Rotating frame nuclear Overhauser effect: a practical tool for the ^{1}H NMR study of peptides in solution

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A new 2D NMR experiment, the rotating frame nuclear Overhauser enhancement (nOe) spectroscopy (ROESY) was recently proposed as an alternative to NOESY for the study of intermediate molecules. Its practical use is here tested on bacillomycin D, a bacterial cyclic lipopeptide. This sequential resonance assignment, based on nOe connectivities at room temperature, shows that the high viscosity which is generally required for conventional nOe measurement is no longer a restriction for choosing a suitable solvent. In comparison to low-temperature NOESY, short internuclear connectivities can be safely demonstrated because of no spin diffusion, for sequential assignment of peptide resonances.

Lipopeptide *Assignment* *2D NMR* *Nuclear Overhauser effect* *Conformational analysis*

1. INTRODUCTION

Resonance assignment is the prerequisite for any further NMR study of biological macromolecules such as peptides and proteins. However, the chemical shift has not yet been rationalized enough for assignment purposes [1] and thus the only alternative relies on the correlation of the interacting spins. Wüthrich and co-workers [2–4] have largely emphasized the use of 2D NMR techniques to map either the through-bond *J*-coupling connectivities (COSY) or the through-space nuclear Overhauser effect (nOe) connectivities (NOESY) on small globular proteins (M_r 5000–6000). The observation of large nOes on narrow lines is actually feasible because of the residual internal flexibility coexisting with the slow molecular tumbling rate. Unfortunately, this straightforward strategy cannot be readily transferred to smaller molecules (such as peptides with 8–10 residues), where the nOe vanishes owing to their intermediate rotational correlation time in common solvents.

To avoid this drawback, 2 palliatives, both based on an increase of the solvent viscosity, were suggested, however at the expense of the resonance linewidth: one can thus use either a solvent with a high viscosity at room temperature (e.g. tetramethylene sulfone [5] instead of Me_2SO) or a solvent, the low-freezing point of which enables one to run low-temperature experiments.

This latter technique was chosen in our recent conformational study of iturin A, an 8-residue bacterial lipopeptide [6]. The internuclear distances derived from NOESY spectra at −20°C and the *J*-coupling measured at 27°C were simultaneously used as input for the conformational optimization.

Unfortunately, this strategy carries with it its own unavoidable penalty in the mandatory temperature modification, although the final structure fortunately did not reveal any inconsistency between the 2 data sets.

In this context, the recently proposed rotating frame NOESY experiment (called ROESY) [7,8] was considered, because sizeable nOe effects are expected even for molecules of intermediate size. Here we report a first practical application of this technique to bacillomycin D, a member of the iturin family, which is soluble in a very restricted range of solvents and which exhibits, at low

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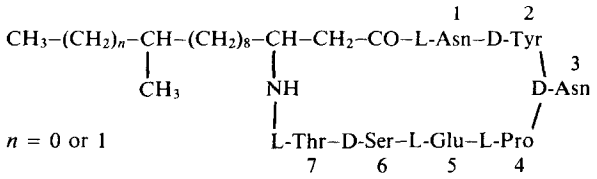
temperature, broad resonances due to a mechanism which has not yet been ascribed. The results will be discussed in view of those obtained on iturin A using the conventional NOESY experiment.

2. MATERIALS AND METHODS

Bacillomycin D, an antifungal antibiotic, was isolated from the culture media of *Bacillus subtilis*:



This lipopeptide was kindly purified by Drs F. Peypoux and G. Michel (University of Lyons I, France) as described [9,10]. A 4 mM sample in pyridine-d_5 was prepared and sealed under vacuum.

NMR experiments were carried out on a Bruker AM 300 WB spectrometer (^1H = 300 MHz). Double quantum filtered COSY (DQF-COSY) were recorded according to the sequence of Piantini et al. [11]. The ROESY pulse scheme is described as [8]:

$$90^\circ_\phi - t_1 - [\text{spin lock}]_{\phi+\pi/2} - \text{acquisition } (t_2)$$

where the spin lock is achieved using the decoupling channel. Because of the digital frequency synthesis, the carrier frequencies of both transmitter and decoupling channels are perfectly synchronized.

As for conventional NOESY, the separation of nOe and J cross-peaks [12] is of the utmost importance. The optimal r.f. strength of the spin lock as well as the carrier frequency were chosen by trial and error according to the following: a weaker field does not give rise to nOe cross-peaks (the sign of which is opposite to that of the diagonal) whereas too high a strength leads to the spurious J cross-peaks described by Bax and Davis [8]. Let us note that only the nOe cross-peaks are almost insensitive to large carrier frequency shifts.

Both DQF-COSY and ROESY experiments were performed using the time-proportional phase increment (TPPI) method [13] and the data displayed in the phase-sensitive mode.

3. RESULTS AND DISCUSSION

The resonance assignment basically entails 2 steps: (i) the identification of the scalar coupled spin systems and (ii) the subsequent use of through-space connectivities for sequential assignment [2]. In the DQF-COSY spectrum, the following spin systems were identified: one prolyl residue evidenced by the lack of NH and 7 residues (arbitrarily labelled I–VII) which bear an amide proton.

The NH-C$^\alpha$H correlation map is shown in fig.1

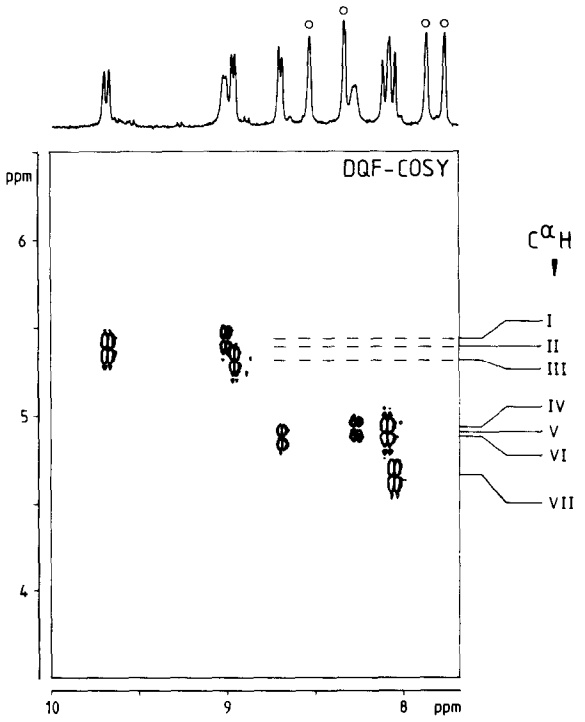


Fig.1. Identification of the scalar coupled spin systems by means of J-correlated spectroscopy (DQF-COSY). The 1D spectrum of the amide region exhibits 7 backbone NH as well as 2 pairs of side-chain NH_2 (identified by an open circle). Even at 300 K, 2 NH already show a partial line broadening which actually extends to all resonance at lower temperature. 7 NH-C$^\alpha$H cross-peaks (arbitrarily labelled I–VII according to the C$^\alpha$H chemical shift) are identified in the correlation map. The side-chain NH_2 are not scalar coupled with any other proton.

beneath the 1D spectrum of the amide protons. From a further inspection of the whole *J*-correlated spectrum, residue VII is assigned to the β-amino acid (its $C^{\beta}H$ is coupled with 4 protons in addition to the NH) and residue VI to D-Ser[6] (the $C^{\beta}H_2$ resonances lie around 4.35 ppm). Because of accidental overlaps, the L-Thr and L-Glu spin systems cannot yet be assigned, in spite of a different geometry.

Through-space connectivities are now required for going on with the assignment. Fig.2, which displays 2 portions of a ROESY spectrum, illustrates how possible artifacts due to *J* cross-peak can merely be identified by a shift of the carrier frequency (cf. insets a and b). However, due to the opposite sign of the *J* and nOe cross-peaks [8], the occurrence of these *J* peaks may only cause a nOe peak to be lost but does not give rise to misleading peaks. Intraresidue connectivities are found between the $C^{\beta}H_2$ of I and III and side-chain NH_2 and also between the $C^{\beta}H_2$ of II and the tyrosyl ring. Next neighbour $C^{\alpha}H$-NH connectivities are drawn in the less crowded part of fig.2 (see legend). The digital resolution, however, is high enough for extending this pathway between the cross-peaks of inset b. The cross-peaks between the $C^{\delta}H_2$ of Pro and the $C^{\alpha}H$ of residue I not only assign the spin system I to D-Asn[3] but also show a Pro *trans* conformation. The resonance assignment, reported in table 1, originates from a cross-check of all independent spin connectivities.

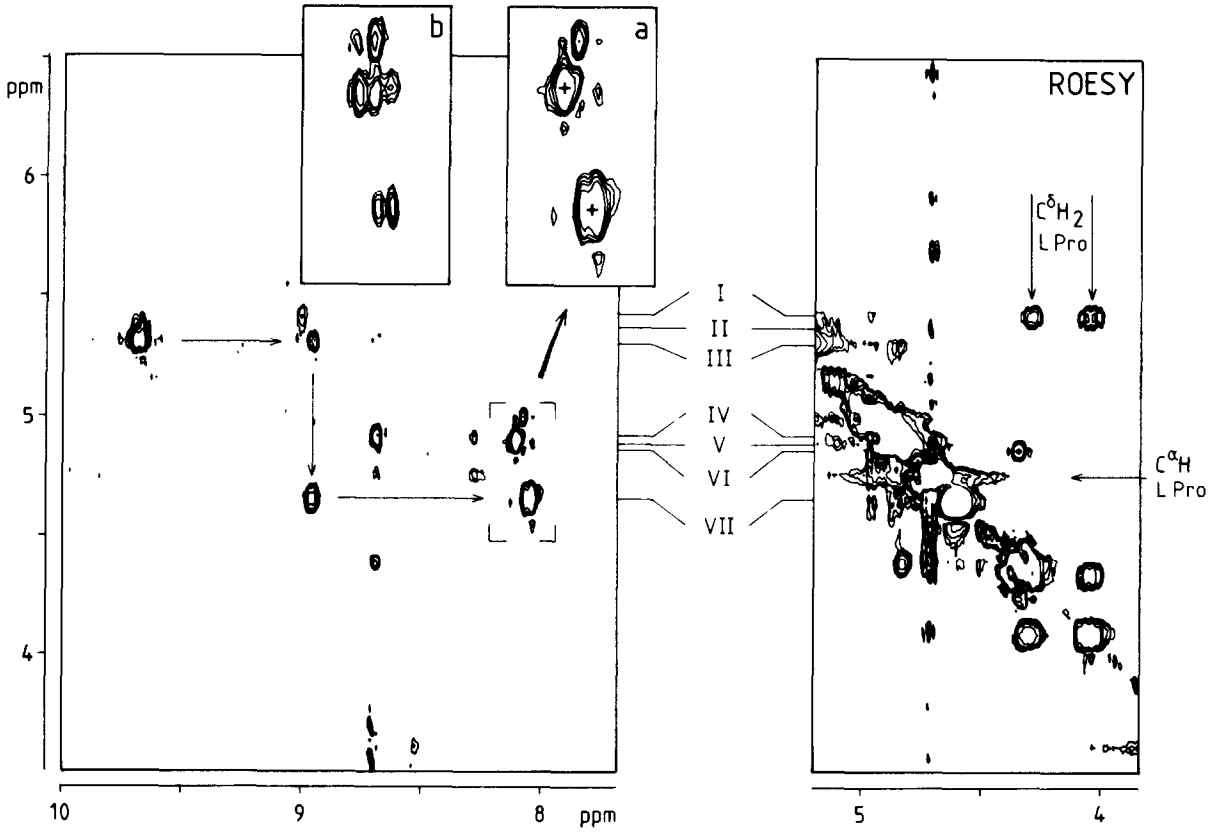


Fig.2. Absorption display on a ROESY spectrum. Both positive (diagonal peaks) and negative levels (cross-peaks unless otherwise stated) are plotted. The mixing – or spin locking – time is 350 ms and the carrier frequency lies at δ = 6.44 ppm. Inset b displays the same area as inset a with the carrier at δ = 4.12 ppm. The positive *J* cross-peaks (Hartman-Hahn effect) shown in inset a are eliminated in inset b merely by shifting the carrier frequency. Light arrows on the left-hand side indicate the assignment (in reverse sequential order) of residues II ⟶ III ⟶ VII to D-Tyr[2] ⟶ L-Asn[1] ⟶ β-amino acid[8].

Table 1

Resonance assignment of bacillomycin D in pyridine-d_5 (temperature = 300 K)

	1 L-Asn	2 D-Tyr	3 D-Asn	4 L-Pro	5 L-Glu	6 D-Ser	7 L-Thr	8 β-a.a.
Spin system label[a]	III	II	I		IV	VI	V	VII
NH	8.95	9.68	9.00		8.28	8.68	8.08	8.03
$C^{\alpha}H$	5.26	5.35	5.39	4.71	4.88	4.83	4.86	2.61 2.41
$C^{\beta}H$	3.05 2.96	3.71 3.37	3.58 3.17	2.09 2.04	2.79 2.63	4.37 4.34	4.96	4.60
$C^{\gamma}H$				1.84 1.67	3.0 2.8		1.33	1.55 1.42
$C^{\delta}H$				4.30 4.04				
$C_{26}H$		7.04						
$C_{35}H$		7.48						
NH_2[b] *trans*	8.32		8.51					
NH_2[b] *cis*	7.75		7.86					

[a] These labels are used in figs 1 and 2 prior to the complete assignment
[b] Amide protons *trans* or *cis* with respect to the C=O

4. CONCLUSION

In comparison with NOESY experiments run on iturin A [6], several conclusions can be drawn with respect to the potentialities of the ROESY technique. The viscosity requirements, as discussed above, no longer narrow the range of possible solvents. This may alleviate the weak solubility of many peptides in organic solvents. On the other hand, an experiment over 20–30 h is obviously more comfortable at room temperature than at below 0°C.

Our purpose is now to evaluate the use of ROESY for distance determination: the measurable range seems to be somewhat reduced as compared to NOESY, no connectivities were observed between successive amide protons which on average are more remote, but, on balance, the problem of spin diffusion is reduced. This difference originates from the relative magnitude of the build-up and decay rates of the cross-peaks: in ROESY these rates are similar whereas, in NOESY, the build-up is much faster than the subsequent decay [14]. Because of its reliability on transverse magnetizations, ROESY is sensitive to any source of line broadening, e.g. an equilibrium at intermediate rate. For example, the low intensity of the connectivities involving the NH of Glu^5 (fig.2) is in accordance with its linewidth (fig.1).

In a similar way to NOESY experiments, the internal flexibility may yield to an erroneous distance determination. According to the equations derived by Tropp [15], the modulation of the internuclear distance r_{ij} induces an nOe averaging involving either $\langle r_{ij}^{-3} \rangle$ or $\langle r_{ij}^{-6} \rangle$ terms, depending on the respective rates of internal and overall motion. Only a discrepancy during the optimization of the structure may reveal such an artifact.

In conclusion, new developments are expected from the ROESY experiment in the field of conformational analysis of intermediate-size peptides. The ^1H resonances can now be sequentially as-

signed in a rigorous manner in any solvent in which the peptide is soluble enough. In addition, both *J*-coupling and nOe-derived distances can be measured under the same conditions. This experimental improvement supports a strategy based on the simultaneous optimization [6] of these 2 efficient structural probes, to investigate the peptide conformation in solution.

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