An NMR strategy for obtaining multiple conformational constraints for ^{15}N–^{13}C spin-pair labelled organic solids

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This work demonstrates a solid-state NMR strategy for extracting multiple interatomic distance and angle constraints from moderately complex solid organic compounds containing a single ^{13}C label and a single ^{15}N label. It is shown that the constraints obtained for the compound cimetidine, with the labels placed at positions directed by the standard synthetic route, are sufficient to provide reliable estimates of 5 of the 8 torsional degrees of freedom in the molecule and are consistent with a molecular conformation close to that determined by crystallography. This strategy will be useful for determining the conformations of sparsely-labelled small molecules in motionally restrained environments, such as within the binding sites of membrane-embedded receptors.

Introduction

Recent developments in cross-polarization magic-angle spinning (CP-MAS) solid-state NMR spectroscopy combined with sophisticated *ab initio* quantum mechanical computational methods have provided new tools that are complementary to X-ray powder diffraction for solving the molecular conformations of small organic molecules in their crystalline forms and in other motionally restricted environments.[1–3] Improvements in methods to facilitate NMR peak assignment and line narrowing have made it possible to measure chemical shielding tensor values for multiple sites in a molecule, each of which contains a wealth of information about bond geometry, hydrogen bonding and π–π interactions.[4] Alongside these advances, measurements of internuclear distances and bond angles from isotope-enriched materials remain widely employed for obtaining structural information by solid-state NMR. This approach is preferable if assignments of peaks from naturally abundant spins are ambiguous or if signal-to-noise is poor, such as when the molecule is bound to an immobilised membrane-embedded protein at low concentration (<200 nmol).[5] A variety of heteronuclear and homonuclear dipolar recoupling methods including rotational resonance and rotational echo double resonance (REDOR) have been developed to measure precise internuclear distances under MAS conditions (reviewed in ref. 6). In addition, N–C–C–N, H–C–C–H and H–C–N–H torsional angles can be measured in experiments that excite double quantum (DQ) coherences in rotating solids.[7–9]

A disadvantage of measuring distances and angles to determine molecular structure is that, in most cases, the compound must be isotope labelled in order to detect the NMR response. Isotope labelling is costly and resource intensive and it is often a challenge to prepare small organic compounds with labels situated at a sufficient number of structurally diagnostic sites to provide enough constraints to solve the molecular conformation. A compound must usually contain a pair of homonuclear (*e.g.*, ^{13}C–^{13}C) or heteronuclear (*e.g.*, ^{13}C–^{15}N) labels in order to measure just a single interatomic distance, yet the value of the structural information obtained from a spin-pair labelled compound may not compensate for the expense and difficulties encountered in the synthesis. Even for a moderately complex molecule in which the isotope labels are separated by 4 or more torsional degrees of freedom, measurement of a single internuclear distance is insufficient to define the molecular conformation unambiguously.

In previous work, the conformation of a double ^{13}C labelled organic solid, the histamine H_2 receptor antagonist cimetidine, was analysed by combining NMR measurements of through space C–C distances with estimates of C–H bond orientations.[10] The single interatomic distance combined with the orientational measurement provided a much stronger constraint on molecular conformation than was provided by the distance alone. Even with the additional constraint, however, several molecular conformations were shown to satisfy the experimental data and it was not possible to distinguish between the various geometries on the basis of other criteria such as energy considerations. Here, this work describes a related, but much more powerful, NMR strategy for measuring multiple geometric parameters from a ^{13}C–^{15}N spin-pair labelled organic solid having several torsional degrees of freedom, in order to provide much stronger constraints on its molecular conformation than are obtained from measuring the C–N interatomic distance alone. The key to this approach is to synthesize the compound with ^{13}C and ^{15}N labels placed several bonds apart, with each label bonded to a single proton. It is then possible to use a series of dipolar recoupling experiments[8,11,12] to measure (i) the C–N distance, (ii) the through space distances between one label and the proton bonded to the other label and (iii) an effective torsional angle defining the relative orientations of the remote C–H and N–H bonds. It is shown that in favourable cases these combined

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measurements are sufficient to provide significant constraints on the molecular conformation of a solid compound having more than 5 torsional degrees of freedom between a pair of labels. The strategy is applied to the conformational analysis of cimetidine crystallised in its commercial anhydrate form A. Cimetidine can be synthesized[13] with ^{13}C in the imidazo ring, using $K^{13}CN$ as the precursor, and ^{15}N in the guanidinium group using $CH_3{}^{15}NH_2$ as the labelling reagent. The protons bonded to the ^{13}C and ^{15}N labels are separated from each other by 9 torsional angles (Fig. 1); ϕ_1 and ϕ_2 are fixed and can be predicted from standard bond geometry, ϕ_3–ϕ_9 can take values from 0 to ±180°. Values of these torsional angles have been estimated from the NMR distance and angle constraints and compared with the values taken from the crystal structure of cimetidine.

Theory

The general case considered is of a single ^{15}N–H group separated from a single ^{13}C–H group by n bonds between the carbon and nitrogen atoms. The relative positions in space of the nitrogen and carbon labels and their two bonded protons are defined by a set of n bond lengths ($\boldsymbol{r}$) and n-1 bond angles ($\boldsymbol{\theta}$) and n-2 torsional angles ($\boldsymbol{\phi}$). The simple example of $n = 5$ is demonstrated in Fig. 2a, showing the torsional angles ϕ_1–ϕ_3 and defining the interatomic distances r_{CN}, $r_{C[HN]}$ and $r_{N[HC]}$ that can in principle be measured by NMR dipolar recoupling methods.[8,11,12] Assuming all bond lengths and angles are known or can be predicted, the absolute value of the torsional angle N–R–R–C (ϕ_2) corresponds to a unique value of the internuclear distance r_{CN} according to

$$\phi_2 = \cos^{-1}\left\{\frac{r_1^2 + r_2^2 + r_3^2 - r_{CN}^2 - 2r_1r_2\cos\theta_1 - 2r_2r_3\cos\theta_2 + 2r_1r_3\cos\theta_1\cos\theta_2}{2r_1r_3\sin\theta_1\sin\theta_2}\right\} \quad (1)$$

where θ_1 and θ_2 are the N–R–R and R–R–C bond angles, respectively. For standard tetrahedral sp^3 geometry, the distance r_{CN} spans the range 2.5–3.8 Å as ϕ_2 rotates through ±180° (Fig. 2b). In the example shown, a measured distance r_{CN} of 3.2 Å corresponds to a torsional angle of ±80° (Fig. 2b, dotted line).

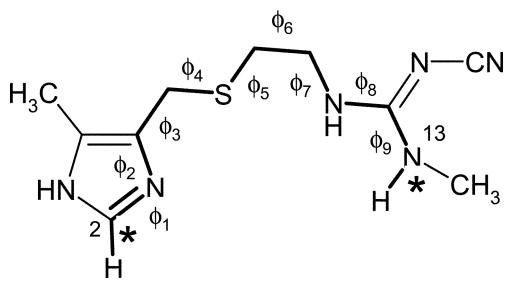

Fig. 1 The chemical structure of [^{13}C,^{15}N]cimetidine showing the positions of the ^{15}N and ^{13}C labels (denoted by asterisks) and the set of torsional angles $\boldsymbol{\phi}$.

The torsional angles H–C–R–R (θ_1) and R–R–N–H (θ_3) are not dependent on r_{CN} but are functions of the internuclear distances $r_{C[HN]}$ and $r_{N[HC]}$ (Fig. 2a). It can be seen from the contour plots in Fig. 2 (panels c and d), however, that even if ϕ_2 is known, the additional distances $r_{C[HN]}$ and $r_{N[HC]}$ cannot provide unique values of ϕ_1 and ϕ_3, but instead correspond to a continuous or discontinuous range of values. When ϕ_2 = 80°, a distance $r_{N[HC]}$ of 3.6 Å, for example, corresponds to ϕ_1 values of 72 and 220° (*i.e.*, –140°) (Fig. 2c, dotted lines) and a distance $r_{C[HN]}$ of 2.8 Å corresponds to values of ϕ_3 of 19 and 285° (−75°) (Fig. 2d, dotted lines). Hence, the three distance constraints, r_{CN}, $r_{C[HN]}$ and $r_{N[HC]}$, are consistent with four possible conformations (and their mirror images) defined by $\{\phi_1, \phi_2, \phi_3\}$ values of {72, 80, 19°}, {72, 80, −75°}, {−140, 80, 19°} and {−140, 80, −75°}. A fourth constraint can be introduced, which is the effective torsional angle H–N–C–H (ϕ_{eff}), where the dashed line indicates an imaginary bond corresponding to the through space N–C interatomic vector (Fig. 2e). From the contour plot shown in Fig. 2f it can be seen that the four sets of $\{\phi_1, \phi_2, \phi_3\}$ values above correspond to ϕ_{eff} values of 180, 80, 20 and 100° (dotted lines). Hence this fourth constraint can in principle identify a unique conformation from the four possibilities, provided ϕ_{eff} can be measured precisely. It can be shown that the set of four constraints $\{r_{CN}$, $r_{C[HN]}$, $r_{N[HC]}$, $\phi_{eff}\}$ is sufficient to uniquely define the vast majority of mirror image conformations of this 5-bond system, although a number of ambiguities may remain depending on the symmetry of the system and precision of the distance and angle measurements.

In practice, r_{CN}, $r_{C[HN]}$, $r_{N[HC]}$ and ϕ_{eff} can be measured from a ^{13}C–^{15}N spin-pair labelled solid compound using MAS dipolar recoupling methods.[8,10,11] The precision of the measurements depends on the internuclear distances; generally the upper limit of detection of the C–N distance is about 4.3 Å, and upper limits of about 6 Å for C–H and 5 Å for N–H are reasonable depending on the homogeneity and size of the sample. This means that as the number of bonds increases above $n = 6$ there are progressively fewer molecular conformations that are accessible to this approach because many conformations will fall outside these limits of detection. Moreover, as n increases, the relationship between r_{CN}, $r_{C[HN]}$, $r_{N[HC]}$ and ϕ_{eff} and the torsional angles is complex, with some combinations of measurements defining unique conformations and others corresponding to a range of conformations. Nevertheless, the strategy described here provides significantly stronger constraints on the molecular conformations of organic solids than can be achieved by measuring a single internuclear distance, thereby improving the cost-to-benefit ratio associated with isotope labelling.

Experimental

All experiments were carried out using a Bruker Avance 400 spectrometer equipped with a Bruker CP-MAS triple resonance probe head operating at frequencies of 400 MHz for ^1H, 100.13 for ^{13}C and 41 MHz for ^{15}N. The cimetidine sample was prepared by co-crystallising from isopropanol a mixture of [^{13}C,^{15}N]cimetidine and a 5-fold excess of unlabelled

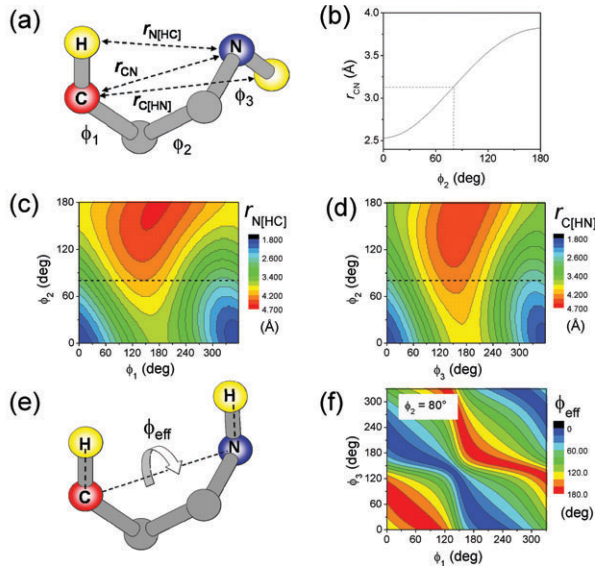

Fig. 2 An overview of the strategy for obtaining multiple distance and angle constraints from a single heteronuclear (*i.e.* ^{13}C–^{15}N) spin pair. The strategy is demonstrated for a 5-bond system with standard sp^3 geometry in which the spatial position of a single, ^{15}N-bonded proton is related to the position of a single ^{13}C-bonded proton through three torsional degrees of freedom. The torsional angles ϕ_1, ϕ_2 and ϕ_3 are related to the interatomic distances r_{CN}, $r_{C[HN]}$, $r_{N[HC]}$ (a). The distance r_{CN} is related to the absolute value of ϕ_2 according to eqn (1). The distance $r_{N[HC]}$ is dependent on the combination $\{\phi_1, \phi_2\}$ as shown by the contour plot (c). Similarly, $r_{C[HN]}$ is related to the combination $\{\phi_3, \phi_2\}$ (d). The effective torsional angle ϕ_{eff} (e) is related to combinations of $\{\phi_1, \phi_3\}$ for any given value of ϕ_2 (f). The dotted lines represent the combinations of distances and torsional angles described in the text.

compound.[14] The solid material (50 mg) was packed without grinding into a 4 mm external diameter zirconium rotor. A ^{13}C CP-MAS spectrum of the sample confirmed it to be pure form A from measurements of isotropic chemical shift values.[14] A two dimensional ^{1}H–^{15}N heteronuclear correlation experiment with 100 μs Lee–Goldberg cross-polarization (LGCP) verified that the labelled ^{13}C and ^{15}N sites were fully protonated (data not presented). All experiments were performed at ambient temperature under MAS, with sample spinning maintained automatically at the desired spinning rate ±1 Hz. In all experiments the recycle delay was 10 s.

The distance r_{CN} was measured in a ^{13}C-observe, ^{15}N-dephase REDOR experiment[15] at sample spinning rate of 4000 Hz. After Hartmann–Hahn cross polarization from ^{1}H to ^{13}C at a proton field of 66 kHz, dipolar dephasing was achieved using a train of rotationally synchronous 15 μs 180° pulses applied at the ^{15}N frequency twice every sample rotation period (with xyxyyxyx phase cycling) to prevent rotational averaging of the C–N dipolar interaction. An 8 μs 180° pulse at the ^{13}C frequency was applied in the centre of the dephasing period to refocus ^{13}C chemical shifts. Continuous wave proton decoupling at 100 kHz was applied during the dephasing period and TPPM decoupling[16] at the same field was applied during acquisition. The extent of dephasing was measured as the ratio of ^{13}C peak intensities recorded with and without the ^{15}N 180° pulses. The total experimental time was 1.3 h.

The distances $r_{C[HN]}$ and $r_{N[HC]}$ could not be measured directly because the strong ^{1}H homonuclear couplings cause a rapid loss of coherence. Instead an experiment was employed which detected ^{1}H dipolar dephasing indirectly by transferring the ^{1}H magnetization to the observed, non-dephasing nucleus.[11,12] Hence, ^{1}H dephasing by the ^{13}C spin was detected through ^{15}N and ^{1}H dephasing by ^{15}N was detected through ^{13}C. Proton homonuclear decoupling during the dephasing interval was achieved with 6 blocks of MREV-8 every sample rotation period, with 3.5 μs 90° pulses and the long window set to 3.5 μs. The sample spinning rate was maintained at 3401 Hz. During the interval two 8 μs 180° pulses per sample rotation period were applied at the frequency of the dephasing spin, ^{13}C or ^{15}N. The ^{1}H magnetization at the end of the dephasing interval was transferred to the observed nucleus (^{13}C when ^{15}N was the dephasing spin or ^{15}N when ^{13}C was the dephasing spin) by 100 μs of LGCP. The total experimental time was 8 h for the $r_{C[HN]}$ measurement and 12 h for the $r_{N[HC]}$ measurement.

The constraint ϕ_{eff} was obtained using the experiment described by Hong and co-workers,[8] in which ^{13}C–^{15}N DQ coherence is excited and allowed to evolve for periods of up to one cycle of sample rotation while proton homonuclear decoupling is applied using MREV-8. The DQ coherence is then reconverted to observable ^{13}C magnetization. The spinning rate in this experiment was 7000 Hz, TPPM decoupling of protons was at a field of 100 kHz, the cross-polarization contact time was 2 ms and all other pulse lengths and conditions were as described above.

Results

A library of cimetidine structures was created by modelling the molecule with standard bond lengths and bond angles and sampling its entire conformational space by varying the torsional angles $\boldsymbol{\phi}$. A total of 322 102 pairs of models, each pair representing mirror image conformations of cimetidine, were generated by rotating each of the 7 non-rigid torsional angles ϕ_3–ϕ_9 separating the ^{13}C and ^{15}N labels through ±180° in regularly spaced angular increments. The geometries of the models were compared against distance constraints obtained in a series of dipolar recoupling CP-MAS NMR experiments on [^{13}C,^{15}N]cimetidine. The results of the experiments to obtain measurements of C–N, C–H and N–H distances are shown in Fig. 3(a–c). The experimental data are expressed as profiles of the extent of dipolar dephasing (S_D) as a function of the dephasing interval. The profiles were compared with numerically simulated curves[11,12,15] to find distances in closest agreement with the experimental data. Comparison of the data with simulations indicated that the precision of the calculated distances was ±0.2 Å or better. After comparison of the modelled cimetidine structures with each of the distances, those models having geometries that were inconsistent with the experimental results were eliminated from the library.

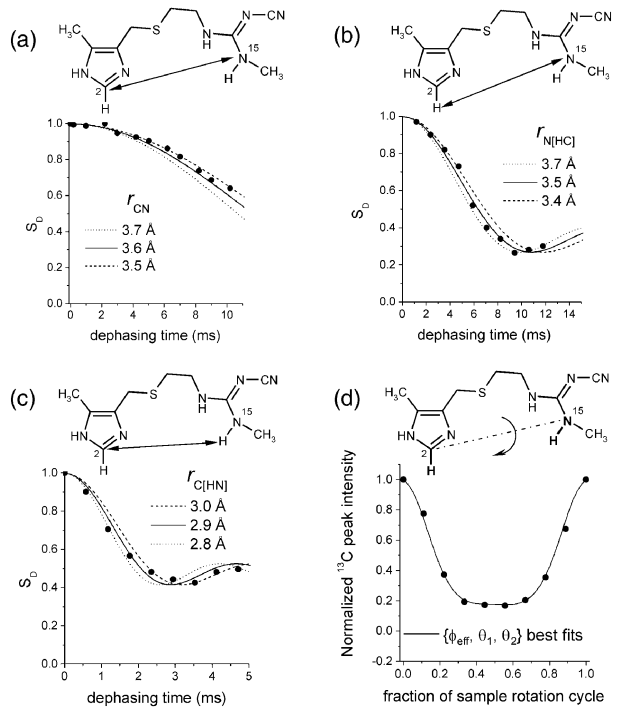

Fig. 3 Results of distance and angle measurements on cimetidine using MAS dipolar recoupling experiments. Experimental data (solid circles) were compared with simulated curves; for the distance measurements, curves corresponding to the upper and lower limits of the experimental data are shown as dashed and dotted lines, respectively, and the best fitting curves are shown as solid lines. Distance r_{CN} was measured to be 3.6 ± 0.1 Å. (a) Distance $r_{N[HC]}$ was measured to be 3.4–3.7 Å (b) and distance $r_{C[HN]}$ was 2.9 ± 0.1 Å. (c) The simulated curves for ^{1}H dephasing were calculated after adjusting the value of the C–H dipolar coupling constant by a factor of 0.55 to take into account the scaling effect of MREV-8 decoupling on the heteronuclear coupling during the dephasing period. The curves were also multiplied by an additional scaling constant to account for non-maximal dephasing because of the effects of accumulated pulse imperfections.[10] To calculate the effective H–C–N–H torsional angle, a plot of normalized ^{13}C peak intensities representing the evolution of ^{13}C–^{15}N DQ coherence at periods of up to one sample rotation period was compared with curves simulated for ϕ_{eff} for the cimetidine conformations that were consistent with the distance constraints obtained in (a)–(c), as described in the text (d). The curves in closest agreement with the data are shown as solid lines.

The effective torsional angle ϕ_{eff} was measured by following the evolution of DQ coherence under the influence of local proton fields.[8] A profile of peak intensities at different DQ evolution periods is a function of r_{CN}, the effective strengths of the bonded N–H and C–H dipolar couplings d (κd_{CH} and κd_{NH}, where κ is a scaling factor) and the effective bond angles HC–N (θ_1) and HN–C (θ_2). These angles are given by

$$\theta_1 = \cos^{-1}\left\{\frac{r_{CN}^2 + r_{HC}^2 - r_{N[HC]}^2}{2r_{CN}r_{HC}}\right\} \qquad (2)$$

and

$$\theta_2 = \cos^{-1}\left\{\frac{r_{CN}^2 + r_{HN}^2 - r_{C[HN]}^2}{2r_{CN}r_{HN}}\right\} \qquad (3)$$

where r_{HC} and r_{HN} are the C–H and N–H bond lengths. Curves were simulated for each set {r_{CN}, θ_1, θ_2, ϕ_{eff}}, representing the geometry of each structure remaining in the library, together with values of κd_{CH} and κd_{NH} measured experimentally from the cimetidine sample as described in ref. 8. The models having geometry consistent with the experimental DQ evolution curves (Fig. 3d) were retained and optimised by local energy minimisation.

The basic distance constraint r_{CN} reduced the number of mirror image pairs of models in the initial library from 322 102 to ~5000 (Fig. 4a). Hence, although this measurement restricts the possible conformations of cimetidine to within <2% of its entire conformational space, its distance alone is clearly insufficient to determine the global structure of cimetidine unambiguously. Measurement of $r_{C[HN]}$ and $r_{N[HC]}$ reduced the number of pairs of models further to 136 conformations and after applying the final experimental filter, ϕ_{eff}, only 12 pairs of models remained in the library (Fig. 4a). The spread of geometries of the models consistent with the distance constraint r_{CN} alone and of the models remaining after applying all 4 constraints was assessed by multivariate (principal component) statistical analysis of the 7 torsional angles $\boldsymbol{\phi}$ for the ~5000 models. A scores plot of the first two principal components (PCs) indicated that the models satisfying the r_{CN} constraint alone covered a large spread of conformations sampling virtually the entire range of possible geometries (Fig. 4b). After applying all 4 constraints, the PC scores plot (representing one mirror image for each model for clarity) indicated that the remaining models clustered into two equally populated groups of conformations, denoted NMR1 and NMR2 (Fig. 4c).

The remaining models all adopted conformations in which torsional angles ϕ_3, ϕ_4, ϕ_6, ϕ_8 and ϕ_9 deviated from the median values by ±30° or less (Fig. 4d). For one group of conformations (NMR1), ϕ_5 and ϕ_7 lay within the range 70 ± 10° and 80 ± 10°, respectively. For the other group (NMR2), the values of ϕ_5 and ϕ_7 were less precise and lay within the range 275 ± 55° and 270 ± 40°, respectively. The models in group NMR1 had the lowest energy of the remaining models and their geometry permitted intramolecular hydrogen bonding between the guanidinium and imidazole moieties. The set of torsional angles $\boldsymbol{\phi}$ obtained from the crystal structure of cimetidine in form A all lay within the range of values spanned by group NMR1 (Fig. 4d, dotted line). The molecular conformation representing the mean of all torsional angles from the lowest energy group NMR1 was in good agreement with the cimetidine conformation determined by X-ray crystallography[17] (Fig. 4e).

Conclusion

This work has demonstrated how multiple C–N, C–H and N–H distances and angles measured from a solid organic molecule containing just one pair of ^{15}N–^{13}C labels provide

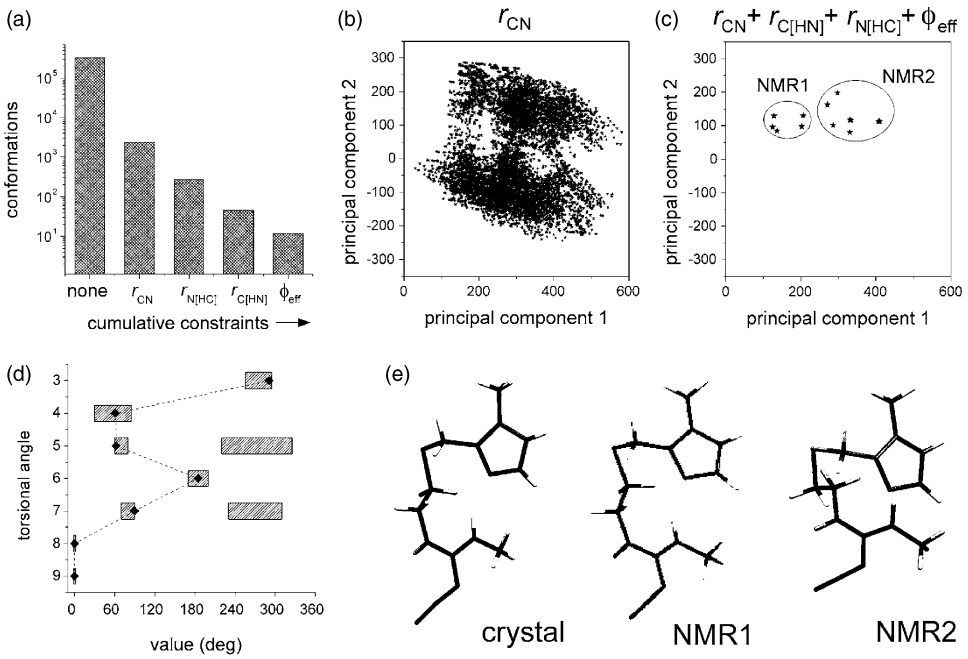

Fig. 4 Conformational analysis of cimetidine form A from the distance and angle constraints obtained by NMR. The application of each of the constraints r_{CN}, $r_{C[HN]}$, $r_{N[HC]}$ and ϕ_{eff} reduced the number of cimetidine conformations in the library from 322 102 pairs of mirror images to 12 pairs. (a) Principal component scores plot of the $\boldsymbol{\theta}$ values for each of the conformations consistent with the constraint r_{CN} (b) and with all four constraints (c) illustrate how r_{CN} alone is a weak constraint whereas the combined measurements restrict the geometry of cimetidine to within two clusters of limited conformational space (NMR1 and NMR2). The clusters of points in (b) represent the mirror images of each conformation but in (c) one set of mirror image points has been omitted for clarity. An analysis of the range of torsional angles $\boldsymbol{\phi}$ for the 12 conformations indicated that ϕ_3, ϕ_4, ϕ_6, ϕ_8 and ϕ_9 fell within a narrow range of $\pm 30^\circ$ from the median values for both NMR1 and NMR2 groups and that the main differences between the NMR1 and NMR2 geometries originated from ϕ_5 and ϕ_7 adopting values within two distinct ranges (d). The filled diamonds and dotted line indicate the values of $\boldsymbol{\phi}$ taken from the crystal structure. Representative conformations of cimetidine from groups NMR1 and NMR2, taken from the median values of $\boldsymbol{\phi}$ are shown alongside the conformation from the crystallographic coordinates (e). Note that the torsional angle ϕ_{10} defining the position of the nitrile group has been set to 180° although the actual value could not be determined experimentally.

substantially stronger constraints on molecular conformation than is provided by measuring the C–N internuclear distance alone. For the example of the histamine receptor antagonist cimetidine, the additional constraints were not sufficient to define a unique conformation of the molecule but were able to restrict the geometry of the molecule to two clusters of closely related conformations that could be evaluated further using additional criteria (*i.e.*, energy considerations). The lowest energy group of conformations was in excellent agreement with the conformation of cimetidine form A determined by X-ray crystallography. The similarity of the NMR-derived and crystal structures indicates that the measured dipolar couplings are very close to the rigid values and were not scaled by internal molecular dynamics. It is noted, however, that the conformational analysis of certain materials such as biological membranes may require the effects of molecular motion to be reduced or eliminated by performing measurements at low temperatures (−50 °C). This approach will be of particular value for determining the molecular conformations of small molecules such as cimetidine in environments that are not readily amenable to analysis by crystallography, such as when bound to membrane-embedded G-protein coupled receptors.

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