(x, -x, -x, x)_{64} (-x, x, x, -x)_{64}]$.

and Opella (1989) to exploit the heteronuclear spreading of proton resonances and to provide a different presentation of the homonuclear 2D NOESY spectrum: if a proton Ha, scalarly coupled to the heteronucleus X (^{13}C or ^{15}N), has a NOE to another proton Hb, a cross peak will be observed at frequency coordinates Hb in F_2 and Xa in F_1 , in addition to the scalar connectivity generated at frequency coordinates (Ha, Xa). Considering the lack of a symmetry plane in such a presentation, with respect to the conventional NOESY spectrum, the resolution advantage is readily seen.

Particular attention should be given to the choice of the heteronuclear magnetization transfer scheme in order to obtain improved F_1 resolution and have longer transverse relaxation times governing t_1 evolution. It has been shown (Bax et al., 1990; Norwood et al., 1990) that experiments involving ^{15}N SQC offer improved resolution compared to the HMQC experiments as the ^1H - ^1H dipolar contribution to the HMQC transverse relaxation is larger than the heteronuclear counterpart to the ^{15}N SQC (antiphase) relaxation rate, and the unresolved J splittings are absent in the F_1 dimension of the SQC spectra. In fact, the heteronuclear antiphase coherences of type $-2I_{kz}S_{my}$, selected in SQC spectra at the beginning of t_1 , are invariant to any $\pi J_{ki}2I_{kz}I_{lz}$ or $\pi J_{kn}2I_{kz}S_{nz}$ rotations.

For ^{13}C the heteronuclear dipolar contribution is much larger, thus ^1H - ^{13}C multiple-quantum relaxation proves slower than ^{13}C transverse relaxation; however, the presence of the contribution of ^1H - ^1H J couplings in the F_1 dimension means that the MQC experiment offers no advantage over experiments that use transverse ^{13}C magnetization during the evolution period.

The two OverBodenhausen INEPT (Bodenhausen and Ruben, 1980) transfer steps, flanking t_1 evolution (Scheme 1), are the best choice when working with biopolymers for two reasons: (i) their duration is shorter in comparison with refocused INEPT or DEPT transfer schemes; (ii) the efficiency of the transfer is independent of the multiplicity n of the $I_n\text{S}$ moiety, a valuable feature especially in ^{13}C spectroscopy. Otting and Wüthrich (1988) included high-power spin-lock purging pulses at the end of the INEPT transfers to improve the elimination of undesired coherences coming from protons not coupled to heterospins, as well as from minor residual transverse components, which survived because of incomplete refocusing of the heteronuclear direct J coupling after the first INEPT transfer. These purging pulses should also destroy any additional contributions developed throughout the INEPT steps, from either the homonuclear or long-range heteronuclear J coupling evolution.

The latter point can be easily shown by analysing the sequence in terms of the product operator formalism (Sørensen et al., 1983). If I_k and S (the proton and the spin $1/2$ heteronucleus, respectively) are directly coupled, the relevant coherence evolution scheme, leading to nonequilibrium proton longitudinal magnetization, is for $\Delta = (4J_{ks})^{-1}$:

$$\begin{aligned}
 I_{kz} &\xrightarrow{(\pi/2)I_x} -I_{ky} \xrightarrow{\Delta-(\pi)_x-\Delta} -2I_{kx}S_z \xrightarrow{\pi/2(I_y+S_x)} -2I_{kz}S_y \xrightarrow{t_1-(\pi I_x)-t_1} \\
 &2I_{kz}S_y \cos(\omega_s t_1) \xrightarrow{(\pi/2)_x} -2I_{ky}S_z \cos(\omega_s t_1) \xrightarrow{\Delta-(\pi)_x-\Delta} I_{kx} \cos(\omega_s t_1) \xrightarrow{(\pi/2)I_y} -I_{kz} \cos(\omega_s t_1)
 \end{aligned}$$

Should a homonuclear scalar coupling operator $2I_{kz}I_l$ be active, the following coherences will be present at the end of the retro-INEPT scheme:

$$\begin{aligned}
 I_{kx} \cos(\omega_s t_1) \cos\left(\frac{\pi J_{kl}}{2J_{ks}}\right) + 2I_{ky}I_{lz} \cos(\omega_s t_1) \sin\left(\frac{\pi J_{kl}}{2J_{ks}}\right) - 2I_{ky}S_x \sin(\omega_s t_1) \cos\left(\frac{\pi J_{kl}}{2J_{ks}}\right) \\
 + 4I_{kx}S_x I_{lz} \sin(\omega_s t_1) \sin\left(\frac{\pi J_{kl}}{2J_{ks}}\right)
 \end{aligned} \quad (1)$$

where also the sin terms from t_1 frequency labelling have been indicated and the ratio $\frac{\pi J_{kl}}{2J_{ks}}$ comes from the total length of the last evolution period $2\Delta = (2J_{ks})^{-1}$.

The experiment is designed to select only the first term of Eq. 1. Although the two rightmost terms can be neglected, since they do not give rise to any observable proton component, it must be remembered that any subsequent transformation of the antiphase component $2I_{ky}I_{lz}$ would give antiphase dispersive I_{kx} and I_{lx} magnetizations. These J contributions are expected to be small in view of the $\sin\left(\frac{\pi J_{kl}}{2J_{ks}}\right)$ factor (ranging from 0.05 to 0.25 for typical ^{13}C or ^{15}N coupling parameters). However, in unfavourable conditions, their addition to direct and ^1H NOE relayed heteronuclear connectivities might appreciably affect the experimental intensities in the final spectrum, also leading to erroneous attributions of dipolar connectivities.

It is clear that the presentation obtained in HMQC or SQC-NOESY is a slice projection of the 3D hetero-NOESY experiment and, while retaining the handiness of 2D data matrices it is still quite adequate for the quantitative purposes that will be described here. In fact, when faced with the crowded 2D homonuclear NOESY matrices of protein or nucleic acid spectra, the detailed analysis of the cross-peak intensities represents an additional time-consuming bottleneck in the spectroscopist's work. The use of 2D heteronuclear resolved NOESY experiments may alleviate some of the problems once the proton spectrum has been almost completely assigned.

An SQC-NOESY is recorded with a mixing time set to zero. The experiment is then repeated with a mixing time sufficient to avoid spin diffusion (50–100 ms). Then, using the volume-ratio method, described for homonuclear chemical exchange spectroscopy (Bodenhausen and Ernst, 1982) and successively applied to the dipolar magnetization exchange (Esposito and Pastore,

1988), the internuclear distances can be easily extracted for resolved cross peaks and 'diagonal' peaks, i.e. ^1H NOE relayed and direct scalar heteronuclear connectivities, respectively.

Compared to the other approximative methods (build-up rate ratio, cross-peak ratio), this approach has two advantages: (i) it is independent of the difference in leakage relaxation of the reference and unknown proton pairs; (ii) it can be applied outside the linear build-up regime when a pairwise interaction model can be assumed. Its main limitation, that the overlap of diagonal peaks hinders its application in conventional homonuclear NOESY spectra of complex molecules, is, to a large extent, removed in the heteronuclear resolved ^1H NOESY presentation. The validity of the cross-peak/diagonal volume ratio method for the SQC-NOESY experiment rests on the consideration that after the evolution interval each heteronuclear labelled antiphase coherence is transferred into proton longitudinal magnetization. Therefore dipolar transfers originate from each heteronuclear labelled I_z proton magnetization, according to the specific coupling network. Except for limiting situations (e.g. the presence of paramagnetic centers), differences in T_2 between NOE-connected proton magnetizations are not expected to impair, in practice, the quantitative analysis; relaxation taking place prior to the mixing time equally affects both 'diagonal' and NOE-relayed cross peaks and their volume ratio affords the interproton distance, according to the following equation (Macura and Ernst, 1980):

$$r_{ij} = \left\{ 2q \left[\frac{6J(2\omega) - J(0)}{R_c} \right] \right\}^{1.6} \quad (2)$$

where $J(n\omega) = \frac{\tau_c}{[1 + (n\omega\tau_c)^2]}$, $q = 0.1;^4 h^2 \left(\frac{\mu_0}{4\pi} \right)^2$, $R_c = \left(\frac{k}{\tau_m} \right) \ln \left[\frac{(1+x)}{(1-x)} \right]$, with $k = 1$ for $\omega\tau_c > 1.12$,

and $k = -1$ for $\omega\tau_c < 1.12$ and x represents the experimental ratio between the cross-peak and diagonal volumes. The definition of the cross-relaxation rate constant, R_c , as well as the theory underlying Eq. 2 is given in detail by Macura and Ernst (1980) and will not be reported here. The heteronuclear SQC-NOESY experiment is not symmetric, whereas its homonuclear counterpart is. Individual cross peaks are expected to reflect the same specific attenuation factors as their 'diagonal' counterpart (i.e. at the same F_1 frequency), responsible for the relaxation of the parent transverse and heteronuclear antiphase coherences during the INEPT transfers and t_1 . Therefore the volume ratio must be calculated using connectivities with the same F_1 frequency. The possibility of using a similar approach recently appeared in the literature (Wagner, 1990), but its practical application has never been reported before.

RESULTS

To apply the discussed method, knowledge of τ_c is necessary. This can be obtained by rearranging Eq. 2, exploiting the known distance of a pair likely to reflect the correlation time of the whole molecule. In the case of a BPTI ^1H - ^{13}C SQC-NOESY experiment, vicinal aromatic protons of tyrosines and phenylalanines cannot be considered for calibration because of their local motions. Likewise, magnetically nonequivalent methylene protons, usually employed for calibrations in conventional NOESY experiments, cannot be used in the heteronuclear version: indeed, the two geminal protons are attached to the same carbon and are therefore not resolved on the heteronuclear

frequency axis, their NOE being mutually superimposed to the heteronuclear scalar connectivity peak. The key to the application of the method we describe is that both members of each pair of cross-relaxing protons are resolved on the heteronuclear axis.

Good calibration can be obtained from the α - β protons of prolines. In the five-membered ring the α and β positions are not appreciably affected by puckering. Thus the two α - β separations are approximately constant (0.23 and 0.27 nm). It is reasonable to expect that the correlation time calculated from the known distances of these protons reflects the correlation time of the molecular backbone, unless the specific chosen proline is located in a mobile fragment. For the shortest α - β distances of P2, P8, P9, and P13 in BPTI (0.228, 0.232, 0.233 and 0.236 nm, respectively) the following τ_c were obtained: 1.7, 3.2, 3.1 and 2.2 ns. These values are in good agreement with those previously reported by Jardetzky (Ribeiro et al., 1980), who estimated a τ_c in the range of 1.66–3.33 ns, and with the value of 4 ns (Richarz et al., 1980). We assumed a mean τ_c value of 3 ns for the interproton distance calculations.

The first check for the reliability of quantitative estimations from SQC-NOESY data using the cross-diagonal volume ratios comes from the analysis of the short $d_{\alpha\alpha}$ contacts, found in the region of the antiparallel β -sheet (Wagner et al., 1987a). The results are listed in Table 1. The corresponding 2D maps are depicted in Figs. 1A and B. The data in Table 1 show that for backbone protons, where a single τ_c can be assumed, the distances agree with the X-ray data. The values obtained from two rows, corresponding to the same pair interaction, are very similar (the same holds for the entire data set); this enables us to feel confident about the reliability of the method, even when only a single cross section can be used due to overlap in the other row. Additional test distances have been calculated for H_α - H_β of Cys, Thr, Asp, Asn, Gln and Arg residues, the data are reported in Table 2.

As expected, larger deviations (≈ 0.04 nm) are observed for mobile side chains, whereas for the more constrained Cys side chains the calculated values are accurate within 0.02 nm. The sequential α P8- δ P9 separation is, on the other hand, well reproduced, as expected for backbone protons. It must be noted that, when dealing with the calculation of the H_α - H_β / H_β distance for resolved β proton resonances, it is possible to distinguish two transfer pathways: α to β and/or β' and β and/or β' to α . In the former case these two interactions are separated and one or two cross peaks may be detected and integrated, while, in the latter, a single cross peak is observed, representing the

TABLE 1
ANALYSIS OF THE SHORT $d_{\alpha\alpha}$ CONTACTS (nm), USING THE CROSS DIAGONAL VOLUME RATIOS FOR THE ^1H ^{13}C SQC-NOESY SPECTRUM OF BPTI

	d(X-ray) ^a	d(SQC-NOESY) ^b
H_α C30- H_α Y23	0.219	0.21 ₁
H_α T11- H_α Y35	0.250	0.23 ₁
H_α I19- H_α V34	0.288	0.28 ₁ , 0.29 ₂
H_α T32- H_α Y21	0.253	0.22 ₁ , 0.23 ₂

^a Wagner et al., 1987.

^b Values obtained from Eq. 1, assuming $\tau_c = 3$ ns. Two values are given when both cross sections in the F_2 dimension exhibit resolved diagonal and cross peaks.

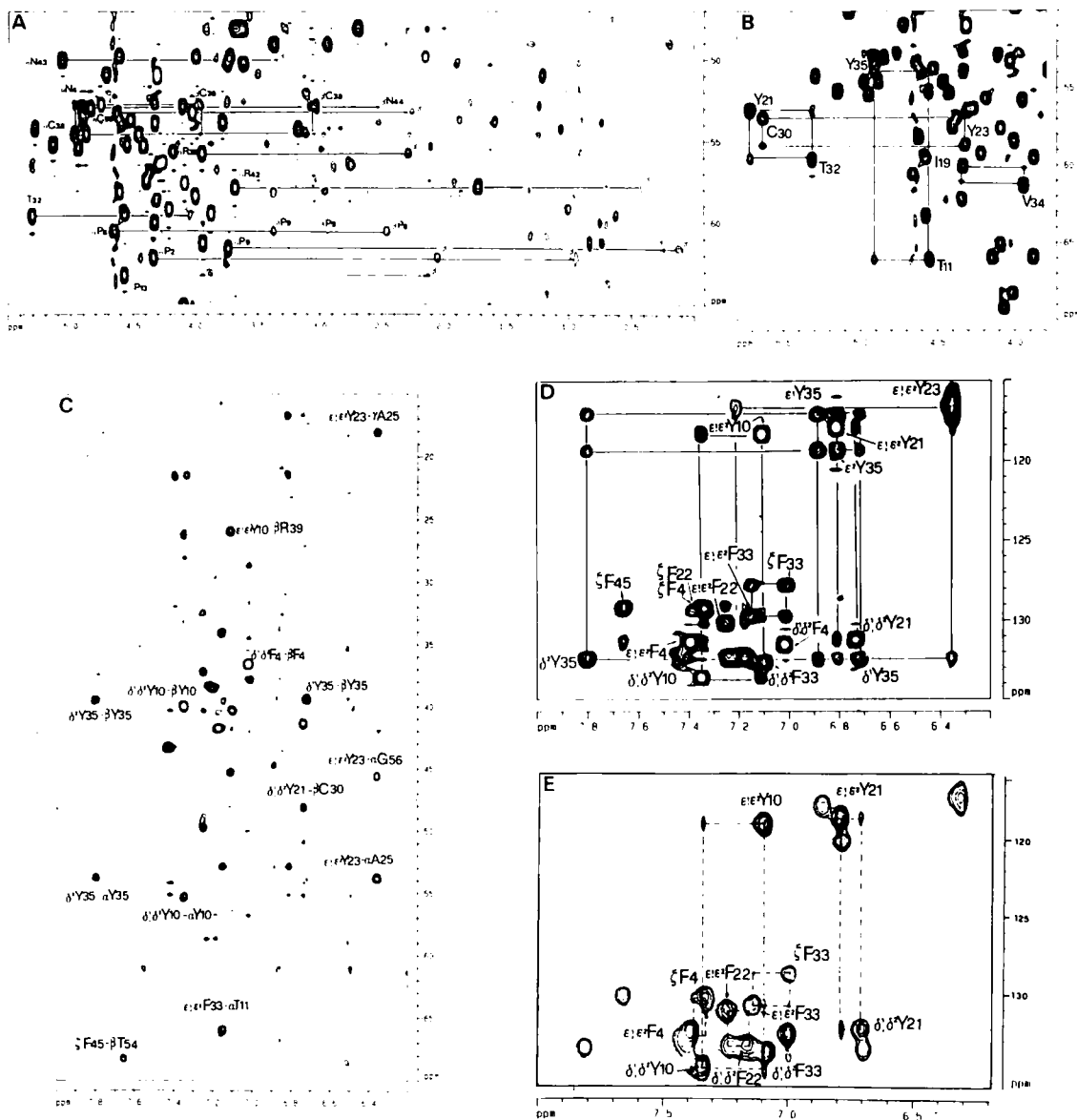


Fig. 1. Phase-sensitive ^1H - ^{13}C SQC-NOESY of a 14 mM solution of BPTI in D_2O at natural abundance, $T = 309$ K, using the pulse sequence of Scheme 1 with ^{13}C decoupling during acquisition. The spectrum was recorded in a 5 mm sample tube without sample spinning on a Bruker AM 500 spectrometer. The delays were tuned to the heteronuclear one-bond coupling constant $J = 143$ Hz, the spin-lock purging pulses were set to 3.5 ms, τ_m was 100 ms. GARP 1 was employed as a decoupling scheme, using a ^{13}C 90° pulse width of 44.5 μs . The acquisition time was 0.141 s in t_2 , 0.013 s in t_1 , and 640 transients were collected for each t_1 increment. The spectrum was processed employing a Lorentzian-Gaussian filter. This experiment was obtained with a high number of transients and experiments: very good results can be obtained with 512 scans per experiment with a total of 300 t_1 values, reducing the acquisition to ca. 60 h. The correlations shown are relative to (A) CH_α - CH_β interactions, (B) CH_α - CH_α interactions, (C) aromatic-aliphatic region, (D) aromatic region, (E) the same experiment with $\tau_m = 5$ μs ; aromatic region. Spectrum E should be compared with Fig. 1D. Dashed lines indicate the presence of cross peaks originating from heteronuclear strong coupling.

TABLE 2
ANALYSIS OF THE $d_{\alpha-\beta}$ CONTACTS (nm), USING THE CROSS/DIAGONAL VOLUME RATIOS FOR THE ^1H - ^{13}C SQC-NOESY SPECTRUM OF BPTI

	d(X-ray) ^a	d(SQC-NOESY) ^b
H _α H _β C30	0.251	0.26 ₆
H _α H _β C38	0.239	0.25 ₇
H _α H _β C51	0.239	0.26 ₆
H _α H _β C55	0.261	0.27 ₂
H _α H _β T11	0.310	0.29 ₇
H _α H _β T32	0.261	0.26 ₁
H _α H _β T54	0.305	0.27 ₉
H _α H _β N43	0.248	0.28 ₄
H _α H _β N44	0.240	0.28 ₄
H _α H _β Q31	0.247	0.28 ₅
H _α H _β R39	0.267	0.24 ₈
H _α H _β R42	0.256	0.24 ₄
H _α H _β R17	0.239	0.23 ₃
H _α P8-CH ₂ βP9	0.241	0.24 ₅

^a Wagner et al., 1987.

^b For those residues exhibiting two resolved β protons, hence two α - β distances, only the shortest value is given, on the assumption that the detectable NOE refers to the shortest distance, unless otherwise indicated.

sum of both dipolar interactions. The β protons 'diagonal' peak volumes also include their reciprocal NOE, and thus should not be used for quantitative purposes. Generally speaking, this feature restricts the applicability of the described calculation method to X-H groups and, using the proper stoichiometric coefficients, to X-H_n groups with equivalent protons.

The issue of local mobility, affecting the accuracy of distance determinations in side chains, can be further explored considering the dipolar interactions between aromatic and aliphatic protons.

In BPTI, three of the eight Phe and Tyr aromatic rings are known to rotate slowly: F45, Y23 and Y35, with flipping rates at 313 K of 1700, 300 and 50 s⁻¹, respectively (Wagner et al., 1976). At 309 K (the temperature of our measurements) only for Y35 is the flipping motion slow enough, on the proton chemical-shift scale, to allow the observation of four resolved resonances. On the carbon chemical-shift scale, however, resolution is observed only for the corresponding C^{ε1} and C^{ε2} resonances (Wagner et al., 1987b). For slowly flipping aromatic residues it is not reasonable to extract internuclear separations without carefully accounting for chemical exchange contributions, unless the rate of the intervening exchange process is known to be significantly slower than the involved relaxation rates. For the remaining aromatic rings the availability of an internal reference pair could be exploited to extract the effective correlation time, accounting for local mobility. Table 3 lists some distances for aromatic nuclei obtained employing both the mean backbone τ_c of 3 ns and the local effective τ_c , as obtained from each pair of vicinal protons (0.248 nm apart) (see Fig. 1C). The mean backbone τ_c works much better in reproducing the corresponding crystallographic separations. This is not surprising because the estimation of the effective τ_c for aromatic internuclear vectors is bound to be noticeably affected by strong coupling effects.

TABLE 3

ANALYSIS OF SOME DISTANCES (nm) INVOLVING AROMATIC NUCLEI USING THE CROSS DIAGONAL VOLUME RATIOS FOR THE ^1H ^{13}C SQC-NOESY SPECTRUM OF BPTI. THE RESULTS ARE COMPARED FOR TWO DIFFERENT τ_c 's, I.E. THE MEAN BACKBONE OF 3 ns AND THE LOCAL EFFECTIVE $\tau_{c,\text{eff}}$ (REPORTED IN PARENTHESES)

	d(X-ray) ^b	d(SQC-NOESY) $\tau_c = 3 \text{ ns}$	d(SQC-NOESY) $\tau_{c,\text{eff}}$ (ns)
C ^o H'sY10 H _o Y10	0.251	0.26 ₄	0.29 ₅ (5.9)
C ^o H'sY10 H _o Y10	0.242	0.23 ₂	0.25 ₆ (5.9)
C ^o HF45 H _o T54	0.305	0.30 ₁	c
C ^o H'sF4 H _o F4	0.230	0.20 ₄	0.18 ₆ (1.9)
C ^o H'sY23 H _o A25	0.254	0.23 ₆	0.21 ₅ (1.7)
C ^o H'sF33 H _o T11	0.258	0.25 ₁	0.31 ₆ (12.7)
C ^o H'sY10 H _o R39	0.278	0.20 ₆	0.23 ₁ (5.9)
C ^o H'sY23 H _o G56	0.289	0.27 ₁	0.24 ₅ (1.7)
C ^o H'sY23 C _H _v A25	0.317	0.30 ₇	0.27 ₇ (1.7)

^a All the distance calculations have been performed keeping the proper stoichiometric coefficients, relative to different populations of the spin species involved in the dipolar interaction (Yip, 1990).

^b Wagner et al., 1987a.

^c At 309 K C^oH's and C^oH resonances are exchange broadened, thus no local τ_c can be calculated.

In heteronuclear systems strong coupling, even if absent in the corresponding homonuclear case, occurs when (Garbow et al., 1982):

$$\left| \omega_K - \left(\omega_I \pm \frac{J_{IS}}{2} \right) \right| \approx |J_{IK}|$$

where I and K are protons and S is the heterospin.

For the aromatic protons of BPTI, at 500 MHz, this is always the case except for F4 ($\Delta\omega = 0.37$ ppm between C^oH's and C^eH's), Y23 ($\Delta\omega = 0.87$ ppm between C^oH's and C^eH's) and Y35 ($\Delta\omega = 1.08$ ppm between C^oH and C^eH and $\Delta\omega = 0.99$ ppm between C^oH and C^eH). In the presence of such strong coupling, the INEPT transfers and the t_1 evolution would no longer be effective in sorting out only antiphase or transverse coherences of the spin directly bound to the heteronucleus; hence the experimental intensities of the aromatic systems do not reflect only dipolar interactions. (A theoretical analysis of the evolution under strong-coupling conditions will be presented in a forthcoming paper.) However, the 'diagonal' connectivity may still be used to roughly calculate NOE involving 'external' nuclei (i.e. not belonging to the strongly coupled system) when the extent of its intensity perturbation, due to strong coupling, takes place before τ_m , and the departure from the isotropic spectral density, due to local motions, is not too severe. Of course, when the homonuclear spin system is inherently strongly coupled, an additional oscillatory perturbation is expected to operate also during the mixing time and t_2 , thus the quantitative analysis may become rather difficult.

For BPTI aromatic systems the intensity deviation of the heteronuclear connectivities due to strong coupling must not be too large because the distances, determined using an isotropic spec-

tral density function with $\tau_c = 3$ ns, are in reasonable agreement with the crystal structure. The largest deviation is observed for Y10 ($\Delta\omega = 0.23$ ppm between $C^{\delta}H$'s and $C^{\epsilon}H$'s). In fact even at negligible values of the mixing time ($\tau_m = 5$ μ s), cross peaks can be recognized between the aromatic pairs ϵ^1, ϵ^2 and δ^1, δ^2 of Y10 (see Fig. 1E). Even for the slowly tumbling Y23 the crystal separations are reasonably reproduced, the residual deviations probably reflecting the inadequacy of the isotropic spectral density in describing the rotational dynamics of the specific internuclear vectors. Indeed, the effective correlation time, 1.7 ns, obtained from the vicinal dipolar interaction in Y23, is not very different from the mean molecular τ_c , as one could expect for ring flipping rates equal to or slower than the overall molecular tumbling rate. This agrees with the results obtained for F4, characterized by a fast flipping rate. Although the effective τ_c for F4 cannot be safely determined, due to the poor resolution on the ^{13}C and on the 1H scale, the deviation obtained for F4 δ - β separation (Table 3) can be ascribed only to local mobility. The motion of F45 is of no consequence to the C^{ζ} dipolar interaction. For Y35 no conclusion can be drawn, because any NOE connectivity will be superimposed on chemical exchange magnetization transfers. $C^{\epsilon^1}H$ and $C^{\epsilon^2}H$ of Y35 show cross peaks to each other. Considering their large separation (0.42 nm), if their long-range J coupling were small the cross-peak intensities would primarily reflect the chemical exchange process. Based on the relative shift differences between δ and ϵ protons (Wagner et al., 1987a) it can be envisaged that the best estimate of the flipping rate would be extracted from the ratio of the $C^{\epsilon^1}H$ cross peak to $C^{\epsilon^2}H$ 'diagonal'. The calculation yields a flipping rate of 14 s^{-1} , i.e. half the value of 30 s^{-1} measured at 309 K by Wagner et al. (1976). Probably strong coupling effects are effective between $C^{\epsilon^1}H$ and $C^{\epsilon^2}H$.

Generally speaking strong coupling effects can be expected also for protons other than the aromatic ones, for example they could occur between β and γ protons of side chains; we avoided considering such cases in our calculations.

Thus it can be stated that the quantitative evaluation of internuclear distances for mobile side chains, using approximative methods, requires a careful consideration of the approximations introduced. Proper spectral density functions should be used, but additional assumptions are needed about the local geometry. On the other hand the model-free approach (Lipari and Szabo, 1982) would require preliminary relaxation studies. For SQC-NOESY spectra, attempts to circumvent the problem by employing effective correlation times, obtained from local reference proton pairs (i.e. aromatic *ortho* protons), are often hindered by proton strong scalar coupling caused by heteronuclear J coupling, thus altering the experimental intensities. Moreover, even when, in the absence of strong coupling, a reasonable effective correlation time is obtained, it cannot be used tout court in isotropic spectral density functions unless the local mobility far exceeds the mean molecular mobility. When local motions are not too slow, rough distance estimations are more safely obtained using the mean molecular τ_c .

In order to check for the accuracy of the distances obtained by 'diagonal'/cross-peak volume ratios, a 1H - ^{15}N SQC-NOESY experiment was also performed on Gramicidin S (in DMSO) where the correlations mainly involve backbone protons such as NH and CH_{α} , thus a single isotropic correlation time can be more safely assumed to describe the system. In this respect the ^{15}N filter is far more informative and unambiguous than the ^{13}C filter, and should be preferred whenever possible. The SQC-NOESY spectrum (not shown) was obtained employing the pulse sequence of Scheme 1, with a mixing time of 200 ms. It is apparent from the data of Table 4 that the 'diagonal'/cross-peak ratio method affords distances that fit the model very well.

TABLE 4
NH DISTANCES (nm) CALCULATED FROM A ^1H - ^{15}N SQC-NOESY EXPERIMENT OF GRAMICIDIN S

	d(SQC-NOESY)	d(Model) ^a
NH Phe- α Leu	0.20 ₀	0.219
NH Orn- α Val	0.20 ₁	0.224
NH Leu- α Orn	0.20 ₄	0.222
NH Val- α Pro	0.31 ₁	0.339
NH Leu- α Leu	0.25 ₆	0.280
NH Phe- α Phe	0.26 ₁	0.271
NH Orn- α Orn	0.25 ₂	0.286
NH Val- α Val	0.27 ₀	0.283
NH Phe- β_1 Phe	0.22 ₉	0.232
NH Phe- β_2 Phe	0.24 ₈	0.237

^a Dygert et al., 1975.

In conclusion SQC-NOESY spectra provide an additional and convenient tool for distance evaluation. While still retaining the advantage of limited size of the 2D matrices, the intensity of direct scalar connectivities can be measured (contrary to the corresponding diagonals of conventional NOESY in crowded spectra). Thus approximation methods can be applied to evaluate interproton distances. The resulting values can either be employed directly as restraints in MD simulations or used as initial guesses for further refinement. A full relaxation matrix treatment of SQC-NOESY data may also be envisaged in suitable systems when all the members of the dipolar network are coupled to the heteronucleus observed in t_1 . Some of the limitations encountered in the quantitative analysis of 2D SQC-NOESY (e.g. degeneracy on the heteronuclear dimension of resolved proton resonances) can be circumvented with the corresponding 3D extension of the experiment, but, regardless of the number of dimensions employed, it must be kept in mind that strong coupling effects, due to heteronuclear scalar coupling, may affect the experimental intensities. The confidence limits of the method tested depend mainly on the τ_c value and on the volume estimation error, provided that systematic errors, such as departure from the pairwise interaction model, spin diffusion or strong coupling effects, are avoided. The accuracy demand for τ_c is not very stringent (Clare, 1985). For instance, in BPTI some 10% volume fluctuation is obtained when the calculation is carried out using the extremes of the range 1.7–4 ns for τ_c . Larger deviations are, of course, expected when the actual τ_c is radically different from the mean backbone value, as in mobile side chains (Nirmala and Wagner, 1988). Provided a sufficient S/N ratio is attained the errors arising from volume estimation can be reduced by using sufficient resolution and removing possible causes of baseline distortions. In our example some 15% fluctuation on the distance values, calculated for weaker connectivities, could be expected when the S/N evaluated for those traces containing the strong connectivities (protons 0.21–0.22 nm apart) is ca. 50/1.

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