Structures of some Hoechst analogues in gas phase and in aqueous solution

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Four Hoechst analogues have been examined in this work to determine their conformational preferences in three different environments, viz. gas phase, within the DNA minor groove and aqueous phase. Rotations about all the three single bonds, namely those connecting the phenol ring to a benzimidazole ring, the two benzimidazole rings, and a benzimidazole ring to the N-methylpiperazine ring have been considered. It is found that the optimum conformation necessary for binding to the DNA minor groove is easily attained, especially in aqueous solution.

Drugs that bind specifically to certain DNA sequences are of great interest, as they may find use in antiviral and anticancer therapy. Of particular interest are minor groove-binding drugs, both natural (netropsin and distamycin) and synthetic. In the latter category 2-(4-hydroxyphenyl)-5-[5-(4-methylpiperazin-1-yl)benzimidazole, commonly known as Hoechst 33258 (Figure 1), is one of the better studied drugs, in relation to its interactions with the DNA double helix. It binds selectively to AT-rich regions. As can be seen from Figure 1, it is an N-methylpiperazine derivative, having two benzimidazole rings and one phenyl ring. In a bid to modify its AT selectivity, several workers have made chemical changes in the benzimidazole rings \(^1,2\), as well as in the phenol group \(^3,4\) to bring about additional sequence determining features to the end of the molecule. In fact, the moving of phenolic hydroxy group from the para- to the meta- position has been found to give rise to an additional hydrogen bond between the meta hydroxy group and DNA \(^4\). Changing the phenolic hydroxy group to an ethoxy group results in a compound, Hoechst 33342, which has a lower affinity to DNA than Hoechst 33258 \(^5\). Both Hoechst 33258 and Hoechst 33342 are commercially available DNA ligands, which have been used for over two decades as fluorescent dyes in a number of cytological techniques. They also possess radioprotective properties. A new analogue, methylproamine (Figure 1), has been synthesized, which is more active than the parent compound, both \textit{in vitro} and \textit{in vivo}\(^6\).

In view of these diverse applications of Hoechst derivatives, we have carried out a thorough quantum mechanical study on the four derivatives, viz. Hoechst 33258, Hoechst 33342, \textit{meta}-Hoechst and methylproamine. Previous calculations on these systems have mainly been performed by methods based on empirical force fields. In contrast, molecular orbital studies have been rather limited \(^7,10\) and have been mostly confined to individual fragments.

Numerous reports of X-ray crystallographic structures of DNA-bound Hoechst 33258 have appeared in the literature \(^11\). One of the main drawbacks of these crystal structures is their relatively low atomic resolution. The electron density in the minor groove corresponding to the drug is also, in general, poorly defined. This leads to poor final
geometries of the crystallographic model and an ambiguous interpretation of the electron density maps at the early stages of the refinement. Such interpretation is more difficult in the flexible parts of the drug, like the piperazine ring and its exocyclic substituents in Hoechst 33258. The results described in this work would provide a useful framework with which future structural determinations of Hoechst 33258 complexed with DNA can be compared.

We have first investigated the gas phase conformational preferences of the four Hoechst derivatives using the semiempirical Austin Model I (AM1) method\textsuperscript{23}. The COSMO (Conductor-like Screening Model) procedure\textsuperscript{24} was then used to account for the influence of the aqueous solvent and to simulate the dielectric environment in the minor groove of the DNA. These calculations provide a model of the complexed Hoechst in three different environments, and are compared with previous \textit{ab initio} calculations\textsuperscript{25} and X-ray crystallographic data. These models provide clues to the molecular recognition and binding processes.

**Computational methods**

As seen from Figure 1, there are three essentially single bonds connecting the four different ring systems in the molecule. We, therefore, concentrated on each torsion angle between two adjacent rings before constructing the whole molecule. The first torsion angle, $\alpha$ (see Figure 1) was studied by carrying out a complete 360° rotation, in steps of 10°, about the single bond connecting the phenol ring with a benzimidazole ring. The structure in each point of the rotational profile was obtained from geometry optimization at a fixed torsional angle value. Since it is this part of the molecule that differs for the four molecules investigated in this work, this fragment was studied in minute detail, both in the gas phase and in aqueous solution, as well as in the dielectric environment of the minor groove.

Similarly, two more such fragments were studied in order to investigate the dependence of the energy on the torsion angles $\beta$ and $\gamma$ (see Figure 1). The fragment consisting of the two benzimidazole rings may also exhibit tautomerism. The barrier separating the two tautomers was also calculated.

Finally, the complete Hoechst 33258 molecule was constructed, using the optimized conformational angles obtained for the fragments in the starting geometry. The geometry was then completely optimized at the AM1 level. To find the global minimum, a molecular dynamics simulation was performed to anneal the system to obtain a lower energy minimum. The heat time was set to 0.1 ps, the temperature step to 30 K and the run time to one ps. A complete energy optimization using the AM1 method was carried out on the final geometry to find the global minimum.

**Gas phase conformations**

All calculations were performed at the SCF level, using the MOPAC 7.0 program system\textsuperscript{26}. The molecular geometries were fully optimized with respect to the energy without any conformational or symmetry restrictions. The keywords PRECISE and GNORM = 0.0 were used in all geometry optimizations. This ensured that, in most cases, a mean gradient value lower than 0.01 kcal mol\textsuperscript{-1} Å\textsuperscript{-1} was achieved.\textsuperscript{1} The vibrational frequencies were calculated to verify that the calculated structures are energy minima on the potential energy surface. The results for the torsional angle, $\alpha$ were compared with the results obtained at the \textit{ab initio} Hartree-Fock 6-31G(d) level by Allem\textsuperscript{2}.

**Aqueous phase calculations**

The energies of solvation were determined using the COSMO (Conductor-like Screening Model) procedure implemented in MOPAC 7.0, with the dielectric constant ($\varepsilon$) taken as 78.5 for water at 298 K, and 20 for simulating the dielectric environment in the minor groove. The geometries were fully optimized with respect to the energy.

**Results and Discussion**

**Fragment 1.** This fragment consists of the phenol ring attached to a benzimidazole ring. The gas and solution phase relative energies and torsional angles of the minima and saddle points obtained at the AM1 computational level for the various substituents are compiled in Table 1. The data in Table 1 reveal that none of the minima corresponds to planar conformations (syn and anti). Rather, there are two minima, \textit{anti-gauche} ($\alpha = 150^\circ$) and \textit{syn-gauche} ($\alpha = 30^\circ$), which are almost isoenergetic. In the case of Hoechst 33258 and Hoechst 33342, the global minimum corresponds to the \textit{anti-gauche} conformation. The saddle point connecting these two minima corresponds to the \textit{gauche-gauche} ($\alpha = 90^\circ$)

\textsuperscript{1} 1 kcal=4.184 kJ
conformation. A schematic picture of the three conformations, namely, syn-gauche, anti-gauche and gauche-gauche, is shown in Figure 2. For the fragments corresponding to meta Hoechst and methylproamine, the syn-gauche conformation is favoured over the anti-gauche conformation. The relative energy between the two minima is also higher (1.9 kJ/mol) for meta substitution, as compared to -0.6 kJ/mol for para substitution. However, while the two para compounds have an energy barrier of approximately 6.0 kJ/mol, and the energy barrier for the meta derivative is also similar (6.3 kJ/mol), the energy barrier is least for the fragment corresponding to methylproamine (2.3 kJ/mol), and the degree of non-planarity is also greater for the latter.

Table I shows the HF/6-31G(d) data. Although the calculated AM1 barriers are clearly underestimated, the AM1 method gives a reasonable representation of both the syn-gauche and anti-gauche conformations; the largest deviation in \( \alpha \) with respect to the HF/6-31G(d) minima is 15\(^\circ\).

Rotational profiles for the four model compounds are quite similar, and the differences are only in the relative energies between the minima and the value of the energy barrier (see Table I). The gas phase and aqueous solution profiles for the fragment corresponding to Hoechst 33258 are shown in Figure 3. Water further destabilizes the planar conformations syn and anti. In fact, the degree of nonplanarity (\( \alpha \)) in the syn-gauche and anti-gauche conformations is also larger in the aqueous phase than in the gas phase, and there is large stabilization of the gauche-gauche conformation, reducing the rotational barrier and facilitating free rotation between the syn-gauche and anti-gauche conformations. This is probably due to the electrostatic factor, as the gauche-gauche conformation has a higher dipole moment.

**Table I**—Energies and torsional angles of Fragment 1 obtained from semiempirical AM1 calculations

<table>
<thead>
<tr>
<th>Compound</th>
<th>Torsion angles (deg.) and energies (kJ/mol) for</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Syn-gauche</td>
</tr>
<tr>
<td></td>
<td>( \alpha )</td>
</tr>
<tr>
<td>( X=OH )</td>
<td>33.3 0.7</td>
</tr>
<tr>
<td>( X=OEt )</td>
<td>32.9 0.7</td>
</tr>
<tr>
<td>( Y=OH )</td>
<td>34.9 0.0</td>
</tr>
<tr>
<td>( X= )</td>
<td>-47.8 0.0</td>
</tr>
</tbody>
</table>

*see Figure 1

Figure 2 — Schematic picture of the anti-gauche, gauche-gauche, and syn-gauche conformations for Fragment 1 of Hoechst 33258. Conformations are displayed with the primary inertial axis of the molecule aligned with the x-axis. Carbons are coloured cyan, nitrogens blue, the oxygen red, and hydrogens are white.

Figure 3 — Rotational profiles for fragment 1 of Hoechst 33258.
corresponds to a reduction of conformational preferences observed dramatically the conformation. This is disfavoured with respect to the global minimum by 6.5 kJ/mol. The two minima are separated by a barrier that energy of the second minimum is about occurs around a torsional angle of corresponds to the greater than that for the

The two minima are now located at global minimum in the gas phase is located around the dihedral angle $\beta$ with sequence C-C-C-N. There are two different tautomers of 2 (I and II), which are shown in Figure 4. Another quinoid form, in which the two benzimidazole hydrogens are on nitrogens in the same ring, has been ignored in this study as this tautomer is likely to be highly unstable. The gas phase and aqueous solution rotational profiles of the experimentally observed tautomer I are similar to those observed for the first fragment. The global minimum in the gas phase is located around $\beta = 153^\circ$ (anti-gauche). This twisted conformation is more stable than the anti conformation ($\beta = 180^\circ$) by 1.9 kJ/mol. A second local minimum, syn-gauche, occurs around a torsional angle of $\beta = 45^\circ$. This energy of the second minimum is about 3.0 kJ/mol greater than that for the anti-gauche conformation. The two minima are separated by a barrier that corresponds to the gauche-gauche ($\beta = 90^\circ$) conformation. This is disfavoured with respect to the global minimum by 6.5 kJ/mol. Water changes dramatically the conformational preferences observed in the gas phase. From the aqueous profile, the global and second minima are now located at $\beta = 130^\circ$ and $\beta = 50^\circ$, respectively. Furthermore, the energy barrier between both minima is 1.3 kJ/mol, which corresponds to a reduction of 5.2 kJ/mol with respect to the gas phase. A comparison between gas phase and solution phase profiles reveals another important difference. As for the first fragment, the bulk water tends to destabilize the syn and anti planar conformations.

Results for tautomer II are similar to those for tautomer I, the minima being located in twisted conformations rather than in planar ones. Thus, the global minimum appears in the anti-gauche conformation ($\beta = 155^\circ$), whereas the local minimum corresponds to the syn-gauche conformation ($\beta = 43^\circ$). The second minimum lies 2.3 kJ/mol above the anti-gauche conformation. The energy barrier between the two minima is 4.1 kJ/mol, being 2.4 kJ/mol lower than that predicted for I. The planar syn and anti conformations are 3.1 and 6.5 kJ/mol less favoured than the global minimum.

The rotational profiles indicate that both I and II do not adopt a planar conformation in the gas phase. This conformational behaviour is similar to that found for other aromatic compounds containing two rings attached by a single bond. Table II summarizes the results of optimized angles and relative energies.

The dihedral angle $\beta$ in both the syn-gauche and anti-gauche minima is very similar for the two tautomers. The energies of tautomerization in the gas phase reveal that I and II are almost isoenergetic in the anti-gauche conformation, whereas both tautomers are slightly disfavoured in the syn-gauche conformation. However, water changes this preference. This is because the syn conformers have higher dipole moments (5.2 Debye of I) than the anti conformers (1.7 Debyes of I). Thus, in aqueous solution, tautomer I in the syn-gauche conformation is favoured by approximately 1.5 kJ/mol with respect to the other possible positions. Since this is the conformer which has the favourable conformation for binding to the DNA minor groove, the results of this study clearly indicate that the polar hydrogens adopt the most favourable binding position in aqueous

**Figure 4** — The two tautomeric forms of fragment 2

![Fragment 2](image)

(3.31 Debye) than the anti conformation (1.75 Debye), and thus interacts more strongly with the polar solvent.

**Table II** — Enthalpies of tautomerization of 2 in gas phase, aqueous solution, and within the minor groove. Tautomers I and II are shown in Figure 4. The two minimum conformations of each tautomer have been considered for $\beta$, and enthalpies are given with respect to the most stable conformation in that phase.

<table>
<thead>
<tr>
<th>Tautomer</th>
<th>$\beta$ (deg.)</th>
<th>$\Delta H_{gs}$ (kJ/mol)</th>
<th>$\Delta H_{aq}$ (kJ/mol)</th>
<th>$\Delta H_{mg}$ (kJ/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>II A</td>
<td>155.1</td>
<td>0.0</td>
<td>1.4</td>
<td>1.3</td>
</tr>
<tr>
<td>I A</td>
<td>152.6</td>
<td>0.3</td>
<td>2.4</td>
<td>1.8</td>
</tr>
<tr>
<td>II B</td>
<td>42.9</td>
<td>2.3</td>
<td>1.2</td>
<td>0.7</td>
</tr>
<tr>
<td>I B</td>
<td>44.5</td>
<td>3.3</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>
solution. In fact, the syn-gauche conformations are the preferred ones in aqueous solution. However, when the drug inserts in the minor groove, it displaces the water molecules already present, so the medium is no longer aqueous. As such, the dielectric constant in the minor groove gets lowered. To confirm that the hydrogens retain the preferred conformation in this region, we have simulated the dielectric environment in the minor groove by carrying out calculations on the relative stabilities of the tautomers at a dielectric constant value of 20, which is roughly the dielectric constant in the minor groove. Again, the preferred conformer is the syn-gauche form of 1 (see Table II), which is the one involved in binding to DNA.

Thus, for both the fragments, water aids the rotation about the bonds connecting the rings by firstly destabilizing the planar structures with respect to twisted ones, and secondly by stabilizing the transition states connecting the two twisted structures, facilitating easy rotation. This helps the drug molecule in adopting a conformation suitable for binding to the DNA minor groove. There is also a change in the relative stability between the syn-gauche and anti-gauche conformations, the former being more favoured in aqueous solution as they have higher dipole moments.

Fragment 3. The spatial arrangement of the piperazine ring in Hoechst 33258 was investigated in the fourth fragment, which contains a benzimidazole ring bonded to a piperazine ring (Figure 1), and the relative orientation between the two rings is described by the dihedral angle $\gamma$ with the sequence C-C-N-C. Before investigating the role of the dihedral angle $\gamma$ on the energy, we investigated whether an axial or an equatorial position is preferred for the $N$-methyl group and the benzimidazole ring. Although crystal and solution structures of Hoechst 33258-DNA complexes were completely optimized. Force constant analyses were carried out to verify the minimum nature of the conformations.

It was found that the conformers with the bulky methyl group attached to the positive nitrogen in an equatorial position are favoured by 6.5 kJ/mol. The axial orientation of this methyl is not favoured due to repulsive steric interactions with the axial hydrogens. This energy difference of 6.5 kJ/mol must, therefore, be associated with the repulsive steric interaction generated by one axial methyl group at the charged nitrogen. The equatorial position of the methyl group is in agreement with the structures determined for the piperazine ring in Hoechst 33258-DNA complexes.

We also compared the energies for the axial and equatorial positions of the benzimidazole group, keeping the methyl group at the positively charged nitrogen in the equatorial position. It was found that the conformer with the benzimidazole group at the axial orientation is preferred by about 8 kJ/mol. This conformer has the two bulky methyl and benzimidazole groups in the equatorial orientation, whereas the hydrogen atom bound to the positively charged nitrogen and the lone pair of the other nitrogen are in the axial orientation. Therefore, it seems to be stabilized mainly by the absence of repulsive steric interactions between the bulky groups and the axial hydrogens. Furthermore, it should be noted that a weak interaction of an electrostatic nature between the lone pair of nitrogen and the axial hydrogen attached to the nearest endocyclic carbon atoms is also possible.

The gas phase rotational profile was plotted for equatorial positions of the methyl and the benzimidazole groups. Two minima were found, the lowest energy minimum at $\gamma \approx 240^\circ$ is favoured over the other minimum by about 3.5 kJ/mol. The latter is a very shallow minimum. A high-energy barrier was found ($\approx 13$ kJ/mol), which is probably due to the repulsive electrostatic interactions induced by the positive charge of the piperazine ring.

The two minimum energy conformations found for this fragment are displayed in Figure 5. The values of the dihedral angle $\gamma$ in the two minima are $-115.6^\circ$ and $19.2^\circ$, the former being 3.0 kJ/mol more stable than the latter. In all cases the piperazine ring retains a chair conformation. A detailed inspection of Figure 5 reveals that the benzimidazole ring is perpendicular to the piperazine ring in the two minimum energy
conformations. The difference between the two minima results from a rotation of 180° of the benzimidazole ring with respect to the chair conformation of the piperazine ring.

Modelling of the Hoechst 33258 molecule

The results obtained for individual fragments 1 – 3 were combined to obtain the final molecular models of the drugs. This was performed by geometry optimization of the full molecule in the gas phase, initially keeping the dihedral angles α, β, and γ at the minimum energy values obtained for the fragments. To find the global minimum, a molecular dynamics simulation was carried out, as described in the computational section, and the resulting minimum energy structure was fully optimized. The fully optimized structure (A) is displayed in Figure 6. The two benzimidazole hydrogens, however, do not have the requisite conformation for forming hydrogen bonds with the DNA bases. The tautomer with the benzimidazole hydrogens facing the concave edge (B) has a higher energy by 9.1 kJ/mol, and this conformation is shown in Figure 6. However, it is to be noted that structure B is likely to be stabilized in aqueous solution, as indicated by our results for the second fragment.

It is interesting to note that, while the individual fragments optimized to structures with high torsion angles, the inter-ring angles are relatively smaller for the optimized structure of the whole molecule. Thus, the three angles, α, β and γ are 177.5°, 145.7° and -57.0°, respectively, for tautomer A, compared with 148.3°, 155.1° and -115.1°, respectively, optimized for the fragments. In fact, the phenol ring becomes almost coplanar with the adjacent benzimidazole ring. For the other tautomer B, the optimized angles are 10.8°, -17.6° and -58.5°, respectively.

Conclusions

The present results indicate that compounds of the Hoechst family are not planar, either in the gas phase or in aqueous solution. Although π-π conjugation between adjacent aromatic rings favours planar conformations, other factors control the degree of nonplanarity. Thus, while the dihedral angles are smaller in the gas phase, there is greater deviation from planarity, in the aqueous phase, suggesting that non-bonded interactions with the solvent are stronger than conjugative interactions.

Comparison between Hoechst 33258, Hoechst 33342, meta-Hoechst, and methylproamine reveals important differences between them. The four compounds have similar conformational preferences in that they are all nonplanar. However, the degree of nonplanarity and the potential energy barriers for rotation around the torsional angle α are different, and this permits an explanation of their different behaviour. Thus, Hoechst 33258 and Hoechst 33342 are similar since they have a low-energy barrier, which makes their para-phenolic group able to flip easily. This is confirmed for Hoechst 33258 by NMR spectroscopy. meta-Hoechst has a slightly higher rotational barrier than the para compound, and, therefore, the ring flipping of the meta-phenolic group must be restricted. This is in good agreement with the NMR study where the ring flipping was found to be
The results shown in Table 1 indicate that the deviations from planarity are much greater in aqueous solution than in the gas phase. This feature must be attributed to the better interaction of the solvent with the folded Hoechst than with the planar Hoechst. It is well known from X-ray and NMR studies that the binding of the drug molecule induces distortions of the DNA double helix. However, the distortions from the planarity of the minor-groove binding drugs have been traditionally attributed to the improvement of the interactions with the DNA. In contrast, our results suggest that when the drug binds to DNA a much more planar conformation is induced. Thus, the drug loses its distorted conformation in order to adopt the irregular form of the minor groove, which requires a more planar arrangement. Furthermore, the nonpolar dielectric environment in the minor groove of Hoechst bound to alternating poly(AT) (dielectric constant $\varepsilon = 20$), is much closer to the dielectric environment of the gas phase ($\varepsilon = 1$) than to that of bulk water ($\varepsilon = 80$).

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References