Molecular-Dynamics and NMR Investigation of the Property Space of the Zwitterionic Antihistamine Cetirizine

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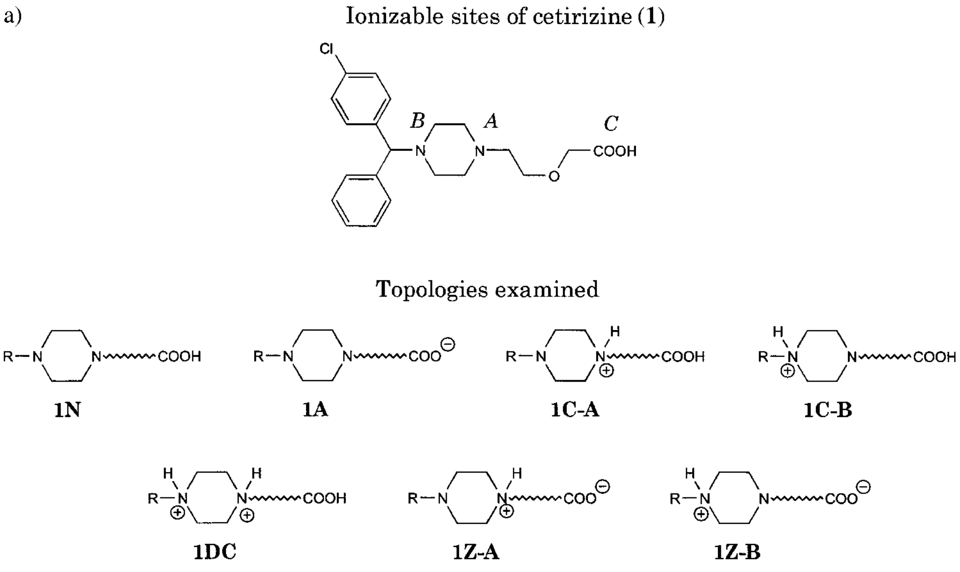
The zwitterionic antihistamine cetirizine and its parent drug hydroxyzine as reference compound were examined for their 3D structures and dynamics. After attributing, by NMR spectroscopy, the two basic pK_a values, the most common conformations for each electrical species were determined by molecular-dynamics simulations and confirmed by NMR measurements. For cetirizine, the results demonstrate that the zwitterion, which is the predominant species at physiological pH, exists as folded conformers able to partly mask polar groups. Extended and folded conformers of similar energy were also found for neutral hydroxyzine, whereas its monocationic species displayed folded conformers stabilized by intramolecular H-bonds. These findings are in full agreement with previous results on the lipophilicity behavior of cetirizine in isotropic solvent systems and, taken together, could explain the favorable pharmacokinetic properties of the drug.

1. Introduction.

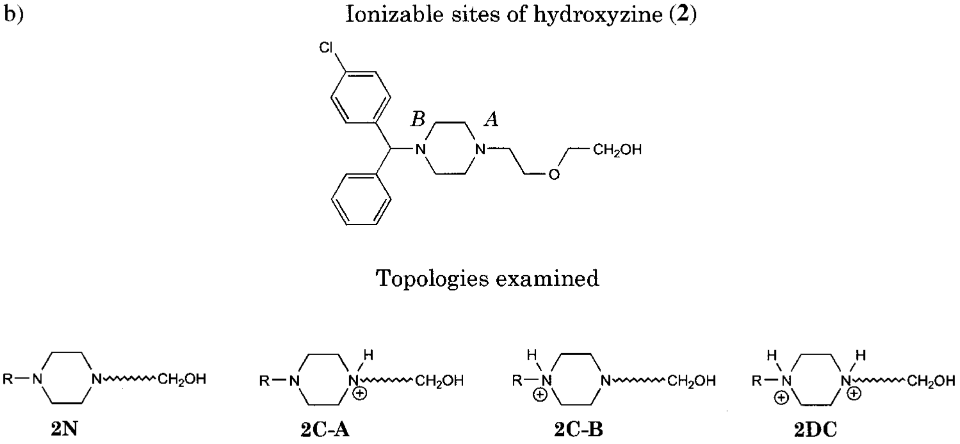
– Cetirizine (*Fig. 1, a*) is a zwitterionic antihistamine compound with peculiar molecular properties that contrast with those of classical antihistamines [1]. A careful analysis of the lipophilicity profile of cetirizine in two isotropic media, namely octanol/H_2O and dodecane/H_2O, as well as molecular-graphics studies, suggested that conformational changes would induce a partial charge neutralization in the predominant zwitterionic species at physiological pH, which could be responsible for the favorable pharmacokinetic behavior of the drug [1]. In an effort to better understand how intramolecular effects act in the partial masking of charges, we undertook a detailed conformational investigation of cetirizine in its zwitterionic state, the predominant electrical species in the physiological pH range (from 2 to 8).

Cetirizine (**1**) may exist in biological media in different electrical states, as determined by its three macro-pK_a values, and influenced by pH [1][2]. The exact values of the three macro-pK_as has been recently reported to be 2.19, 2.91, and 8.00 [1], but the attribution of the two basic pK_as of the piperazine N-atoms was not elucidated. This attribution is reported here and allows a detailed investigation of the structural and conformational behavior of cetirizine (**1**). The present study was thus performed in two steps, namely a) the attribution, by NMR spectroscopy, of the two pK_a values on the piperazine N-atoms to elucidate the exact structure of the electrical species predominating at physiological pH, and *b*) an in-depth analysis of the conformational behavior of cetirizine by molecular-dynamics studies, confirmed by NMR whenever possible.

Taken together, the results clearly indicate that zwitterionic cetirizine exists in folded conformations able to partly mask the polarity of the molecule and decrease its hydrophilicity, so accounting for its favorable pharmacokinetic behavior.


a) Ionizable sites of cetirizine (**1**)

Topologies examined


b) Ionizable sites of hydroxyzine (**2**)

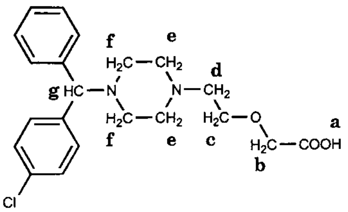
Topologies examined

Fig. 1. a) *Various electrical states of cetirizine* (**1**) *examined by molecular-dynamics simulations.* b) *Various electrical states of hydroxyzine* (**2**) *examined by molecular-dynamics simulations*

Results and Discussion. – 2.1. *Attribution of the Two Basic* pK_a *values by NMR Spectroscopy.* The detailed investigation of the ^{1}H-NMR spectra in the wide pD range considered (from 2 to 8) allowed the elucidation of the relative basicity of the N-atoms A and B, and thus the assignment of their respective pK_a values (*Fig. 1, a*).

The structural assignment of the ^{1}H-NMR spectra of cetirizine (**1**) in aqueous solution was based on chemical shifts (*Table 1*), multiplicity patterns, and NOE experiments. *Fig. 2* shows the NMR spectra at pD 2.23, 5.04, and 8.95. The spectrum at pD 2.23 shows narrow and well-separated peaks in the aliphatic region, allowing easy assignment of most resonances. The correct attribution of the resonances of the

Table 1. *^{1}H-NMR Chemical Shifts of Cetirizine* (**1**) *at Different pD Values*



pD[a])	**b**	**c**	**d**	**e**	**f**	**g**	Arom. H[b])
1.36	4.28	3.99	3.64	3.82	3.67	5.58	7.70–7.50
2.23	4.25	3.97	3.55	3.68	3.38	5.44	7.70–7.50
3.55	4.08	3.87	3.35	3.35	2.73	4.49	7.50–7.30
4.33	4.03	3.84	3.31	3.25	2.63	4.38	7.50–7.30
5.04	4.02	3.84	3.30	3.25	2.60	4.35	7.40–7.20
6.11	4.01	3.84	3.30	3.25	2.61	4.35	7.40–7.20
7.07	4.00	3.80	3.23	3.16	2.57	4.32	7.40–7.20
8.04	3.98	3.75	3.08	2.97	2.50	4.26	7.40–7.20
8.95	3.95	3.65	2.81	2.72	2.43	4.23	7.40–7.20
13.70	3.70	3.40	2.40	2.30	2.30	4.10	7.30–7.10

[a]) pD Values were obtained by adding 0.4 to the value read on the pH-meter.

[b]) Chemical shifts of the aromatic H-atoms are reported as a ppm range due to the overlapping of the signals.

piperazine-ring protons (indicated in *Fig. 2* as **e** and **f**) was based on NOE enhancement of the *multiplet* at 3.38 ppm, following the irradiation of the benzhydrylic CH at 5.44 ppm. Thus, the signal at 3.38 ppm was assigned to the piperazine protons indicated as **f** in *Fig. 2*. Finally, ^{1}H-NMR assignments were back-validated by 2D-HETCOR experiments, for which ^{13}C-NMR assignments (*Table 2*) were based on the chemical shifts of ^{1}H-decoupled ^{13}C-NMR spectra or DEPT (data not shown).

To help relate the p*K*a values to protonation sites, the distribution of ionic species of cetirizine (**1**) in H_2O as a function of pH must be considered, as shown in *Fig. 3* [1]. The pD dependence of the chemical shifts of cetirizine protons is depicted in *Fig. 4*. From pD 1.36 to pD 13.70, all signals were shifted upfield. However, as expected, the protons close to the piperazine N-atoms (namely **d**, **e**, **f**, and **g**) were more strongly affected by the pD change, undergoing an upfield shift in the range of 1.2–1.4 ppm. Following a first upfield shift, the chemical shifts of **d**, **e**, **f**, and **g** reached a plateau in the isoelectric region, namely pH 3.5–7.5 (the isoelectric pH of cetirizine is 5.46 [1]) before moving again upfield for $pD > 8$, where the anionic form of cetirizine predominates. Interestingly, the relative upfield shifts of the protons close to the piperazine N-atoms were not identical. In fact, moving from acidic to isoelectric pD, protons **f** and **g** appear shifted upfield by 1.18 and 1.34 ppm, respectively, whereas protons **d** and **e** show smaller upfield shifts (0.67 and 0.62 ppm, respectively). Beyond the zwitterionic plateau, the signals of **d** and **e** underwent a further upfield shift of 0.67 and 0.65 ppm, respectively, whereas the signals of **f** and **g** were barely affected.

The pD dependence profile of the chemical shifts in *Fig. 4* indicates that the preferred protonation site, namely the stronger basic center, is the N-atom A (see *Fig. 1, a*). In fact, the observed changes in chemical shift (*Table 1*) are in keeping with a

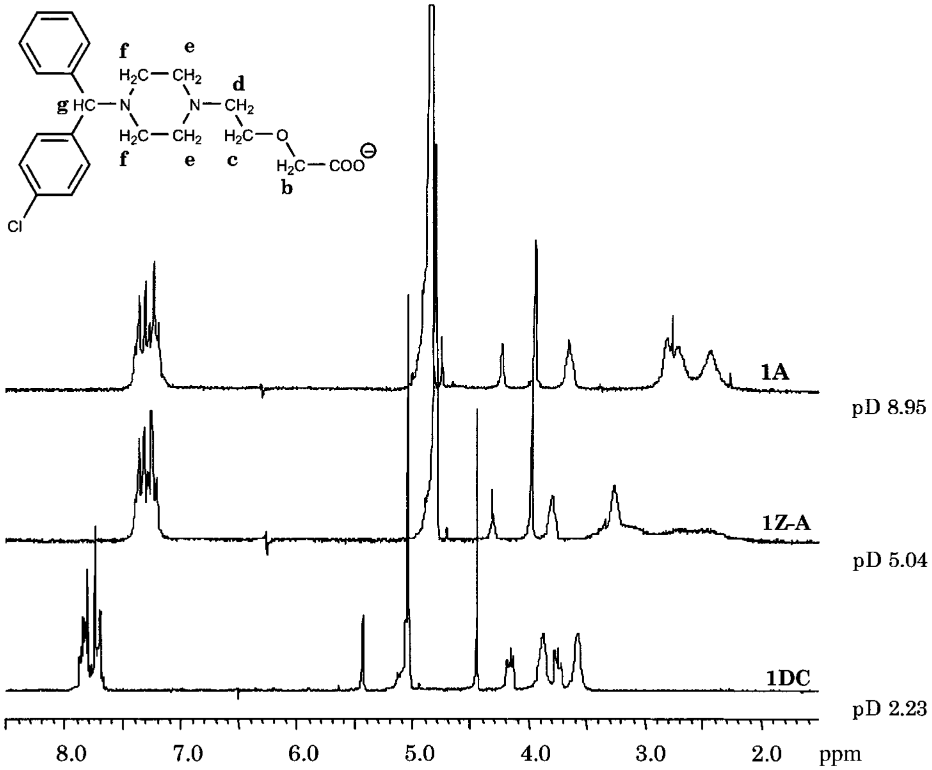


Fig. 2. *^{1}H-NMR Spectra of cetirizine* (**1**) *registered at pD 2.23* (bottom), *5.04* (middle), *and 8.95* (top)

Table 2. *^{13}C-NMR Chemical Shifts of Cetirizine* (**1**) *at various pD Values.* Resonances are labeled according to *Table 1.*

pD[a])	**a**	**b**	**c**	**d**	**e**	**f**	**g**	Arom. C
1.36	171.8	64.8	61.2	53.3	46.2	45.8	72.2	125.4; 127.0; 127.3; 127.4; 127.5; 129.3; 130.5; 132.8
2.23	172.8	65.5	61.4	53.2	47.8	45.7	71.6	125.2; 126.3; 126.7; 126.9; 131.2; 133.9; 134.8
3.55	174.0	66.4	61.5	53.1	48.8	45.7	71.3	125.8; 125.4; 126.4; 130.1; 131.7; 136.9; 137.6
4.33	174.9	67.1	61.4	53.1	49.0	45.7	71.3	125.1; 126.3; 126.5; 129.8; 137.6; 138.2; 140.6; 143.4
5.04	175.2	67.3	61.3	53.1	49.0	45.7	71.3	125.0; 126.3; 126.5; 129.8; 136.7; 138.3
6.11	175.2	67.3	61.4	53.1	49.1	45.8	71.3	125.1; 126.3; 126.5; 129.8; 137.7; 138.3
7.07	175.4	67.3	61.8	53.2	49.2	46.0	71.5	125.1; 126.5; 129.7; 137.8; 138.4
8.04	175.1	67.3	61.2	53.4	49.3	46.5	71.7	125.1; 126.4; 129.7; 138.0; 138.5
8.95	175.1	67.3	62.8	53.5	49.4	47.2	72.1	125.1; 126.3; 126.6; 129.6; 138.0; 138.7

[a]) pD Values were obtained by adding 0.4 to the value read on the pH-meter.

first proton loss from the N-atom B, the weaker basic center, as expected because of the strong steric hindrance and electron withdrawal caused by the benzhydryl group.

The smaller shield effect observed for protons **d** and **e**, and the negligible effect on **g** and **f** following the second proton loss could be due to a partial intramolecular

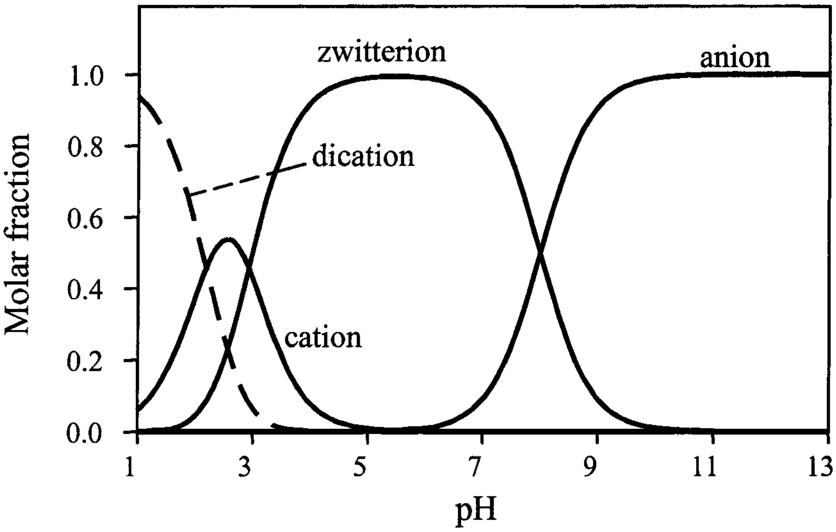


Fig. 3. *Distribution of electrical species of cetirizine* (**1**) *as function of pH* (modified from [1])

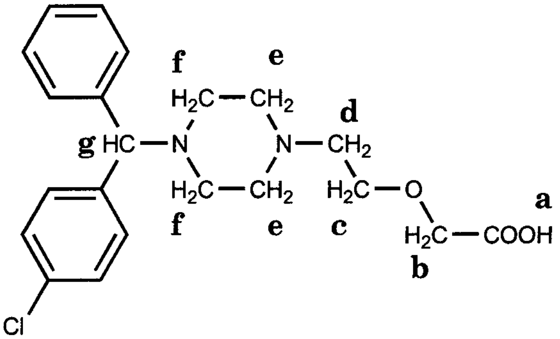
neutralization existing in folded conformations of zwitterionic cetirizine, as suggested in [1] and as confirmed below.

Finally, a pD-dependence of the shape of the proton signals close to the piperazine N-atoms was observed. In the acidic range (pD from 1.36 to 2.23), where the dicationic form predominates (*Fig. 3*), all signals were quite narrow, indicating a high degree of molecular mobility. On approaching the pD range where the zwitterion predominates, the signals tended to broaden. This was true in particular for the piperazine ring protons, where signals at isoelectric pD showed very broad peaks at 200 MHz, which split into four different *multiplets* at 500 MHz (see *Fig. 5*).

2.2. *Conformational Study by QMD.* Quenched Molecular Dynamics (QMD) applied to the various states of cetirizine (**1Z-A**, see *Fig. 1, a*) generated an ensemble of conformers that could be analyzed in terms of the three structural parameters depicted in *Fig. 6* and designated L, d_{A1}, and d_{A2} (for details see the *Exper. Part*). The 52 final conformers were grouped into four main clusters by a Cluster-Analysis (CA) procedure (*Fig. 7*) as shown by the dendogram in *Fig. 8*. Three clusters are characteristic of folded conformers, namely clusters 2, 3, and 4. The fourth, cluster 1, is an isolated point corresponding to an extended conformation.

The single conformer (No. 52) constituting cluster 1 was the only extended one. Here, obviously, the formation of an intramolecular H-bond is impossible. Interestingly, conformer 52 is energetically very unfavorable under vacuum (*Fig. 9*).

The conformers belonging to cluster 3 are characterized by a short d_{A1} distance, implying a H-bond between the proton on the N-atom A and the O1 of the carboxylate group. Cluster 4 is conceptually similar to cluster 3 except that O1 is replaced by O2. Conversely, for all conformers grouped in cluster 2, no H-bond can be formed, but, again, folding takes place and demonstrates that, for zwitterionic cetirizine **1Z-A**, folded conformations are always predominant even when no intramolecular H-bonds exist.



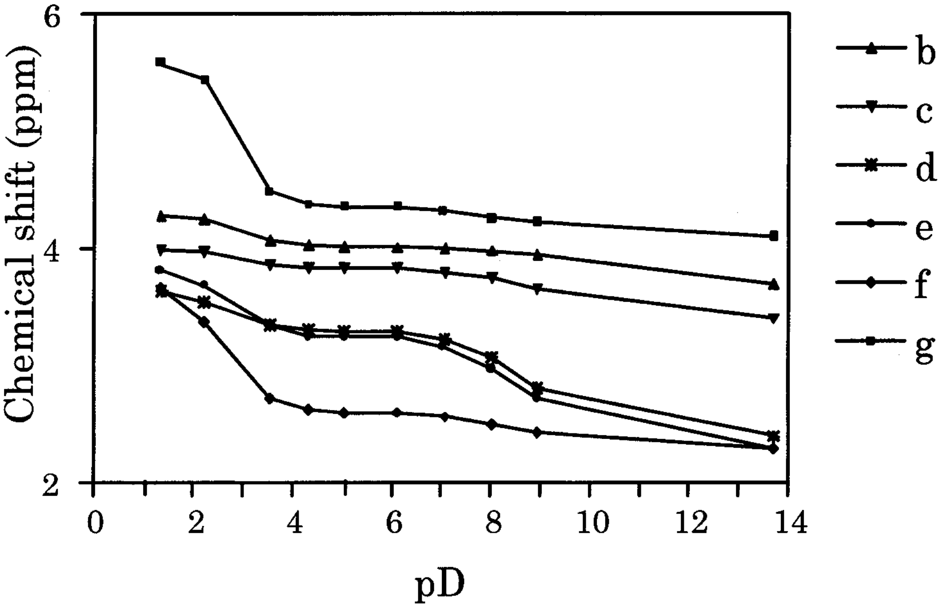


Fig. 4. *^{1}H-NMR Chemical shifts of cetirizine* (**1**) *as a function of pD*

Finally, it is interesting to note that the conformers in clusters 2, 3, and 4 exhibit only partial folding (see *Fig. 9*). In fact, the conformations having energies of less than 3 kcal/mol above the minimum have *L* values ranging from 6.4 to 6.7 Å, meaning that, for lower *L* values, the increment in steric hindrance becomes predominant and cannot be compensated by H-bonding.

All electrical states of cetirizine (**1**) depicted in *Fig. 1, a*, were also considered, regardless of a low probability of existence under physiological conditions. The results are briefly summarized below (data not shown). For the neutral species (**1N**), the clusters containing low-energy conformations are distributed over the entire *L* range. A folded conformer is preferred by 3 kcal/mol over the most stable extended conformers. For the dicationic species (**1DC**), it was necessary to consider distances able to describe the interaction of the carboxylic acid moiety and the ether O-atom with both

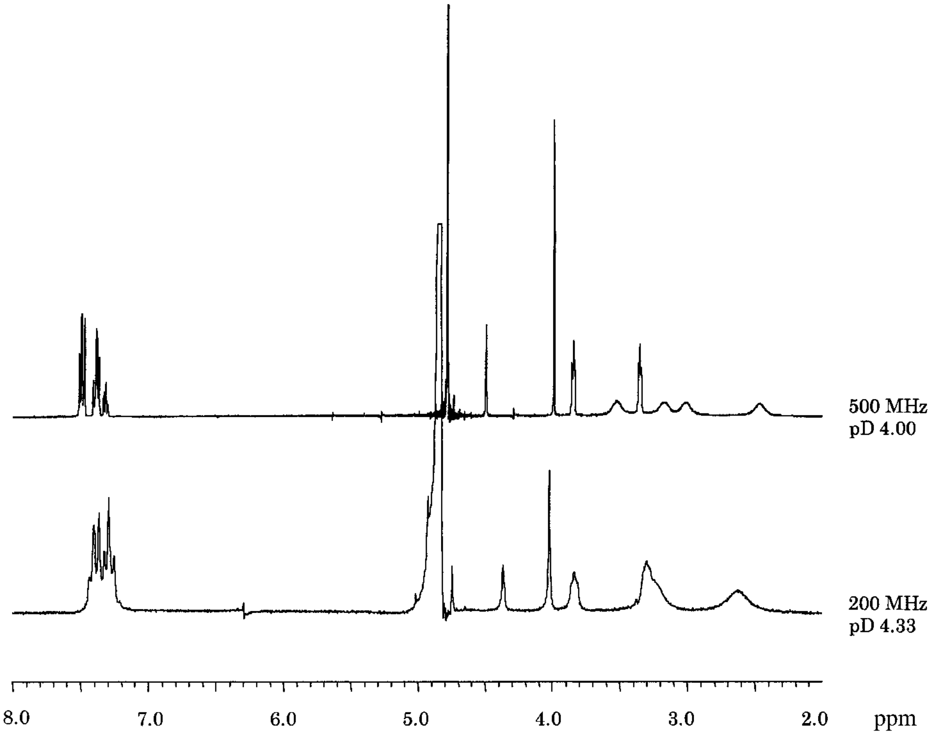


Fig. 5. *^{1}H-NMR Spectra of cetirizine* (**1**) *recorded at 200 and 500 MHz under similar conditions of pD* (*ca.* 4.0)

protonated sites A and B. The folded conformers stabilized by H-bonds are the preferred ones, as extended conformers having higher energies. Interestingly, the most stable conformers of the monocation **1C-A** has the ether O-atom close enough to the H-atom on site A to form an intramolecular H-bond. Folding in the other conformers is produced by a H-bond between the carboxylic acid moiety and the H-atom on site A. The anionic form **1A** is characterized by the absence of H-bonds; hence, folded conformers are only slightly preferred over extended ones. The cationic form **1C-B** and the zwitterionic form **1Z-B** confirm the tendency of cetirizine to adopt folded conformations, especially conformations with internal H-bonds. These forms display a higher energy than the corresponding tautomers **1C-A** and **1Z-A**.

The same theoretical approach was applied to hydroxyzine **2N** (*Fig. 1,b*). Interestingly, its extended conformers have the lowest energy although the difference in energy with folded ones can be as small as 1–2 kcal/mol. The most stable conformers of monocationic hydroxyzine **2C-A** (*Fig. 10*) are folded by H-bond interactions between protonated site A and the hydroxy or ether O-atoms. These intramolecular interactions are similar to those already described for cetirizine (**1**).

Globally, QMD calculations combined with CA gave a good selection of conformers possibly present in aqueous solutions at all pH values. Under vacuum, cetirizine seems to prefer folded conformations regardless of its electrical state. Under these conditions, however, some artefacts due to the presence of electrical charges

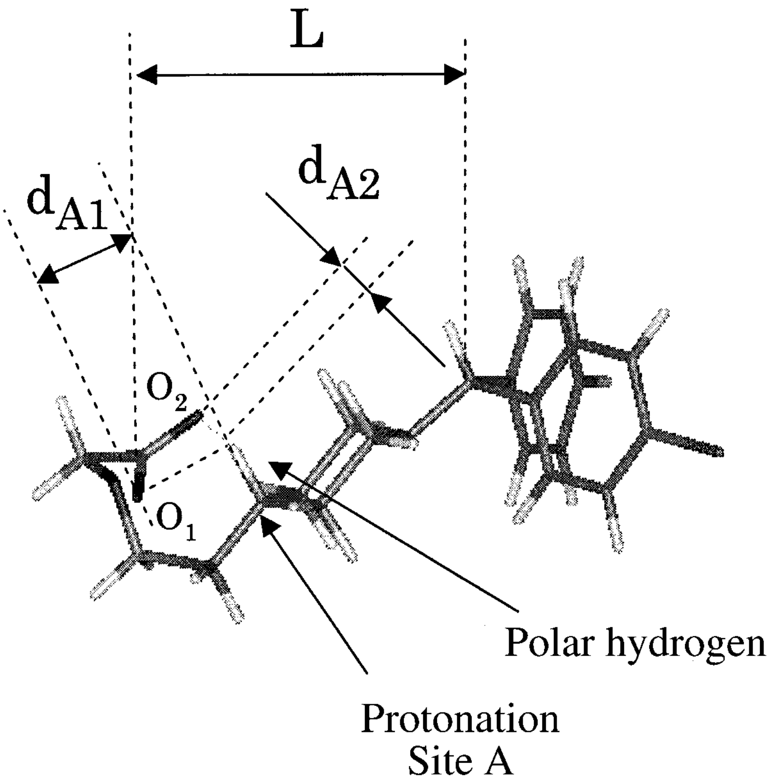


Fig. 6. *Distances used in the cluster analysis (CA)*

could favor folded conformations, as suggested by the observation that extended conformers are energetically favorable for neutral cetirizine.

In aqueous solution, the different shapes of the peaks observed in ^{1}H-NMR spectra at low and isoelectric pD indicate different degrees of molecular mobility (*Fig. 2*). At low pD in particular, cationic species have a great conformational mobility that decreases with increasing pD. At isoelectric pD, cetirizine shows the maximum conformational restriction (as indicated by the marked broadening of the piperazine signals). This behavior could be explained by the preference of the energetically favorable folded conformation of the zwitterionic species even in polar media. A similar picture was also observed at low temperatures. In fact, cetirizine spectra at 253 and 273 K in a 80:20 DMSO/D_2O cryoprotective mixture show a general broadening of all signals, as well as a marked separation of the piperazine protons into four broad *multiplets*.

The NOESY spectrum recorded at 500 MHz and at isoelectric pH (*Fig. 11*) shows a number of cross-peaks due to ^{1}H,^{1}H dipolar couplings at distances shorter than 3 Å. However, these distances are common both to the extended and the folded conformers, as indicated by QMD analysis, and no additional H $\cdots$ H distance < 3 Å was observed in the most stable folded conformers. Thus, NOE experiments, under our experimental conditions, are not able to discriminate between different conformers of cetirizine.

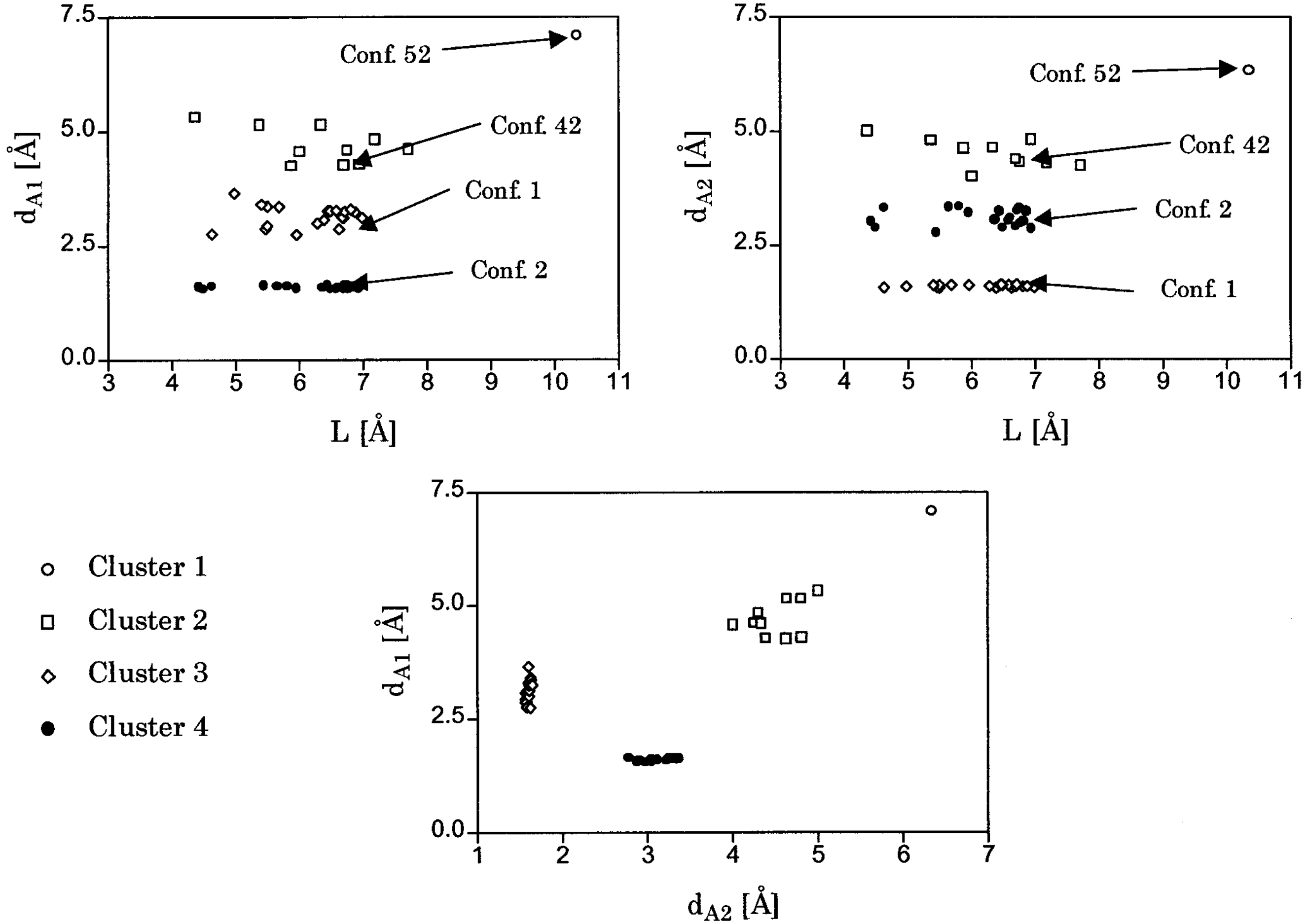


Fig. 7. *QMD Results combined with CA for* **1Z-A.** Clusters are represented in d_{A1}-d_{A2}, d_{A1}-L, and d_{A2}-L planes to give physical meaning to cluster subdivision. In each cluster, the number of the most stable conformer is noted.

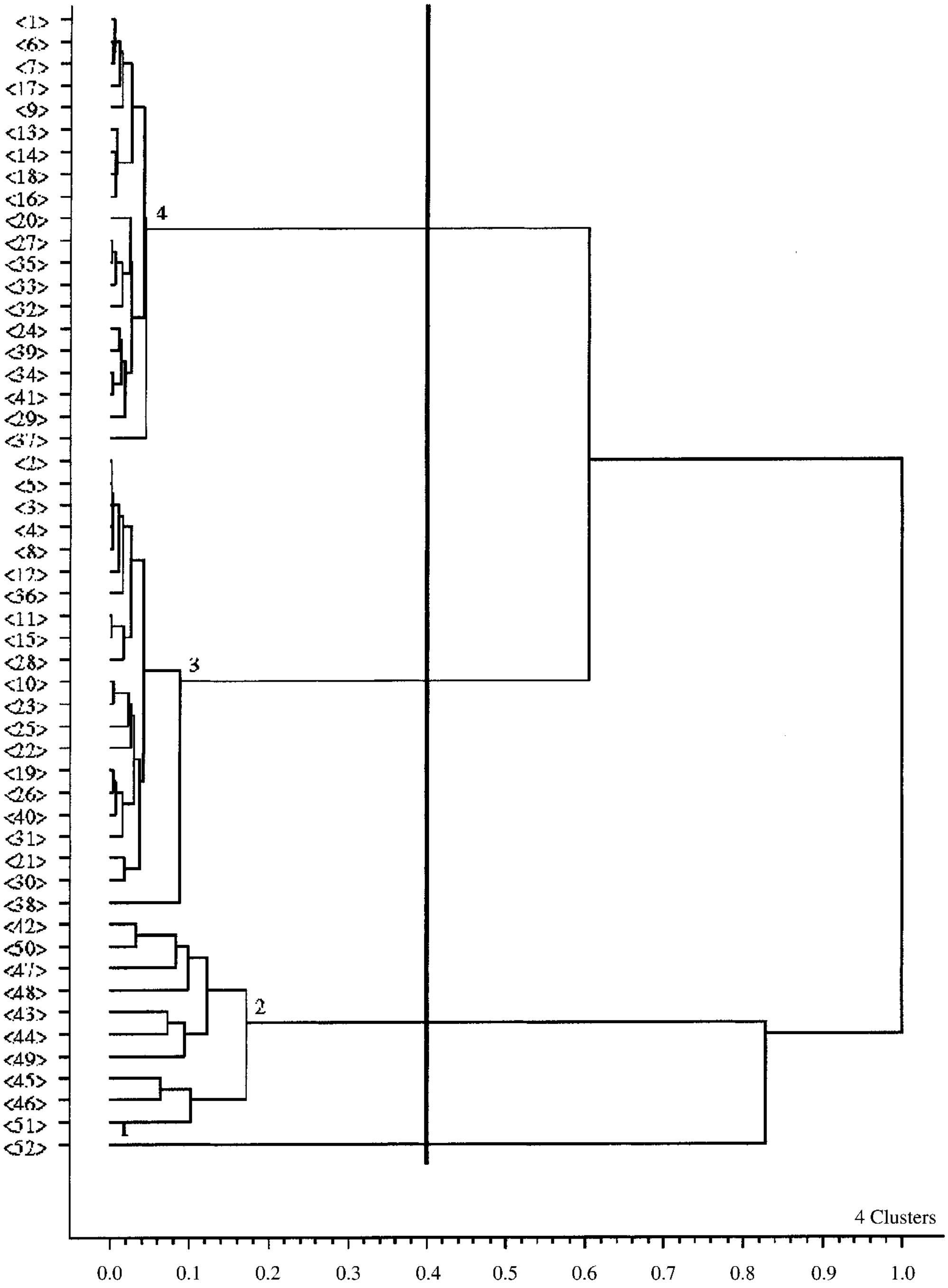


Fig. 8. *Dendogram obtained for the* **1Z-A** *electrical form of cetirizine.* The line defining the four clusters was chosen empirically by testing different distance values.

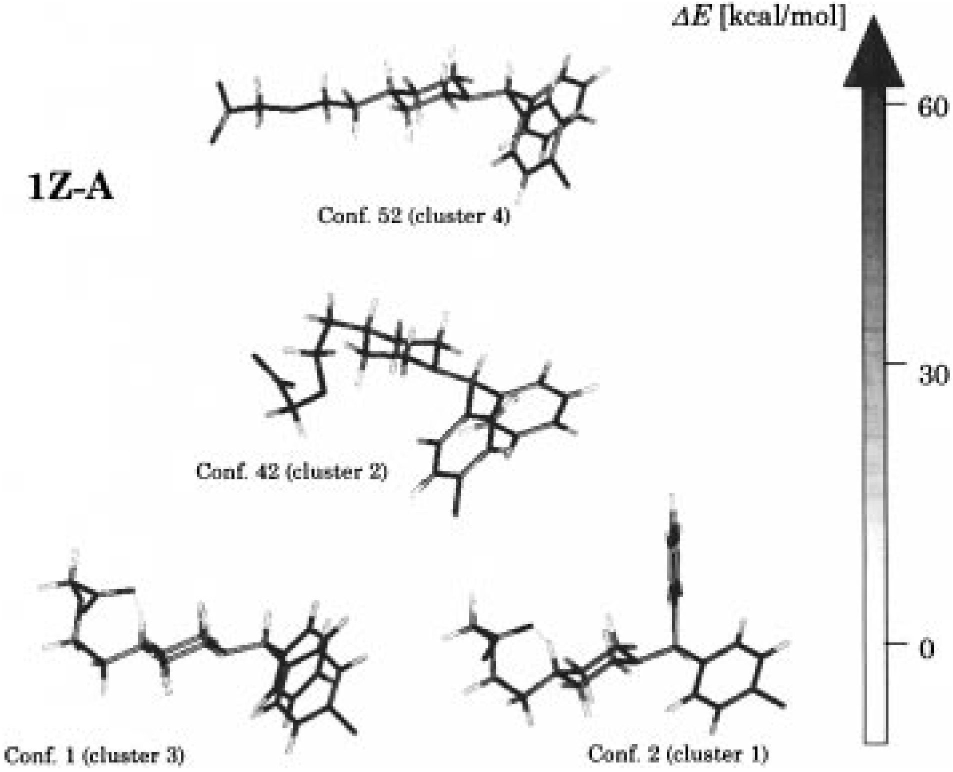


Fig. 9. *The most stable conformer of each cluster is depicted together with the relative position on the energy scale*

3. Conclusions and Perspectives. – The results presented here demonstrate that, for cetirizine, the electrical species predominant at physiological pH is the zwitterion, with preferred protonation of the piperazine N-atom linked to the alkyloxy chain. For this electrical species, folded conformers of low energy are preferred over extended ones. Such a conformational behavior accounts for the partial masking of the polarity of zwitterionic cetirizine and offers a sound support to recent lipophilicity studies in isotropic media [3].

Investigations of the interaction between cetirizine and phospholipid vesicles (liposomes) are in progress to gain further information on its conformational behavior and lipophilicity profile in anisotropic media.

Experimental Part

Compounds and Reagents. Deuterated solvents were purchased from *Aldrich Chemical Co.* (Milwaukee, WI, USA). Cetirizine · 2 HCl and hydroxyzine · 2 HCl were supplied by *UCB* (Braine-l'Alleud, Belgium), and acrivastine was kindly provided by *The Wellcome Foundation Ltd.* (Dartford, United Kingdom).

NMR Experiments. Standard ^{1}H-NMR and decoupled ^{13}C-NMR spectra of cetirizine (**1**) were obtained either in aq. soln. or in different deuterated solvents. Experiments (see below) were also performed with hydroxyzine only when comparison was necessary to solve structural ambiguities. The concentrations of the

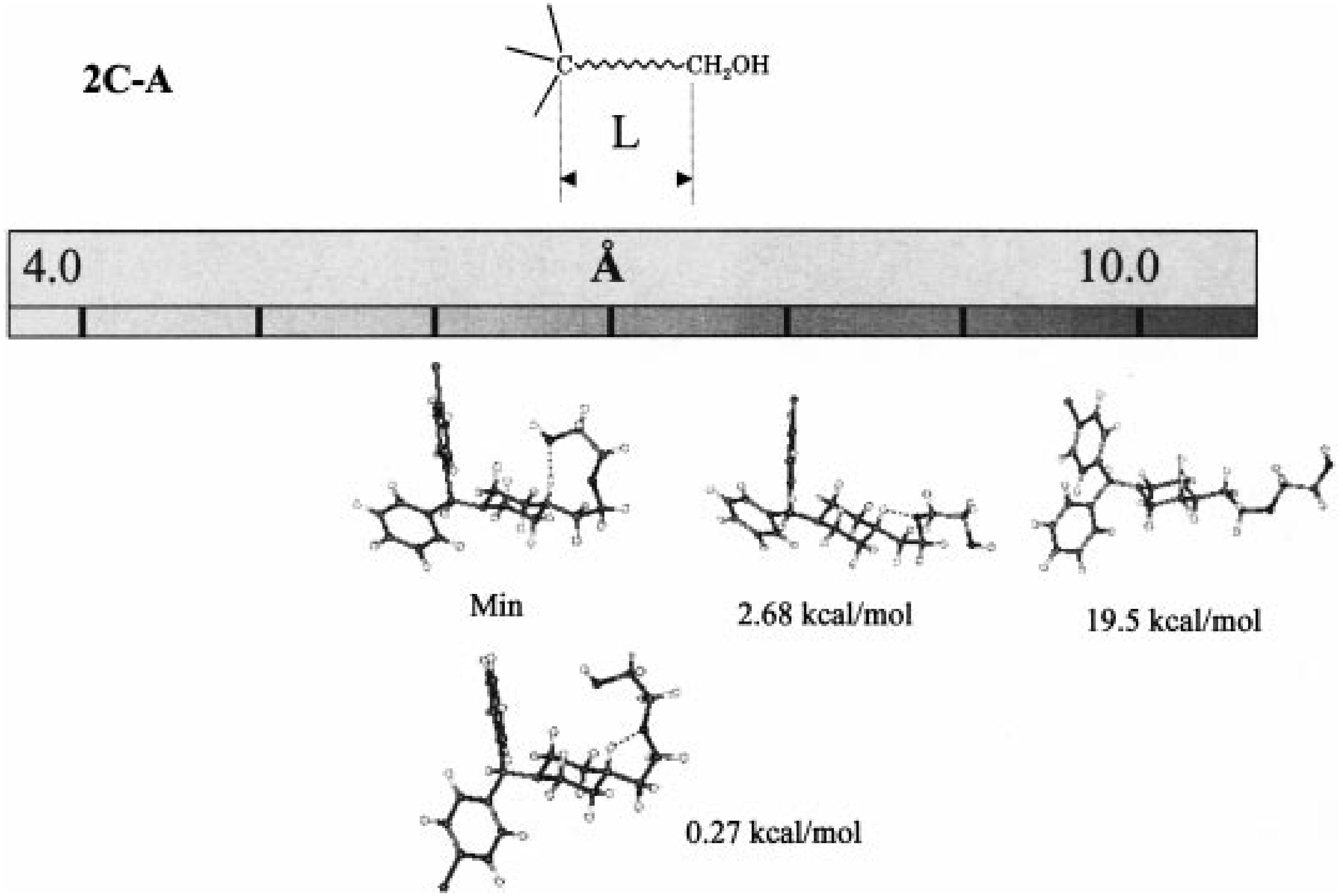


Fig. 10. *QMD Results combined with CA for hydroxyzine* **2C-A**. In the figure, the conformers are reported on the *L* scale. For each conformer, the difference in energy relative to the most stable conformer is reported.

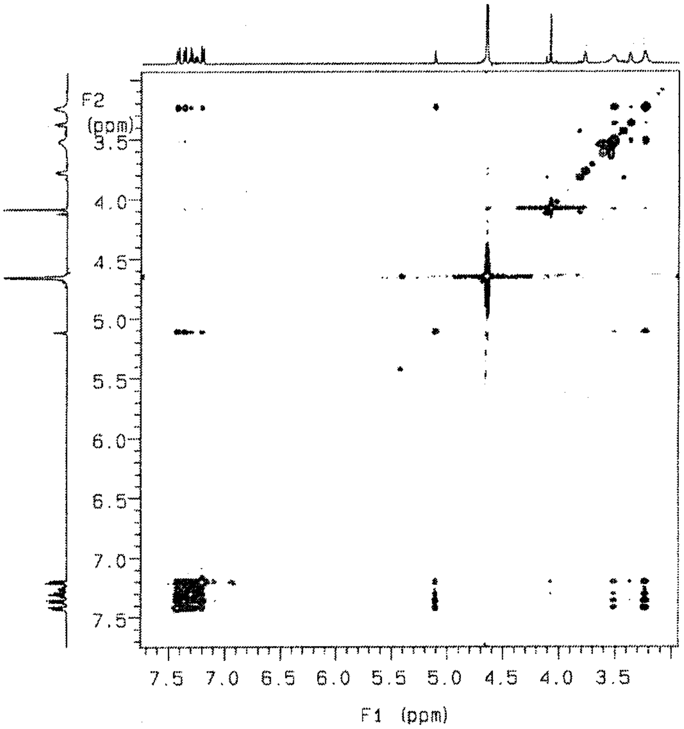


Fig. 11. *NOESY Experiment for cetirizine in deuterated water* (pD 2.23)

drugs were *ca.* 15 mм, and spectra were recorded at 298 K with sodium (trimethylsilyl)propanoate (STP) and tetramethylsilane (TMS) as internal standards.

The temp. dependence of the ^{1}H-NMR spectra of cetirizine was checked with a 16.2 mм soln. of cetirizine base in 1 ml of 8:20 DMSO/D_2O mixture, a typical cryoprotective solvent which, at subzero temp., has properties close to those of H_2O at r.t. [4]. In such a soln., the measured pD was within the isoelectric range (4.0–6.0).

The variation of chemical shifts *vs.* pD was determined by recording spectra at pDs ranging from 1.36 to 13.70. The samples were prepared starting from a 15 mм stock soln. The final solns. were prepared by placing 0.7 ml of the stock soln. in each NMR tube and by adjusting the pD with NaOD or DCl. The pH values were measured at r.t. (25°) with a *Metrohm 654* pH-meter (Herisau, Switzerland) coupled with a glass microelectrode and calibrated with standard aq. buffers. The pD values were controlled after recording each spectrum. The pD values were obtained by adding a correction factor of 0.4 to the measured pH values [5].

Distortionless enhancement by polarization-transfer (DEPT-135) experiments were optimized with 16 K time domain data points, 10000-Hz spectral width, 2-s relaxation time, $\theta = 135°$, $\Delta 1 = 1/2\,J = 0.0038$ s [6].

^{13}C,^{1}H Heterocorrelated spectra (2D-HETCOR) were optimized with 10000-Hz spectral width, 1-K F_2 time-domain data points (^{13}C frequencies), 2000-Hz spectral width, 512W F^1 time domain data points (^{1}H frequencies), 2-s relaxation time, $\Delta_1 = 1/2\,J = 0.0038$ s and $\Delta_2 = 1/4\,J = 0.0019$ s.

Steady-state NOE difference spectra [7] were performed for cetirizine over the entire pH range by irradiating the benzhydryl, piperazine, and methylene protons. Spectral conditions were optimized with 16-K time-domain data points, 4000-Hz spectral width, 700-ms mixing time, 25-s relaxation delay, and 128 transients.

A NOESY experiment was performed for cetirizine in deuterated water of pD 2.23. Spectral conditions were optimized with 2772.1-Hz spectral width in both dimensions, 1 K in F_1 and F_2 time-domain data points, 10-s relaxation time, 8 repetitions, 1.5-s mixing time.

The NMR spectra were recorded on a *Bruker AC-200* spectrometer operating at 200 MHz and on a *Varian Unity-500* spectrometer operating at 500 MHz.

Molecular Modeling. The conformational hypersurface of cetirizine (**1**) and hydroxyzine (**2**) in the vacuum was explored by Quenched Molecular Dynamics (QMD) as described in [8–10]. For **1**, the dynamics were performed for seven different topologies (*Fig. 1*) in order to consider the neutral form and all possible electrical states. For each topology, six different geometries were chosen as starting points. QMD applied to **1** and **2** produced for each electrical state a number of conformers (ranging form 50 up to 60). The final geometries were classified by cluster analysis as described below. The same calculations were also performed for the four possible electrical states of **2** (*Fig. 1, b*).

For cetirizine (**1**) and hydroxyzine (**2**), conformational analysis by Quenched Molecular Dynamics (QMD) and analyses of conformers by Cluster Analysis (CA) were combined to obtain additional insights. The first step of CA consisted in chosing some numerical variables to adequately represent the conformers. For **1**, a few variables indicating favorable H-bond interactions and a variable representing the 'length' of the molecule seemed to produce a useful clustering of the conformations. These variables were the distances between the H-atoms bound to polar atoms and potential H-bond acceptor atoms (d_{A1} and d_{A2} as represented in *Fig. 6* for **1Z-A**,), and the distance between the C-atom of the carboxyl group and the benzhydrylic C-atom (L in *Fig. 6*).

The distances (d_{A1}, d_{A2}, and L for **1Z-A**) were then standardized by mean and standard deviation according to *Eqn. 1*

$$x = \frac{d_{i,\alpha} - \overline{d_{\alpha}}}{\sigma_{\alpha}} \tag{1}$$

where $d_{\mathrm{i},\alpha}$ is one of the chosen distances α (*i. e.*, d_{A1}, d_{A2}, or L for **1Z-A**) for the generic i conformer, and σ_{α}^{-} are σ_{α} are the mean and standard deviations for the distance α, respectively, calculated over all conformers, and $x_{i,\alpha}$ is the standardized distance.

The *Eulerian* distance $\delta_{i,j}$ (*Eqn. 2*) was adopted to calculate proximity or distance between pairs of data points (here, data points represent conformers)

$$\delta = \sqrt{\sum_{\alpha=1}^{n} (x_{i,\alpha} - x_{\alpha,j})^{2}} \tag{2}$$

where α is one of the n distances (3 for **1Z-A**) that characterize the conformers i and j.

Finally, clusters were assembled by an agglomerative, hierarchical method. The minimum distance between data points was the criterion followed to agglomerate clusters, meaning that two points were assembled in the same cluster if the distance between them was smaller than the distance with all others.

The dendogram for a zwitterionic species **1Z-A** is shown in *Fig. 8*. A series of clusters were obtained by selecting a minimum distance. The choice was made empirically by testing different values. For instance, the **1Z-A** species was rationalized with an 'amalgamation' distance (distance where the final clusters were created) of 0.4.

Molecular modeling was performed on *Silicon Graphics* workstations (*Indigo R4400*) with the Sybyl [11] and TSAR [12] softwares.

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