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# Intercalation of Ethidium and Analogues with Nucleic Acids: a Molecular Orbital Study

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**Abstract**—Semiempirical calculations suggest that the intercalation complexes of phenanthridinium cations **1–4** with G-C/C-G and **1** with A-U/U-A are stabilized by frontier orbital interactions between the LUMO of the intercalator and the HOMOs of the adjacent purine bases. The charge on the ring nitrogen of **1–4** appears to be necessary for the orbital interactions, lowering the LUMO, facilitating mixing of this orbital with the HOMOs of the adjacent purine bases to give an extended HOMO stabilizing the complex and resulting in the bathochromic shift in the electron absorption spectrum. Noncationic phenanthridine **5** shows no frontier orbital interactions in the forced intercalation complex with G-C/C-G. The results of the calculations parallel experimental $T_m$ values. 

## Introduction

The expression of activity of many antiviral, antiparasitic, and anticancer agents involves interaction with nucleic acids.[1] A fundamental mode of the interaction is intercalation, defined as the insertion of a planar or nearly planar aromatic molecule between adjacent Watson–Crick base-pairs of DNA.[1,2] Intercalation is known to occur without interfering with hydrogen bonding of the base-pairs and obeys the nearest neighbor exclusion principle. When DNA is saturated with intercalators, every second potential intercalation site on the helix remains empty. In order to relieve stress caused by the insertion of an aromatic molecule between adjacent base pairs in DNA, the double helix undergoes partial left-handed unwinding.[1–3]

In spite of the wealth of structural data known for intercalation complexes, the origin of the intercalation forces is much less understood. Intercalation has been generally considered to be the result of a hydrophobic interaction in which a hydrophobic aromatic molecule is drawn to a hydrophobic environment of the base-pairs from the hydrophilic aqueous environment.[4] An increase in complex stability is observed when the aromatic intercalator is a cation. This has traditionally been considered to result from electrostatic interaction between the cation with the anionic DNA backbone or a negative potential associated with the DNA groove. Specific hydrogen bonding interactions and a favorable orientation of dipoles of the base-pairs and a polar intercalator molecule are considered important in stabilizing the complex because many DNA intercalator complexes have well-defined stereochemistry.[5] There are, however, puzzling experimental results that cannot be explained in terms of these factors alone. For example, binding of ethidium (**1**), a classical intercalator, with DNA results in a 40 nm bathochromic shift in the electronic absorption spectrum and a 20-fold increase in the fluorescence quantum yield.[6] These spectral changes cannot be induced in ethidium (**1**) in the absence of DNA by changes in solvent varying in hydrophilicity from water to mixtures of ethanol with hexanes.[6]

Even more striking are the results obtained for binding of **1** with a histone octamer. The interaction of **1** with a hydrophobic moiety of the protein gave only a 4 nm bathochromic shift in the electronic spectrum and an insignificant change in the fluorescence intensity.[7] It has also been shown that an electrostatic interaction of **1** with poly(vinyl sulfate) quenches fluorescence of **1** without affecting electronic absorption.[8] These results show that binding of **1** with DNA is complicated and cannot be accounted for solely by hydrophobic and electrostatic interactions. Furthermore, hydrogen bonding and dipole–dipole interactions that may be involved in the complex stabilization, as already mentioned, are considered unlikely to contribute significantly to the observed spectral changes of **1** upon complexation with DNA.

We consider it likely that molecular orbital interactions between ethidium and the bases of nucleic acids can provide an additional stabilization of the intercalation complexes and may be responsible for the observed spectral properties of the complexes. In this paper, we report molecular orbital calculations on intercalation complexes of **1** and analogues **2–5** with nucleic acids. The computational results are compared to the experimental complex stabilities as obtained from thermal melting experiments.

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## Methodology

### Molecular orbital calculations

The molecular orbital computations were run on a Silicon Graphics 4d/380s, Indigo workstation or IBM Risc 320H with the program AMPAC 2.1 (ACPE Program 506, Indiana University) using the AM1 Hamiltonian. The van der Waals surface calculation was performed using the program MACROMODEL. The AMPAC input files for calculations on the intercalation complexes of **1** (vide infra) were prepared by reading the crystal structure files of dinucleotide complexes[2] in PCmodel and adding hydrogens. These files were output as SYBYL files and the Cartesian coordinates were taken from the SYBYL files and edited for AMPAC input.

### Models of the intercalation complexes for MO calculations

Since molecular orbital calculations are demanding in terms of required memory and processing time, computations of entire dinucleotide intercalation complexes are not practical. The structures were therefore edited to a complex of intercalator and immediately adjacent base pairs. Elimination of the sugar–phosphate moieties from the complex reduces the size of the structure by more than 50% and semiempirical calculations become practical. In this study the calculations of the intercalation complexes of **1** were performed using the crystal structures coordinates reported by Jain et al.[2] for the intercalator of **1** and bases G-C/C-G or A-U/U-A.

Since crystal structures of the intercalation complexes of **2–5** are not available, the corresponding model complexes were similarly derived from the above crystal structures. For studies where the sugar–phosphate moieties are removed from dinucleotides or a substituent is removed from **1** and replaced by hydrogen, the following procedure was used. The coordinates of the X-ray structures were read into PCmodel and the sugar–phosphate moieties removed and replaced by hydrogens. All heavy atoms were fixed and the hydrogen positions optimized using AMPAC. All calculations were conducted with nonprotonated forms of **1–5** because these molecules exist largely as free bases under physiological pH conditions. In particular, the highest $pK_a$ value of 5.82 for **5** is for protonation of the phenanthridine ring nitrogen.[9] The protonated forms of the monophosphates were used in the calculations because most of the charge of nucleic acid in solution is neutralized by tight association with counter ions.[10]

### Heat of formation for intercalation

For comparison to the experimental $T_m$ values (vide infra) the calculated heat of formation for intercalation ($\Delta E$) was defined as follows:

$$\Delta E = E(\text{intercalation complex}) - [E(\text{base-pair}) - E(\text{intercalator})],$$

where $E$(intercalation complex) is the calculated heat of formation for the simplified intercalation complex (without a sugar–phosphate backbone), $E$(base-pair) is the calculated heat of formation for the base aggregate without the intercalator separated at the intercalation distance, and $E$(intercalator) is the calculated heat of formation for the intercalator.[11]

### $T_m$ determinations

The experiments were conducted with the oligomer $d$(G-C)$_4$ (Sigma) that was purified and characterized as previously described.[12,13] Ethidium (**1**) and 3,8-diamino-5-ethylphenanthridinium bromide (**4**) were obtained from Sigma and May and Baker Co., respectively. 8-Amino-5-ethyl-6-phenylphenanthridiniumbromide(**2**), 3-amino-5-ethyl-6-phenylphenanthridinium bromide (**3**) and 3,8-diamino-6-phenylphenanthridine (**5**) were a gift from Dr David Graves, the University of Mississippi. Compounds **1–5** were purified by crystallization before use.

Thermal melting curves for $d$(G-C)$_4$ and complexes with **1–5** were obtained under the previously described conditions.[13] The experiments were conducted in a buffer containing 0.01 M piperazine-*N*,*N*-bis(2-ethanesulfonic acid), 0.001 M EDTA, 0.2 M NaCl, and adjusted to pH 7.0. Compounds **1–5** were compared by the increase in $T_m$ [$(\Delta T_m = T_m(\text{complex}) - T_m(\text{free DNA})$] they produced in the PIPES buffer at saturating amounts of the compound (a molar ratio of 0.3 of compound-to-nucleic acid bases). The estimated errors in the $\Delta T_m$ values are $\pm 0.4$ °C.

## Results and Discussion

### Preliminary calculations

The possibility that the frontier molecular orbitals of the bases are affected by deletion of the sugar phosphate moieties and their replacement by hydrogens even in the absence of intercalators was investigated in the following way. Single point calculations of each of the four bases along with the connected sugar-phosphate, with coordinates taken from the X-ray structures, were compared with similar calculations of the bases after removal of the sugar–phosphates and replacement by hydrogens. The coordinates of the hydrogens were optimized as described previously. The calculations showed that with guanine the energy of the HOMO in the presence of the sugar–phosphate was $-8.58$ eV and when the sugar–phosphate was replaced by hydrogen the energy was $-8.68$ eV. The orbital coefficients changed by less than 3% when the sugar–phosphate was replaced by hydrogen. Similar results were obtained with the other bases.

To examine the effect of base pairing (hydrogen bonding) on the frontier orbitals, the orbital coeffi-

cients from single point calculations on the individual bases were compared with similar calculations on the G-C base-pair. The HOMO of the G-C base-pair contained contributions solely from guanine. Compared with calculation of the individual guanine the orbital coefficients of the HOMO of the G-C base-pair differed by less than 3.5%. The HOMO-1 (next to HOMO) of the G-C base-pair contained contributions solely from the cytosine. Compared with calculations of the individual cytosine the orbital coefficients of the HOMO-1 of the base pair were not changed by more than 3%. The frontier orbital energy of the G-C base-pair was changed by less than 2% when compared with single point calculations of the individual bases. The study shows that base-pairing and the sugar–phosphate link in DNA do not significantly affect the frontier occupied orbitals of the base-pairs.

The $\Delta E$ values for the complexes of **1**–**5** with G-C/C-G were compared with experimentally determined $\Delta T_m$ values for the interaction of **1**–**5** with $d(\text{G-C})_4$ (Table 1). The values of $\Delta E$ parallel the order of experimental values of $\Delta T_m$: **1**>**2**>**3**>**4**>**5**. Both the $\Delta E$ values obtained from the calculations and the $\Delta T_m$ measurements are consistent with stabilization of the adjacent G-C/C-G base-pairs by interaction with the intercalators **1**–**4**. Also, the calculations and the experimental $\Delta T_m$ values suggest that the noncationic phenanthridine **5** is not an intercalator. DNA is destabilized by forcing interaction with this compound. Interestingly, a significant correlation [eq. (1)] was obtained between the experimental $\Delta T_m$ values and the calculated $\Delta E$ values for the intercalation complexes of **1**–**4**. This correlation strongly suggests that interaction of **1**–**4** with adjacent G-C base-pairs is of primary importance for stabilization of the intercalation complexes.

$$\Delta T_m = -1.40(\pm 0.1\ SE)\Delta E + 5.19(\pm 0.54\ SE) \quad (1)$$

$n = 4$ (compounds **1**–**4**), $r^2 = 0.99$.

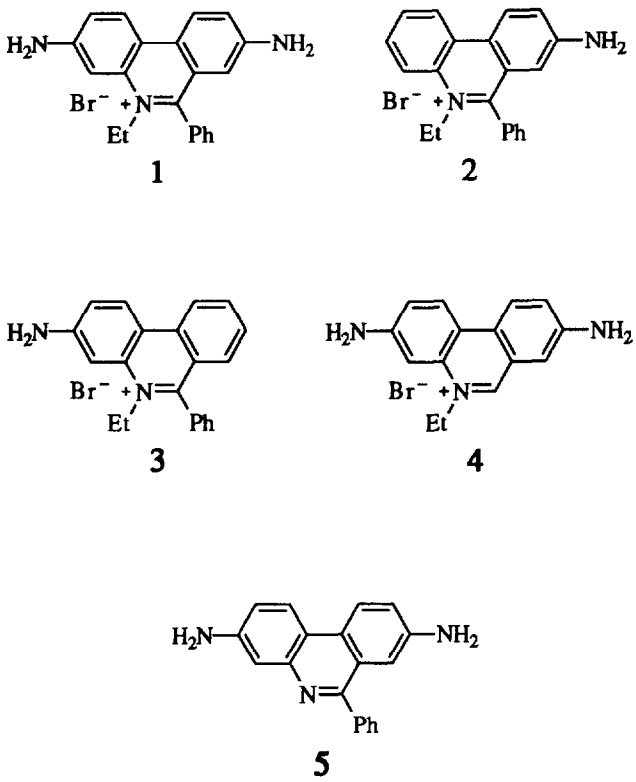
## Molecular orbital calculations

The energy calculations discussed above involve many terms including frontier orbital interactions. It was of interest, therefore, to analyze in more detail the frontier orbitals of base-pairs and their intercalation complexes. This problem has not been addressed previously.

The calculated coefficients of the frontier orbitals for an isolated G-C base-pair having the coordinates as defined from the X-ray structure, but with the sugar–phosphate moieties replaced by H (followed by optimization of the hydrogen coordinates) changed less than 3% compared with corresponding structures of two adjacent G-C/C-G base pairs separated by 3.4 Å. The coordinates for the latter were taken from the X-ray structure with the intercalator removed and the base-pairs positioned to have a separation of 3.4 Å and a relative orientation with respect to each other of the standard Watson–Crick model. These calculations suggest that there is little, if any, molecular orbital interactions in the HOMO between the bases of stacked pairs in the ground electronic state when separated by 3.4 Å. The two G-C/C-G base-pairs unwind and are separated in the intercalation complex by a distance of 6.7 Å. In contrast to the results above the HOMO of the intercalation complex of ethidium (**1**) with G-C/C-G, but in the absence of the sugar–phosphate groups contains contributions from both guanines on opposite faces of the intercalator. More specifically, the HOMO of the complex involves both purines and **1** (Fig. 1). The less stable intercalation complexes with **2**–**4** contain HOMOs which involve the intercalator and at least one guanine. It should be noted that all intercalators **1**–**4** are phenanthridinium cations. Interestingly, the electrically neutral phenanthridine **5**, which is classified as a nonintercalator on the basis of $\Delta T_m$ measurements and $\Delta E$ calculations,

**Table 1.** Changes in $T_m$ (°C) upon interaction of compounds **1**–**5** with the oligomer $d(\text{G-C})_4$, predicted increases in $T_m$ for intercalation of **1**–**4** with $d(\text{G-C})_4$ [eq. (1)], and calculated heat of formation for intercalation $\Delta E$ (kcal/mol) of **1**–**5** with G-C/C-G

| Compound | $\Delta T_m$ | | $\Delta E$ |
|---|---|---|---|
| | Experimental | Predicted | |
| **1** | 15.4 | 15.6 | −7.4 |
| **2** | 12.9 | 12.6 | −5.3 |
| **3** | 12.4 | 12.3 | −5.1 |
| **4** | 9.9 | 10.1 | −3.5 |
| **5** | −2.6 | | 2.6 |



shows no orbital interaction in the hypothetical intercalation complex (Fig. 1).

A similar calculation of the intercalation complex of **1** with A-U/U-A in the absence of sugar–phosphate groups (Fig. 2) was carried out using the structural coordinates from the X-ray structure of the complex.[2] The calculation shows the HOMO of the complex contains contributions from both adjacent electron-rich adenines on opposite faces of the intercalator. This parallels the results found for the complex of **1** with G-C/C-G, where the HOMO shows contributions from the electron-rich adjacent guanines on both faces of the intercalator. These results show the importance of frontier orbital interactions between the intercalator and adjacent electron-rich base-pairs in stabilizing complex formation. The resulting HOMOs make an important contribution to stabilization of the intercalation complex.

## Conclusions

The computational results are consistent with mixing of the frontier orbitals between phenanthridinium cations **1–4** and purine bases in the intercalation complexes. This effect is considered to contribute to stabilization of the intercalation complexes. This contrasts to the widely accepted view that electrostatic interaction of the positive charge on the aromatic system of **1** and analogues is responsible only for the electrostatic interaction with nucleic acids. The results reported herein show the phenanthridinium cation is necessary to allow the frontier orbitals of the bases and intercalator to

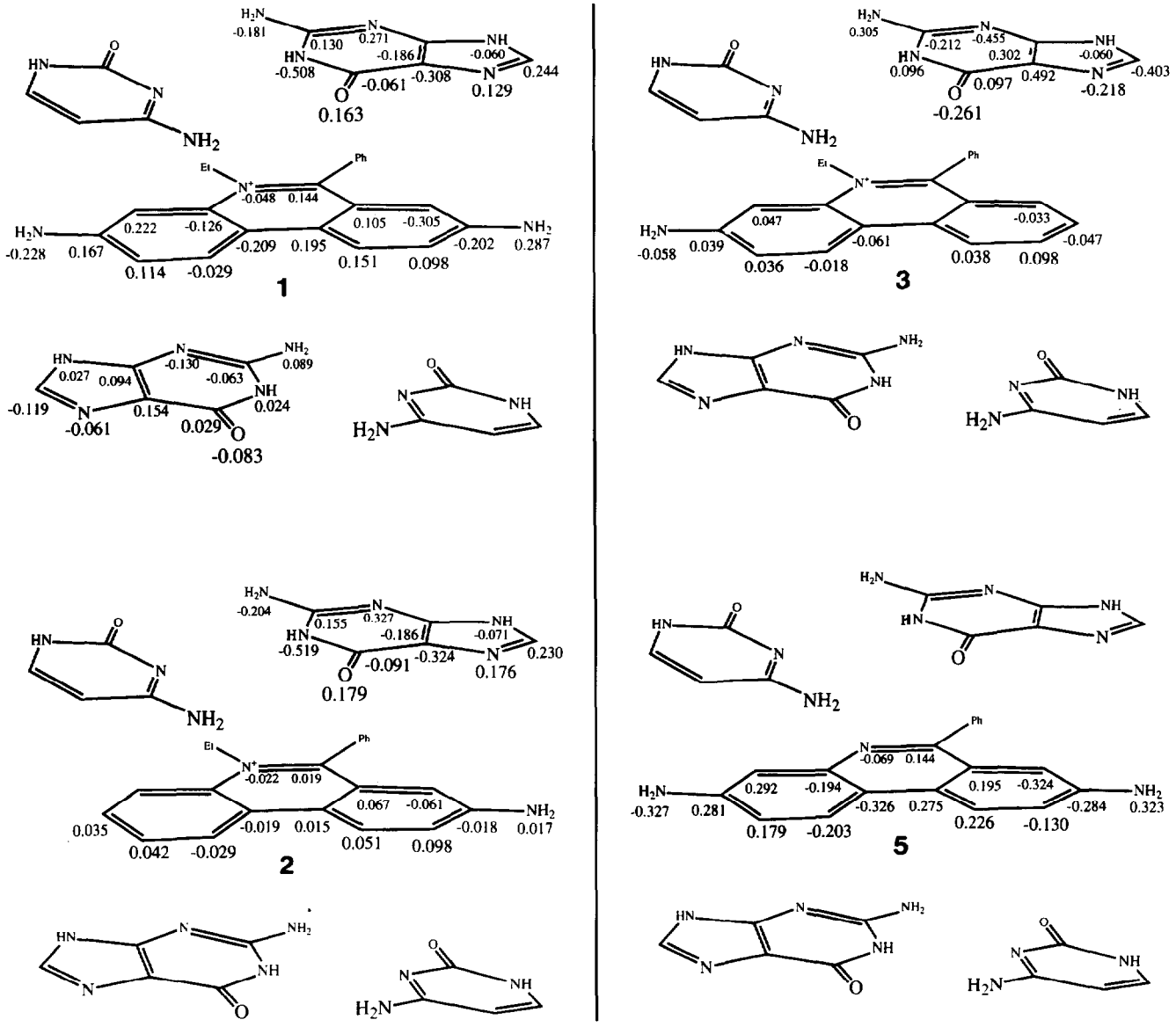



**Figure 1.** Calculated HOMO coefficients (listed below the corresponding atoms) for G-C/C-G intercalation complexes with **1–3** and a forced intercalation complex with **5**. The HOMO of the intercalation complex of G-C/C-G with **4** shows strong contributions from **4** and the lower guanine and a small contribution from the upper guanine (not shown).

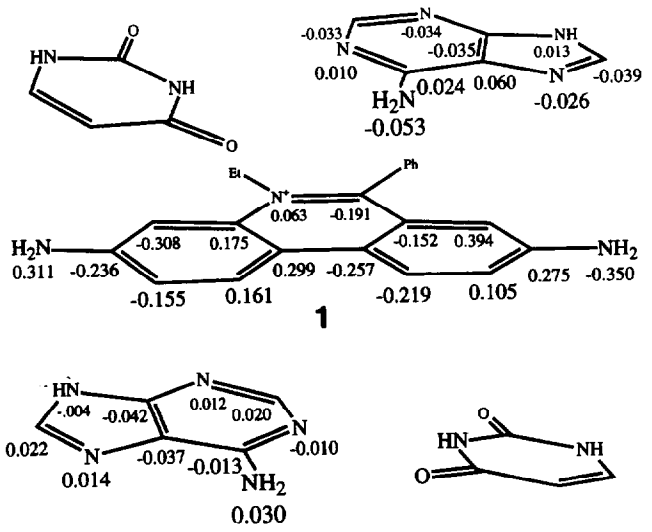



**Figure 2.** Calculated HOMO coefficients for the A-U/U-A intercalation complex with **1**.

mix producing an extended HOMO for the complex along with concomitant lowering of the LUMO. These changes will result in a bathochromic shift in the electronic absorption. The cation lowers the LUMO of the intercalator facilitating mixing of this orbital with the HOMOs of the adjacent and most electron rich bases (i.e. those having the highest HOMOs). The resulting extended HOMO of the complex stabilizes the complex.

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