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Determination of cross relaxation rates by 3D NOE-NOE spectroscopy

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SUMMARY

A method for quantitative determination of cross-relaxation rates of macromolecules in solution is developed. The method is based on the analysis of the intensities of cross peaks in 3D NOE-NOE spectra. The linear combination of the intensities of 3D peaks (spin-diffusion peaks, back-transfer peaks) results in an expression directly proportional to the cross-relaxation rate. The proposed approach allows to determine interproton distances in macromolecules more accurately.

INTRODUCTION

The nuclear Overhauser effect plays a major role for structure determination of biomolecules in solution (Macura and Ernst, 1980; Ernst et al., 1987; Neuhaus and Williamson, 1989). The interproton distances derived from NOE data have been used as main experimental constraints carrying out distance geometry and molecular dynamics calculations to obtain the spatial structure of macromolecules (Kaptein et al., 1985; Wüthrich, 1986). Several different methods are in use to transform the information contained in 2D NOESY spectra into distance constraints. The most widely used approach to obtain the interproton distances from cross-relaxation rates, is based on the isolated two-spin approximation (ISPA). This approach utilizes the initial build-up rate of the NOE, which is proportional to the cross-relaxation rate, measured by NOESY spectra recorded at short mixing times. For longer mixing times, however, spin diffusion causes systematic errors in calculated distances (Macura and Ernst, 1980; Kaptein et al., 1985; Wüthrich, 1986; Ernst et al., 1987; Madrid et al., 1989; Neuhaus and Williamson, 1989). Different relaxation rates of the various protons introduce additional problems. Several proposals have been made to isolate the cross-relaxation rates from unwanted contributions (Wagner and Wüthrich, 1979; Kumar et al., 1981). This also includes scaling of cross-peak intensities to the intensity of the corresponding diagonal peaks (Fejzo et al., 1989) and the more promising method of the back-calculation of the

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relaxation matrix via an iterative relaxation matrix approach (IRMA, CORMA) (Boelens et al., 1989; Borgias et al., 1990). Recently 3D NOE-NOE spectroscopy was introduced in this field (Boelens et al., 1989a; Breg et al., 1990). This technique presented an elegant way to interpret and to differentiate the spin-diffusion paths by such spectra. Here we propose a new approach to determine the cross-relaxation rates directly from the 3D NOE-NOE spectra of molecules in the spin-diffusion limit. This method is based on the observation and quantification of the additional peaks present in 3D NOE-NOE spectra. The intensity of a 3D cross peak (**a-a-b**) is proportional to the cross-relaxation rate between protons **a** and **b** and other terms that are caused by spin diffusion. Spin diffusion can be evaluated directly, using the corresponding 'spin-diffusion peaks'. Under the condition of slow tumbling, the relaxation rate of a proton is approximately equal to the negative of the sum of the cross-relaxation rates to its neighbors (when leakage is neglected). This means, that all flow of magnetization from proton **a** to protons **n** is reflected in the intensities of cross peaks in the 3D spectrum (including spin-diffusion and back-transfer peaks) involving **a**. Therefore a correction of a direct peak (**a-a-b**) to pure cross-relaxation is possible by utilizing the intensities of spin-diffusion and back-transfer peaks. This allows the determination of cross-relaxation rates for macromolecules by the analysis of NOE cross-peak intensities of 3D NOE-NOE spectra at longer mixing times, even when severe spin diffusion occurs.

THEORY

Cross-relaxation in a multispin system can be described by the generalized Bloch equations (Abragam, 1978; Keepers et al., 1984A; Ernst et al., 1987). The time dependence of the peak volumes in a 2D NOE spectrum is given by:

$$\mathbf{A}(\tau_m) = \exp\{-\tau_m \mathbf{R}\}\mathbf{A}(0) \tag{1}$$

$\mathbf{A}(0)$ is the diagonal matrix of peak volumes at zero mixing time $\tau_m = 0$, representing the Boltzmann distribution of proton magnetizations. $\mathbf{R}$ is the relaxation matrix that contains the elements $R_{ii} = \rho_{ii}$ and $R_{ij} = \sigma_{ij}$,

$$\mathbf{R} = \begin{bmatrix} \rho_{11} & \sigma_{12} & \sigma_{13} & \cdot & \cdot & \cdot \\ \sigma_{21} & \rho_{22} & \cdot & \cdot & \cdot & \cdot \\ \cdot & \cdot & \cdot & \cdot & \cdot & \cdot \\ \cdot & \cdot & \cdot & \cdot & \cdot & \cdot \\ \cdot & \cdot & \cdot & \cdot & \cdot & \cdot \\ \rho_{n1} & \cdot & \cdot & \cdot & \cdot & \rho_{nn} \end{bmatrix}$$

with the relaxation rates:

$$\rho_{ii} = \gamma^4 \hbar^2 (1/r_{ij}^6)(0.1J(0) + 0.3J(\omega_0) + 0.6J(2\omega_0)) \tag{2}$$

and the cross-relaxation rates:

$$\sigma_{ij} = \gamma^4 \hbar^2 (1/r_{ij}^6)(0.6J(2\omega_0) - 0.1J(0)) \tag{3}$$

where r_{ij} is the distance between the protons i and j and ω_0 is the proton Larmor frequency. The $J(\omega)$'s are the spectral densities, that depend on the molecular motion. For isotropic motion they can be expressed as simple Lorentzians:

$$J(\omega) = \frac{\tau_c}{1 + (n\omega_0\tau_c)^2} \tag{4}$$

The $n\omega_0$ are the frequencies of the fluctuating magnetic field responsible for relaxation of the spins and τ_c is the effective correlation time. The matrix equation can be numerically solved after diagonalization of the rate matrix **R** (Keepers and James, 1984):

$$\mathbf{A}(\tau_m)/\mathbf{A}(0) = \mathbf{U}\exp\{-\mathbf{D}\tau_m\}\mathbf{U}^{-1} \tag{5}$$

where **D** is the eigenvalue matrix of **R** and **U**, $\mathbf{U}^{-1}$ are the matrices of eigenvectors and its inverse, respectively.

The intensity of cross peaks a_{ji} in a 2D NOESY spectrum can be approximated by a power series expansion:

$$a_{ji} = \{\delta_{ji} - R_{ji}\tau_m + 0.5\sum_n R_{jn}R_{ni}\tau_m^2 + \ldots \quad \}a_{ii}(0) \tag{6}$$

The R's are the elements of the relaxation matrix, δ_{ji} is the Kronecker symbol.

For 3D spectra the matrix of peak intensities is a three-dimensional array. Alternatively the elements of matrix $\mathbf{A}(\tau_m)$ obtained after evaluation of peak volumes after the first mixing time of a 3D NOE-NOE experiment can be arranged into n diagonal matrices, that represent the distribution of proton magnetization before the second mixing period:

$$\mathbf{A}_n(i,i) = \mathbf{A}(n,i) \tag{7}$$

The second mixing period can then be described as:

$$\mathbf{A}_n(\tau_m^{(2)}) = \exp\{-\tau_m^{(2)}\mathbf{R}\}\mathbf{A}_n(\tau_m^{(1)}) \tag{8}$$

The intensity of a peak at the frequency coordinates i,j,k in the 3D spectrum is then given by the product of the two individual mixing amplitudes and $a_{ii}(0)$:

$$a_{kji} = \{\delta_{kj} - R_{kj}\tau_m^{(2)} + 0.5\sum_n R_{kn}R_{nj}\tau_m^{(2)2} + \ldots\} \times \{\delta_{ji} - R_{ji}\tau_m^{(1)} + 0.5\sum_n R_{jn}R_{ni}\tau_m^{(1)2} + \ldots\}a_{ii}(0) \tag{9}$$

This yields for one-step transfer peaks, when only linear and quadratic terms in $\tau_m^{(1)}$ and $\tau_m^{(2)}$ are retained:

$$a_{jii}(\tau_m^{(1)}, \tau_m^{(2)}) \approx \{-R_{ji}\tau_m^{(2)} + 0.5\sum_{n \neq i,j} R_{jn}R_{ni}\tau_m^{(2)2} + R_{ii}R_{ji}\tau_m^{(1)}\tau_m^{(2)} + 0.5R_{ji}(R_{ii} + R_{jj})\tau_m^{(2)2}\}a_{ii}(0) \tag{10}$$

Note the difference to 2D NOESY spectra, where the intensity for a_{ji} is given by (again retaining only linear and quadratic terms in τ_m):

$$a_{ji} \approx \{ -R_{ji}\tau_m + 0.5 \sum_{n \neq i,j} R_{jn}R_{ni}\tau_m^2 + 0.5R_{ji}(R_{ii}+R_{jj})\tau_m^2 \} a_{ii}(0) \tag{11}$$

We assume that for molecules in the spin-diffusion limit ($\omega_0\tau_c \gg 1$) the dominant relaxation source (and therefore the major part of the relaxation rates) is the interaction with neighboring spins, and neglect all other contributions:

$$R_{ii} \approx \sum_{j \neq i} -R_{ij} \tag{12}$$

After substitution of Eq. 12 into Eq. 10 for a_{jii} the auto-relaxation terms R_{ii}, that are usually difficult to evaluate, are completely eliminated and substituted by measurable quantities: intensities of 3D cross peaks and back-transfer peaks. It can be shown that (for $\tau_m^{(1)} = \tau_m^{(2)} = \tau_m$) it is possible to rewrite Eq. 10:

$$\begin{aligned} a_{jii}(\tau_m) = \{ & -R_{ji}\tau_m + 0.5 \sum_{n \neq i,j} R_{jn}R_{ni}\tau_m^2 - 1.5 \sum_{n \neq i,j} R_{ji}R_{in}\tau_m^2 - 0.5 \sum_{n \neq j,i} R_{ij}R_{jn}\tau_m^2 \\ & - 1.5R_{ji}R_{ij}\tau_m^2 - 0.5R_{ij}R_{ji}\tau_m^2 \} a_{ii}(0) \end{aligned} \tag{13}$$

The first term on the right side of Eq. 13 is directly proportional to the cross-relaxation rate. The second term corresponds to the spin diffusion, while the third and fourth terms represent magnetization transfer to the surrounding protons (i→n, j→n). The last two terms represent back-transfer for spins i and j. Analogous expressions hold for other one-step transfer peaks. To obtain the cross-relaxation rate the additional contributions to the intensity of the cross peak a_{jii} simply need to be added or subtracted, depending on their sign in Eq. 13. This leads to the final expression for the cross-relaxation rates:

$$\begin{aligned} -R_{ij}\tau_m a_{ii}(0) = a_{iij}(\tau_m) & - 0.5 \sum_{n \neq i,j} R_{jn}R_{ni}\tau_m^2 a_{ii}(0) + 1.5 \sum_{n \neq i,j} R_{ji}R_{in}\tau_m^2 a_{ii}(0) + 0.5 \sum_{n \neq j,i} R_{ij}R_{jn}\tau_m^2 a_{ii}(0) \\ & + 1.5R_{ji}R_{ij}\tau_m^2 a_{ii}(0) + 0.5R_{ij}R_{ji}\tau_m^2 a_{ii}(0) \end{aligned} \tag{14}$$

It is assumed, that the quadratic terms on the right side of Eq. 14 correspond to cross peaks in the 3D NOE-NOE spectrum, so that for example $R_{in}R_{nj}$ can be directly expressed by $a_{jni}(\tau_m)$. These cross peaks can be found in two different 2D planes of the 3D NOE-NOE spectrum (Fig. 1). The spin-diffusion peaks (i→k→j) are located at the frequency coordinates (F1 = **i**/F2 = **k**/F3 = **j**). 'Leakage peaks' (i→j→k and j→i→k) can be found at (F1 = **i**/F2 = **j**/F3 = **k**) and (F1 = **j**/F2 = **i**/F3 = **k**). It should be pointed out, that equivalent peaks can be found in other 2D planes also.

RESULTS

We have simulated the intensities of the cross peaks in 2D NOESY and 3D NOE-NOE spectra for a 5-proton system (Fig. 2) with the spatial arrangement typical for a β-turn region in polypep-

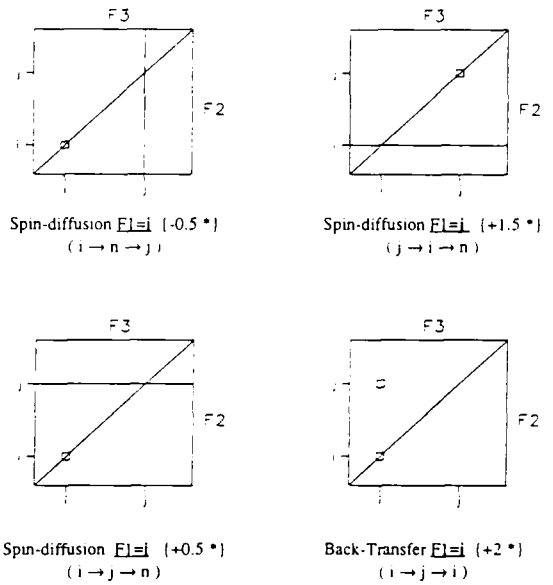


Fig. 1. Schematic 2D planes of a 3D NOE-NOE spectrum. The 3D diagonal peak is marked on the cross-diagonal of the plane. To correct the cross-peak intensities $a_{jii}(\tau_m^2)$, all 3D cross peaks and back-transfer peaks on the specified lines must be added (multiplied with the corresponding factor, written at the bottom of each plane) to the intensity of that cross peak.

tides to demonstrate the usefulness of our approach. A homewritten program in FORTRAN 77 was used to carry out the calculations. The distances between the nuclei are chosen as shown in Table 1. From these distances, the relaxation matrix for the system was calculated and the cross-peak intensities were obtained according to Eqs. 5, 7, 8 and 14. We assumed isotropic motion with equal correlation times for all nuclei and used correlation times of 5 and 10 ns, which are typical for very large biomolecules in the range 10-60 kDa. The dependence of a_{jii} (corrected as described above) with τ_m, is linear even at very long mixing times (up to 200 ms)(Figs. 3 a,b). This means, that the simple evaluation of distances by $r_{ij} = r_{ref}(a(corr.)_{ref}/a(corr)_{jii})^{1/6}$ is possible at much longer mixing times than suitable for the initial rate approximation. For very long mixing times, however, the contributions of higher-order spin-diffusion terms cannot be neglected. The distances derived by this method are compared to the values that would be obtained with a single 2D NOESY spectrum (Table 1). The quality of the distances is strongly dependent on the geometry and reference distance used for calibration. For both methods, the values that lie within the range of the calibration distance are in good agreement with the target values. For longer distances, however, the correction from 3D NOE-NOE data gives far better results than 2D NOE data, especially in the range 400–500 pm.

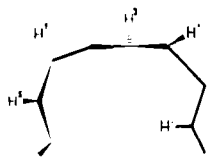


Fig. 2. Geometry of the five protons used in the simulation of 2D NOESY and 3D NOE-NOE cross-peak intensities. The arrangement is similar to a β-turn structure often found in polypeptides.

TABLE I

DISTANCES DERIVED FROM THE THEORETICAL 2D NOESY AND CORRECTED THEORETICAL 3D NOE-NOE CROSS-PEAK INTENSITIES CALCULATED WITH $r_{ij} = r_{ref}(a_{ref}/a_{ij})^{1/6}$

Distance	Target value (pm)	10 ns/100 ms mixing time		5 ns/100 ms mixing time	
		Distance from 2D NOESY	Corrected from 3D NOE-NOE	Distance from 2D NOESY	Corrected from 3D NOE-NOE
1-2	380	293	350	320	369
1 3	220 (ref.)		-	-	
1 4	530	367	468	409	506
1 5	350	328	346	338	348
2 3	250	243	248	246	249
2-4	450	373	434	403	445
2-5	540	471	536	504	539
3-4	310	296	307	303	309
3 5	550	407	504	450	534
4-5	850	664	877	748	860

The mixing time was set to 100 ms for the 2D NOESY simulation and 2 × 50 ms for 3D NOE-NOE. Values at top of the colums are the correlation times and mixing times, used in the simulations.

To verify experimentally that the proposed method gives reliable results, a 3D NOE-NOE spectrum of an 11 mM solution of the peptide antibiotic Ro 09-0198 (Kessler et al., 1988) bound to SDS-micelles in H_2O/D_2O (9:1) at 600 MHz and 317 K was recorded. The pulse sequence was: Relaxation delay-$90°(\varphi_1) - t_1 - 90°(\varphi_2) - \tau_m^{(1)} - 90°(\varphi_3) - t_2 - 90°(\varphi_4) - \tau_m^{(2)} - 90°(\varphi_5)$ – acquisition(φ_6); $\varphi_1 = (x, -x)$, $\varphi_2 = (x)$, $\varphi_3 = (x)$, $\varphi_4 = (x,x,-x,-x)$, $\varphi_5 = (x)$, $\varphi_6 = (x,-x,-x,x)$. The mixing time was 165 ms for $\tau_m^{(1)}$ and $\tau_m^{(2)}$. The water resonance was suppressed by low-power irradiation during the relaxation delay and both mixing times. 256 × 192 × 512 data points were taken, with TPPI applied in the F1 and F2 dimensions. Acquisition times in F1, F2 and F3 were 42.5 ms, 32 ms and 85 ms respectively. To achieve axial peak suppression in both dimensions, four scans were taken for each transient, resulting in a total measuring time of 64 h. Coherence transfer suppression was done by applying a strong homo-spoil pulse (20 ms) during both mixing times. The 3D spectrum was processed on a Silicon Graphics IRIS 4D 240 with the FELIX software of Dr. Dennis Hare*. The data size was 256 × 256 × 256 real points. Integration of cross peaks was carried out with the software written by Christian Cieslar on a Convex C5. Here linear prediction was used for processing, with a final size of 1k real data points in each dimension (the spectrum was processed in separate parts). Instead of summing up the intensity around a local maximum, we have used the maximum intensity of a cross peak. In this way problems resulting from partially overlapping peaks could be handled. However, one should bear in mind that this method is only valid when all peak shapes in the spectrum are comparable. The comparison of selected integrals and the corresponding peak maxima showed that in our case no serious error is introduced by this approach. To illustrate the application of the method we have chosen as an example two proton pairs (Val^{13}-C^2H/Val^{13}-NH and $Val^{13}C^2H$/S-Abu^{14}-NH) of the peptide that are separated by a

* Hare Research, Inc., 14810 216th Avenue NE, Woodinville, WA 98072, U.S.A.

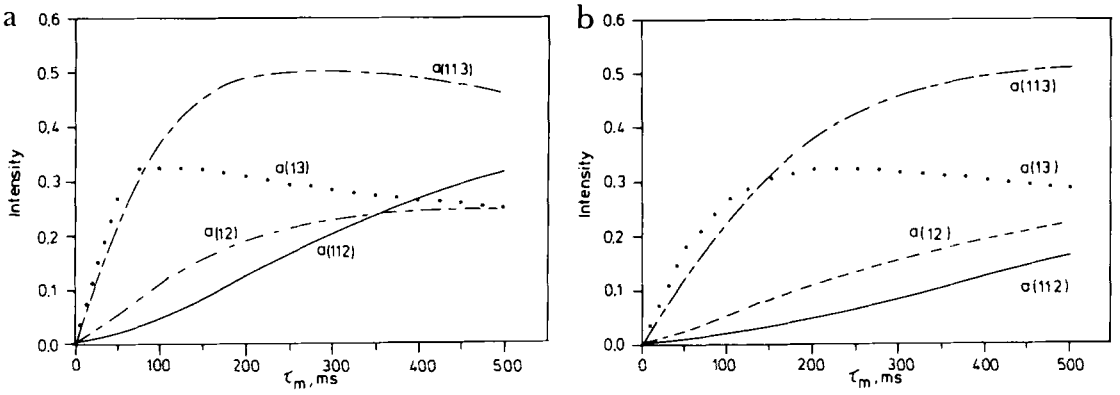


Fig. 3. Dependence of the cross-peak intensities from the mixing time for short and long distances at different correlation times. a) Shows data for 10 ns correlation time; b) for 5 ns correlation time. Calculations were done on the five-spin system shown in Fig. 2. The geometry of this spin system is similar to that found in β-turns. The curves that belong to a(112) and a(113) were derived from the corrected intensities of 3D NOE-NOE simulations, whereas a(12) and a(13) represent intensities from 2D NOESY simulations.

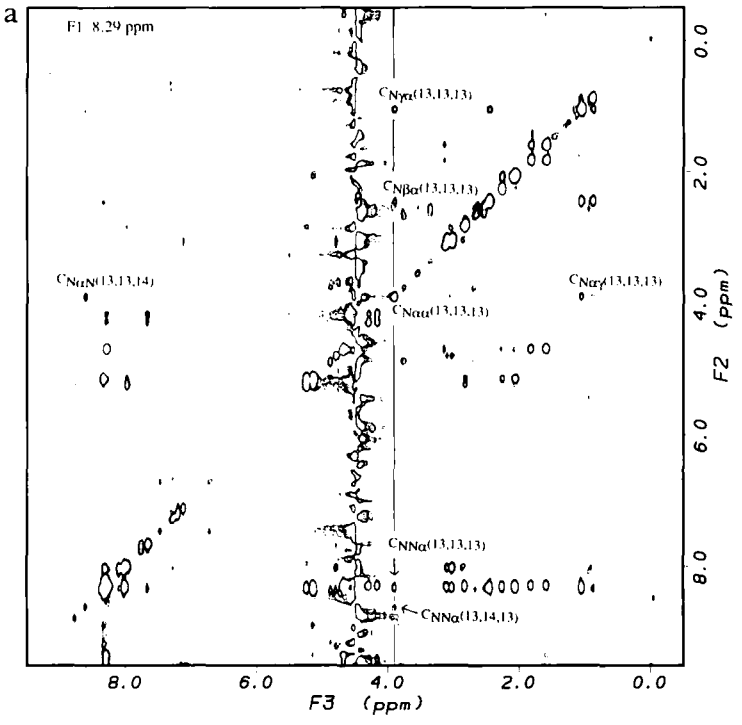
distance of 200–300 pm. It was obvious that experimental 2D NOESY data, even with measuring NOE buildup-rates (data not shown), gave wrong results: <200 pm for the interresidue H^{α}-NH distance and >320 pm for the intraresidue H^{α}-NH distance, both impossible in peptide molecules. The corrected intensities for the direct peaks ($a_{jii}(\tau_m)$) were evaluated. Table 2 presents the

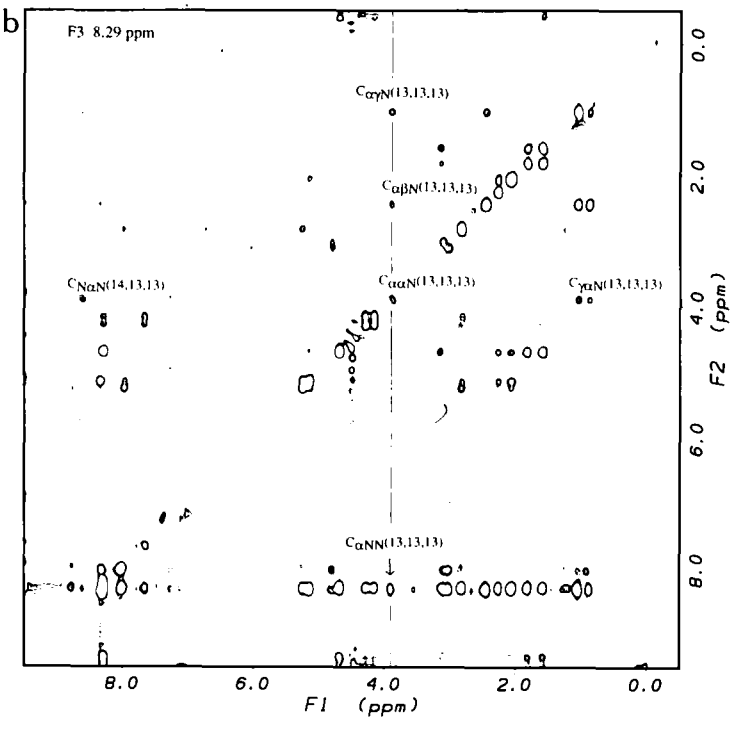
TABLE 2

INTENSITIES OF THE 3D NOE-NOE CROSS PEAKS FOR THE PROTON PAIRS Val^{13} $C^{\alpha}H$-HN AND $Val^{13}C^{\alpha}H$-HN S-Abu^{14}

3D NOE-NOE cross peak	$Val^{13}C^{\alpha}H$-HN-Val^{13}	Intensity	$Val^{13}C^{\alpha}H$-HN S-Abu^{14}	Intensity
1. One-step transfer	$C_{\alpha_2N}(13,13,13)$	0.45	$C_{\alpha_2N}(13,13,14)$	1.25
2. Back-transfer	$C_{\alpha N_2}(13,13,13)$	0.41	$C_{\alpha N_2}(13,14,13)$	2.10
3. Leakage	$C_{NN_2}(14,13,13)$	0.20	$C_{NN_2}(13,14,13)$	0.18
	$C_{\gamma 1N_2}(13,13,13)$	0.27	$C_{\gamma 1N_2}(13,14,13)$	1.14
	$C_{\beta N_2}(13,13,13)$	0.13	$C_{\beta 1N_2}(14,14,13)$	0.26
	$C_{\gamma N_2}(12,13,13)$	0.12	$C_{\gamma 2N_2}(13,14,13)$	0.65
			$C_{\beta 2N_2}(14,14,13)$	0.29
			$C_{\beta N_2}(13,14,13)$	0.23
			$C_{\alpha N_2}(14,14,13)$	0.11
4. Leakage	$C_{N_2N}(13,13,14)$	0.08	$C_{N_2\beta}(14,13,13)$	0.23
	$C_{N_2N}(12,13,13)$	0.14	$C_{N_2\gamma 1}(14,13,13)$	0.14
	$C_{\gamma_2N}(13,13,13)$	0.18	$C_{N_2\gamma 2}(14,13,13)$	0.08
	$C_{\beta_2N}(13,13,13)$	0.13	$C_{N_2N}(14,13,13)$	0.08
5. Spin diffussion	$C_{N\gamma 1_2}(13,13,13)$	0.33	$C_{N\gamma 1_2}(14,13,13)$	1.71
	$C_{N\gamma 2_2}(13,13,13)$	0.20	$C_{N\gamma 2_2}(14,13,13)$	1.06
	$C_{NN_2}(13,14,13)$	0.18	$C_{N\beta_2}(14,13,13)$	0.13
	$C_{N\beta_2}(13,13,13)$	0.13	$C_{NN_2}(14,13,13)$	0.02

Shown are all the peak intensities needed for evaluation of the cross-relaxation rates. These peaks can be found in the two planes shown in Fig. 3. The nomenclature used is taken from Borgias et al. (1990).


a
F1 8.29 ppm
CNγα(13,13,13)
CNβα(13,13,13)
CNαN(13,13,14)
CNαγ(13,13,13)
CNαα(13,13,13)
CNNα(13,13,13)
CNNα(13,14,13)
8.0
6.0
4.0
2.0
0.0
F3 (ppm)
F2 (ppm)


b
F3 8.29 ppm
CαγN(13,13,13)
CαβN(13,13,13)
CNαN(14,13,13)
CααN(13,13,13)
CγαN(13,13,13)
CαNN(13,13,13)
8.0
6.0
4.0
2.0
0.0
F1 (ppm)
F2 (ppm)

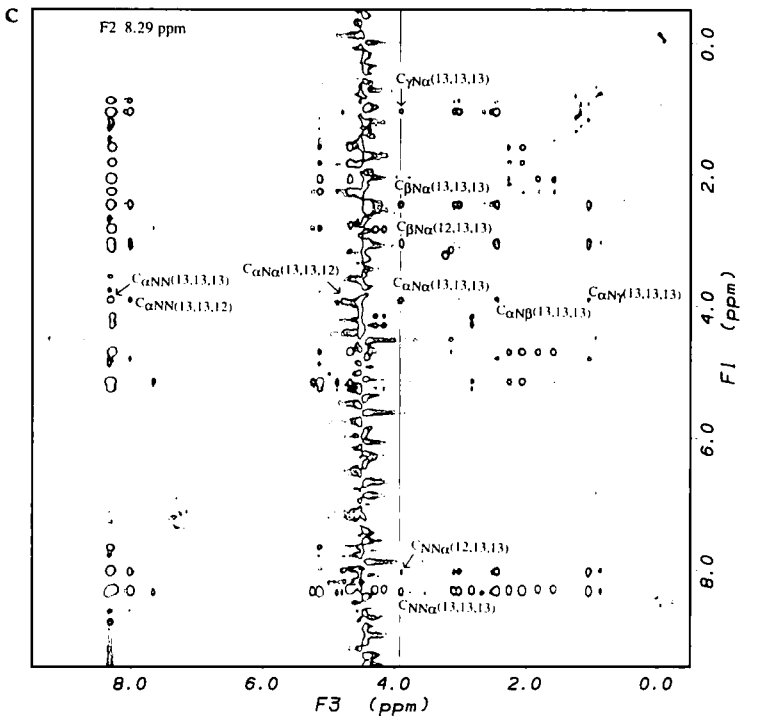


Fig. 4. Sections from the 3D NOE-NOE spectrum of Ro 09-0198. Assignment of the relevant peaks is given. Shown are sections perpendicular to the F1, F2 and F3 dimensions of the 3D spectrum. a) F2/F3 2D plane at the resonance of Val^{13}-NH (8.29 ppm) in F1. Along the line at 3.95 ppm are spin-diffusion peaks. b) F1/F2 2D plane at the resonance of Val^{13}-NH in F3. c) F1/F3 2D plane at the resonance of Val^{13}-NH in F2.

experimentally found values of all peaks needed for the calculation. As a reference distance we used the corrected 3D NOE-NOE cross-peak intensity of geminal protons of a proline residue (Pro^9-H^{β}-$H^{\beta'}$). The final distances of the proton pairs {Val^{13}-$C^{\alpha}H$-Val^{13}-NH} and {Val^{13}-$C^{\alpha}H$-S-Abu^{14}-NH}, obtained by the ratio $r_{ij} = r_{ref}(a(corr.)_{ref}/a(corr.)_{jii})^{1/6}$ are 281 pm and 227 pm respectively. These distances are in good agreement with the turn structure derived for this region. Problems that must be addressed in this context are errors due to apodization, especially in the less-well-digitized frequency domains and the partial saturation of resonances due to fast pulsing. Both these errors could lead to wrong integrals and therefore bad distance values. These errors can be estimated by analysing the intensities of symmetric 3D cross peaks, for example back-transfer peaks a_{iji} and a_{jij}. We have found some differences between the integral values of these peaks which were up to 20%. In our case these errors affected mainly the cross peaks belonging to methyl groups and could be avoided by choosing a longer relaxation delay (if spectrometer time is available). Another problem is the choice of a reasonable peak-picking level. In order to avoid that too many noise peaks (especially in the vicinity of the water resonance) are included in the peaklist, we set the threshold limit relatively high. Therefore some small peaks which were just above the noise level could be lost. So, for example, the $C_{\alpha}NN(13,13,12)$ cross peak is visible in Fig. 3c but was not used in the correction of the corresponding cross peak.

Of course, to use such an approach for all proton pairs in a macromolecule would be a time-

consuming procedure, so that automated sorting and correction is inevitable. Such routines are currently in development.

We have shown that the determination of distances from 3D NOE-NOE data is a viable alternative to the methods used up to now to evaluate 2D NOESY data. Two approximations have been used for this approach: (i) retaining only the linear and second-order terms in the power series expansion of $a(\tau_m)$ (higher-order terms are neglected) and (ii) the long correlation time limit. Spin-diffusion effects that are present in NOE spectra can be corrected by simple experimental procedures, yielding more exact distances in the presence of spin diffusion. Therefore, longer mixing times can be used, which means that larger distances can be observed at a much better S/N ratio. One important requirement for the application of this method is appropriate software for 3D computation and integration as well as large data storage capacity, because high resolution is needed for successful application of this method. The problem of accurate 3D-peak volume-integration is, to our knowledge, not yet solved. The manual evaluation of all the information present in a 3D NOE-NOE spectrum is practically impossible, so that automatic peak-picking and integration routines are highly desirable.

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