On the Electronic Structure of Ethidium

Nathan W. Luedtke,* Qi Liu, and Yitzhak Tor*[a]

Abstract: The electronic structure of the common intercalating agent ethidium bromide (3,8-diamino-5-ethyl-6-phenylphenanthridinium bromide) is dominated by an interplay of electron donating and withdrawing effects mediated by its nitrogen atoms. X-ray crystallography, UV/Vis and IR absorption, fluorescence emission, and NMR spectroscopy are used to probe the electronic properties of the phenanthridinium “core” of ethidium as well as its exocyclic amines and 6-phenyl groups. Interestingly, despite its positive charge, most of ethidium’s aromatic carbon and hydrogen atoms have high electron densities (compared to both 6-phenylphenanthridine and benzene). The data suggest that electron donation by ethidium’s exocyclic amines dominates over the electron withdrawing effects of its endocyclic iminium in their combined influence on the electron densities of these atoms. Ethidium’s nitrogen atoms are, conversely, electron deficient where the 5-position is the most electropositive, followed by the 3-amino, and lastly the 8-amino group. These results have been used to generate an empirically-based π-electron density map of ethidium that may prove useful to understanding its nucleic acid binding specificity.

Introduction

Ethidium bromide (3,8-diamino-5-ethyl-6-phenylphenanthridinium bromide) is a common fluorescent stain for nucleic acids.[1] It is reported to have significant anti-tumor,[2,3] and anti-viral properties.[4,5] Its potential applications in human therapy are prevented, however, due to its mutagenic and carcinogenic activities in model organisms.[6,7] These activities are thought to be a direct consequence of ethidium’s high affinity for DNA.[8–10] We recently derivatized the amines at the 3- and 8-positions of ethidium to tune its nucleic acid affinity and binding specificity.[11] The resulting compounds exhibit a number of interesting trends in their photophysical characteristics and DNA binding properties.[11] A potential relationship between these properties has prompted us to explore the forces involved in the binding of ethidium to various nucleic acids.

Under physiologically relevant ionic strengths, ethidium binds to duplex nucleic acids by intercalation between base pairs.[8,9,12] Ethidium binds to different duplex nucleic acids with a free energy of about 5–9 kcal mol⁻¹, depending on the identity of the nucleic acid.[13a,14] Both the binding mode (surface association versus intercalation) and the free energy of ethidium–nucleic acid complexes are highly sensitive to the ionic strength of the solution.[9,13] This raises the possibility that ethidium’s base stacking propensity and intercalation site specificity may also depend upon electrostatic interactions.[15,16] Electrostatic complementation is known to be an important factor in π-stacking interactions.[17,18] For phenyl–phenyl stacking, electron-withdrawing groups lower Coulombic repulsion between the π electrons of each ring.[18] Attraction is observed when electron-rich and electron-poor rings are stacked face-to-face.[17,20] Gas phase computational studies based upon electrostatic surface potentials,[15] atom-centered charges,[16] and HOMO–LUMO overlap,[21] have been used to model the stacking interactions between ethidium and one or more base pair(s). These studies were based upon the premise that the phenanthridinium “core” of ethidium is an electron-poor polycyclic system that, depending on geometric constraints, can stack face-to-face with the relatively electron-rich nucleobases.[15,16,21] These studies concluded that dispersion forces likely provide the main energetic driving force for intercalation,[16,18] but that electrostatic complementation may play an important role in determining ethidium’s site specificity and binding orientation.[15]

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We have accumulated data using high resolution X-ray crystallography, UV/Vis and IR absorption, fluorescence emission, NMR spectroscopy, chemical reactivity, and acid-base titrations that have facilitated a broad survey of ethidium’s electronic features. We have used the data to estimate the charges on each of its nitrogen atoms and to generate an empirically-based π-electron density map that is scaled to ethidium’s +1 charge. A comparison of these results with previous theoretical studies is presented, and the ways that ethidium’s electron distribution may influence its binding interactions with nucleic acids are discussed.

Results and Discussion

Chemical reactivity: We have recently reported a modular synthetic scheme that facilitates the protection and modification of ethidium’s exocyclic amines.\[11\] By reacting one equivalent of benzyl chloroformate with ethidium bromide (1), a mixture of benzyl carbamate (Cbz)-containing products is obtained that contains five-times more “8-Cbz-ethidium chloride” (3) as compared to “3-Cbz-ethidium chloride” (2) (Figure 1). Weaker electrophiles exhibit little if any reactivity towards ethidium’s exocyclic amines (especially at the 3-position) even at elevated temperatures and extended reaction times.\[11\] Similar trends in relative reactivity of the 3- and 8-amines have also been reported by another group.\[23\]

The differences were explained as a combination of two effects: the electronic inactivation of the 3-position by resonance with the 5-iminium group as well as the steric constraints imposed on ethidium upon its dimerization in DMF.\[23\] For the studies reported here, dimerization is not expected to play a role since ethidium exhibits only a very weak association constant in water (\(K_{\text{eq}} = 30–178 \text{m}^{-1}\)), and shows no dimerization in DMSO.\[24\] We believe that the trends in the chemical reactivity of ethidium’s exocyclic amines (as well as their \(pK_a\) values, NMR chemical shifts, and nitrogen-carbon bond lengths) are a consequence of two or more electronic effects presented below. These same effects may also play an important role in the photophysical properties of ethidium and its derivatives.

Photophysical properties: We have used the Cbz-protected compounds 2 and 3 as precursors for the synthesis of a family of ethidium derivatives (Table 1).\[23\] The trends in the photophysical properties of the resulting derivatives provide insight into the distinct influences that the 3- and 8-positions have on the ground and excited states of ethidium. Aqueous solutions of ethidium display an absorption band at 486 nm that possesses charge-transfer character.\[26\] This transition is highly sensitive to the identity and combination of the functional groups at the 3- and 8-positions (Table 1). For all ethidium derivatives that have a lower electron donation capacity at these positions (relative to ethidium’s unmodified amines), a blue-shift in \(\lambda_{\text{max}}\) is observed (compare ethidium (1) to compounds 2–11, Table 1). The only derivative that has augmented electron donation at these positions relative to ethidium (compound 12, Table 1) has a red-shifted absorption maximum. These same derivatives also exhibit variable fluorescence properties. All bis-substituted derivatives that have diminished electron donation capacities (4, 7, 10 and 11), have both a higher quantum yield and a smaller Stokes shift relative to ethidium. The only derivative that augments electron donation, on the other hand, exhibits a much lower quantum efficiency as compared to ethidium (compound 12, Table 1). Taken together, these observations suggest that electron donation from ethidium’s exocyclic amines is likely to facilitate a non-radiative decay of its excited state. This is a very different quenching mechanism compared with the one proposed by Olmstead and Kearns for ethidium, which relies upon the water-mediated deprotonation of ethidium’s exocyclic amines in its excited state.\[27\] While it is possible that this type of proton transfer may influence the photophysical properties of ethidium, it cannot explain the low quantum yields of ethidium derivatives that lack NH-containing functional groups at the 3- and 8-positions (including compounds 12 and 15, Table 1).

For compounds 2–14, the independent modification of the 3-position of ethidium consistently leads to a larger blue shift in both absorption and emission maxima compared with the modification of the 8-position (compare compounds 2 to 3, 5 to 6, 8 to 9, and 13 to 14, Table 1). Other groups have also reported different photophysical properties for 3-versus 8-modified derivatives of ethidium, and have attributed the differences to the different influence each position has on the LUMO energy level of ethidium.\[28\] Our data indicate that significant differences in the charge-transfer propensities of these positions also exist in the ground state of ethidium (presented below). The trends in the photophysical data, therefore, likely reflect significant changes to both the

Figure 1. Selective protection of the exocyclic amines of ethidium (where \(R = \text{NHCO}_2\text{CH}_2\text{Ph}\)) is accomplished by treatment with benzyl chloroformate in buffered DMSO/water 1:1.\[25\] Isolated yields are indicated. A 1:5 ratio of compounds 2 and 3 is also observed in the crude reaction mixture (determined by NMR integration). Unambiguous assignment of each isomer was accomplished using X-ray crystallography (see Figure 11 and Supporting Information).
Ethidium

HOMO and LUMO energy levels of each derivative upon modification of each amine.

**pKₐ values of ethidium and selected derivatives**: The UV/Vis absorbance spectrum of ethidium changes as a function of pH. A plot of λ_max (or, alternatively, ΔAbs) versus the pH of the solution reveals a fully reversible, biphasic curve between pH 0.5 and 3.0 (Figure 2). To assist in assigning each transition, we have conducted a similar study of compounds 5–9 (see Figure 3 for representative titrations and Figure 4 for a summary of pKₐ values). In the pH range of 4–11, both 3-guanidino-ethidium (5) and 8-guanidino-ethidium (6) have monophasic titration curves (Figure 3a). pKₐ values of 9.2 and 9.9, respectively, are measured for these compounds by taking the inflection point of each curve as equal to the pKₐ of the guanidinium group. Interestingly, 3,8-bis-guanidino-ethidium (7) exhibits a biphasic titration curve very similar in shape to that of ethidium, where the “first” protonation (pKₐ₁) causes a larger change in λ_max compared with the “second” protonation (pKₐ₂) (compare Figure 2 and Figure 3b). Since 8-guanidino-ethidium (6) is more basic than 3-guanidino-ethidium (5), the inflection points at pH 6.2 and 9.5 in Figure 3b are assigned to the 3- and 8-guanidino groups of 7, respectively. The mono-urea derivatives 8 and 9 have pKₐ values very similar to the values measured for ethidium (Figure 4). We have, therefore, assigned the 8-urea ammonium of ethidium as having a pKₐ₂ = 2.0 (similar to 3-urea ethidium); and the 3-amine of ethidium as having a pKₐ₁ = 0.8 (similar to 8-urea ethidium). This assignment is consistent with the differences in nucleophilicity of these two positions (Figure 1), as well as the predicted assignment of these values by Zimmermann and Zimmermann using computation.\[28\] Importantly, the exocyclic amines of ethidium are 400–6300 times less basic than aniline (pKₐ 4.6 for the anilinium ion).\[29\] This indicates that ethidium’s exocyclic amines (especially at the 3-position) are very electron poor. This conclusion is

<table>
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<th>Compound</th>
<th>R¹</th>
<th>R²</th>
<th>λ_max [nm]</th>
<th>λ_em [nm]</th>
<th>f_rel</th>
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<td>468</td>
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<td>N⁻O⁻Ph</td>
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<td>603</td>
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<td>N⁻O⁻</td>
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<td>510</td>
<td>57</td>
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<td>593</td>
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<td>504</td>
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<td>-N⁻</td>
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<tr>
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<td>-N⁻</td>
<td>428</td>
<td>504</td>
<td>1.2</td>
</tr>
</tbody>
</table>

[a] All values determined using 10μM of each compound in 50mM sodium phosphate (pH 7.5). The counter ion (X) is phosphate for all photophysical measurements presented above, and chloride for all NMR studies (presented below).
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Figure 2. Changes in the $\lambda_{\text{max}}$ of ethidium (1) as a function of pH. By taking the pH at each inflection point as equal to $pK_a$, values of 2.0 and 0.8 are measured for $pK_a$ and $pK_{diss}$, respectively.

Figure 3. a) Changes in the $\lambda_{\text{max}}$ of 3-guanidino-ethidium (5) and 8-guanidino-ethidium (6). b) Changes in the $\lambda_{\text{max}}$ of 3,8-bis-guanidino-ethidium (7) versus pH. Unlike ethidium, both 5 and 6 exhibit a monophasic transition between pH 4 and 11 (data not gathered for these compounds below pH 4). As a control, 3,8-bis-urea-ethidium (10) was also monitored, and shows no changes in its absorption spectrum over this range. The errors for all pH titrations are approximately $\pm 1$ nm and $\pm 0.05$ pH units for each data point. Titrations were conducted using 20 mM of each compound in aqueous buffer (either 50 mM sodium phosphate or potassium chloride/HCl) and adding HCl or NaOH to adjust the pH.

consistent with the weak nucleophilicity of these amines, as well as NMR and crystallographic data (below).

$^1$H NMR spectroscopic analysis of ethidium and selected derivatives: A comparison of the proton NMR spectra of ethidium’s uncharged analogue (3,8-diamino-6-phenylphenanthridine) with ethidium allows one to assess how ethidium’s positive charge influences the chemical environment of each of its hydrogen atoms (compare Figure 5a with b). Relative to 3,8-diamino-6-phenylphenanthridine, most of ethidium’s protons are shifted downfield by 0.2 to 0.5 ppm. There are two notable exceptions: proton H$_7$ is shifted upfield by 0.8 ppm, and the protons on the 3-amine are shifted downfield by 1.0 ppm. Interestingly, both exocyclic amines in 3,8-diamino-6-phenylphenanthridine have very similar chemical shifts (Figure 5a). Consistent with this finding, the exocyclic amines of 3,8-diamino-6-phenylphenanthridine exhibit similar chemical reactivity towards electrophiles, including Boc anhydride (not shown). The exocyclic amines of ethidium, on the other hand, are separated by 0.5 ppm (Figure 5b). Taken together, these data indicate that ethidium’s charged quaternary center is essential for the differences in electron densities and chemical reactivities of the 3- and 8-amines.

We have fully assigned ethidium’s $^1$H NMR spectrum (including the hydrogen atoms of each amine) using $^1$H,$^1$C heteronuclear multiple bond correlation (HMBC) and the ethyl protons H$_{15}$ as an initial starting point for the assignment (summarized in Figure 5b). Previous groups have assigned ethidium’s $^1$H NMR spectrum using the highly shielded proton H$_7$ as their initial reference (Figure 5). It was assumed that the phenyl ring at the 6-position shields the H$_7$ proton via a ring current effect. Interestingly, this does not appear to be the primary origin of this phenomenon. Our results indicate that both the positive charge afforded by the ethidium’s quaternary nitrogen and electron donation from its 8-amino group are necessary for the high field shift of proton H$_7$. Ethidium’s uncharged analogue (3,8-diamino-6-phenylphenanthridine) shows no unusual shielding of H$_7$ (Figure 5a). While the presence or absence of the ethyl group at the 5-position could, in theory, change the conformational dynamics of the 6-phenyl group relative to the phenanthridine “core”, we have found that the 8-amino modified derivatives of ethidium (3, 4, 6, 7, 9, 10, 11, 14, and 15 Table 1), show no unusual shielding of proton H$_7$ (where perturbation of the conformation of the 6-phenyl ring by these modifications is unlikely). The chemical shift of H$_7$ is highly sensitive to modifications of the 8-position, but insensitive to modifications made to the 3-position (see Figure 6a–c for $^1$H NMR data of compounds 1–4). Supporting Information for compound 11, ref. [11] for compounds 5–10, and 13–15, and ref. [34] for 12. The conversion of the 8-amino of ethidium into any functional group that reduces electron donation from that nitrogen results in a downfield shift of H$_7$ to a more typical aromatic frequency ($\Delta d = 7.8$ ppm). Methylation of the amine at 8-position, on the other hand, results in an upfield shift of H$_7$ ($\Delta d = 0.2$ ppm for compound 1 minus 12). Taken together, this indicates that the H$_7$ proton is shielded ($\Delta d = 1.3$ ppm for 1 minus 11) by electron density originating primarily from the amine at 8-position. $^{13}$C NMR experiments (presented in the next section) provide similar results. Notably, however, even in the absence of exocyclic amines, the presence of the positively charged quaternary nitrogen also shields proton H$_7$ ($\Delta d = -0.5$ ppm). This effect is apparent by comparing the $^1$H NMR spectra of the two ethidium analogues 6-phenyl
phenanthridine and 5-ethyl-6-phenyl phenanthridium chloride (see Supporting Information, Figure S9.1b).\textsuperscript{[33]} It is unclear at this time if this (minor) effect is related to electronic polarization or changes in the conformational dynamics of the 6-phenyl ring.

Upon Cbz modification of ethidium/C29s amines, protons H4 and H 7 are shifted downfield much more than H 1, H 2, H 9, and H 10 (Figure 6a–d). Protons H 1 and H 10 are meta to each amine and exhibit little sensitivity to Cbz modification. Protons H2, H 4, H 7, and H 9, on the other hand, are all ortho to the exocyclic amines; but protons H 4 and H 7 are shifted downfield by 1.4 ppm, while H 2 and H 9 are only shifted downfield by 0.7 ppm upon Cbz modification of the neighboring amine (compare compounds 2 and 3 to compound 1, Figure 6a, b, and d). This same effect is also apparent in the 13C NMR spectra of these compounds (next section). This suggests that electron density from both the 3-amino group and the 8-amino, is drawn preferentially towards the positive center, thus shielding H 4 and H 7 much more than H 2 and H 9. There are, however, different bond orders observed between carbons C2–C3 versus C3–C4, and C7–C8 versus C8–C9 that may also contribute to this same effect (presented below and in Figure 12).

13C NMR spectroscopical analysis of ethidium and selected derivatives: Similar to its 1H NMR spectrum, ethidium’s 13C NMR spectrum exhibits a wide dispersion of chemical shifts in its aromatic region (Figure 7a). We have used distortionless enhancement by polarization transfer (DEPT), carbon–hydrogen correlation spectroscopy (HETCOR), and HMBC experiments to fully assign the 13C spectrum of ethidium.\textsuperscript{[32]} Our assignment, summarized in Table 2, is consistent with the partial one previously reported.\textsuperscript{[35]} We have used these same NMR techniques to fully assign the 13C NMR spectra of three related phenanthridine derivatives: 5-ethyl-6-phenanthridinium chloride (11), 3,8-di-
amino-6-phenylphenanthridine (16), and 6-phenyl-phenanthridine (17) (Figure 8 and Table 2). We have also partially assigned the mono Cbz derivatives 2 and 3 (Figure 7b and c). Consistent with the trends observed in their $^1$H NMR spectra (Figure 6), the modification of ethidium’s 3-amino or 8-amino with Cbz results in a large downfield shift ($\Delta \delta = +10$ ppm) of the C$_4$ and C$_7$ carbon atoms, respectively (Figure 7a–c). A similar trend was also observed upon protonation of these amines.[35] This indicates that the high electron densities at C$_4$ and C$_7$ depend on electron donation from the neighboring exocyclic amines. Interestingly, both $^1$H NMR and $^{13}$C NMR spectroscopy indicate that the 3-amine shields the 4-position by about the same amount as the 8-amine shields the 7-position. This suggests that these interactions are not responsible for the different electron densities on the 3- and 8-amines of ethidium.

The $^{13}$C chemical shifts of most carbon atoms in ethidium’s phenanthridinium “core” are upfield of typical aromatic carbon atoms, while the carbon atoms in its 6-phenyl ring have about the same chemical shift (~128 ppm) as benzene (Figure 7a). The carbon atoms C$_3$ and C$_8$ of ethidium at 97 and 107 ppm, respectively, are in the same chemical shift region as aromatic carbanions (see Supporting Information and ref. [36–39]). The analogous carbon atoms to C$_4$ and C$_7$ in the compound 6-phenyl phenanthridine (17) have, on the other hand, about the same chemical shift as benzene (Table 2). This suggests that, despite ethidium’s positive charge, these positions in ethidium possess very high electron densities. Similar conclusions are reached by comparing the $^1$H NMR spectra of these compounds.[33] The only carbon atoms in the $^{13}$C spectrum of ethidium that are significantly downfield of benzene, are the four carbons that are bound directly to nitrogen atoms (Figure 7a). Carbon C$_3$ and C$_8$ have chemical shifts very similar to the nitrogen-bound carbon of aniline (148 ppm).[39] Compared with this value, the chemical shift of C$_6$ is significantly downfield, and C$_3$ is found upfield (Figure 7a). By comparing the $^{13}$C chemical shifts of ethidium’s carbon atoms to those of...
the related phenanthridine-containing compounds presented in Figure 8 and Table 2, it is apparent that the relatively high-field shift of C\textsubscript{13} is dependent on the presence of the quaternary nitrogen at the 5-position, and that this effect is independent of the electron donation capacity of the groups at the 3- and 8-positions. The chemical shift for C\textsubscript{6}, on the other hand, is very similar for all four compounds (Table 2). This is consistent with earlier work that shows the phenyl ring at the 6-position of ethidium is responsible for the low-field shift of C\textsubscript{6}.\[35\]

13C NMR spectroscopy provides a direct method to probe the $\pi$-electron densities of aromatic carbon atoms.\[36–39\] A linear correlation between $\pi$-electron charge densities and 13C chemical shifts ($\delta$) of aromatic compounds is well established (see Supporting Information and ref. \[36–39\]). This provides a framework for understanding the $\pi$-electron charge distribution in the phenanthridinium core of ethidium. NMR spectroscopy is, however, highly sensitive to other properties of the molecule, including bond order, hybridization, paramagnetic coupling, and electronegativities.\[38,39\] To systematically dissect the electronic contributions made by ethidium’s positive charge and its exocyclic amines, we have conducted an “NMR difference analysis” of the phenanthridine derivatives shown in Figure 8 (see Figure 9 for the differences in 1H chemical shifts of these compounds, and Supporting Information for a similar analysis of the 1H spectra).\[33\] By calculating the differences in the chemical shifts at each position, the factors that are common to each compound should, for the most part, cancel out. Thus, by making a carbon-by-carbon comparison of ethidium chloride (I) to 5-ethyl-6-phenylphenanthridinium chloride (II), the $\pi$-electron charge distribution originating from ethidium’s

Table 2. Summary of 13C NMR chemical shifts for ethidium chloride and three related phenanthridine-containing compounds. See Figure 7 for the numbering scheme.

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<tr>
<th>Carbon</th>
<th>Ethidium chloride (I)</th>
<th>5-Ethyl-6-phenylphenanthridinium chloride (II)</th>
<th>3,8-Diamino-6-phenylphenanthridine (II)</th>
<th>6-Phenylphenanthridine (I)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>123.7</td>
<td>124.7</td>
<td>122.1</td>
<td>122.5</td>
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<tr>
<td>2</td>
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<td>128.0</td>
<td>127.8</td>
<td>127.9</td>
</tr>
<tr>
<td>20</td>
<td>129.9</td>
<td>132.1</td>
<td>127.8</td>
<td>128.3</td>
</tr>
</tbody>
</table>

[a] n.a. = not applicable.
By comparing $^{13}\text{C}$ NMR chemical shifts of ethidium chloride (1) to $3,8$-diamino-6-phenylphenanthridine (16), the effect of introducing a positive charge into ethidium can be examined (the difference in chemical shifts for each carbon atom in these two molecules is presented in Figure 9b). As expected, the introduction of a positive charge deshields most carbon atoms (positive values, Figure 9b). There are, however, a small number of carbon atoms that become more shielded in the presence of the positive charge ($C_4$, $C_6$, and $C_{17}$). To evaluate what effect, if any, the exocyclic amines of ethidium have upon this phenomenon, the chemical shift differences between 5-ethyl-6-phenylphenanthridinium (11) and 6-phenylphenanthridine (17) have also been determined (Figure 9c). These differences assess the impact of introducing a positive charge into the system in the absence of the exocyclic amines. For most carbon atoms, the differences between 1 and 16 (Figure 9b) are very similar to the differences between 11 and 17 (Figure 9c). This suggests that for most positions, the positive charge afforded by the quaternary nitrogen has little impact on the magnitude of electron donation by the exocyclic amines. Some carbon atoms, however, do receive more electron density from the exocyclic amines when the positive charge is present (including $C_4$, $C_6$, and $C_{17}$, compare Figure 9b and c). This suggests that the positive charge of ethidium pulls additional electron density from its exocyclic amines into the π-orbitals of these particular carbon atoms. Unlike the differences seen in the presence/absence of the exocyclic amines (Figure 9a), the carbon atoms in the 6-phenyl ring of ethidium are sensitive to the presence/absence of the charge at the 5-position (Figure 9b and c). The positive charge polarizes the 6-phenyl ring, resulting in a relatively high electron density at $C_{17}$ and a lower electron density at $C_{20}$.

Importantly, for all carbon atoms in the phenanthridinium “core” of ethidium, the magnitudes of $^{13}\text{C}$ chemical shift differences are much larger in the presence/absence of the exocyclic amines (Figure 9a) as compared with the presence/absence of the positive charge (Figure 9b, c). This indicates that electron donation by ethidium’s exocyclic amines has a greater effect on the overall π-electron charge density of the phenanthridinium system than the electron withdrawing effect of the quaternary nitrogen. Taken together, these results can be rationalized if one considers the phenanthridinium “core” of ethidium to be an iminium-bridged biphenyl (presented in the next section).

By calculating the differences in $^{13}\text{C}$ NMR chemical shifts between ethidium chloride (1) and 6-phenylphenanthridine (17), the combined contributions of both electron donation and withdrawing effects are established (Figure 9d). Importantly, the resulting values (Figure 9d) are equal to a summation of the individual contributions made by electron donation from the amines (Figure 9a) plus the contributions made by the introduction of the positive charge (Figure 9b, c). These effects, therefore, exhibit simple additivity, and the magnitudes of the individual “difference maps” are scaled the same (Figure 9a–d). These properties are consistent with the linear relationship between the electron densities of exocyclic amines has been evaluated. The differences in $^{13}\text{C}$ NMR chemical shifts ($\Delta\delta$) of these two compounds are summarized in Figure 9a. As expected, the majority of electron density from these amines is directed to carbon atoms that are ortho/para to them ($C_2$, $C_4$, $C_7$, $C_9$, $C_{11}$, and $C_{17}$) (see Figure 9a). Consistent with trends observed for the Cbz-modified derivatives (presented above), the ortho positions closer to the quaternary nitrogen ($C_4$ and $C_7$) receive much more electron density than the ortho positions further from the positive center ($C_2$ and $C_9$). The carbon atoms in the 5-ethyl and 6-phenyl groups of ethidium, on the other hand, are relatively insensitive to the presence/absence of ethidium’s exocyclic amines (Figure 9a). This indicates that, for the most part, these carbon atoms are electronically isolated from the exocyclic amines.
simple aromatic anions and cations and their $^{13}$C chemical
shifts (see Supporting Information and ref. [36–39]). Inter-
estingly, the net effect of introducing both the positive
charge and the exocyclic amines into the 6-phenylphenan-
thridine scaffold is to increase the electron density at most
of the aromatic carbon atoms in ethidium (negative values,
Figure 9d). This same conclusion is reached by conducting
the same type of “NMR difference analysis” of the $^1$H NMR
spectra of these four compounds.[33]

X-ray crystallography of ethidium and selected derivatives:
To relate the electronic features of ethidium to the bond
orders between its C and N atoms, X-ray crystallography
was used to solve the 3D structure of ethidium chloride (1),
and the Cbz derivatives 2 and 3 (Figures 10 and 11). The C–
C bond lengths observed in the phenanthridinium core of
ethidium chloride are very similar to those reported for
both phenanthridine and 5,6-dimethylphenanthridinium
chloride (Figure 10a–c).[41,42] The $\pi$-bond orders of ethidium
are, therefore, similar to those previously reported for phe-
nanthridine (Figure 12).[42,43] For all crystal structures of
phenanthridine-containing compounds (Figures 10 and 11),
the carbon–carbon bond lengths in the phenanthridine “core”
alternate between relatively short (≈1.35 Å) and relatively
long (≈1.42 Å) bonds (for comparison, benzene’s C–C bond
lengths are 1.39 Å). More double-bond character, therefore,
is found between the C$_3$–C$_4$ and C$_7$–C$_8$ ethidium carbon
atoms as compared to C$_2$–C$_3$ and C$_8$–C$_9$. This may, in part,
explain the greater magnitude of electron donation from the
exocyclic amines to its 4- and 7-

positions versus its 2-
and 9-

positions (Figure 9a). Importantly,
the bond between N$_5$ and C$_6$ of
ethidium is almost a full double
bond (Figure 12).[43] This con-

clusion is consistent with the
gentle conditions under which
this bond is reduced.[24a] as well
as the strong IR absorbance
peak of ethidium at 1620 cm$^{-1}$
(typical for an iminium N–C
stretch).[44–46] The phenanthri-
nium “core” of ethidium can,
therefore, be regarded as an
iminium-bridged biphenyl
(Figure 13). The electronic “iso-
lation” of ethidium’s iminium
from the biphenyl system may
explain why the $\pi$-electron den-
sity in its biphenyl “core” is
dominated by its exocyclic amines,
as well as why the chemical
shifts of the 6-phenyl ring are
sensitive to changes at the 5-

position but not to changes at the
3-

and 8-

amines (Figures 5, 6,
Figure 9).

Crystallographic data indicate that both phenanthridine
and 5,6-dimethyl phenanthridinium chloride (Figure 10) are
planar.[41,42] In contrast, ethidium is found to have a 4$^\circ$ twist
1H downfield shifts of ethidium/C29’s 3- and 8-amino groups and H9/H10, relative to the central axis of the biphenyl the midpoints of the two vectors defined by protons H2/H1 in its phenantheridinium core (the dihedral angle between electronic structure. Order relationships indicate that the depictions near the bottom of this Figure contribute most to the actual Figure 13. All possible Clar and Kekulé depictions of ethidium/C29’s phenanthridinium “core”. Bond-length/bond-order relationships indicate that the depictions near the bottom of this Figure contribute most to the actual electronic structure.

Figure 12. A summary of phenantheridium’s π-bond orders as determined by Kiralj et al. Pauling bond orders were calculated by analyzing the bond lengths in a high-resolution crystal structure of phenantheridium. We have found that the phenantheridinium cores of both ethidium chloride and 5,6-dimethylphenantheridinium chloride each have approximately the same bond lengths as those observed for phenantheridine (Figure 10). and therefore, each have similar bond orders as those shown above.

in its phenantheridinium core (the dihedral angle between the midpoints of the two vectors defined by protons H1/H2 and H3/H4 relative to the central axis of the biphenyl “core” of ethidium). Similar to ethidium, the phenantheridinium cores of both 2 and 3 are also not planar. The twist angle is, however, different in the crystal structure of each of these Cbz derivatives. 3-Cbz ethidium chloride (2) has about the same twist as the parent compound (4%). 8-Cbz ethidium chloride (3), however, shows a greater twist of the phenantheridine core (8%). Taken together, this indicates that some sp3 character is introduced into the phenantheridinium core of ethidium by its exocyclic amines. Interestingly, the C–N bond lengths for the free amines of 2 and 3 are shorter than the analogous bonds in the parent structure (compare Figures 10c and 11). This indicates that upon blocking one amine (by Cbz protection) the amine opposite to it compensates by donating more of its electron density into the phenantheridinium core. This is consistent with the 1H downfield shifts of ethidium’s 3- and 8-amino groups upon Cbz modification of the other amine (Figure 6a, b, d). These effects may, in part, explain the differences in the relative quantum efficiencies of emission for compounds 2–13 (Table 1) where the independent modification of the 8-position (compounds 3, 6, 9, and 14) leads to a significant (5–15 fold) decrease in emission intensity as compared to ethidium, while modification of the 3-position shows little or no effect for compounds 2, 5, 8 and 13 (Table 1).

Consistent with an early crystal structure of ethidium,[47] differences in the C–N bond lengths of ethidium’s two exocyclic amines are observed. A 0.01 Å shorter C–N bond length is found for the amine at the 3-position as compared to the 8-position (Figure 10c). A 0.01 Å difference is also observed between the C–N bond lengths for the “free” amines of the two mono Cbz derivatives 2 and 3 (Figure 11).

Taken together, this indicates that more C–N double-bond character, and hence, more positive charge resides at the 3-amine of ethidium as compared to the 8-amine. Both amines, however, have shorter C–N bond lengths than that of aniline (1.4 Å), and are, therefore, more electropositive than aniline. These results are consistent with the 1H NMR chemical shifts (Figure 6), pK values (Figure 4), and the chemical reactivities of these amines (Figure 1).

π-Electron density map of ethidium: We have used the 13C NMR “total” difference map (Figure 9d) in conjunction with bond-length/bond-order relationships to generate an empirical π-electron density map of ethidium that is scaled to its +1 charge (Figure 14a, b). Since the π-charges on ethidium’s nitrogen atoms are proportional to their π-bonding with carbon atoms, the partial charges on these atoms are estimated from known N–C bond lengths (Figure 10c). Due to experimental errors associated with bond lengths (+0.003 Å), and the somewhat arbitrary assignments made for bond-order/charge relationships, these values will serve as estimates. Despite this, the trends between like atoms should be reliable. For the quaternary nitrogen, the N–C bond length is used to assign its charge by comparing its length to standard N–C bond lengths from crystal structures of analogous nitrogen-containing molecules with known charges.[50] The N–C bond length of N,N-dimethylisopropylideneammonium tetraphe- nylborate (1.30 Å) is used as a standard equal to +1 (a full double bond).[51] The N–C bond length of 7-(α-(2-methoxy- carbonylaminobenzyl)quinoline-8-ol is taken as a single bond standard (at 1.47 Å) and equal to zero charge.[52] Using these standards, a linear correlation is used to assign a charge of +0.76 for the quaternary nitrogen (Figure 14a).[50,53] A similar method is used to estimate the charges on ethidium’s 3- and 8-amino groups. As a standard...
for these exocyclic amines, the N–C bond lengths for the Cbz-modified amino groups of 2 and 3 (1.40 Å) are taken to equal zero charge (Figure 11). This is the same bond length as aniline, and, therefore, the charges assigned to these atoms are with respect to aniline. The N=C bond length of both N,N-dimethyl-isopropylideneammonium tetraphenylborate and 9-(10-methyl)acridinium iodide (both 1.30 Å) are taken as the standards for a double bond and are taken as a +1 charge. Using these standards, charges of +0.28 and +0.18 have been assigned to the 3- and 8-amines, respectively (Figure 14a). An early computational study by Giacomoni and Le Bret also predicted positive ρ-charges for all of ethidium’s nitrogen atoms. Their study utilized a very early and possibly less accurate crystal structure of ethidium. Using a Pariser–Parr calculation they predicted that the ethidium’s exocyclic amines have the same partial charges and that its positive charge is highly delocalized over many different atoms (see Supporting Information for a comparison of the Le Bret model with ours). While the RESP atomic charges reported in a more recent computational study do not have any physical meaning in the absence of a force field, similar trends in these values are also reported. Our results, on the other hand, indicate that the positive charge of ethidium is highly localized at N and that its exocyclic amines have substantially different charges.

The total sum of charge carried by the three nitrogen atoms of ethidium equals +1.22 (Figure 14a). To maintain a total charge of +1.0, the charge on all carbon atoms is taken as equal to −0.22. The magnitude and sign of this charge is consistent with the preponderance of highly-shielded carbon atoms in the 13C NMR spectrum of ethidium, and it is distributed according to the differences in 13C NMR chemical shifts between ethidium chloride and 6-phenyl phenanthridine (Figure 9d). The reported charge density on each carbon atom is, therefore, relative to 6-phenyl-phenanthridine (very similar to benzene) for most of its carbon atoms, see Table 2. 13C NMR experiments indicate that the differences between ethidium chloride and 6-phenyl phenanthridine (Figure 9d) are a simple summation of the electronic contributions made by its exocyclic amines (Figure 9a) and the positive charge afforded by the quaternary nitrogen (Figure 9b, c). The total sum of all the differences in 13C NMR chemical shifts (due to the exocyclic amines and quaternary nitrogen of ethidium) is −50 ppm (Figure 9d). This total difference is taken as equal to the total “excess” charge originating from all three amines (−0.22). Important-

ly, this charge to 13C chemical shift ratio (at 0.005 charge units per ppm) is approximately the same relationship already established for simple aromatic anions and cations (at 0.006 charge units per ppm; see Supporting Information and ref. [36–39]). This provides substantial evidence that the assumptions made in assigning the charges of nitrogen atoms are appropriate, and that the reported ρ-charges on the nitrogen and carbon atoms are scaled the same (Figure 14). Since the relationship between Δρ and ρ-electron charge density is known to be linear (see Supporting Information and ref. [36–39]), we have assigned the partial charge on each of the carbon atoms of ethidium by taking its fractional contribution to the total difference between the 13C NMR spectra of ethidium and 6-phenylphenanthridine (ΔρC/−50 ppm) multiplied by (−0.22). The resulting charges on each atom are summarized (Figure 14a), and are presented in a space-filling model of ethidium where ρ-electron densities are indicated by the color and intensity of each atom (Figure 14b).

A number of theoretical studies portray ethidium as having a relatively uniform, electron-poor polycyclic system, and, therefore, an electron “acceptor” in its π-stacking interactions with nucleic acid base pairs. In contrast, we find that the ρ-electron distribution of ethidium is highly irregular and that high electron densities are found on most of ethidium’s carbon atoms, and low ρ-electron densities on its nitrogen atoms (Figure 14a, b). The magnitudes and distribution of these charges form a unique and different nature of the π-electron charge distributions.
pattern within the phenanthridinium “core” of ethidium that shares many important similarities to a semiempirical SCF-Cl-PPP computational study reported by Zimmermann and Zimmermann. Their model, however, likely underestimates the differences in charges at the 3- and 8-amin, and little agreement is observed for the atoms in the 6-phenyl ring of ethidium (see Supporting Information for a comparison of the Zimmermann model with our results). Numerous potential reasons exist for the differences between our results and the various theoretical studies mentioned throughout this report, including: solvation and counterion effects, the accuracy of the molecular coordinates, and validity of the models used.

Implications

The detailed study of an intercalating agent’s π-electron distribution may provide a framework for understanding its nucleic acid binding specificity. For nucleic acids, π-stacking interactions between base pairs are known to have a profound effect on both the geometry and thermodynamic stability of helical structures. The combined electrostatic effects of both π-electron density and lone pair localization are taken as important factors in mediating these base-stacking interactions. The π-electron density and lone pair delocalization of ethidium should, therefore, also be important determinants in its affinity for different intercalation sites. We have, for the first time, measured these parameters using high resolution experimental data and relatively simple data analysis.

Our results prove that compared with the 8-amino derivative, the 3-amino is significantly more electron poor, and that both amines are electropositive (relative to aniline). The electronic differences between the 3- and 8-positions of ethidium are consistent with simple resonance theory. The positive charge on ethidium’s quaternary nitrogen can delocalize to the 3-nitrogen but not to the 8-nitrogen (Figure 15a). It should be noted, however, that even in the absence of the exocyclic amines, the charge afforded by the quaternary nitrogen polarizes the phenanthridine core of ethidium more at C2-C8 as compared to C3-C7 (according to the differences in the 13C NMR chemical shifts of 5-ethyl-6-phenylphenanthridinium chloride versus 6-phenylphenanthridine, see Figure 9c). This suggests that two or more different electronic effects make the 3-amino of ethidium more electropositive than the 8-amino.

Unlike a number of previous theoretical studies, we conclude that most carbon and hydrogen atoms in ethidium have relatively high electron densities. This makes it less likely that these groups will exhibit energetically favorable π-staking interactions with nucleobase pairs. It is possible that some degree of π-electron repulsion may actually decrease ethidium’s intercalation energy at some sites more than others and thus exhibit influence on its observed binding site specificity (electronic negative readout).

As expected, the exocyclic amines of ethidium donate electron density to the carbon atoms that are ortho/para to them (Figure 9a). Interestingly, a disproportionate amount of electron density is found at the two ortho positions located closer to the positive center (carbon atoms C4 and C7). This feature is also apparent in two theoretical models. We have found that the high π-electron densities at these carbons depend upon the electron donation capacity of the neighboring exocyclic amine. The ability of electron density from these amines to flow to C4 and C7 through resonance is apparent by a comparison of the relative C7-C8 and C4-C7 bond lengths in compounds 2 and 3. Upon “blocking” each amine by Cbz modification, a shorter bond length is observed in the neighboring C–C bond found closer to the positive center while other C–C bond lengths are largely unaffected (Figure 11). Taken together, these results indicate that at least two different charge-separated resonance structures make important contributions to the ground-state electronic structure of ethidium (Figure 15b).

The exocyclic amines of ethidium are known to be important for DNA binding. We have found that the conversion of these amines into other functional groups dramatically affects both the nucleic acid affinity/specificity, and the electronic structure of the resulting derivatives. It has been proposed that the partial positive charges carried by ethidium’s exocyclic amines are important for mediating electrostatic attraction and hydrogen bonding interactions with DNA’s phosphate groups. Indeed, these phosphate and amino groups are within hydrogen bonding distances in a crystal structure of ethidium intercalated into nucleotide di- phosphates. Our results support the potential importance of this electrostatic interaction, and are in stark contrast to a
recent computational study that portrays these amines as being electron rich (with low electrostatic potentials compared with the aromatic carbon atoms of etidium). It is currently unknown how the different changes that ethidium’s 3- and 8-amines influence its binding orientation and/or specificity. Unfortunately, there is crystallographic evidence for the intercalation of ethidium into duplex DNA from both “approaches” (i.e., the phenyl residing in both the major and minor grooves). This ambiguity currently limits the reliability of even simple electrostatic models of etidium bound by DNA, since the geometric constraints of complexes formed by the intercalation of ethidium into long duplex DNA are currently unknown.

Much work remains to fully understand and predict the π-stacking interactions between ethidium and nucleic acids. A complex interplay of multiple energetic contributions (dispersion, electrostatic, dipole-multipole, etc.), combined with the geometric constraints of the particular ethidium–nucleic acid complex, are likely responsible for ethidium’s binding specificity. We have presented, for the first time, an empirically-based π-electron distribution map of etidium. The ways in which this electronic structure contributes to the total binding energy of various intercalation complexes are currently under investigation and will be reported in due course.

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[32] Coupling between the methylene hydrogens of ethidium’s ethyl group and C6 (apparent by HMBC experiments) allows unambiguous assignment of C6 at 157.5 ppm (see Figure 7a). This provides the starting point for the 1H and 13C NMR assignments reported here.
[33] See Supporting Information for the full 1H NMR assignments and δ difference maps of ethidium chloride (1), 3,8-diamino-6-phenylanthridine (16), 5-ethyl-6-phenylanthridinium chloride (11), and 6-phenylanthridinium (17).
[40] The δ at C6 and C7 may be dominated by differences in electronegativities of nitrogen versus hydrogen and may, therefore, not be indicative of the π-electron densities at these positions.
[43] The N=C bond length between N6 and C7 is longer in ethidium than in phenanthridine because the theoretical bond length for a full N=C double bond is longer in a fully substituted iminium than in a partially substituted iminium (computational studies not shown). Indeed, both the chemical reactivity and IR absorption of this bond indicate that for ethidium, the N6 to C7 bond is, in fact, nearly a full double bond.
[44] Another group has assigned this peak to an N=C exocyclic amine.[11] This does not, however, appear to be correct since tetramethyl-ethidium chloride (12) and 5-ethyl-6-phenylanthridinium chloride (11) lack N–H bonds but show absorban-
[45] See Supporting Information for IR spectra of ethidium chloride (1), tetramethyl-ethidium chloride (12), and 5-ethyl-6-phenylanthridinium chloride (11).
Early theoretical studies claim that the 6-phenyl ring of ethidium is responsible for its lack of planarity (see ref. [26]). A survey of the Cambridge Crystallographic Database, however, suggests that electron donating groups break the planarity of aromatic systems more effectively than phenyl rings. This can be observed for the following series of phenanthrine-based compounds (twist angles indicated in parenthesis): phenanthrine (0.8), 6-napthyl-phenanthrine (0.9), 6-(N,N-dimethyl aniline)-phenanthrine (2.0).

The atomic coordinates of the hydrogen atoms were determined using electron difference maps. See Supporting Information for the ORTEP drawing and crystallographic parameters for compounds 1–3. CCDC-247564 (1), -247565 (2), and -247566 (3) contain the atomic coordinates for these structures. These data can be obtained free of charge via www.ccdc.cam.ac.uk/conts/retrieving.html (or from the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: (+44) 1223-336-033; or deposit@ccdc.cam.ac.uk).

The C_{13}-N_5 bond has not been used as part of the charge calculation of N_5, since little p-bonding between these atoms is observed (Figures 12 and 13). In addition, since the N-C bond length in aniline (1.40 Å) has been used as the standard length equal to no charge for the exocyclic amines, the C_{13}-N_5 bond (also 1.40 Å) has no effect on computed charge of N_5.


A linear correlation between bond-length/bond-order has already been established for the nitrogen–carbon bonds of phenanthidine-containing molecules (see ref. [42]).


The C_3 and C_8 carbons of ethidium have 13C chemical shifts very similar to the nitrogen-bound carbon of aniline. This supports the premise that the contributions made by N–C polarization and hybridization should not have a significant influence on the charges calculated at these positions.


Hydrogen atoms have been omitted from this analysis. Similar conclusions, however, are reached using 1H NMR difference analysis of the same compounds presented in Figure 9 (see ref. [33]). This analysis also indicates that the electron donating effects of ethidium’s exocyclic amines dominate over the electron withdrawing effects of ethidium’s quaternary nitrogen, and that the 4- and 9-positions are the most electron-rich.

Due to the sensitivity of NMR to the changes in orbital hybridizations of N_5 upon alkylation, our NMR methodology likely overestimates the electron density at C_6. This position is expected to be electropositive (see Supporting Information and refs. [22] and [59]).


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