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Refined procedures for accurate determination of solution structures of nucleic acids by two dimensional nuclear magnetic resonance spectroscopy

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Abstract. New procedures have been described for accurate determination of solution structures of nucleic acids. These are two fold; new two dimensional nuclear magnetic resonance techniques and better approaches for interpretation of nuclear magnetic resonance data for structure determination purposes. The significant development in two dimensional nuclear magnetic resonance techniques for this purpose are ω_1 -scaling and recording of pure phase spectra. Use of ω_1-scaled correlated and nuclear Overhauser effect spectra for estimation of interproton distances and ^{1}H-^{1}H coupling constants has been described. Computer simulation procedures for exact determination of structure have been described. Experimental spectra demonstrating the application of new procedures have been presented.

Keywords. Nucleic acids; ω_1-scaling; 2D NMR. computer simulation; inter proton distances.

Introduction

During the last 5 years, two-dimensional nuclear magnetic resonance (2D NMR) spectroscopy has been extensively used in an endevour to determine 3-dimensional structure of nucleic acids in aqueous solutions (Feigon *et al.,* 1983a,b; Haasnoot *et al.,* 1983; Hare *et al.,* 1983; Scheck *et al.,* 1983; Broido *et al.,* 1984; Clore and Gronenborn, 1985; Weiss *et al.,* 1984; Govil *et al.,* 1985; Hare *et al.,* 1985; Ravikumar *et al.,* 1985; Chazin *et al.,* 1986; Frechet *et al.,* 1983; Hosur, 1986; Hosur *al.,* 1985a, 1986a,b; Chary *et al.,* 1987; Sheth *et al.,* 1987a, b). A fair amount of success has been achieved and it has been possible to assess qualitatively the sequence-specific structural features in oligonucleotides of lengths of 10–15 units. In short the procedure for structure determination involves (i) the use of cross peak positions or coordinates in 2D J-correlated (COSY) spectra (Jeener, 1971; Aue *et al.,* 1976) and nuclear Overhauser effect (NOE) correlated (NOESY) spectra (Jeener *et al.,* 1979; Anil Kumar *et al.,* 1980; Macura and Ernst, 1980; Macura *et al.,* 1982) to obtain sequence-specific resonance assignments of protons in DNA fragments and (ii) interpretation of relative intensities of cross peaks in the two types of spectra to derive structural information about the molecule. The NMR parameters relevant for this purpose are the coupling constants and NOE intensities and these are related to dihedral angles and interproton distances, respectively.

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Abbreviations used: 2D NMR, Two-dimensional nuclear magnetic resonance spectroscopy; COSY, correlated spectroscopy; NOE, nuclear Overhauser effect; NOESY, NOE spectroscopy.

The J-correlated spectra provide information about J-coupling networks and enable identification of chemical shifts of sugar ring protons. In addition the H6 and CH_3 protons of thymines and H6 and H5 protons of cytosines in the oligonucleotide can be readily identified. The NOE correlated spectra on the other hand, reflect through-space interactions between protons and thus show distance correlations between protons on the same nucleotide as well as between protons on adjacent nucleotide units. These correlations are useful for obtaining sequence-specific assignments.

The intensities of cross peaks in NOE correlated spectra primarily depend on the interproton distances. They also depend to a substantial extent on the experimental parameters. Of particular importance is the mixing time (τ_m) used in the pulse sequence of NOESY. During this time, magnetization transfer occurs between protons coupled through dipolar interactions (D). If the system consists of only two cross relaxing protons, then the intensity of the cross-peak increases with mixing time till a steady state is reached. However, when the system consists of more than two protons, it constitutes a network of D-coupled spins and a more complicated diffusion of magnetization occurs. This is referred to as spin diffusion. Under such conditions, the relative intensities of NOESY cross peaks are not a true measure of the distances between the protons they connect. Therefore, experiments should be performed with low mixing time for reliable use of intensities for distance estimation. Under these conditions, appearance of a cross peak implies an interproton distance of less than 4 A.

Another important factor which affects intensities is the line shape. The line shapes are distorted when complex data manipulation procedures are employed, and in the event of poor multiplet resolution, component intensities cancel each other, resulting in poor overall intensities. Procedures have been evolved to overcome these problems and some success has been achieved in getting reasonable estimates of interproton distances (Hare *et al.,* 1985, 1986a, b; Chary, K. V. R., Hosur, R. V. Govil, G. and Miles, H. T., unpublished results). Methodologies are being still developed for obtaining more accurate distance estimates. Overlap of cross peaks has often restricted the number of measurable distances in the molecule.

In the case of J-correlated COSY spectra, the cross peaks which contain the coupling constant information have anti-phase character. Under conditions of low resolution in the spectrum (caused by limited disk storage space, spectrometer time, etc), the component intensities cancel resulting in poor overall intensities. Since the cancellations depend upon the magnitudes of the coupling constants involved, the relative intensities in a given COSY spectrum can be used to derive qualitative information about the magnitudes of the coupling constants. The vicinal coupling constants are related to the dihedral angles in the sugar ring and thus help in fixing the geometry of the sugar ring.

A more precise definition of the structure of a DNA segment can be obtained by extracting detailed NMR information and following the procedures outlined below.

(i) Exact knowledge of coupling constants to fix the sugar geometries,

(ii) Accurate knowledge of a large number of interproton distances from NOESY spectra. This in turn requires improvements in estimation procedures, better separation between cross peaks, better digital resolution, etc.

(iii) Computer simulation to match experimental spectra with simulated spectra.

(iv) Energy minimisation to remove short contacts and obtain the preferred conformation.

While considering any of the 4 steps mentioned above, one has to keep in mind the dynamics of the molecule. The observed parameters are time averages and therefore the data will have to be analyzed in terms of contributions from individual conformers. However, it is observed that in large oligonucleotides (10–15 units long), the central units do not exhibit too much motion in the sense that only one conformer makes a major contribution to the observed properties. In the following, we describe new developments in our laboratories which will take us a long way in the determination of 3D structures of nucleic acids in aqueous solutions. We first describe the new 2D NMR techniques termed "ω_1-scaling techniques" which have helped in achieving sensitivity and resolution enhancement in 2D NMR spectra. This is followed by methodologies for interproton distance estimation and measurement of coupling constants. Finally, we discuss computer simulation of NOESY spectra for overall comparision of spectra. Clearly these steps must follow the resonance assignment, procedures for which are fairly well established.

New techniques in 2D NMR

Among the recent developments that have taken place in 2D NMR, two important ones have proved very useful in the study of biological molecules in solution.

(i) Recording of phase sensitive spectra instead of absolute value plots has greatly improved resolution (States *et al.,* 1982; Marion and Wuthrich, 1983). In this process the dispersive components which have very long tail and therefore hamper resolution are eliminated.

(ii) *ω_1-scaling in 2D NMR spectroscopy:* This concept has originated very recently (Brown, 1984; Hosur *et al.,* 1985b) but it has already found wide application (Gundhi *et al.,* 1985; Ravikumar *et al.,* 1986; Sheth *et al.,* 1986; Hosur *et al.,* 1987a). It can be used for sensitivity enhancement, resolution enhancement, ω_1 -decoupling, narrowing of diagonals, measurement of coupling constants, long range correlation, etc.

A combination of these two developments has several advantages and will be the technique of choice in the future. For example, figure la shows the basic ω_1-scaling pulse scheme which can be incorporated in all forms of correlated spectroscopy such as spin echo correlated spectroscopy (SECSY) (Nagayama, 1980), relayed COSY (Eich *et al.,* 1982), NOESY, Z-COSY (Oschkinat *et al.,* 1986), etc. and also in 2D multiple quantum spectroscopy (Braunschweiler *et al.,* 1983). In every case, the evolution period including the two end radiofrequency pulses is replaced by the pulse scheme of figure 1a. The experimental procedures such as phase cycling have to be suitably modified. The pulse scheme of figure la achieves chemical shift scaling by a factor α and J-scaling by a factor γ along ω_1-axis of the 2D spectrum. It is obvious from the scheme that γ must necessarily be larger than α. Within this constraint, both the factors can be selected in a manner most appropriate for the experiment being performed. For example, selecting γ to be less than unity in NOESY results in both resolution and sensitivity enhancements, whereas in COSY it can lead to loss of cross peak intensities. For purposes of coupling constant measurements, α should be less than 1 and γ greater than 1. Similarly in ω_1-scaled 2D double quantum spectroscopy (Majumdar and Hosur, 1987) selection of $\gamma < 1$ is preferable since it results in both

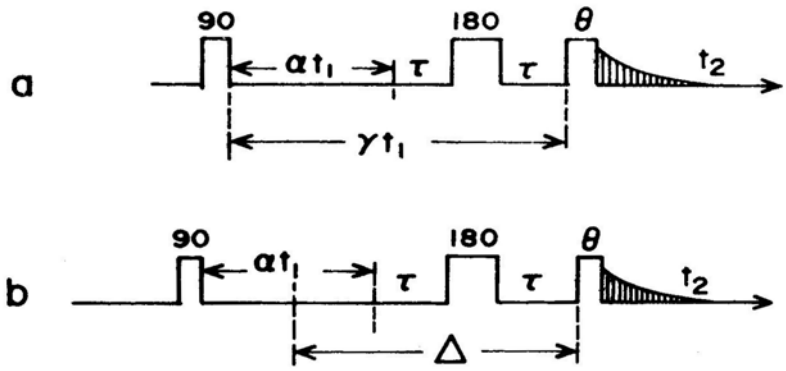

Figure 1. Pulse schemes for phase sensitive ω_1-scaled COSY (a) and exclusive shift scaling (b). The latter is termed as COSS (Hosur *et al.,* 1985c). α and γ are chemical shift and J-scaling factors respectively, τ is a delay which changes with t_1, Δ is a fixed delay and θ is the flip angle of the mixing pulse.

resolution and sensitivity enhancements. In general, $\alpha<1$ helps in increasing multiplet resolution within the peaks in the 2D spectrum. However, it reduces the separation between the peaks and therefore α has to be optimally chosen so as not to merge the peaks.

Figure 2 shows the improvement in multiplet resolution obtained by the ω_1 –scaled phase sensitive COSY (figure 2b) over the conventional absolute value COSY (figure 2a) spectrum for the oligonucleotide d-CTCGAGCTCGAG. From figure 2b it is possible to measure coupling constants as indicated in the figure. Both spectra have been recorded in about the same experimental time (about 15 h). Figure 3 shows the result of incorporation of the scheme of figure 1 in the NOESY pulse scheme. This ω_1-scaled phase sensitive NOESY spectrum has significantly higher resolution and sensitivity compared to conventional absolute value NOESY spectra. This allows recording of good quality spectra with a large number of cross peaks even at very low mixing times. This is a crucial factor for structure elucidation purposes. In the spectrum of figure 3, mixing time is 200 ms and a large number of intra and internucleotide cross peaks are seen. The cross peak regions have been identified by the proton labels in the figure.

Figure l b shows a pulse scheme (correlation with shift scaling, COSS) (Hosur *et al.,* 1985c) for exclusive scaling up of shifts in the 2D correlated spectrum, which is not possible with the pulse scheme of figure 1a. Here again the shift scaling factor can be chosen arbitrarily. The purpose of this experiment is to increase the separation between cross peaks in the 2D spectrum. Since the J-values are not scaled, the sizes of the cross peaks are not altered significantly except for small changes due to slight increases in linewidths as a result of scaling. This effectively results in an increase in the separation between the peaks. As far as dispersion of the peaks is concerned the experiment is equivalent to performing a COSY experiment on a higher field spectrometer.

The parameter Δ in figure l b alters the phase characteristics of the cross and diagonal peaks. It can be optimised in the case of nucleic acids to suppress the diagonal peaks in the (H2′, H2″) region of the spectrum. Figure 4 shows comparison of COSY and COSS spectra in two different regions. The advantages of COSS are

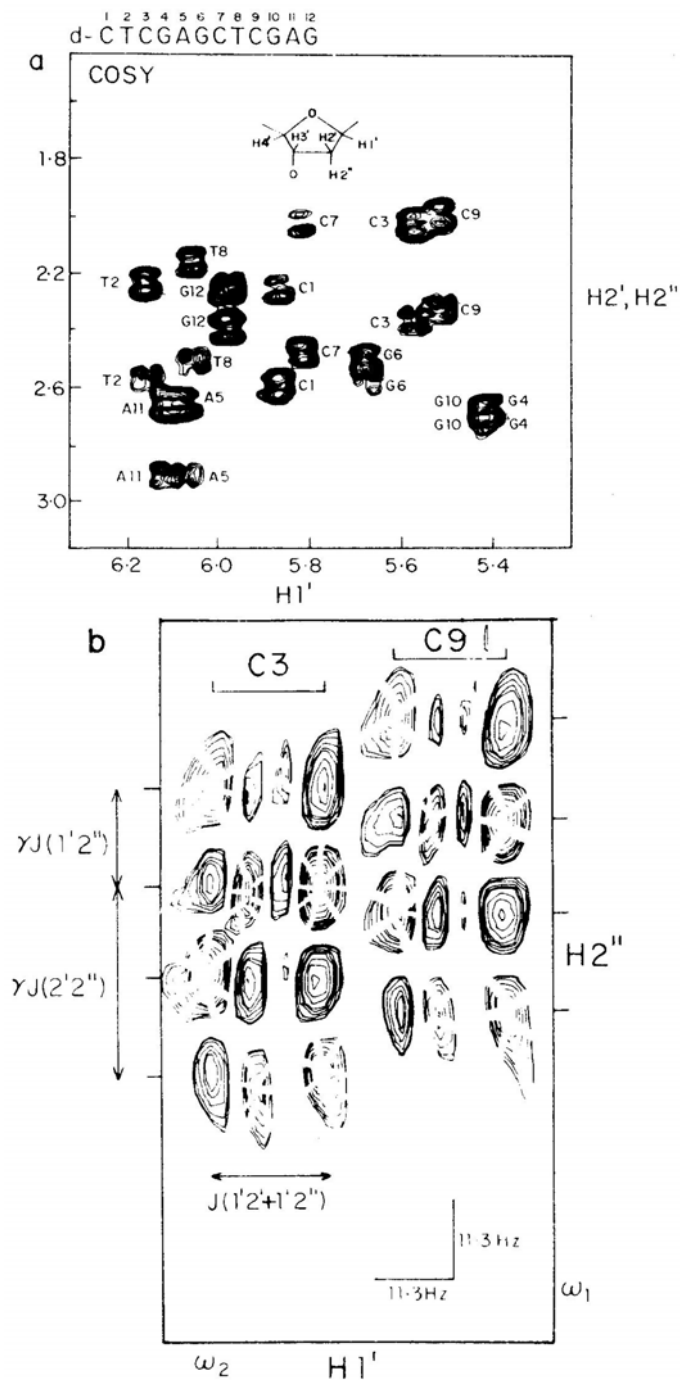

Figure 2. Comparison of absolute value COSY (a) and ω_1-scaled phase sensitive COSY (b) in selected spectral regions of d-CTCGAGCTCGAG. Spectra have been recorded on Bruker AM 500 spectrometer with 5 mM sample in D_2O at 25°C, pH 7.2. Spectrum b shows better multiplet resolution. Dashed contours are negative peaks and the others are positive peaks.

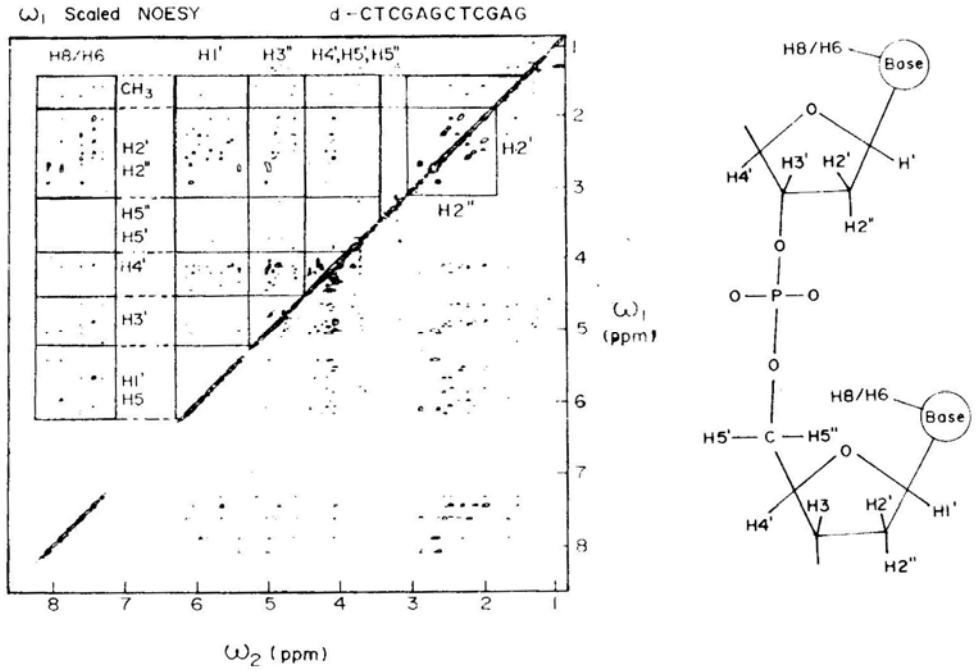

Figure 3. ω_1-scaled NOESY spectrum of the same sample as in figure 2. The cross peak regions have been identified by the hydrogen labels given near the individual boxes. A dinucleotide fragment showing nomenclature of atoms is also included. Many of the boxes contain cross peaks between protons on the same nucleotide as well as between adjacent nucleotide units.

obvious. However, the COSS technique has one disadvantage which arises because of the alteration of phase characteristics of the peaks. Both diagonal and cross peaks acquire contributions from dispersive as well as absorptive components. The dispersive components tend to reduce the resolution within the peaks, and this is undesirable for obtaining J coupling information. Therefore the application of COSS will be in resolving ambiguities due to overlap of cross peaks rather than in improving multiplet resolution which is the case with ω_1 -scaled phase sensitive COSY.

Estimation of interproton distances

As mentioned in the introduction, the intensity of a NOESY cross peak at low mixing time (below the spin diffusion limit) depends directly on the interproton distance. Quantitatively, the intensity is proportional to cross relaxation rate σ_{ij} between the two protons i and j and this is inversely proportional to the sixth power of distance r_{ij} as shown below

$$I_{ij} \propto \sigma_{ij} = \frac{f(\omega, \tau_c, \tau_m)}{r_{ij}^6}, \qquad (1)$$

Where $f(\omega, \tau_c, \tau)$ is a function of correlation time τ_c, mixing time τ_m and frequency ω.

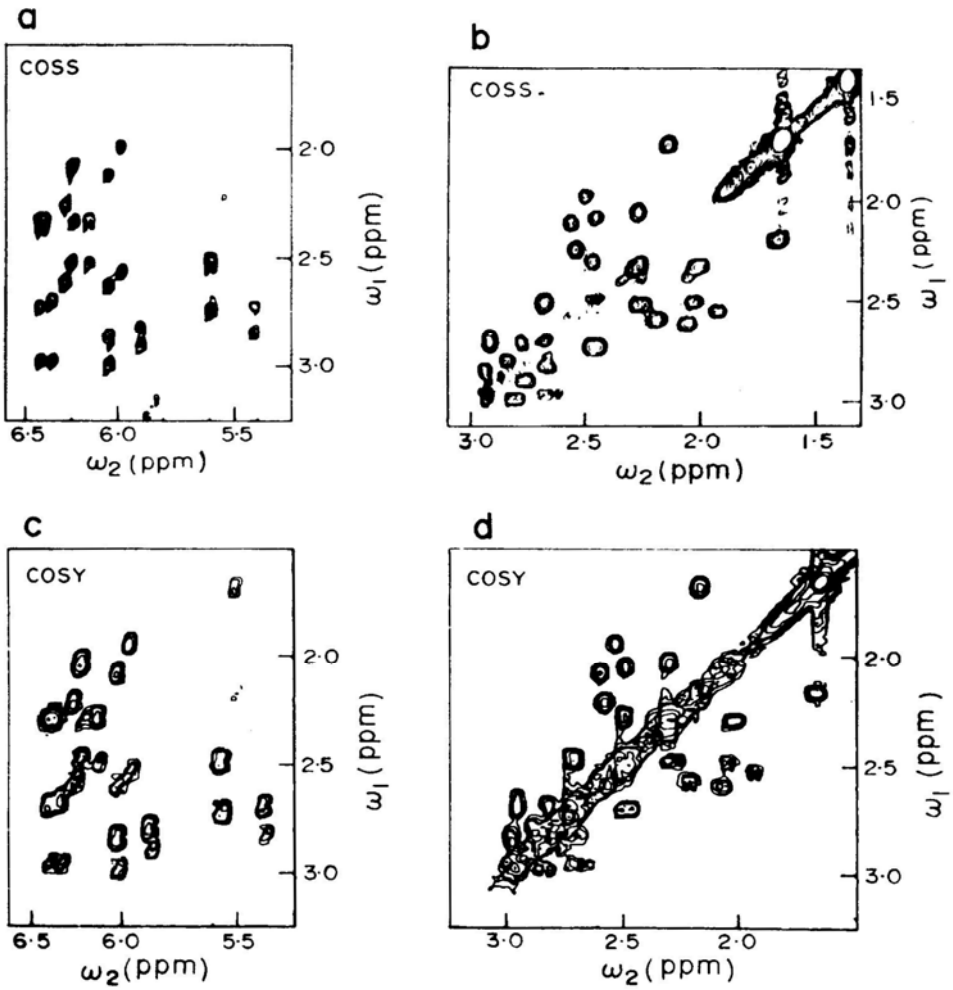

Figure 4. Comparison of 500 MHz absolute value COSY (c, d) and COSS (a, b) spectra in two different regions. Improved resolution and narrow diagonal can be seen in COSS spectra (a and b). Value of α is in 1·5 in 'a' and 2 in 'b'.

Under the conditions of slow motion ($\omega\tau_c \gg 1$) prevalent in biological macromolecules, a simplification arises and the intensity is given by (Ernst *et al.,* 1987)

$$I_{ij} \propto \frac{\tau_c \tau_m}{r_{ij}^6}. \tag{2}$$

Assuming that the correlation time τ_c is the same for all proton pairs, one can obtain a relation

$$\frac{I_{ij}}{I_{kl}} \cong \left(\frac{r_{kl}}{r_{ij}}\right)^6, \tag{3}$$

which enables measurement of ratios of interatomic distances from a given spectrum. By choosing a reference peak corresponding to a known distance, for example, H5-H6, H2'-H2" or H6-CH_3, estimates of the other distances can be obtained.

The entire procedure thus begins with the establishment of the spin diffusion limit, which in principle can be different for different protons. NOESY experiments must be performed with various mixing times and NOE build-up curves obtained as functions of mixing time. In the 2D spectra, NOE intensities correspond to volumes of the peaks and different kinds of approaches have been adopted to estimate the volumes of the peaks in the 2D NOESY spectrum. The simplest of these is indicated in figure 5a. It is assumed that the peaks have absorptive Lorenzian line shapes along both the axes, an assumption which is valid under the conditions in which phase sensitive spectra are recorded employing weak data apodization functions. Volume is then calculated as

$$V\alpha\pi\times L_1 \times L_2 h, \tag{4}$$

where h is the height of the peak and $2L_1$ *and* $2L_2$ are the widths at half-heights along the ω_1 and ω_2 axes respectively (figure 5a). Figure 5b shows typical cross sections along the ω_1 and ω_2 axes through a few cross peaks in the phase sensitive NOESY spectrum of d-GGTACGCGTACC.

In the absence of spin diffusion there will be linear dependence of NOE on τ_m . As spin diffusion sets in, different types of NOE build-up curves are obtained. Figure 6 shows some illustrative curves obtained in the case of d-GGTACGCGTACC and it is seen that the patterns are different for different proton pairs. From these curves it is clear that one has to use a τ_m of less than 80 ms to draw meaningful conclusions about distances. Following the procedure described above several interproton distances have been measured and some of these are shown in figure 7. Details will be published elsewhere.

Measurement of ^{1}H-^{1}H-coupling constants

The protons of d-ribose rings constitute a complex network of coupled spins and each one of them has a complex multiplet structure. There are 6 observable coupling constants, namely J(1'2'), J(1'2"), J(2'3'), J(2"3'), J(3'4') and J(2'2"). Their values depend critically on the geometry of the sugar ring, and the relationship has been analyzed earlier (Hosur, 1986; Hosur *et al.,* 1986a, b; Rinkel and Altona, 1987).

The best method to obtain coupling constants is from the analysis of cross peak multiplet patterns in the ω_1 -scaled phase-sensitive COSY spectrum (Hosur *et al.,* 1987b). The expected patterns for a few illustrative cases are shown in figure 8; these are valid for $\theta = \pi/2$ in figure la. If $\theta < \pi/2$, then the cross peaks look simpler with fewer components, but this simplification is at the cost of some sensitivity in the spectrum. Thus the ω_1-scaled COSY should be optimised with judicious choice of θ, α and γ depending upon the needs.

Figure 9 shows H1'-(H2', H2") spectral regions of ω_1-scaled phase sensitive COSY spectrum of d- CTCGAGCTCGAG, with $\theta = \pi/4$. The cross peak components are seen to be well resolved enabling measurements of J(1'2"), J(1'2') and J(2'2"). It is apparent that J (2"3') is less than the width of each peak along the ω_1-axis. J (2'3') can be obtained indirectly from H1'-H2' cross peaks (upfield peak for every H1' except

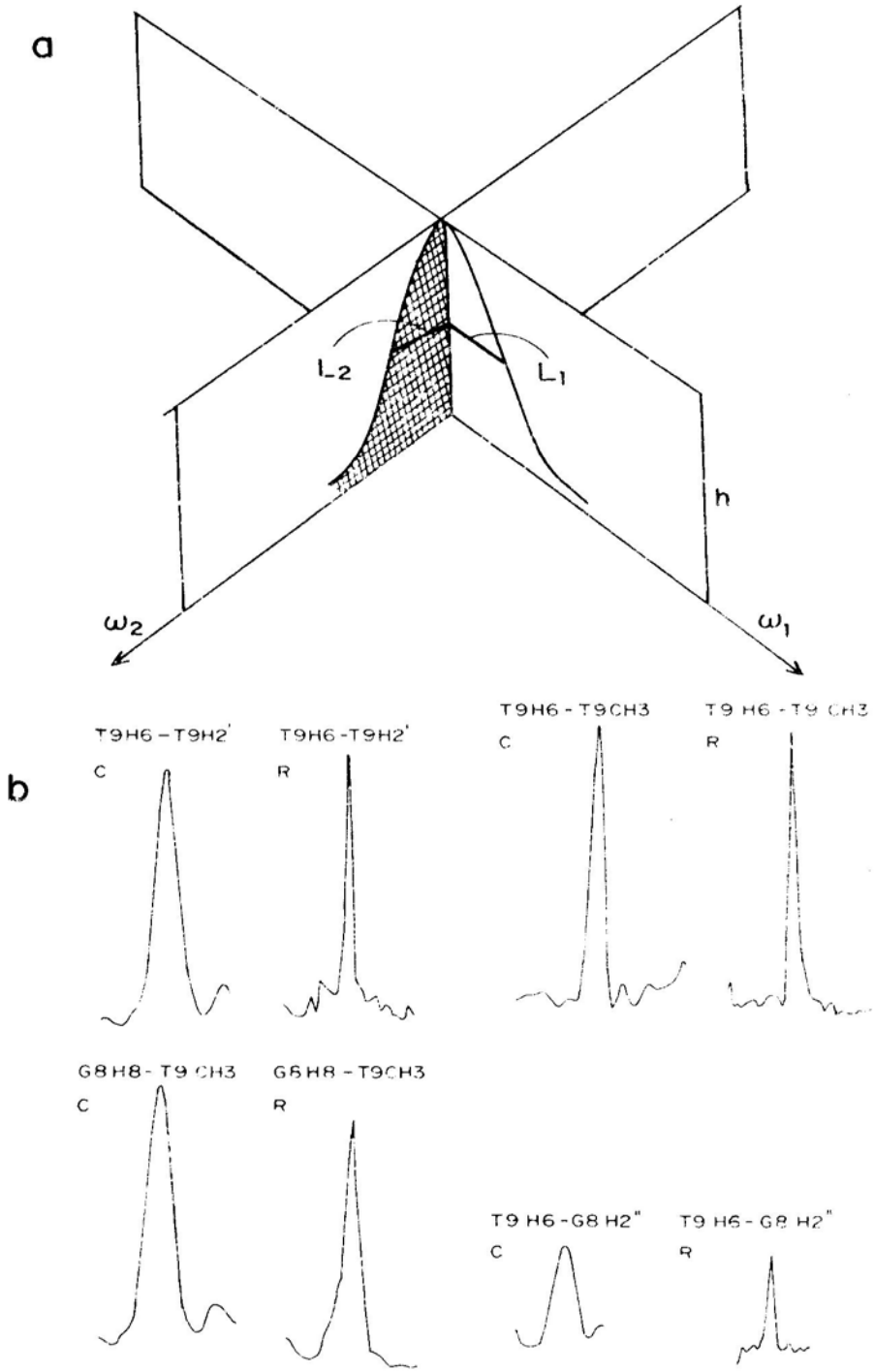

Figure 5.a. Procedure for estimation of cross peak volumes in NOESY spectra, **b.** Illustrative cross sections through some cross peaks to show the line shapes. in experimental spectra. C and R refer to ω_1 and ω_2 cross sections, respectively.

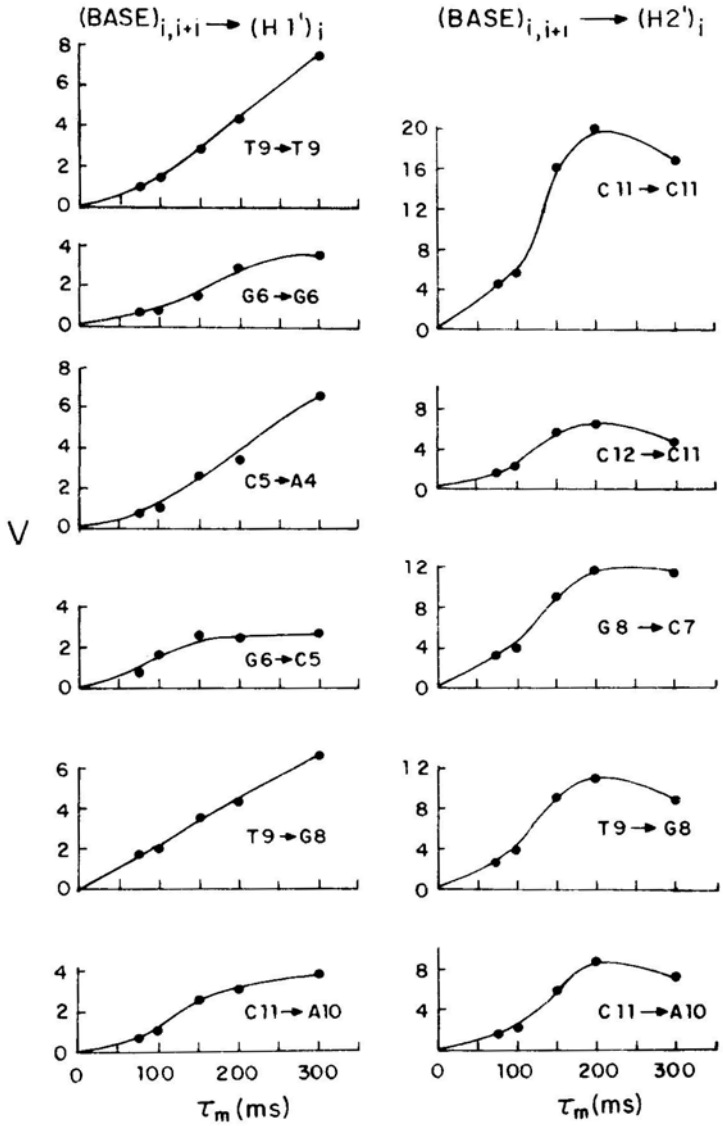

Figure 6. Volumes of a few NOESY cross peaks, in arbitrary units, plotted as a function of mixing time for the oligonucleotide d-GGTACGCGTACC.

for G 12 (Sheth *et al.,* 1987a), since in these peaks the separation between the farthest components yields the sum J(l'2') + J(2'2") + J(2'3'). Extraction of J(3'4') is still difficult because of the extreme complexity of H3'-H4' cross peak structure. However a rough estimate of it can be obtained from the relative intensities of the various peaks in a low resolution COSY spectrum as discussed elsewhere (Sheth *et al.,* 1987a). Such an approach has yielded precise information on sugar geometries in two oligonucleotides d-CTCGAGCTCGAG and d-GGTACGCGTACC, and their details will be published elsewhere.

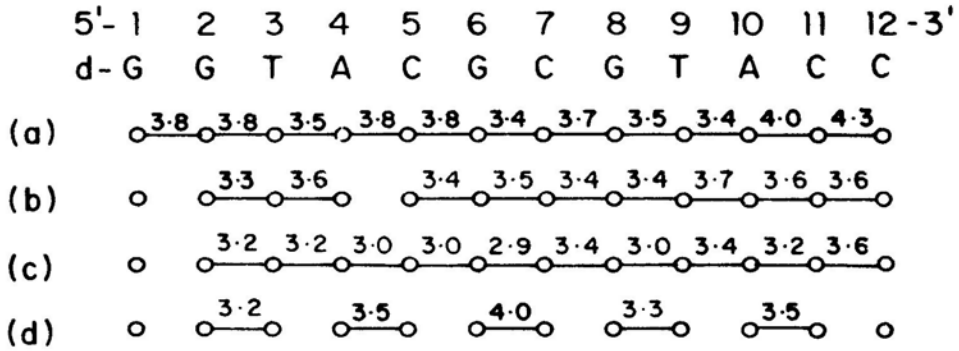

Figure 7. Distances between protons on adjacent nucleotide units, estimated from the volumes of the peaks according to the procedure described in the text. TH_6-TCH_3 distance of 3A has been used for reference a, b, c and d refer to the distances $(H8/H6)_n$ $(H1')_{n-1}$, $(H8/H6)_n$-$(H2')_{n-1}$, $(H8/H6)_n$-$(H2')_{n-1}$ and $(H8/H6)_{n\pm1}$, respectively. Mixing time used in the NOESY experiment was 75 ms.

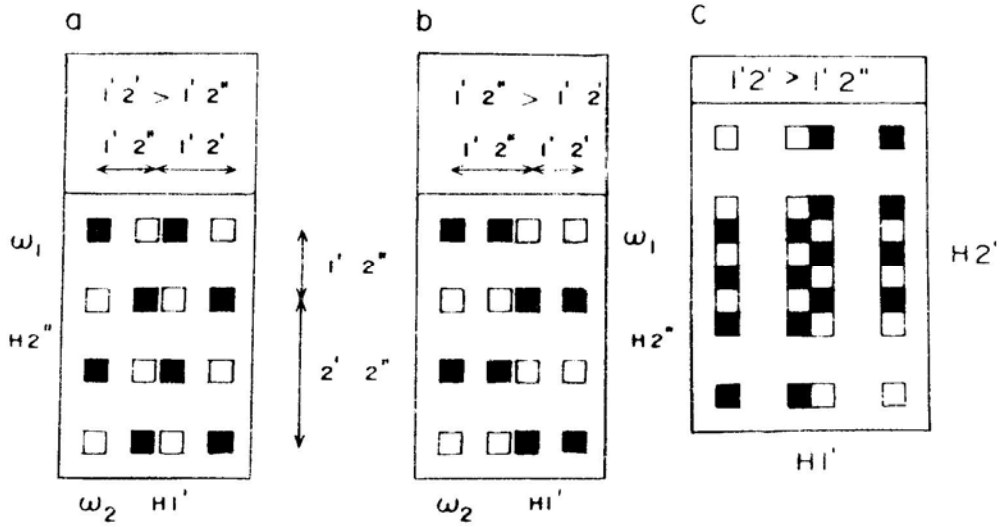

Figure 8. Illustrative cross peak multiplet patterns in COSY spectra of oligonucleotides. The patterns depend on the relative magnitudes of the coupling constants. Filled and open squares refer to positive and negative peaks.

Computer simulation of NOESY spectra

The NOESY spectrum as a whole contains the totality of information about all the short interproton distances within the molecule. However, as must be evident from the preceding discussion, only a limited number of these distances can be measured from the experimental spectrum, since the cross peaks which overlap with other cross peaks cannot be used to estimate the distances. Under such conditions, the obvious way to extract distance information is to simulate NOESY spectra with different structures and match the integrated intensities with the intensities in the experimental spectrum. The correct structure would be the one for which the simulated spectrum

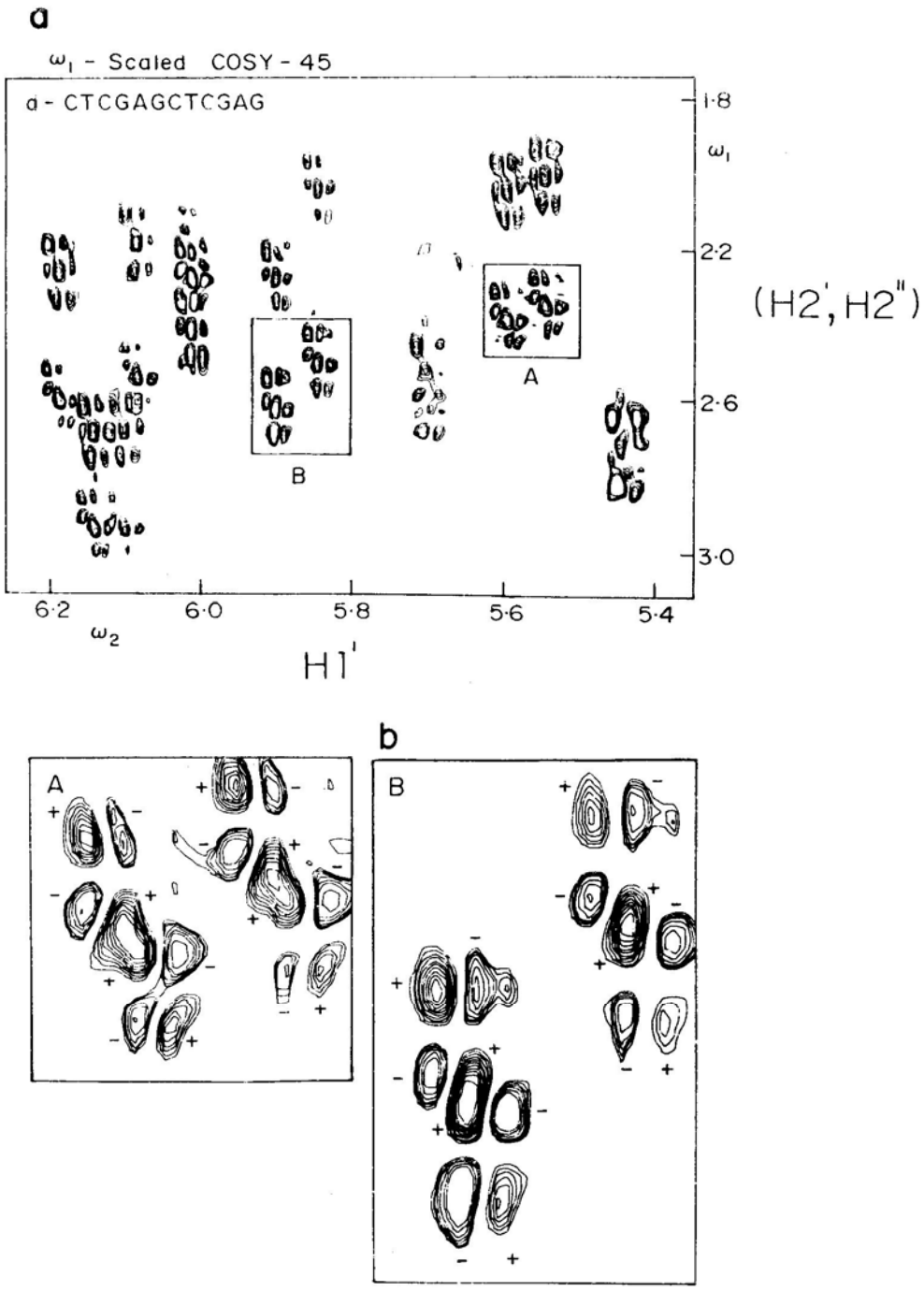

Figure 9. a 500 MHz ω_1-scaled phase sensitive COSY spectrum of d-CTCGAGCTCGAG recorded with a mixing pulse of 45°. **b.** Blow ups of boxes A and B in 'a' ' + ' and ' - ' indicate positive and negative peaks.

matches with the experimental spectrum in all the regions. Figure 10 shows the flow chart of the simulation procedure (Sheth *et al.,* 1987b), which includes a substantial amount of molecular modelling in an interactive fashion. It is obvious that the number of structures that can be generated is enormous, because of the large number of dihedral angles which can be varied. However, the intranucleotide distance estimates and the individual sugar ring geometries obtained from coupling constant data impose several constraints on the conformational space to be scanned. Besides, the overall pattern of the cross peaks in the NOESY spectrum also provides substantial constraints on the backbone dihedral angles, and these can be used to broadly classify the structure as belonging to the known families of DNA conformations. As illustrated in figure 11, the right- and left-handed structures have distinctly different

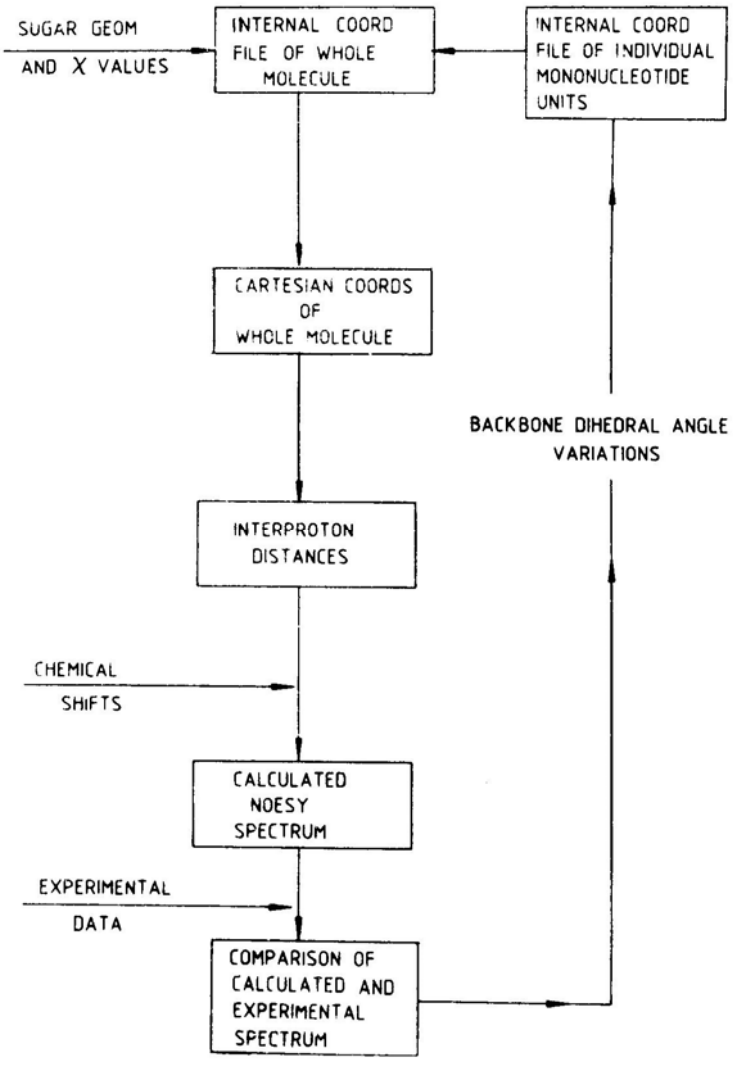

Figure 10. Flow chart of the iterative computer simulation procedure for NOESY spectra.

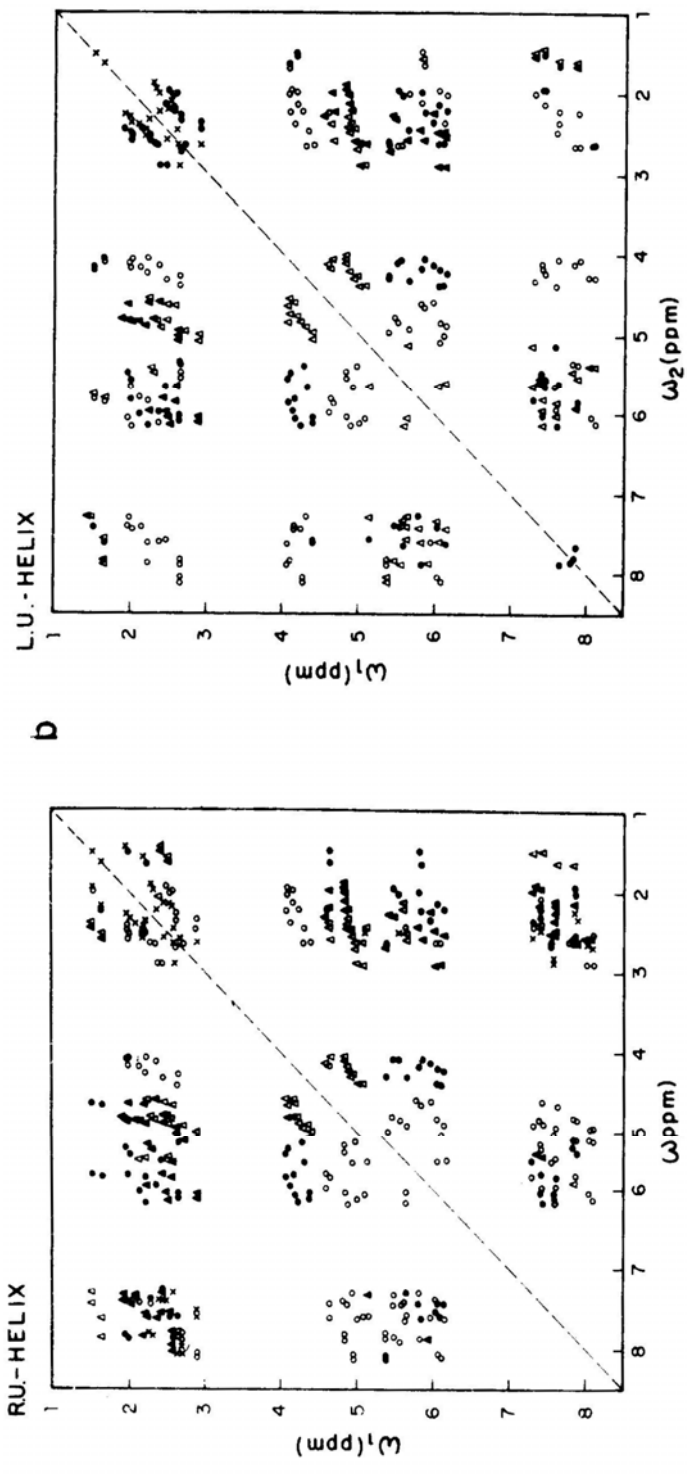

Figure 11. Simulated spea using the assignments of d-CTCGAGCTCGAG for two standard DNA geometries.he patterns of the cross peaks can be used to identify the overall family of DNA struure from the experimental NOESY spectrum. The different symbols represent different ırproton distances (d) and thus indicate the relative intensities of the cross peaks. (X), $1{\cdot}5 < d < 2{\cdot}0$; (▲), $2{\cdot}0 < d < 2{\cdot}5$; (△), $2{\cdot}5 < d < 3{\cdot}0$; (●), $3{\cdot}0 < d < 3{\cdot}5$; (○), $3{\cdot}5 < d < 4{\cdot}0$.

patterns. This of course does not mean that a completely new structure cannot be identified. Detailed analysis- of several oligonucleotides using this approach and energy considerations is in progress in our laboratory.

From spectroscopy to molecular structure

Two dimensional NMR spectroscopy has taken us a long way towards determination of molecular structures of nucleic acids in solution. Sequence-specific resonance labels can be obtained for the various protons; interproton distances within the same nucleotide and between adjacent nucleotide units can be measured; spin-spin coupling constants can be measured. These basic inputs are useful in determining the various structural parameters such as helicity, extent of base pairing, sugar geometries, glycosidic dihedral angles and base stacking in the DNA segment. With this knowledge, computer simulation procedures can help in fixing the backbone dihedral angles, using the concept that the NOESY spectrum is a fingerprint of the DNA structure. Finally, energy minimisation can help in further refining the structure.

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