NMR Delineation of Inner and Outer Protons from Paramagnetic Relaxation Perturbations in 1D and 2D Spectra of Peptides

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The use of soluble spin labels to filter out the cross-peaks of outer proton nuclei in 2D NMR spectra is proposed as a general method of obtaining structural information about complex molecules. Gramicidin S, a decapeptide of well defined and stable structure, has been used as a model system to correlate the paramagnetic effects observed in 1D and 2D spectra and the peptide solution conformation. The method appears particularly suited to obtaining information about the hydrogen bonding of backbone amide protons.

Nowadays the ^{1}H NMR spectrum of a protein can be analysed in the multidimensional frequency domain obtained by use of one of the various pulse sequences normally available for standard spectrometers.[1–3] Information on structure, dynamics and biological mechanisms may be obtained from 2D and 3D NMR techniques which are efficient tools for simplifying the wealth of dipolar and/or scalar connectivities encoded in a complex spectrum.[4] Multiple-quantum and X filterings are typical examples of efficient strategies to reduce the spectral complexity which can often inhibit a detailed analysis of the NMR parameters. Thus, simplification may be achieved on the basis of the specific spin coupling patterns in a way which is controlled only by the physical behaviour of the investigated spin system, regardless of any stereochemical feature of the protein in solution.[5,6] A different type of simplification could arise from the presence of aminoxyl stable spin-labels bound to proteins or nucleic acids, as proposed by several authors.[7–10] In this way, the use of this intrinsic probe to study the environment of the unpaired electron allows the mapping of the ligand binding sites through 1D and 2D methodologies.

Soluble free radicals such as TEMPO or its derivatives, have also been used[11–13] for spin-labelling of the solvent. In this case, information was obtained on the accessibility of the proton nuclei of the aminoxyl and solvent molecules to small, but structured, molecules. The complete absence of any change in the EPR spectrum of the aminoxyl upon addition of gramicidin S suggested that the solution conformation governs the formation of weak collisional adducts between the aminoxyl and the diamagnetic solute and these interactions determine the extent of the paramagnetic perturbation on the observed nuclear relaxation rates.[12]

In the present paper we extend the previous studies to the analysis of 2D spectra of gramicidin S obtained in the presence of aminoxyls in order to correlate observed paramagnetic filters and tridimensional structures of complex spin systems.

While this manuscript was in preparation a paper appeared suggesting an approach for the identification of protein surface nuclei which is very similar to the present one.[14] Our observations and conclusions will be discussed also taking into account the data and analysis reported in that paper.

Experimental

Gramicidin S, obtained from Sigma, was used without further purification and dissolved in [$^2H_6$]DMSO to obtain a 30 mmol dm^{-3} solution. TEMPOL, the 4-hydroxy derivative of TEMPO (2,2,6,6-tetramethylpiperidin-1-yloxy), was purchased from Sigma and dissolved in the same solvent yielding a 6 mol dm^{-3} stock solution. Micro-additions of this aminoxyl solution were performed for spin-labelling of the peptide samples. All ^{1}H NMR measurements were performed with a Varian XL-200 spectrometer. To generate the COSY and phase-sensitive NOESY spectra, 256 and 400 1K FIDs were collected with 80 and 256 scans, respectively. All the 2D experiments were zero-filled to obtain a 1K × 1K data matrix. A line broadening of 0.1 Hz was given to both dimensions of the NOESY spectra. To restore an absorptive shape in the magnitude of the COSY spectrum, pseudo-echo shaping of the COSY FIDs was performed by setting the RE and AF processing parameters of the Varian VXR software at the values: RE = 0.014; RE2 = 0.006; AF = 0.054; AF2 = 0.028. The reported paramagnetic relaxivities from the COSY and phase-sensitive NOESY spectra, S_C and S_N, respectively, were calculated from the intensities of the diagonal and cross-peaks. These measurements were carried out on the integrals of the cross-sections of the individual signals obtained from the rows in the F_2 dimension which allowed a better signal digitilization of the 1D traces. By following this procedure a greater reproducibility of the data was attained than by using peak volumes, probably owing to problems of the volume integration routine of the VXR Varian software in the presence of broad lines. Spin–lattice relaxation measurements were performed with the standard inversion recovery pulse sequence: 32 scans and 11 different values for the variable delay were used to obtain the partially relaxed 1D spectra of gramicidin S both in the diamagnetic and the paramagnetic solutions. The paramagnetic relaxation rates, R_p, were calculated by subtracting the experimental relaxation rates measured in the absence of TEMPOL from the ones measured in the paramagnetic solutions. The S_p parameters, reported in Table 1, are the molar relaxivities calculated for the selected protons, as described in the caption to Fig. 1 (later). The experimental errors of the 1D- and 2D-derived data should be considered to range within 10 and 20% confidence limits, respectively.

Results and Discussion

The paramagnetic perturbation induced by the TEMPOL aminoxyl has been monitored with the 1D procedure previously

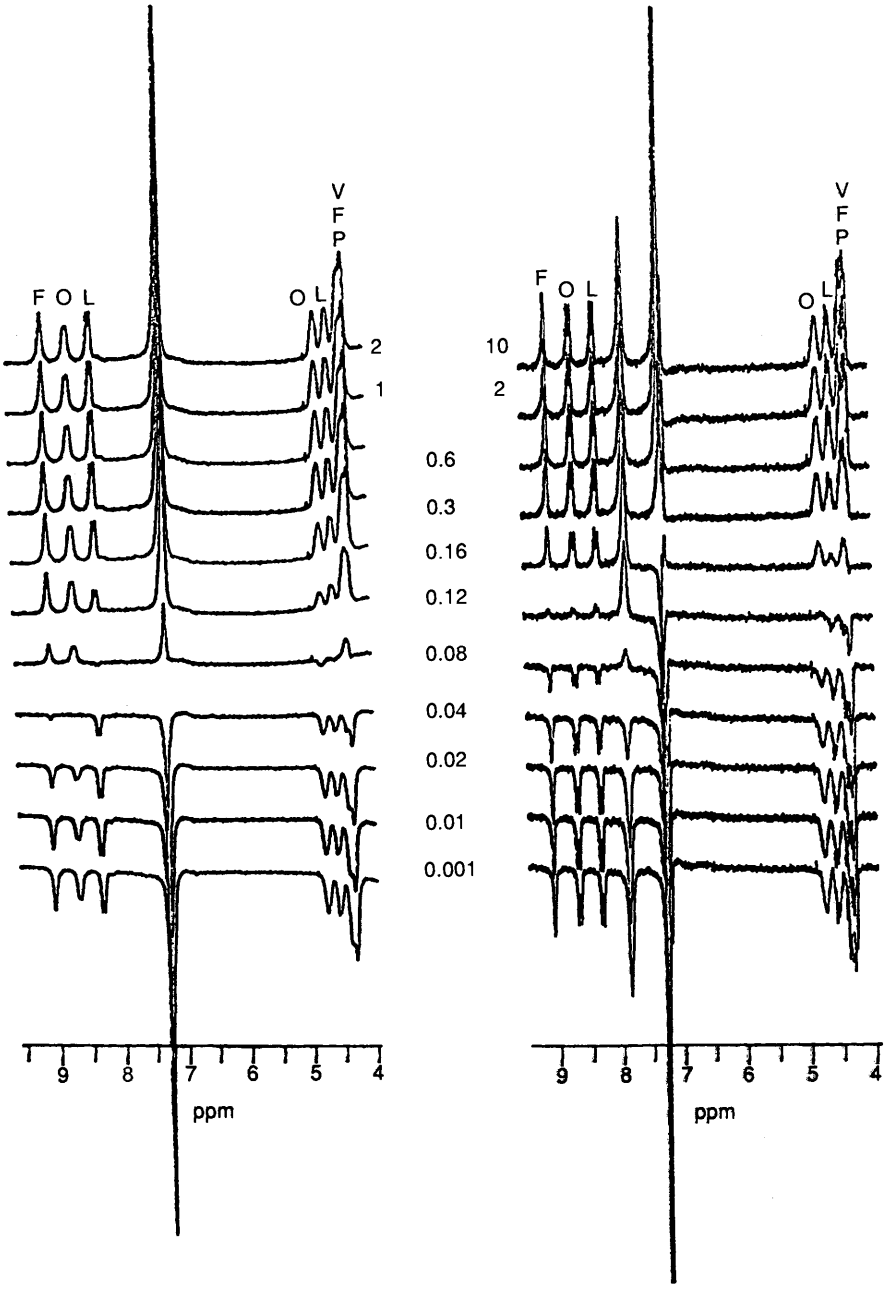

Fig. 1 Partially relaxed spectra of the amide and H_α proton region of gramicidin S 30 mmol dm^{-3} in [$^2H_6$]DMSO solution at 25 °C; traces shown on the right-hand side were obtained in the diamagnetic solvent, while those on the left-hand side were recorded in the presence of 15 mmol dm^{-3} TEMPOL. The delay times, τ, used in the inversion recovery pulse sequence are shown in s.

Table 1 Molar relaxivities, S_p, calculated for selected protons

H	δ (ppm)	S_{pc} [a]	S_{pd} [b]
F NH	9.11	487	1537
O NH	8.62	436	1715
L NH	8.31	98	404
O H_α	4.94	324	542
L H_α	4.64	574	986

[a] S_{pc}: paramagnetic enhancement per mole of spin-label, obtained from slopes of plots of R_p *vs.* TEMPOL concentration in the range 3–15 mmol dm^{-3} for 30 mmol dm^{-3} gramicidin S DMSO solution. [b] S_{pd}: calculated slopes of plots of R_p *vs.* TEMPOL concentration measured at different dilutions for a 30 mmol dm^{-3} gramicidin S–15 mmol dm^{-3} aminoxyl initial solution (see Fig. 1). δ In ppm from external TMS.

described [11] (see Fig. 1), and the results are reported in Table 1. It can easily be seen that the use of TEMPOL for the DMSO spin-labelling yields the same effects as observed on the R_p values of gramicidin S protons in the presence of TEMPO.[11]

The peptide solution conformation, shown in Fig. 2, is the main source of modulation for the dipolar interaction between the aminoxyl unpaired electron and the gramicidin S proton nuclei, since the solvent-exposed amide hydrogens of F and O residues are *ca.* four times more affected than the hydrogen bonded L NH. The same trend is observed also for the α-hydrogens, as the solvent-exposed L H_α has an S_{pc} which is twice that measured for the solvent-shielded O H_α. The same relaxation behaviour can be observed from a different experimental approach, that is by measuring the different R_p values of these protons at different dilutions of a gramicidin S–TEMPOL 2:1 solution. Furthermore, the calculated S_{pd} are *ca.* two and four times higher, respectively, than the corresponding H_α and amide protons S_{pc} values. All these features are consistent with the proposed dynamic model of interaction of the peptide with the aminoxyl in DMSO, where the formation of

Table 2 Gramicidin 2–TEMPOL system investigated by COSY and phase-sensitive NOESY spectroscopy

	S_C		S_N	
	d	cp	c	cp
F NH–H_α	5.4	6.3	56	56.6
O NH–H_α	6.5	4.0	59	53.3
L NH–H_α	0.1	0.7	23	11.9
V NH–H_α	—	1.5		
P H_α–H_β	—	4.6		
O H_α–H_β	0.1	1.0		
L H_α–H_β	4.6	3.5		
V H_α–H_β	—	10.7		
P H_α–$H_{\beta u}$	—	6.6		
P H_α–$H_{\beta d}$	—	5.4		
O H_δ–H_γ	—	2.0		
P H_δ–H_β	—	7.1		

S_C and S_N, the slopes of diagonal (d) and cross-peaks (cp), were obtained by plots of the integrals of cross-sections of COSY and NOESY spectra, respectively. In the case of S_N, the cps of the amide protons are F NH–L H_α, O NH–V H_α and L NH–O H_α.

the weak collisional adducts is influenced by the different solvation at the various molecular sites. Hydrogen bonding of the *N*-oxyl group of the spin-label to amide and amine protons and hydrophobic forces are the two main factors which control the formation of these bimolecular adducts.[12] The fundamental role of the solvent–aminoxyl interaction is suggested by the dilution study, where a strong competition of DMSO with TEMPOL occurs at the exposed phenylalanyl and ornithyl amides, reducing the effects of a relevant mechanism of interaction of this class of proton with the unpaired electron. This dynamic picture is also confirmed by the behaviour of the leucyl amide proton which is already involved by an intramolecular hydrogen bond and exhibits an S_{pd} very similar to the one observed for the solvent-shielded ornithyl H_α.

Four preliminary conclusions can be drawn: (i) each class of hydrogen interacts in a dipolar fashion with the unpaired electron of an aminoxyl in a way which is primarily determined by its solvation behaviour; (ii) within each class of hydrogen, the different paramagnetic relaxivities reflect the location in the molecule; (iii) intramolecular hydrogen bonding of amide protons is efficiently recognised; (iv) the 1D spin–lattice relaxation study is limited by the spectral dispersion of the NMR signals and, therefore, structural analysis by this method of confined to small molecules.

The possibility of exploiting the aminoxyl perturbation to discriminate between surface and core nuclei in more complex molecules implies study of the information flow of the paramagnetic relaxation in multidimensional NMR spectroscopies.

The gramicidin S–TEMPOL model system, under the same conditions, of solvent, concentration and temperature as in the 1D relaxation study, was investigated by COSY and phase-sensitive NOESY 2D spectroscopy and the results are summarized in Table 2.

It is apparent from Fig. 2 and the data of Table 2 that the hydrogen-bonded amide protons of L and V have cross-peaks in the COSY spectrum whose intensities are less affected by TEMPOL than those of all the other signals. The difference of the paramagnetic slopes, S_C, calculated from the integrals of the cross-section measured in the F_2 frequency dimensions, is also a direct consequence of the different effect of the weighting functions used on various signals for obtaining the absolute value representation of the 2D data matrix (see the Experimental section). This FID processing markedly reduces the peak intensity of those signals with shorter T_2 and an amplification of the paramagnetic effects has to be expected.

From analysis of the S_C values of the aliphatic protons, the low aminoxyl dependence of the ornithyl correlation intensity compared with all the other α–β cross-peaks is evident, in agreement with the results of the 1D relaxation data. V H_α–H and O H_δ–H_γ cross-peaks have, respectively, the highest and lowest S_C values, suggesting for the former solvent-exposed pair of protons an increased spin-label accessibility. This finding can be explained if the low hindrance of the proximal side-chains of O and P residues to the approaching aminoxyl is considered. Consistent with the proposed[15–17] hydrogen bonding of the O amine group with the F carbonyl, and the consequent reduced exposure of O H_γ protons, is the low S_C value calculated for the O H_γ–H_δ correlation. From a single inspection of the spectra shown in Fig. 3, it is apparent that the intensity changes of $L_{\gamma\delta}$ and $V_{\beta\gamma}$ cross-peaks are much smaller than the TEMPOL-induced effects observed for all the other intra-side-chain *J* correlations. The fact that L and V side-chains are located in external regions of the molecule excludes any possible hindrance to the spin-label approach. Note that the extensive internal rotations which occur within these two side-chains[18] can cause a decrease of the effective correlation times which modulate the nucleus–electron dipolar interactions. This feature may yield less effective dipolar couplings and, hence, paramagnetic filters. The opposite behaviour of the two locked side-chains of the prolyl and ornithyl residues indicates that the peptide conformation controls the extent of the paramagnetic filter also for the aliphatic protons. In fact, only the former side-chain, solvent exposed, has all its cross-peaks characterized by strongly reduced intensities in the presence of TEMPOL. The different chemical nature of the various residues of gramicidin S seems not to give relevant modulation of the spin-label-induced effects, but differential internal reorientations must be carefully considered whenever side-chain correlations are analysed, as in the previous similar report.[14]

The use of phase-sensitive 2D spectroscpy may solve the problem of relaxation-dependent effects of FID manipulations, as mentioned above. Furthermore, the in-phase character of the NOESY cross-peak multiplets prevents cancellation effects which can be found among the various antiphase components of COSY cross-peaks. This *J*-dependent cancellation, to be avoided for a quantitative structural analysis of the paramagnetic effects, does not give any contribution to the S_N values shown in Table 2.

The molecular tumbling of the decapeptide in DMSO is such that small negative NOEs are observed at our proton operating frequency.[19] Among all the observed NOEs only three could be determined at different TEMPOL concentrations for comparison with the 1D- and COSY-derived results. A similar ratio of paramagnetic relaxivities for solvent shielded/exposed protons can be seen from NOESY and T_1 measurements. The higher discrimination observed for the COSY-derived results can therefore be ascribed totally to the T_2 amplification effect of the paramagnetic perturbation induced by the FID 2D processing. A good correlation between these data and those derived from the 1D relaxation study is apparent.

It can be concluded, therefore, that the solution conformation controls the intensity changes of scalar and dipolar correlations of gramicidin S in DMSO solution and a quantitative analysis of the observed effects can be made from phase-sensitive multidimensional NMR spectra where an in-phase multiplet structure of the cross-peaks can be analysed. In NOESY- and TOCSY-type spectra any *J*-dependent contribution to the intensity variations caused by the presence of the aminoxyl can be ruled out.

The ambiguity induced by nucleus–electron dipolar interactions modulated by different correlation times suggests that this experimental approach should be confined to backbone protons. Of these, amide protons seem to exhibit a large

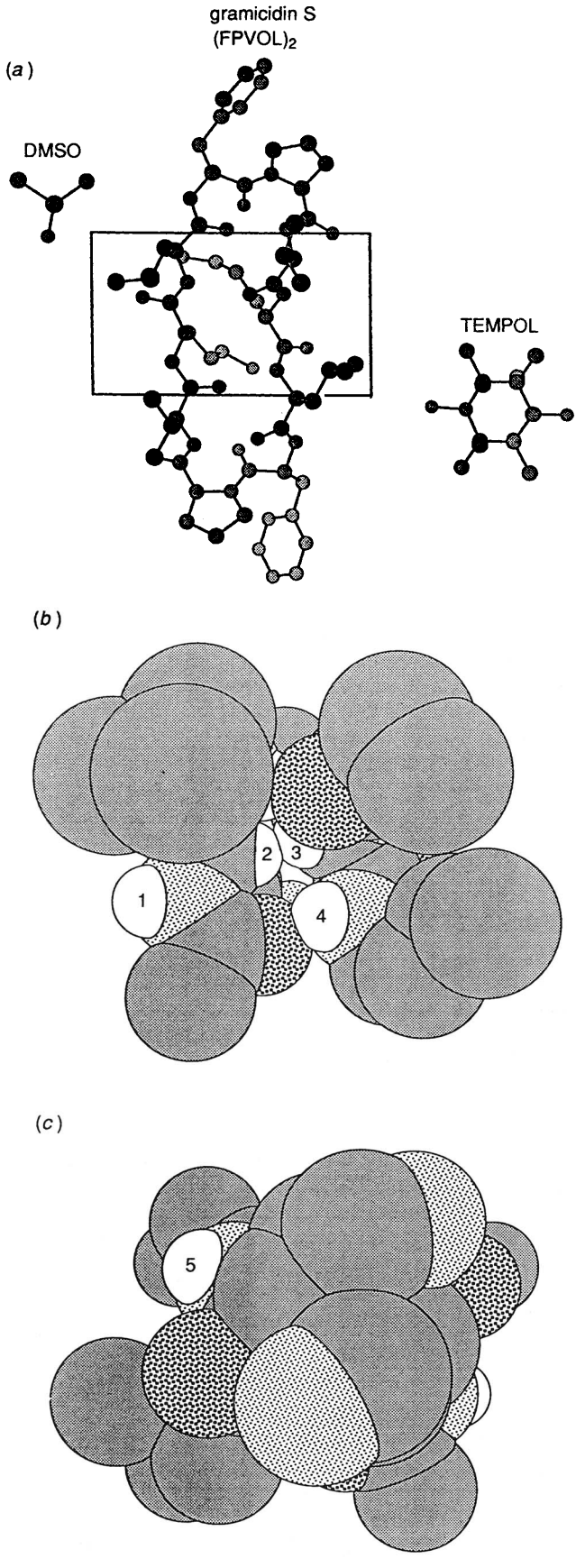

Fig. 2 (*a*) Gramicidin S structure as proposed from the X-ray structure of ref. 15. The inset shows the molecular fragments of Fig. 2(*b*) and 2(*c*) which differ for a 180° rotation along the antiparallel β-pleated sheet direction. Amide hydrogens 1, 4 and 5 are the O (ornithine) of one strand and L and O of the other strand, respectively. Atoms 2 and 3 are the H_α protons of the O of the two strands.

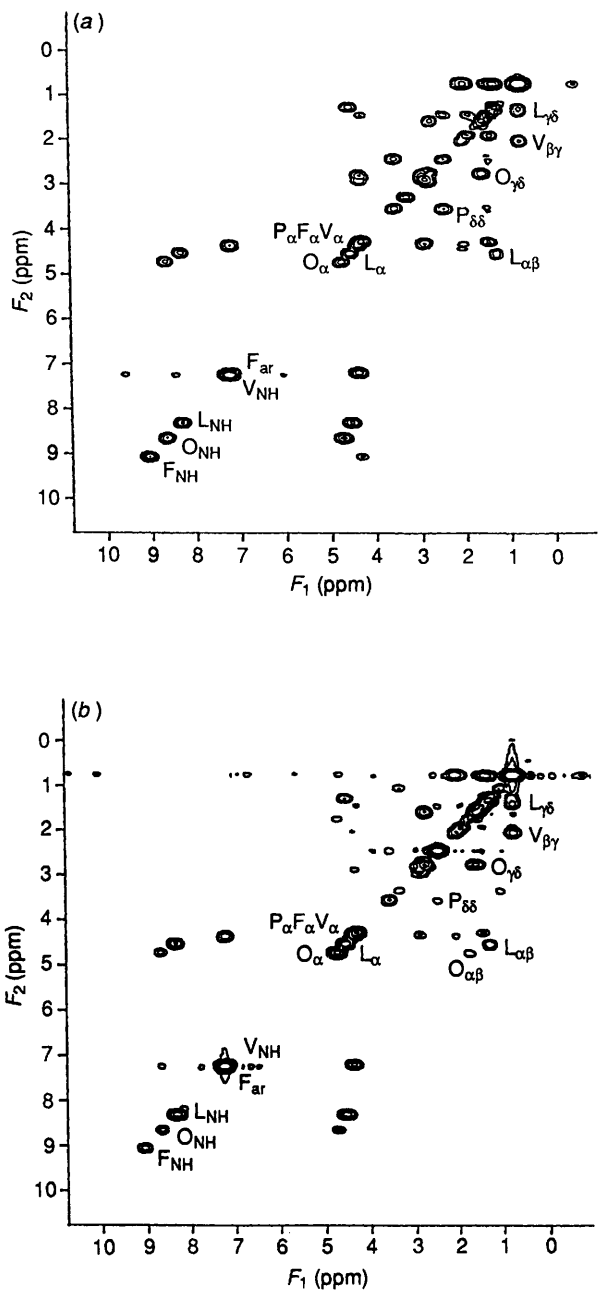

Fig. 3 Magnitude 2D COSY spectra of gramicidin S obtained under the same experimental conditions as described for Fig. 1; (*a*) diamagnetic solution; (*b*) in the presence of 15 mmol dm^{-3} TEMPOL

differentiation in the observed effects for hydrogen-bonded and non-bonded nuclei. Thus, this method looks particularly suited to obtain this relevant structural information.

The use of paramagnetic filters generated by soluble spin-labels may be proposed as a general method to investigate the location of individual amide protons within the molecular framework to obtain subspectra of the inner and, by 2D difference spectroscopy, outer part of a complex biomolecule.

The use of soluble aminoxyl for the investigation of a protein surface composition with a particular emphasis on side-chain protons[14] seems to be limited and ambiguous results can be obtained if the side-chain dynamics are not *a priori* known.

As a final remark, the use of non-square data matrices, *i.e.* with a large difference in the acquisition times t_1 and t_2, may resolve the ambiguity of the cross-peaks of nuclear pairs with a large difference in aminoxyl exposures. In this way, the T_2

contribution to the cross-peak intensity of each nucleus may be differently propagated in the two frequency dimensions.

Acknowledgements

Thanks are due to the Italian CNR and MURST for financial support. Thanks are also due to Professor P. Temussi (University of Naples) and A. M. Lesk (MRC, Cambridge) for helpful suggestions.

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Paper 1/00138H
Received 11*th January* 1991
Accepted 2*nd May* 1991