A Quantitative approach in conformational analysis by 2D-INVERSION RECOVERY DIFFERENCE SPECTROSCOPY

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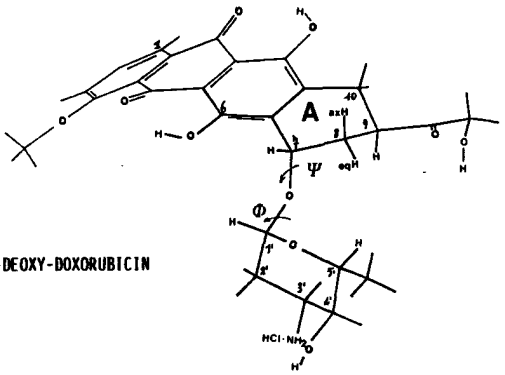

9-DEOXY-DOXORUBICIN

2D phase sensitive NOESY spectra are an invaluable tool in the structure determination of molecules in solution.It has been shown (1) how, starting from a model geometry, it is possible to calculate the complete relaxation matrix Γ, under a suitable motional model, and then fit the molecular geometry by directly comparing experimental and calculated 2D NOESY diagonal- and cross-peak intensities.

The intensities of 2D-NOESY spectra can be accurately calculated by solving the coupled differential equations, which describe the relaxation of a multi-spin system:

1) $d[M]/dt = -[\Gamma][\Delta M]$

In eq. 1) , [M] is the magnetization vector and [Γ] is the relaxation matrix, containing the relaxation rates ϱ_{ij} and the cross-relaxation rates σ_{ij} .
They can be calculated from the spectral densities as:

2) $A_{ij}=1/20(\mu/4\pi)^2\hbar^2\gamma^4 r^{-6}$
$\varrho_{ij}=\Sigma_j A_{ij}[3J(w)+6J(2w)+J(0)]$
$\sigma_{ij}=A_{ij}[6J(2w)-J(0)]$

Diagonalization of the [Γ] matrix is the first step in the solution of the differential equations 1):

3) $[D] = [T]^{-1}[\Gamma][T]$

the NOE intensities are then calculated as:

4) $[M] = [T][\exp(-Dt_{mix}][T]^{-1}$

The main advantage of this method is that spin-diffusion is automatically accounted for; furthermore,the choice of the experimental mixing time is not restricted to short values. Errors commonly introduced with the "two-spin" approximation are thus minimized.

METHOD AND RESULTS

Recently, difference 2D NOESY techniques were devised (2), in order to minimize t1-noise and interference with weak cross-peaks of the more intense diagonal peaks. In the Inversion Recovery Difference method (IRD), four separate FIDs are acquired by using the following pulse sequences, for each t1 value, in the order I, II, I, III :

I) 90(+x)-t1-90(+x)-tmix-
90 (+x) (ADD)
II) 90(+x)-t1-90(+x)-
90(+x) (SUBTRACT)
III) 180(+x)-tmix90(+x)-t1-
90(+x)-90(+x) (ADD)

Co-addition of the four serial files results in the IRD 2D-spectrum, whereas coaddition of the first and third serial files

results in the common 2D-NOESY spectrum.
Intensities of cross- and diagonal- peaks of an IRD spectrum are :

$IRD_{ij} = -\sigma_{ij}.t_{mix}$
$IRD_{ii} = -\Sigma_j IRD_{ij} = -\Sigma_j \sigma_{ij}.t_{mix}$
(given to a first order approximation).
Advantages of this method are that the diagonal of IRD-spectrum is reduced and negative. The cross-peaks near the diagonal become more easily measured; t1-noise and solvent peak are also reduced (see Fig.1 and Fig. 2). The main disadvantage is that , since pulse sequence (II) and (III) do not contribute to cross-peak intensities, the total time of the experiment is doubled, for the same S/N of the corresponding NOESY.
We have applied this methodology to the conformational study of 9-deoxydoxorubicin, a derivative of Adriamycin, a potent antitumour drug (3). We were interested in the preferred orientation of the sugar moiety with respect to the aglicone,as measured by the torsional angles about the glycosidic bonds, Φ =H(1')-C(1')-O(7)-C(7) and Ψ =C(1')-O(7)-C(7)- H(7). NOes of these compounds in aqueous solution display strong negative peaks and extensive spin-diffusion, suggesting non-extreme narrowing conditions ($w\tau_c >> 1$) and, therefore, slow molecular motions, due to the great tendency of this class of compounds to aggregate, forming high molecular weight species with a marked increase in the correlation time . The complete relaxation matrix analysis of NOESY data was necessary in order to take into account this marked spin- diffusion.
Conformation of the saturated anthracycline ring A is better determined by analyzing the proton-proton coupling constants. The experimental J-couplings resulted (4) very similar to those obtained for other analogues (5) and indicate a $^{2}H_{5}$ conformation .

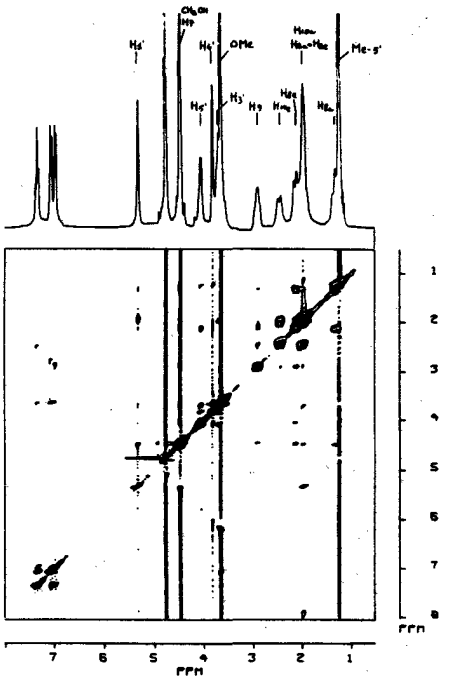

Fig.1: 2D-NOESY (t_{mix}=0.1s)

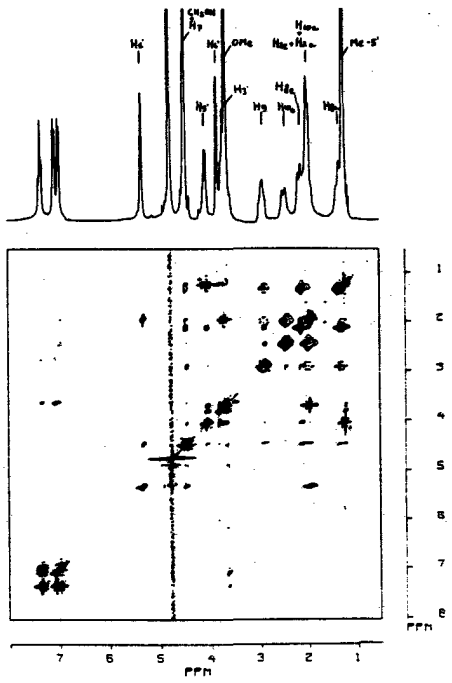

Fig.2: 2D-IRD (t_{mix}=0.1s). Positive levels only

A model geometry of 9-deoxydoxorubicin was built with standard bond lengths and valence

angles and energy minimized with Allinger's MMPI molecular mechanics program (6). The conformation of ring A and of the sugar were driven to a half-chair $^{5}H_{8}$ and chair $^{1}'C_{4}$, conformations, respectively.

All the cross-peaks in the 2D-IRD spectrum were normalized to the diagonal, as $I_{ij}=n_{ij}/\Sigma n_{ij}$ (where n_{ij} are the experimental cross-peaks volumes). The cross-peaks correlating H1' with H7, H5' with H8ax ,H9 and H8eq were used for the determination of the torsional angles. The [Γ] matrix was constructed, with the aid of the model geometry, by calculating the relaxation rates with the equations 2) and 3).

The molecular τ_c was determined by minimizing the r.m.s. error between experimental and calculated 2D-NOESY values involving protons at fixed distances.

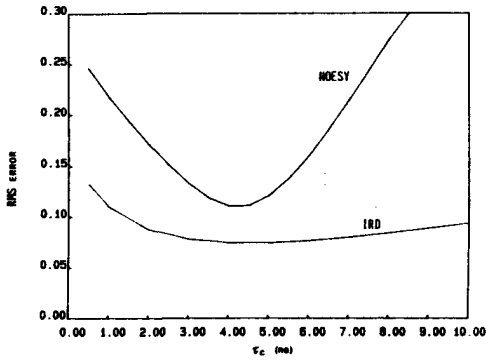

Fig. 3: r.m.s. error between calculated and experimental 2D-NOESY and 2D-IRD data at t_{mix}=0.1 s , as function of τ_c.

Although a knowledge of τ_c is necessary in order to construct the [Γ] matrix, the normalized IRD values are less dependent on the correlation time, than the corresponding NOESY values (see Fig.3). Errors introduced in the determination of τ_c, therefore, are less likely to affect the results.

Torsional angles Φ and Ψ were then obtained by varying systematically their values and finding the minimum error between experimental and calculated IRD values through the use of eq.1-4 The IRD cross-peak intensities, normalized to the diagonal, were calculated as $I_{ij}=m_{ij}/\Sigma m_{ij}$ (where m_{ij} are the intensities of cross-peaks calculated with eq.4) and compared with the experimental ones. As it can be seen from fig.s 4 and 5, the calculated values all agree for a narrow region of and angles. A global minimum of the error between calculated and experimental I_{ij} was found for Ψ =18±10° and Φ=48±15°.

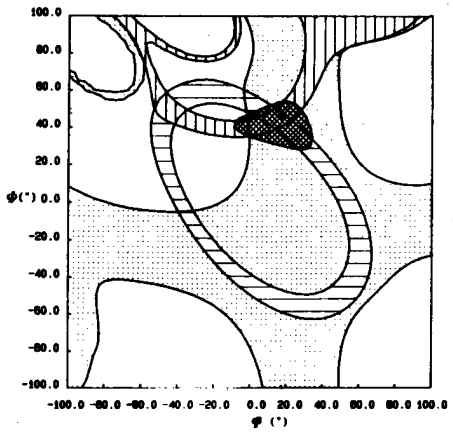

Fig. 4: Calculated Φ and Ψ torsional angles, corresponding to the experimentally determined IRD values for H9-H5' (▨),H8eq-H5' (▥) and H7-H1' (▬) interactions. The plotted regions correspond to Φ , Ψ values where the error is within the double of the r.m.s. minimum. The hatched region corresponds to the most probable values for Φ and Ψ angles.

The analysis of 2D-IRD relied on the assumption of isotropic motions. $1/T_2$ measurements were performed on the same sample, in order to check the validity of this assumption. An inspection of table 1. shows that $1/T_2$ values, and hence correlation times, for

the sugar-ring A moiety are fairly homogeneous.

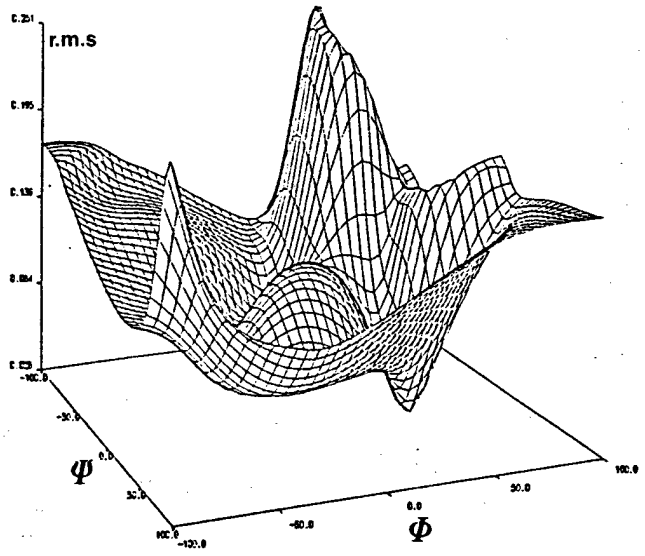

Fig.5 "three-dimensional" plot of the r.m.s. error between experimental and calculated IRD values as function of Φ and Ψ .

TABLE 1 :

Experimental ^{13}C transverse relaxation rates $1/T_2$ (s^{-1}) for 9-deoxydoxorubicin

carbon	$1/T_2$	carbon	$1/T_2$
C-1	25.12	C-10	20.42
C-2	23.56	C-11	4.17
C-3	25.12	C-11a	6.28
C-4	4.71	C-12	9.43
C-4a	9.49	C-13	1.57
C-5	3.14	C-14	(26.70)
C-5a	4.71	C-1'	10.89
C-6	3.14	C-2'	31.42
C-6a	6.28	C-3'	14.14
C-7	12.56	C-4'	17.28
C-8	29.85	C-5'	17.28
Me(5')	4.71	OMe	6.28

EXPERIMENTAL

10 mg of 9-deoxy-doxorubicin were dissolved in 0.5 ml D2O 99.99%, unbuffered solution. All NMR experiments were performed with the same sample using a BRUKER AC-250 at probe temperature.

Phase sensitive 2D-NOESY and 2D-IRD spectra were acquired, in TPPI, by using the pulse sequences I,II and III as described. 256x1K FIDs were acquired (TPPI mode) and fourier-transformed after mild Gaussian-Lorentzian resolution enhancement and zero-filling to 1Kx1K complex data-points.

Cross- and diagonal- peak volumes have been measured by addition of selected rows of two-dimensional spectrum, followed by base-line correction and integration with standard 1D routines. The normalization of 2D-data was done by dividing the cross-peak intensities by the corresponding diagonal peaks. In cases where this was not feasible, because of large overlapping with other resonances, the diagonal peak intensity was estimated by taking the sum of cross-peaks along f2 axis.

^{13}C transverse relaxation rates were measured from experimental line-widths of BB-decoupled 13C resonances,after correction of the applied line-broadening.

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