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Synthesis, Photophysical Properties, and Nucleic Acid Binding of Phenanthridinium Derivatives Based on Ethidium

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Abstract—A series of substituted phenanthridine derivatives has been synthesized by converting the amines at the 3- and 8-positions of ethidium bromide into guanidine, pyrrole, urea, and various substituted ureas. The resulting derivatives exhibit unique spectral properties that change upon binding nucleic acids. The compounds were analyzed for their ability to inhibit the HIV-1 Rev–Rev Response Element (RRE) interaction, as well as for their affinity to calf thymus DNA. One derivative (3,8-bis-urea-ethylenediamine-5-ethyl-6-phenylphenanthridinium trifuroracetate) has an enhanced affinity and specificity for HIV-1 RRE as compared to ethidium bromide. These results indicate that the nucleic acid affinity and specificity of an intercalating agent can be tuned by synthetic modification of its exocyclic amines.

Introduction

Ethidium bromide is the common name for 3,8-diamino-5-ethyl-6-phenylphenanthridinium bromide (**1**). First reported in 1952, it was developed by the Boots company as an anti-trypanosomal agent.[1] Ethidium is a common laboratory stain for double-stranded DNA and RNA,[2] but it is also known to possess significant anti-cancer,[3,4] and anti-viral activities.[5–7] Ethidium's potential applications in human treatment have been prevented, however, due to its mutagenic activities in model systems.[8–11] Despite this, ethidium is still marketed by Laprovet as a safe and inexpensive treatment for cattle suffering from trypanosome infections.[12]

A possible relationship between ethidium's nucleic acid binding and its biological activities have been examined by a number of groups both in vivo and in vitro.[13–17] Sea urchin eggs exposed to water containing 50 μM or more of ethidium develop chromosomal abnormalities and fail to divide normally.[15] Experiments reported by Nass indicated that the growth of both mouse fibroblasts and hamster kidney cells are inhibited by 0.3–13 μM of ethidium, and that mitochondrial, not nuclear, DNA synthesis was inhibited by ethidium.[16] A separate study showed that ethidium accumulates in isolated rat mitochodrion and interferes with the metabolic activities related to respiration.[17] Ethidium is, however, only moderately toxic to mammals, with an LD_{50} in mice of ~300 μM (100 mg/kg, subcutaneous),[18] and is an effective trypanocide in cattle at ~3 μM (1 mg/kg, intravenous).[1,12]

The in vitro study of ethidium-nucleic acid binding can be conducted by monitoring the photophysical changes of ethidium upon addition of nucleic acids.[13,14] Ethidium binds to DNA and RNA duplexes, depending on the sequence, with good to moderate affinities (K_d = 1–500 μM) and with variable stoichiometry (2–5 equivalents of ethidium per helical repeat).[19] LePecq and Paoletti first proposed that ethidium binds to nucleic acids via two distinct modes: at low ionic strengths it binds to the surface of nucleic acids through electrostatic interactions, and at higher, physiologically relevant ionic strengths it intercalates between base pairs.[14] Crystallographic and NMR studies have subsequently confirmed ethidium's ability to bind to nucleic acids through these two distinct modes.[20–22]

We have recently described the ability of ethidium bromide to bind to the HIV-1 Rev Response Element (RRE) with high affinity.[23] Subsequent evaluation for its ability to inhibit HIV-1 gene expression indicates that ethidium is a potent inhibitor of HIV replication,

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with an $IC_{50} \cong 0.2$ μM (8×10^{-5} g/L).[23] The Rev–RRE interaction is a protein–RNA interaction essential for the replication of HIV.[24] The Rev protein binds to the RRE and facilitates the export of the viral transcript from the nucleus, while protecting it from the cell's splicing machinery.[24] Without Rev–RRE binding, the proteins needed for viral production are never translated.[25] The Rev binding site on the RRE is found to be highly conserved even between different groups of HIV isolates (bold bases, Fig. 1).[23,26] Compounds that inhibit HIV replication by binding to the RRE and displacing Rev are expected, therefore, to retain activity across genetically diverse HIV infections.[23] The potent anti-HIV activity of ethidium can, in principle, be related to the inhibition of one or more essential steps of the viral lifecycle including: reverse transcription,[27–29] DNA integration,[7,30] RNA synthesis,[31–33] protein synthesis,[23,34] viral packaging, etc. Ethidium cannot be approved for human use, however, due to its ability to cause frame shift mutations in *Salmonella typhimurium* following metabolic activation (the Ames test).[8–10]

To date, a relatively small number of ethidium derivatives has been reported in the literature.[33,35–46] Early modifications included variation of the alkyl chain with groups other than ethyl (methyl, propyl, etc.).[35] Compared to ethidium, these derivatives have approximately the same DNA affinity,[36] but are significantly more toxic.[1] The 6-position of ethidium has been substituted with various groups (4-amino phenyl, 4-nitro phenyl, methyl, napthyl, etc.).[36,37] Again, similar DNA affinities were measured for these derivatives.[36] Ethidium's exocyclic amines have been converted to azido (N_3), leading to highly reactive photo-crosslinking agents.[38,39] These compounds also have a similar DNA affinity as ethidium,[38] and are also highly mutagenic.[40] Amino acids have been conjugated to ethidium through its exocyclic amines and through its phenyl ring, but the DNA affinities of these derivatives have not yet been reported.[33,41] Surprisingly, the exocyclic amines of ethidium have not, until now, been systematically substituted with other functional groups. Modification of one, or both, of these amines provides a 'modular' approach for introducing new chemical diversity onto the phenantridinium core of ethidium. These modifications dramatically affect the electronic structure of ethidium,[47] as well as the nucleic acid affinity and specificity of the resulting derivatives.

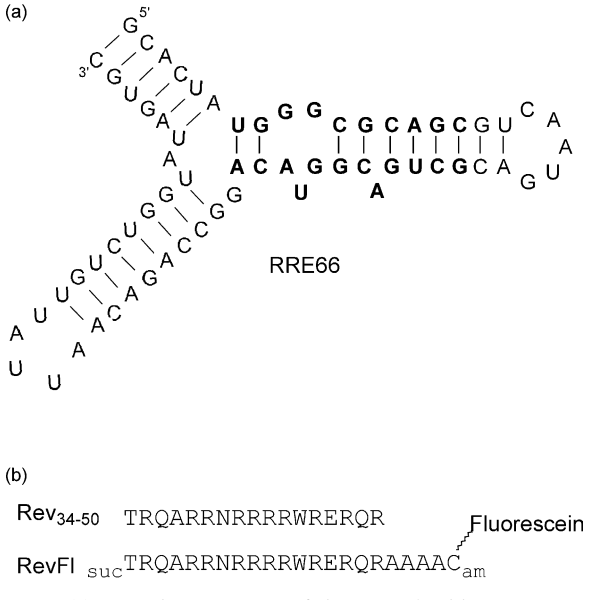

Figure 1. (a) Secondary structure of the 66 nucleotide Rev Response Element 'RRE66' from the HIV-1 isolate HXB3. The high affinity Rev binding site is shown in bold.[24] (b) The RRE binding domain 'Rev_{34-50}' from the HIV-1 Rev protein.[24] RevFl is used for fluorescence anisotropy displacement experiments and has an RRE affinity similar to that of the Rev protein.[23]

In an attempt to *decrease* its DNA affinity (and hopefully its toxic and mutagenic activities as well) we have synthesized a small library of phenathridinium derivatives by modifying the exocyclic amines of ethidium bromide. We report the synthesis, characterization, and spectroscopic properties of these new derivatives. A preliminary survey of the nucleic acid specificity of these compounds is conducted by measuring the apparent affinity of each compound to calf thymus DNA as compared to the Rev binding site on the RRE. By decreasing the DNA affinity of ethidium, it is hoped that the some derivatives will be more selective for viral RNA sites and will, consequently, have better anti-viral potency and decreased mutagenic activities.

Synthesis

The exocyclic amines of ethidium are poor nucleophiles and only weakly basic ($pK_{a1} = 0.8$, $pK_{a2} = 2$).[47,48] The electron withdrawing effect of the phenanthridinium core requires the use of highly reactive electrophiles to modify ethidium's exocyclic amines. Until now, no general method for the systematic protection and 'modular' modification of ethidium's exocyclic amines has been reported. Each of the ethidum's exocyclic amines can be protected in a one-pot reaction with benzyl chloroformate (cbz-chloride). By using one equivalent of cbz-chloride, a mixture of products is obtained that can be purified using standard silica gel chromatography (Fig. 2). The main products are 8-cbz-ethidium chloride (50%) and 3-cbz-ethidium chloride (10%). The remainder is 3,8-bis-cbz ethidium chloride (4%) and unreacted starting material (Fig. 2). The assignment of each mono-protected cbz compound (**2**) and (**3**) has been confirmed using X-ray crystallography.[47] Other research groups have also reported a greater reactivity of the exocyclic amine at the 8-position (relative to the 3-position) and have attributed the difference to steric constraints imposed by dimerization of ethdium in solution.[41] It is more likely, however, that the inherent electronic characteristics of ethidium are responsible for the differences in reactivity of its exocyclic amines.[47]

Guanidinylation of ethidium was conducted using three different methods (Fig. 3).[49–51] The best reagent for guanidinylation of ethidium proved to be *N,N′*-bis-Boc-*S*-methyl-isothiourea (activated with mercury (II) chloride) which afforded a 70% isolated yield of the protected product (3-diBoc-guanidino-8-cbz ethidium

chloride) (Fig. 3).[51] Removal of both the cbz and Boc groups was performed simultaneously by refluxing 3-diBoc-guanidino-8-cbz-ethidium chloride in 6 M HCl/MeOH for 1 h. Reversed-phase chromatography was used to purify the desired product **6** that was obtained in 70% yield. The other guanidino ethidium derivatives, **8** and **9**, were synthesized in similar yields using the same method (Fig. 3).

Urea is a non-charged isostructural analogue of guanidine, and provides an important comparison for guanidinium-containing ethidium derivatives. Once again, the limited reactivity of ethidium's electron-poor exocyclic amines rendered some urea-forming reagents ineffective.[52] By using phenyl chloroformate, however, ethidium's exocyclic amines can be activated for subsequent urea formation. This two-step approach allows for facile synthesis of substituted and unsubstituted ureas.[53] Displacement of phenol by ammonia produces the unsubstituted urea derivatives **11**, **13**, and **15** (Fig. 4). Displacement of phenol by other amines yields the substituted ureas **16**–**19** (Fig. 5).

The pyrrole-containing ethidium derivatives **20**, **21** and **22** have been prepared using 2,5-dimethoxytetrahydrofuran (Fig. 6).[54] The poor yields obtained for the mono-substitued derivatives **20** and **21** are due to problems associated with the removal of cbz using H_2/Pd. We have found that the phenanthridinium core of ethidium is susceptible to reduction and degradation under

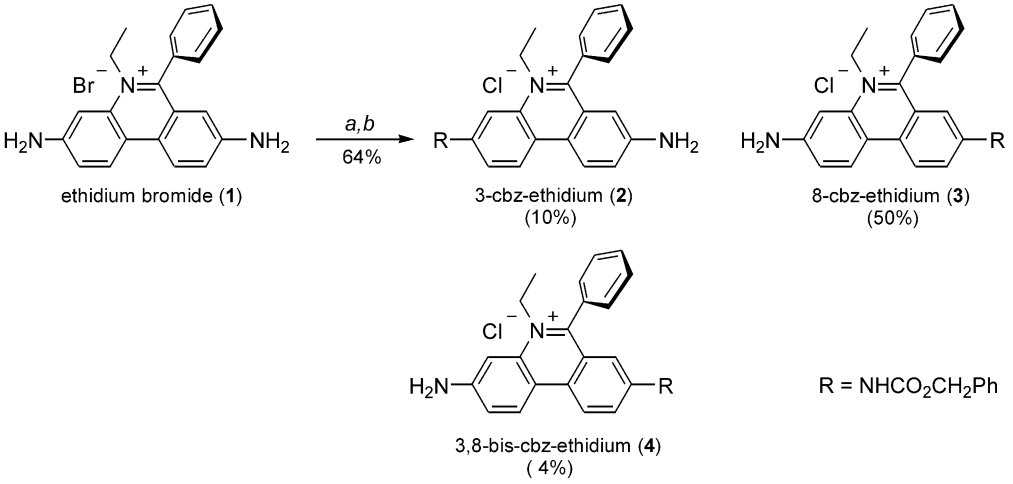

Figure 2. Protection of ethidium bromide with benzyl chloroformate. Reagents and conditions: (a) benzyl chloroformate, acetone, aqueous buffer pH 6.6; (b) AG1-X4 (Cl^-) ion exchange resin. Note: Trivial names are used for all compounds.

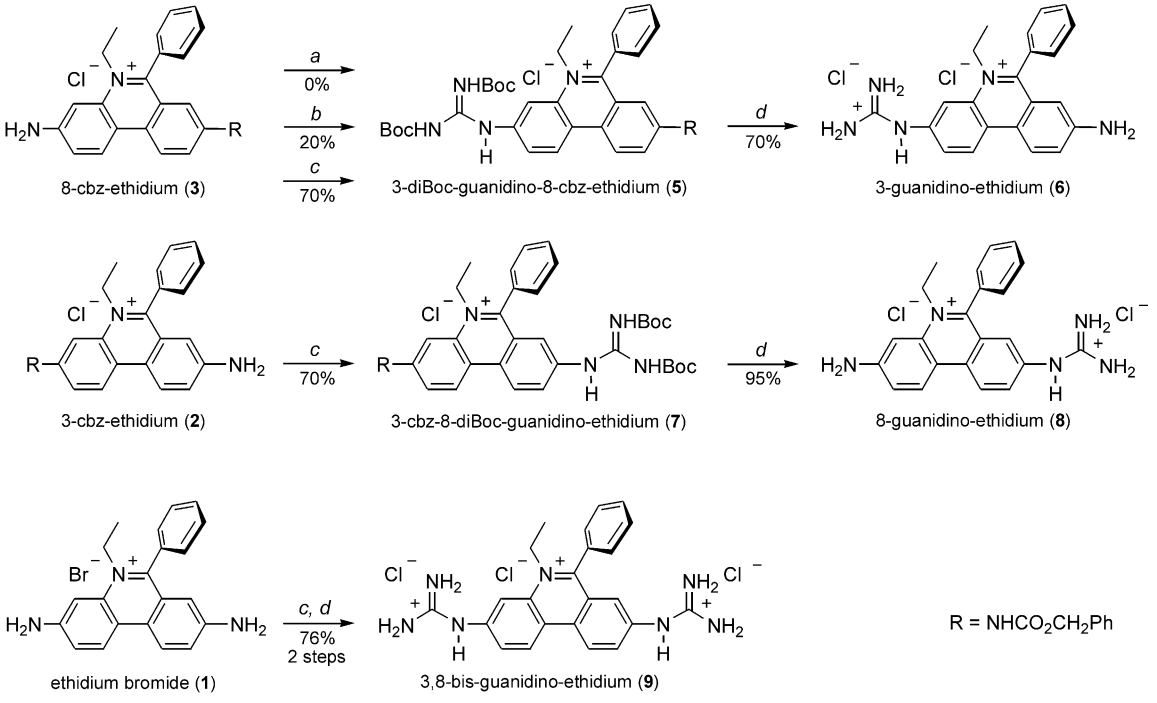

Figure 3. Synthesis of guanidino derivatives of ethidium. Reagents and conditions: (a) *N*,*N*′-diboc-*N*″-triflylguanidine;[49] (b) *N*,*N*′-di-*tert*-butoxycarbonyl-5-chloro-1H-benzotriazole-1-carboxamidine;[50] (c) *N*,*N*′-bis-boc-*S*-methyl-isothiourea, mercury dichloride, and 2,4,6-collidine;[51] (d) 6 M HCl/methanol, 100 °C.

these conditions. Better yields can be obtained by refluxing the protected products in 6 M HCl/methanol to remove cbz (step b, Fig. 6).

RRE inhibition and DNA affinity

We have used fluorescence anistropy to monitor the formation and subsequent inhibition of a Rev-RRE complex.[23] The anisotropy of a fluorescent Rev peptide 'RevFl' increases upon titration of the RRE66 (Fig. 7A). Analysis of this isotherm yields a dissociation constant of 5±1 nM.[55] This is similar to the affinity reported for Rev protein itself.[56] Upon titration of an inhibitory ligand, RevFl is displaced from the RRE into solution, and the anisotropy value decreases back to the value of the free peptide (Fig. 7B). The concentration of each inhibitor needed to displace 50% of RevFl from the RRE (the IC_{50} value) is given in Table 1. From the IC_{50} value, the apparent binding affinity (K_i) for each compound at or near the Rev binding site can be calculated.[55] This value assumes a single binding site for the small molecule is responsible for displacing Rev, hence the term *apparent* affinity is used (Table 1). Most derivatives tested have a significantly lower RRE affinity at

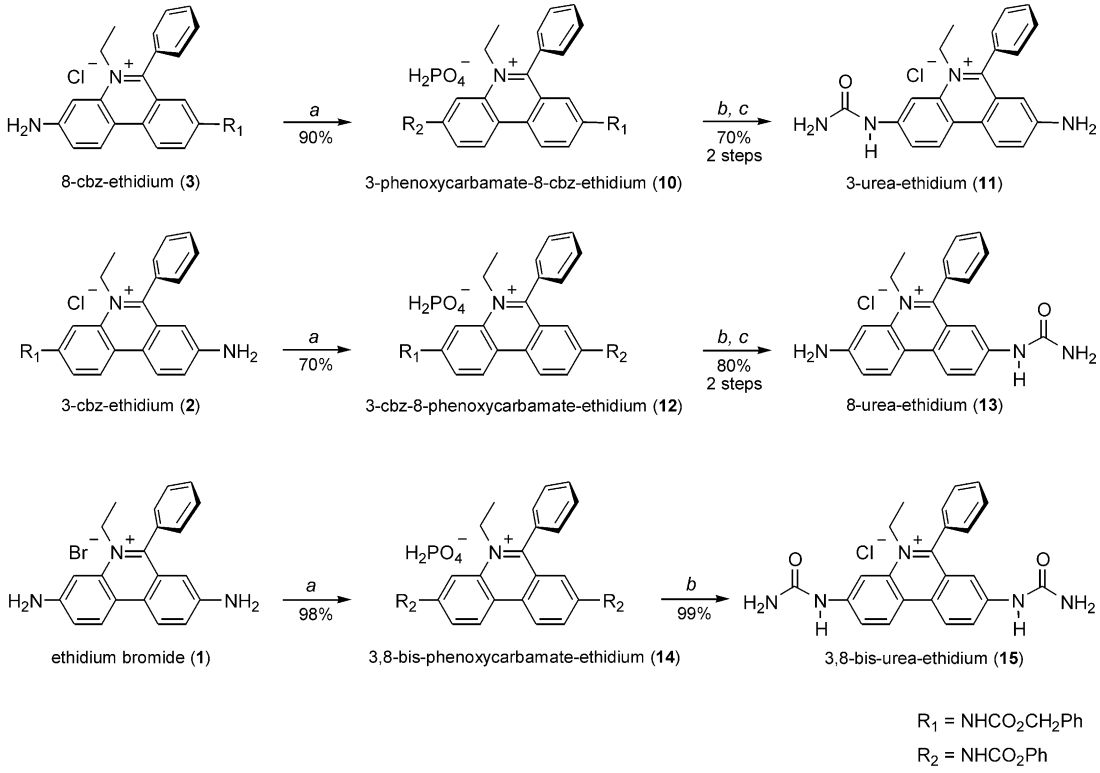

Figure 4. Synthesis of unsubstituted urea derivatives of ethidium. Reagents and conditions: (a) phenyl chloroformate, acetone, aqueous buffer pH 6.6; (b) methanolic ammonia, 76 °C; (c) 6 M HCl/methanol, 100 °C.

Table 1. Summary of RevFl displacement experiments (by anisotropy) and of direct binding experiments with calf thymus (CT) DNA

Compound (trivial names)	Rev-RRE IC_{50} (μM)[a]	Apparent RRE K_i (μM)[b]	CT DNA C_{50} (μM)[c]	Apparent CT DNA K_d (μM)[d]	RRE selectivity ratio[e]
Ethidium (**1**)	0.2	0.05	14	2.3	46
3-Guanidino-ethidium (**6**)	4.1	1.0	30	5.5	5.5
8-Guanidino-ethidium (**8**)	8.1	2.0	120	24	12
3,8-Bis-guanidino-ethidium (**9**)	11	2.8	70	14	5
3-Urea-ethidium (**11**)	0.4	0.10	120	24	240
8-Urea-ethidium (**13**)	4.0	1.0	60	12	12
3,8-Bisurea-ethidium (**15**)	>1[f]	>0.25[f]	350	70	<280[f]
3,8-Bis-urea-ethylenediamine-ethidium (**18**)	0.1	0.02	200	40	2000
3,8-Bisurea-2-DOS-ethidium (**19**)	0.2	0.05	20	3.5	70
3-Pyrole-ethidium (**20**)	0.6	0.15	40	7.5	50
8-Pyrole-ethidium (**21**)	0.4	0.10	20	3.5	35
3,8-Diamino-6-phenyl-phenanthridine	>4[f]	>1[f]	120	24	<24[f]

[a]10 nM each RevFl and RRE66, approximate error ±30% of the reported value.
[b]Apparent $K_i = ((IC_{50} - 0.007)/4)$. See ref 55.
[c]Concentration of CT DNA (in b.p.) needed to bind 1/2 of a 1 μM solution of each ligand.
[d]Apparent $K_d = ((C_{50} * 0.2) - 0.5)$. See Experimental.
[e]Ratio of (CT DNA K_d/Rev–RRE K_i).
[f]Fluorescence interference with RevFl allows only a limit to be reported.

or near the Rev binding site of the RRE as compared to ethidium bromide (Table 1). One exception is the substituted urea derivative 3,8-bis-urea-ethylenediamine-5-ethyl-6-phenylphenanthridinium trifluoroacetate (**18**). This compound has a modest (2-fold) higher apparent RRE affinity as compared to ethidium bromide (Table 1). A much larger difference in RRE specificity is, however, observed for **18**.

To evaluate the RRE specificity and mutagenic potential of each compound, the binding affinity to calf thymus (CT) DNA has been determined. The fluorescence emission spectrum of each derivative shows unique changes upon binding CT DNA (see Fig. 8 for a representative titration, and Table 2 for a summary of the spectral changes of each compound). The concentration of DNA (in base pairs) needed to bind 50% of each compound (the C_{50} value) is measured by assuming that the change in fluorescence intensity of each compound is proportional to the fraction of the compound bound by DNA (see inset of Fig. 8). From each C_{50} value, the apparent binding affinity (K_d) is calculated by assuming that the binding stoichiometry established for ethidium bromide and CT DNA (0.2 ethidium molecules per base pair),[55] holds for all compounds tested (Table 1). By taking the apparent affinity of each compound to CT DNA divided by its apparent affinity to the Rev binding site, an RRE selectivity ratio is calculated (Table 1). The higher this ratio is, the more selective each compound is for the RRE (relative to CT DNA). Interestingly, all the compounds evaluated exhibit a higher affinity to the RRE as compared to CT DNA (Table 1). This is consistent with the observation that intercalating agents have a higher affinity to duplex regions that contain bulged bases and other imperfections as compared to unperturbed duplexes.[32,57]

3,8-Diamino-6-phenylphenanthridine, an uncharged analogue of ethidium, has at least a 10-fold lower affinity to both the RRE and CT DNA (Table 1).[58] This suggests that the positive charge afforded by ethidium's quarternary amine is important for its high-affinity binding of DNA and the RRE. It was hypothesized that the conversion of the amino groups on ethidium into guanidinium would increase its total charge and, therefore, increase its affinity to the RRE. Indeed, at pH 7.5, the guanidino derivatives **6**, **8**, and **9** each have a 2+ charge, while ethidium has a charge of 1+.[47] According to RevFl displacement experiments, the three guanidino derivatives **6**, **8**, and **9** have a 20–60-fold *lower* RRE affinity as compared to ethidium (Table 1). It is possible, that the lower RRE affinity of these guanidinium derivatives is due to unfavorable steric interactions. To test this, the urea derivatives **11**, **13**, and **15** were evaluated

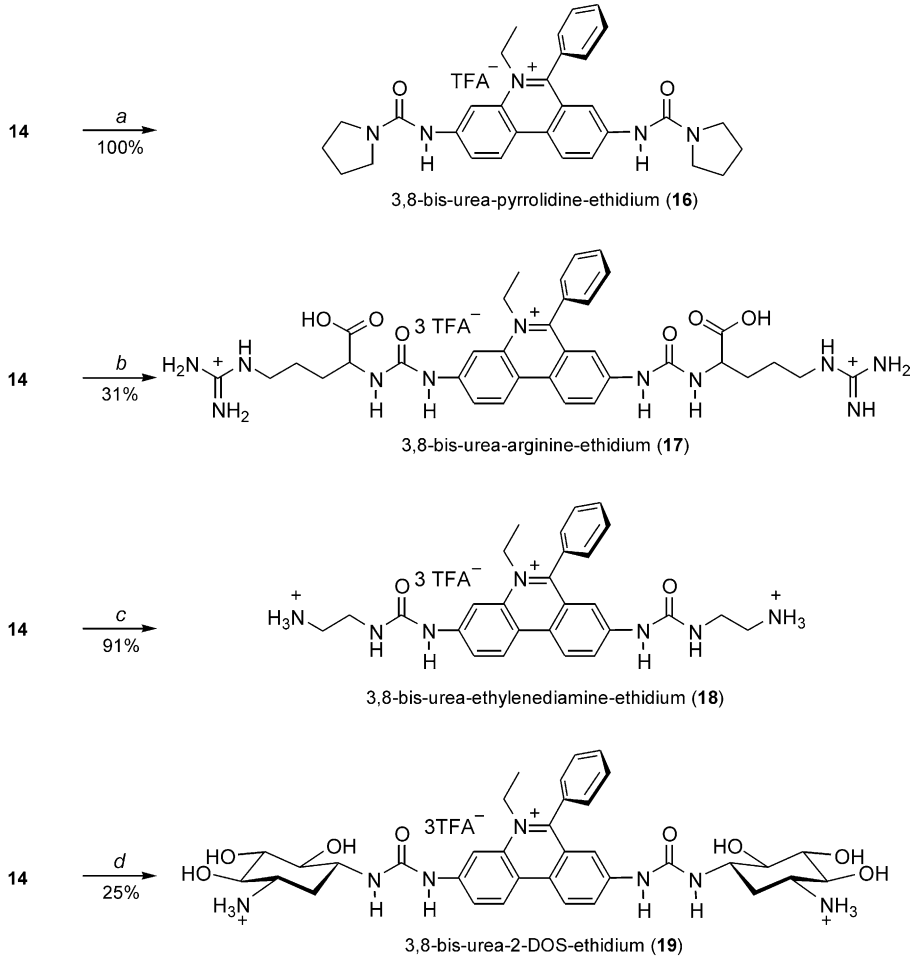

Figure 5. Examples of ethidium-urea conjugates. Reagents and conditions: (a) pyrrolidine, DMSO, 90 °C; (b) L-Arg, water/DMSO, 2,4,6-collidine, 90 °C; (c) ethylene diamine, DMSO, 85 °C; (d) 2-deoxystreptamine, water/DMSO, phenol, Na_2CO_3, 90 °C.

for RRE affinity (Table 1). Interestingly, the urea derivatives have much better RRE affinities as compared to the corresponding guanidino derivatives (Table 1). It appears, therefore, that the additional positive charge of the guanidino derivatives actually *decreases* their RRE affinity. This is the opposite trend as observed for compounds that bind to the surfaces of RNA and DNA.[59,60] It is possible that the introduction of an additional charged group disrupts the charge distribution on the core of ethidium, resulting in less favorable base stacking interactions.[47] Alternatively, the desolvation of the guanidinium group upon intercalation may impose a significant energetic penalty for binding.[61] Interestingly, the urea derivatives **11** and **15** have lower affinities to CT DNA as compared to the corresponding guanidino derivatives **6** and **9** (Table 1). This is the opposite trend as observed for the RRE. Despite their differences in affinity, the changes in spectral properties of 3,8-bis-guanidino ethidium (**9**) (upon saturation with CT DNA) are very similar to the changes observed for bis-urea-ethidium (**15**), suggesting a common binding mode for these derivatives (Table 2). It is possible that neither of these compounds actually intercalates into CT DNA and that the higher positive charge afforded by **9** gives it a higher affinity to the surface of CT DNA when compared to **15**.

Ethidium bromide is very selective for the RRE, exhibiting almost a 50-fold higher affinity to the RRE as compared to CT DNA. The unsubstituted urea derivative, 3-urea ethidium (**11**), is even more selective, exhibiting a 240-fold higher affinity to the RRE as compared to CT DNA (Table 1). **11** has a modestly lower RRE affinity when compared to ethidium, but it has a much lower affinity to CT DNA, resulting in a 5-fold higher RRE selectivity ratio than ethidium (Table 1). A number of other compounds, including **8**, **15**, and **18** also have substantially lower affinities to CT DNA as compared to ethidium (Table 1). This should, in theory, decrease the mutagenic potential of these compounds. One compound, 3,8-bis-urea-ethylene-diamine-ethidium (**18**) has both a higher RRE affinity *and* a lower DNA affinity as compared to ethidium. **18** has about a 2-fold higher RRE affinity and a 20-fold lower DNA affinity as compared to **1** (Table 1). It is possible that the guanidinylation of **18** will improve its RRE affinity and specificity.[60] Interestingly, a related compound 3,8-bis-urea-2-DOS-ethidium (**19**) shows a less promising RRE selectivity as compared to **18** (Table 1). Despite their chemical similarities, **19** has a 10-fold higher DNA affinity when compared to **18**. These compounds also show different spectral changes upon saturation with CT DNA (Table 2). The fluorescence intensity of **19** increases upon binding CT DNA, while the intensity of **18** decreases (Table 2). This may indicate different binding modes (surface binding versus intercalation) of these compounds.

Implications

The discovery of new ligands that possess both high affinity and high specificity for a therapeutically impor-

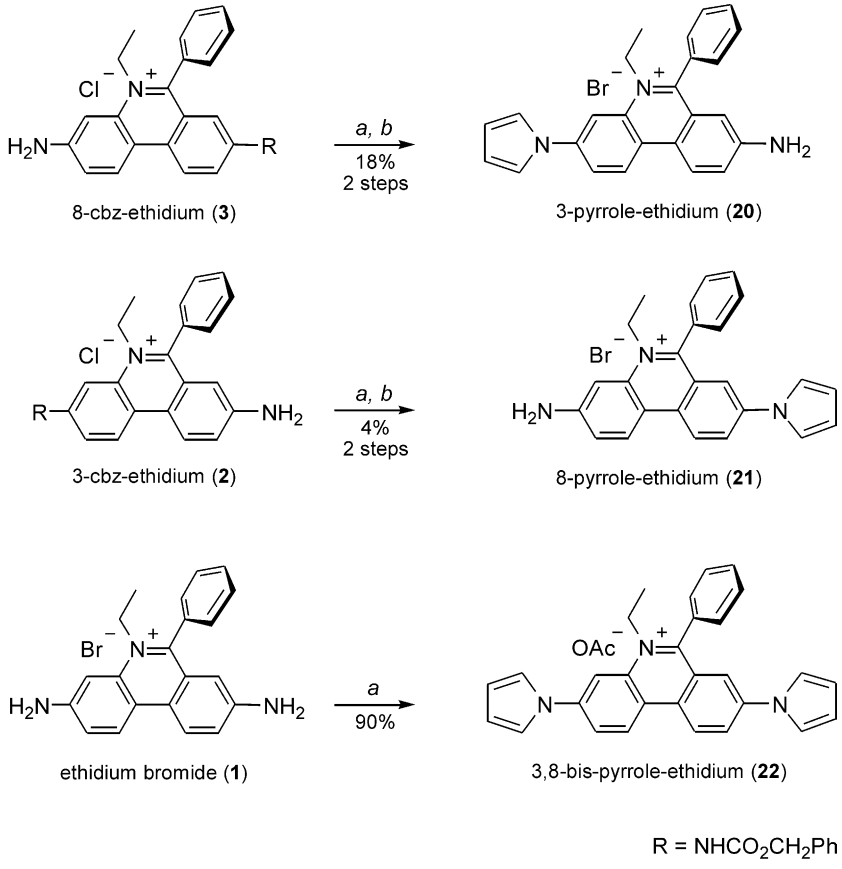

Figure 6. Synthesis of pyrrole derivatives of ethidium. Reagents and conditions: (a) 2,5-dimethoxy tetrahydrofuran, acetic acid, 120 °C; (b) H_2/Pd.

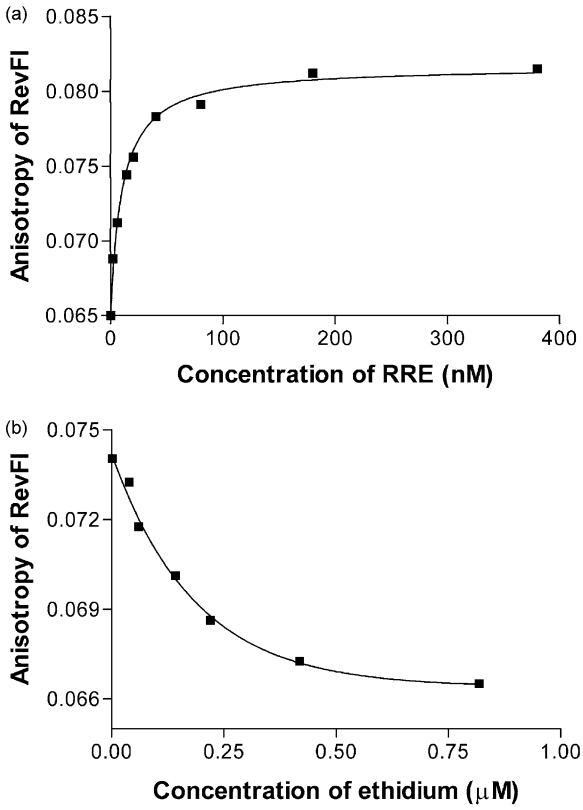

Figure 7. Examples of RevFl-RRE association and inhibition. (**A**) The fluorescence anisotropy of a 10 nM solution of RevFl is monitored as the RRE66 is titrated. (**B**) Upon mixing 10 nM each of RevFl and RRE66, ethidium bromide (**1**) is titrated while monitoring the fluorescence anisotropy of RevFl (**B**).

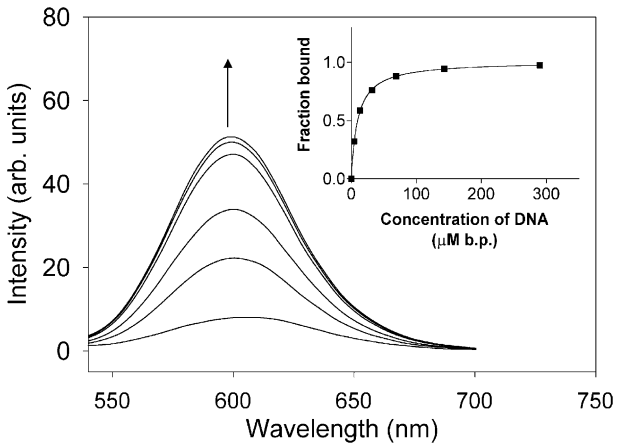

Figure 8. The binding of ethidium bromide (**1**) by calf thymus (CT) DNA. The fluorescence intensity of a 1 μM solution of ethidium in aqueous buffer increases upon addition of CT DNA (excitation 480 nm). By assuming a linear relationship between fluorescence intensity (606 nm) and the fraction bound, a simple binding isotherm is revealed.

tant RNA site is a challenging goal.[55] The synthetic modification of ethidium's exocyclic amines has, for one compound (**18**), accomplished this aim. Interestingly, the metabolic activation of ethidium's exocyclic amines by at least three separate enzymes is known to be important for its mutagenic activities in vivo.[62,63] Most of the novel derivatives presented here (including **15**–**19**) should *not* be recognized by enzymes that modify aromatic amines.[63] In addition, most of these novel derivatives possess significantly lower DNA affinities when compared to ethidium bromide. Taken together, this suggests that these new derivatives will have significantly lower mutagentic activities than ethidium. These properties, along with the anti-HIV activities and other potential therapeutic applications are currently being investigated.

Experimental

Nucleic acids binding

A solution of sonicated CT DNA was purchased from Gibco BRL and quantified using a molecular extinction coefficient of 13,100 cm^{-1} M^{-1} per base pair.[19] All titrations and photophysical properties were measured at 22 °C in a buffer containing 30 mM HEPES (pH 7.5),

Table 2. Summary of the maximum wavelength of absorbance (λ_{max}), maximum wavelength of emission (λ_{em}), and change in emission intensity upon saturation with CT DNA[a]

Compound	λ_{max} (nm)	Δ in λ_{max} (nm) with DNA[b]	λ_{em} (nm)	Δ in λ_{em} (nm) with DNA[b]	Change in emission intensity[c] (%)
Ethidium (**1**)	480	+40	606	−7	+520
3-Guanidino-ethidium (**6**)	444	+31	590	−4	+40
8-Guanidino-ethidium (**8**)	454	+36	605	+7	+150
3,8-Bis-guanidino-ethidium (**9**)	397	+24	500	0	−50
3-Urea-ethidium (**11**)	458	+34	587	−17	+300
8-Urea-ethidium (**13**)	463	+31	601	−13	+570
3,8-Bis-urea-ethidium (**15**)	434	+21	520	0	−63
3,8-Bis-urea-ethylene-diamine-ethidium (**18**)	438	+22	522	−2	−11
3,8-Bis-urea-2DOS-ethidium (**19**)	438	+22	522	−4	+30
3-Pyrole-ethidium (**20**)	454	+39	592	−18	+300
8-Pyrole-ethidium (**21**)	462	+36	603	−10	+580
3,8-Bis-pyrole-ethidium (**22**)	429	+18	502	0	0
3,8-Diamino-6-phenyl-phenanthridine[d]	402	+111	533	0	−75

[a]10 μM of each compound in aqueous buffer pH 7.5 (see Experimental).
[b]Difference upon saturation with calf thymus DNA.
[c]Percent change in total emission intensity upon saturation with CT DNA, relative to the intensity of the compound in buffer only (excitation at λ_{max}).
[d]Becomes protonated upon binding DNA, see ref 58.

KCl (100 mM), sodium phosphate (10 mM), NH_4OAc (20 mM), guanidinium HCl (20 mM), $MgCl_2$ (2 mM), NaCl (20 mM), EDTA (0.5 mM), and Nonidet P-40 (0.001%). Fluorescence anisotropy experiments were conducted and K_i values calculated as described.[55] CT DNA affinities were measured by exciting a 1 μM solution of each compound at the appropriate wavelength (λ_{max}) then adding small aliquots of concentrated CT DNA and monitoring the emission intensity of the compound at the appropriate wavelength (λ_{em}). K_d values are calculated from C_{50} values based upon the definition of K_d:

$$K_d = ([\text{dye}]^*[\text{nucleic acid}])/[\text{complex}] \quad (1)$$

Once the C_{50} value is reached, the concentration of free dye [dye] is equal to the concentration of bound dye [complex], so that the K_d = the concentration of free nucleic acid in available binding sites [nucleic acid]. This value is equal to the total concentration of nucleic acid (in base pairs) multiplied by the binding stoichiometry (0.2 equivalents of compound per base pair),[55] minus the concentration of complex (0.5 μM). Hence, under these conditions, the $K_d = ((C_{50}*0.2)-0.5)$.

3-cbz-Ethidium · Cl (2), 8-cbz-ethidium · Cl (3), and 3,8-bis-cbz-ethidium · Cl (4). Ethidium bromide/8% water (4.13 g, 9.64 mmol) was dissolved in 0.2 M sodium phosphate pH 6.6 (100 mL), acetone (80 mL) and warmed to 32 °C. To this, a solution of benzyl chloroformate (1.43 mL, 10 mmol, 1 equiv) in acetone (20 mL) was slowly added and the reaction warmed to 40 °C for 20 min. AG1-X4 (Cl^-) ion exchange resin (20 g, 70 mmol, 7 mequiv) was then added and stirred 5 min, 40 °C. The slurry was loaded onto a column containing another 20 g (7 mequiv) of AG1-X4 (Cl^-) ion exchange resin and the eluent collected. The resin was washed with 30 mL of 1:1 water/acetone, and the eluents were combined and reduced to a solid under reduced pressure. The products were separated on silica gel using three consecutive columns (8–10% MeOH/CH_2Cl_2, 5–12% MeOH/CH_2Cl_2, and 10% MeOH/CH_2Cl_2). The pure fractions from each column were combined to yield: 0.48 g of the orange-red solid, **3-cbz-ethidium · Cl (2)** (10%). R_f = 0.5 (20% MeOH/$CHCl_3$). ^{1}H NMR (400 MHz, DMSO-d_6, 25 °C): δ 10.61 (s, 1H), δ 8.92 (d, J = 9.2 Hz, 1H), δ 8.76 (d, J = 9.2 Hz, 1H), δ 8.67 (s, 1H), δ 7.97 (d, J = 9.2 Hz, 1H), δ 7.72–7.79 (m, 5H), δ 7.58 (dd J_1 = 9.2 Hz, J_2 = 2.2 Hz, 1H), δ 7.36–7.48 (m, 5H), δ 6.38 (d, J = 2.2 Hz, 1H), δ 6.22 (s, 2H), δ 5.24 (s, 2H), δ 4.54 (q, J = 7.0 Hz, 2H), δ 1.45 (t, J = 7.0 Hz, 3H). ESI MS calcd for $C_{29}H_{26}N_3O_2$: 448.2, found 448.3 $[M]^+$. UV–vis (50 mM sodium phosphate pH 7.5): λ_{max} (nm) and ε (cm^{-1} M^{-1}): 212 (4.1×10^4), 284 (5.6×10^4), 454 (4.9×10^3). 2.3 g of the purple solid, **8-cbz-ethidium · Cl (3)** (50%). R_f = 0.38 (20% MeOH/$CHCl_3$). ^{1}H NMR (400 MHz, DMSO-d_6, 25 °C): δ 10.31 (s, 1H), δ 8.83 (d, J = 9.6 Hz, 1H), δ 8.78 (d, J = 9.2 Hz, 1H), δ 8.11 (dd J_1 = 9.6 Hz, J_2 = 1.6 Hz, 1H), δ 7.71–7.77 (m, 5H), δ 7.64 (d, J = 1.6 Hz, 1H), 7.44 (s, 1H), δ 7.36–7.42 (m, 6H), 6.66 (s, 2H), 5.08 (s, 2H), δ 4.49 (q, J = 7.2 Hz, 2H), δ 1.42 (t, J = 7.2 Hz, 3H). ESI MS calcd for $C_{29}H_{26}N_3O_2$: 448.2, found 448.3 $[M]^+$. UV–vis (50 mM sodium phosphate pH 7.5): λ_{max} (nm) and ε (cm^{-1} M^{-1}): 214 (3.4×10^4), 286 (4.4×10^4), 460 (4.5×10^3). 0.23 g of the yellow solid, **3,8-bis-cbz-ethidium · Cl (4)** (4%). R_f = 0.67 (20% MeOH/$CHCl_3$). ^{1}H NMR (400 MHz, DMSO-d_6, 25 °C): δ 10.76 (s, 1H), δ 10.45 (s, 1H), δ 8.85 (d, J = 9.2 Hz, 1H), δ 9.03 (d, J = 9.2 Hz, 1H), δ 8.78 (s, 1H), δ 8.28 (dd J_1 = 9.2 Hz, J_2 = 2.0 Hz, 1H), δ 8.10 (d, J = 9.2 Hz, 1H), δ 7.74–7.81 (m, 6H), δ 7.33–7.49 (m, 10H), δ 5.26 (s, 2H), 5.10 (s, 2H), δ 4.62 (q, J = 7.0 Hz, 2H), δ 1.49 (t, J = 7.2 Hz, 3H). ESI MS calcd for $C_{37}H_{32}N_3O_4$: 582, found 582 $[M]^+$.

3-diBoc-guanidino-8-cbz-ethidium · Cl (5). 8-cbz Ethidium · Cl (**3**) (48 mg, 100 μmol, DMF (4 mL), *N*,*N*′-bis-Boc-*S*-methyl-isothiourea (145 mg, 500 μmol, 5 equiv), and mercury (II) chloride (227 mg, 837 μmol, 8.4 equiv) were combined and sonicated. 2,4,6 collidine (177 μL, 1.34 mmol, 13.3 equiv) was added dropwise and the reaction was stirred at rt for 15 min with occasional sonication. The reaction mixture was then dissolved in $CHCl_3$ (150 mL) and washed with 0.1 M citric acid (4×40 mL), brine (40 mL), dried over sodium sulfate and concentrated to a solid under reduced pressure. The product was purified on a short (2-inch) silica gel column (20 mL) using a gradient (0–5% MeOH/$CHCl_3$) to yield 47 mg of a solid yellow product (70%). ^{1}H NMR (400 MHz, DMSO-d_6, 25 °C): δ 11.32 (s, 1H), δ 10.51 (s, 1H), δ 10.44 (s, 1H), δ 9.23 (s, 1H), δ 9.06–9.09 (m, 2H), δ 8.26 (d, J = 9.2 Hz, 1H), δ 8.17 (d, J = 8.4 Hz, 1H), δ 7.77–7.80 (m, 7H), δ 7.35–7.38 (m, 5H), δ 5.10 (s, 2H), δ 4.68 (q, J = 7.2 Hz, 2H), δ 1.33–1.56 (m, 21H).

3-Guanidino-ethidium · 2HCl (6). 3-diBoc-guanidino-8-cbz-ethidium · Cl (**5**) (20 mg, 29 μmol) was dissolved in methanol (2 mL) and saturated HCl (2 mL), and heated at 120 °C for 40 min. The reaction flask was then cooled on ice and 2 M NaOH was added dropwise until the yellow solution started to turn orange. The solution was loaded directly onto an activated Water's 'Sep-pack' C-18 reversed-phase column (activated with 10 mL acetonitrile, 10 mL water) and washed with water (5 mL). The product was eluted with 20–30% acetonitrile/water (0.01 M HCl) and lyophilized to yield 13 mg (99%) of an orange solid. ^{1}H NMR (300 MHz, DMSO-d_6, 25 °C): δ 10.61 (s, 1H), δ 9.01 (d, J = 9.0 Hz, 1H), δ 8.85 (d, J = 9.3 Hz, 1H), δ 8.34 (s, 1H), δ 7.74–7.91 (m, 10H), δ 7.62 (dd J_1 = 9.0 Hz, J_2 = 2.1 Hz, 1H), δ 6.43 (d, J = 2.1 Hz, 1H), δ 6.35 (br s, 2H), δ 4.66 (q, J = 6.9 Hz, 2H), δ 1.43 (t, J = 7.1 Hz, 3H). FAB MS calculated for $C_{22}H_{22}N_5$: 356.1876, found 356.1892 $[M]^+$. UV–vis (50 mM sodium phosphate pH 7.5): λ_{max} (nm) and ε (cm^{-1} M^{-1}): 214 (3.3×10^4), 284 (4.8×10^4), 444 (4.1×10^3).

3-cbz-8-diBoc-guanidino-ethidium · Cl (7). 3-cbz-Ethidium · Cl (**2**) (37 mg, 76 μmol, DMF (4 mL), *N*,*N*′-bis-Boc-*S*-methyl-isothiourea (112 mg, 290 μmol, 3.8 equiv), and mercury dichloride (175 mg, 645 μmol, 8.5 equiv) were combined, sonicated, and 2,4,6-collidine (136 μL, 1.03 mmol, 13.5 equiv) was added dropwise. The reaction was stirred at rt for 30 min with occasional sonication. The reaction was then diluted into $CHCl_3$ (200 mL) and washed with 0.1 M citric acid (3×50 mL),

brine (50 mL), dried over sodium sulfate and concentrated to a solid under reduced pressure. The product was purified on a short (2-inch) silica gel column (20 mL) using a gradient (0–5% MeOH/$CHCl_3$) to yield 40 mg of a solid yellow product (76%). ^{1}H NMR (300 MHz, DMSO-d_6, 25 °C): δ 10.92 (s, 1H), δ 10.74 (s, 1H), δ 10.14 (s, 1H), δ 9.13 (d, J = 9.3 Hz, 1H), δ 9.04 (d, J = 9.3 Hz, 1H), δ 8.78 (s, 1H), δ 8.31 (dd J_1 = 9.0 Hz, J_2 = 2.1 Hz, 1H), δ 8.08 (d, J = 8.7 Hz, 1H), δ 7.85 (d, J = 2.1 Hz, 1H), δ 7.75–7.77 (m, 5H), δ 7.37–7.49 (m, 5H), δ 5.26 (s, 2H), δ 4.59 (q, J = 6.3 Hz, 2H), δ 1.31–1.51 (m, 21H).

8-Guanidino-ethidium · 2HCl (8). 3-cbz-8-diBoc-guanidino-ethidium · Cl (**7**) (6 mg, 8.7 μmol) was dissolved in 6 M HCl (2 mL) and heated to 100 °C for 1 h. The reaction flask was then cooled on ice and $NaHCO_3$ was added until the yellow solution turned orange. The solution was loaded directly onto an activated Water's 'Sep-pack' C-18 reversed-phase column (activated with 10 mL acetonitrile, 10 mL water). The column was washed with 1 M NaCl (5 mL), water (5 mL) and the product eluted with 25% acetonitrile/water (0.01 M HCl) and lyophilized to yield 3.5 mg (95%) of an orange solid. ^{1}H NMR (300 MHz, DMSO-d_6, 20 °C): δ 10.28 (s, 1H), δ 8.91 (d, J = 9.6 Hz, 1H), δ 8.88 (d, J = 9.3 Hz, 1H), δ 8.02 (dd J_1 = 9.3 Hz, J_2 = 2.1 Hz, 1H), δ 7.72–7.85 (m, 9H), δ 7.47 (s, 1H), δ 7.43 (d, J = 8.7 Hz, 1H), 7.02 (d, J = 2.1 Hz, 1H), δ 6.78 (br s, 2H), δ 4.52 (q, J = 6.9 Hz, 2H), δ 1.43 (t, J = 6.9 Hz, 3H). FAB MS calculated for $C_{22}H_{22}N_5$: 356.1876, found 356.1862 $[M]^+$. UV–vis (50 mM sodium phosphate pH 7.5): λ_{max} (nm) and ε (cm^{-1} M^{-1}): 213 (2.8×10^4), 242 (1.5×10^4), 288 (3.6×10^4), 453 (4.4×10^3).

3,8-Bis-guanidino-ethidium · 3HCl (9). Ethidium bromide/8% water (**1**) (30 mg, 70 μmol) was dissolved in DMF (3 mL), brought to 0 °C, and *N*,*N*′-bis-Boc-*S*-methyl-isothiourea (180 mg, 0.62 mmol, 8.8 equiv), mercury dichloride (282 mg, 1.04 mmol, 15 equiv) and 2,4,6-collidine (220 μL, 1.67 mmol, 24 equiv) were added. The reaction was slowly warmed to rt, stirred for an additional 12 h, then diluted into 150 mL $CHCl_3$ and washed with 0.1 M citric acid (3×50 mL), 50 g/L of EDTA (40 mL), brine (40 mL) dried over sodium sulfate and concentrated to a solid under reduced pressure. Silica gel (40 mL) was used to purify the BOC-protected product (1.5–5% MeOH/$CHCl_3$) to yield 40 mg of a yellow solid. The product was then deprotected by adding TFA (4 mL, containing 2.5% (v/v) of triisopropylsilane) and mixing for 1 h at rt, it was then diluted into 150 mL water and washed with diethyl ether (3×40 mL) and $CHCl_3$ (3×40 mL). The aqueous phase was concentrated to a solid, then dissolved in water (5 mL) and treated with AG1-X4 (Cl^-) ion exchange resin (1.5 g, 5.2 mmol, 74 mequiv) for 5 min at rt. It was then filtered over an activated Water's 'Sep-pack' C-18 reversed phase column (activated with 10 mL acetonitrile, 10 mL water), the remainder of the product eluted from the column using 20% acetonitrile/water and lyophilized to 25 mg of a yellow solid (76%). ^{1}H NMR (300 MHz, D_2O, 25 °C): δ 8.90 (d, J = 9.0 Hz, 1H), δ 8.83 (d, J = 9.3 Hz, 1H), δ 8.22 (d, J = 1.8 Hz, 1H), δ 7.96 (dd J_1 = 8.7 Hz, J_2 = 2.1 Hz, 1H), δ 7.84 (dd J_1 = 9.0 Hz, J_2 = 1.8 Hz, 1H), δ 7.57–7.65 (m, 3H), δ 7.42–7.46 (m, 2H), δ 7.32 (d, J = 2.4 Hz, 1H), δ 4.73 (q, J = 7.2 Hz, 2H), δ 1.39 (t, J = 7.2 Hz, 3H). ESI MS calculated for $C_{23}H_{24}N_7$: 398.2, found 398.3 $[M]^+$. UV–vis (50 mM sodium phosphate pH 7.5): λ_{max} (nm) and ε (cm^{-1} M^{-1}): 213 (3.6×10^4), 278 (5.4×10^4), 398 (5.0×10^3).

3-Phenoxycarbamate-8-cbz-ethidium · H_2PO_4 (10). 8-cbz-ethidium · Cl (**3**) (180 mg, 372 μmol), acetone (12 mL), and 500 mM sodium phosphate pH 6.6 (4 mL) were combined and phenyl chloroformate (200 μL, 1.58 mmol, 4.2 equiv, diluted into 2 mL of acetone) was added dropwise and stirred for 2 h at rt. Water (5 mL) was then added dropwise and the precipitate was collected by vacuum filtration. The precipitate was then washed with water (10 mL), 3:1 water/acetone (10 mL) and eluted from the filter with methanol (200 mL). The methanolic fraction was then concentrated to a solid under reduced pressure to give 211 mg of a yellow solid (90%). ^{1}H NMR (400 MHz, DMSO-d_6, 20 °C): δ 11.20 (s, 1H), δ 10.46 (s, 1H), δ 9.13 (d, J = 8.8 Hz, 1H), δ 9.05 (d, J = 9.2 Hz, 1H), δ 8.82 (d, J = 1.2 Hz, 1H), δ 8.29 (dd J_1 = 9.2 Hz, J_2 = 2.0 Hz, 1H), δ 8.14 (dd J_1 = 8.8 Hz, J_2 = 1.2 Hz, 1H), δ 7.76–7.81 (m, 6H), δ 7.45–7.49 (m, 2H), δ 7.30–7.39 (m, 8H), δ 5.10 (s, 2H), δ 4.62 (q, J = 7.2 Hz, 2H), 1.49 (t, J = 7.6 Hz, 3H). ESI MS calcd for $C_{36}H_{30}N_3O_4$: 568.2, found 568.3 $[M]^+$.

3-Urea-ethidium · Cl (11). In a 15 mL pressure tube, 3-phenoxycarbamate-8-cbz-ethidium · H_2PO_4 (**10**) (40 mg, 60 μmol) and methanol (8 mL) were mixed and brought to −78 °C whereupon approximately 3 mL of ammonia was added (by bubbling in ammonia gas). The pressure was tube sealed and allowed to warm to rt. The reaction was then heated at 76 °C for 2 h, cooled back to −78 °C, the tube opened and the ammonia was out-gassed by passing argon into the solution as it slowly warmed to rt. All volatiles were then removed under reduced pressure. The solid yellow product was then dissolved in 8 mL of 1:1 mixture of methanol and saturated HCl (in water) and heated at 96 °C for 1 h. The reaction was then concentrated to a solid under reduced pressure and purified by reversed-phase chromatography (C-18 silica gel 60). The column was conditioned with methanol, water, and the crude product was then loaded in water (0.01 M HCl) and a methanol gradient [0–10% methanol/water (0.01 M HCl)] was used to separate ethidium chloride (elutes first) from the desired product (elutes second) to yield 16.5 mg of an orange solid (70%). ^{1}H NMR (400 MHz, DMSO-d_6, 20 °C): δ 9.58 (s, 1H), δ 8.83 (d, J = 9.2 Hz, 1H), δ 8.75 (d, J = 1.2 Hz, 1H), δ 8.73 (d, J = 9.2 Hz, 1H), δ 7.85 (dd J_1 = 9.2 Hz, J_2 = 2.0 Hz, 1H), δ 7.73–7.78 (m, 5H), δ 7.57 (dd J_1 = 8.8 Hz, J_2 = 2.4 Hz, 1H), δ 6.35 (d, J = 2.0 Hz, 1H), 6.29 (br s, 2H), 6.04 (br s, 2H), δ 4.53 (q, J = 7.6 Hz, 2H), δ 1.45 (t, J = 7.0 Hz, 3H). ESI MS calcd for $C_{22}H_{21}N_4O$: 357.2, found 357.3 $[M]^+$. UV–vis (50 mM sodium phosphate pH 7.5): λ_{max} (nm) and ε (cm^{-1} M^{-1}): 214 (2.4×10^4), 284 (3.5×10^4), 458 (3.1×10^3).

3-Cbz-8-phenoxycarbamate-ethidium · H_2PO_4 (12). 3-cbz-ethidium · Cl (**2**) (180 mg, 372 μmol), acetone (10

mL), and 500 mM sodium phosphate pH 6.6 (4 mL) were combined and phenyl chloroformate (200 μL, 1.58 mmol, 4.2 equiv, diluted into 2 mL of acetone) was added dropwise and stirred for 30 min at rt. Water was then added (6 mL) dropwise and the precipitate collected by vacuum filtration and washed with water (20 mL). The precipitate was dried under reduced pressure to yield 195 mg (79%) of a yellow solid. ^{1}H NMR (300 MHz, DMSO-d_6, 20 °C): δ 10.88 (s, 1H), δ 10.74 (s, 1H), δ 9.11 (d, J = 9.3 Hz, 1H), δ 9.07 (d, J = 9.0 Hz, 1H), δ 8.29 (dd J_1 = 9.0 Hz, J_2 = 1.8 Hz, 1H), δ 8.09 (d, J = 8.4 Hz, 1H), δ 7.74–7.76 (m, 5H), δ 7.37–7.49 (m, 7H), δ 7.16–7.29 (m, 3H), δ 5.26 (s, 2H), δ 4.62 (q, J = 7.5 Hz, 2H), 1.49 (t, J = 7.2 Hz, 3H). ESI MS calcd for $C_{36}H_{30}N_3O_4$: 568.2, found 568.3 $[M]^+$.

8-Urea-ethidium · Cl (13). In a 15 mL pressure tube, 3-Cbz-8-phenoxycarbamate-ethidium · H_2PO_4 (**12**) (45 mg, 67 μmol) and methanol (8 mL) were mixed and brought to −78 °C whereupon approximately 2 mL of ammonia was added (by bubbling in ammonia gas). The pressure tube was sealed and allowed to warm to rt. The reaction was then heated at 80 °C for 1 h, cooled back to −78 °C, the tube opened and the ammonia was out-gassed by passing argon into the solution as it slowly warmed to rt. All volatiles were then removed under reduced pressure. The solid yellow product was then dissolved in 10 mL of 1:1 mixture of methanol and saturated HCl (in water) and heated at 96 °C for 1 h. The reaction was then concentrated to a solid under reduced pressure and purified by reversed-phase chromatography (C-18 silica gel 60). The column was conditioned with methanol, water, and the crude product was loaded in 2% methanol/water (0.01 M HCl) and a methanol gradient [2–10% methanol/water (0.01 M HCl)] was used to separate ethidium chloride (elutes first) from the desired product (elutes second), to afford 21 mg of an orange solid (80%). ^{1}H NMR (300 MHz, DMSO-d_6, 20 °C): δ 9.12 (s, 1H), δ 8.76 (d, J = 9.0 Hz, 2H), δ 8.22 (dd J_1 = 9.0 Hz, J_2 = 2.1 Hz, 1H), δ 7.71–7.77 (m, 5H), δ 7.33–7.39 (m, 5H), δ 6.55 (br s, 2H), 5.97 (br s, 2H), δ 4.48 (q, J = 7.2 Hz, 2H), δ 1.41 (t, J = 6.9 Hz, 3H). ESI MS calcd for $C_{22}H_{21}N_4O$: 357.2, found 357.3 $[M]^+$. UV–vis (50 mM sodium phosphate pH 7.5): λ_{max} (nm) and ε (cm^{-1} M^{-1}): 286 (4.9×10^4), 464 (4.7×10^3).

3,8-Bis-phenoxycarbamate-ethidium · H_2PO_4 (14). Ethidium bromide/8% water (**1**) (200 mg, 466 μmol), 500 mM sodium phosphate pH 6.6 (5 mL), and acetone (8 mL) were combined, and phenyl chloroformate (587 μL, 4.66 mmol, 10 equiv, pre-dissolved in 2.5 mL acetone) was added dropwise. After 10 min at rt the reaction was cooled to −80 °C and vacuum filtered. The precipitate was washed with 20% acetone/water (10 mL), 100% acetone (−80 °C, 10 mL), and dried under reduced pressure to yield 300 mg (98%) of a yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6, 20 °C): δ 11.36 (s, 1H), δ 10.98 (s, 1H), δ 9.17 (d, J = 9.2 Hz, 1H), δ 9.12 (d, J = 8.8 Hz, 1H), δ 8.87 (s, 1H), δ 8.36 (dd J_1 = 9.2 Hz, J_2 = 2.0 Hz, 1H), δ 8.22 (d, J = 9.2 Hz, 1H), δ 7.85 (d, J = 2.4 Hz, 1H), δ 7.77 (s, 5H), δ 7.38–7.50 (m, 4H), δ 7.24–7.43 (m, 4H), δ 7.15–7.19 (m, 2H), δ 4.64 (q, J = 7.6 Hz, 2H), 1.48 (t, J = 7.2 Hz, 3H). ESI MS calcd for $C_{36}H_{30}N_3O_4$: 554.2, found 554.3 $[M]^+$.

3,8-Bis-urea-ethidium · Cl (15). In a 15 mL pressure tube, 3,8-bis-phenoxycarbamate-ethidium · H_2PO_4 (**14**) (48 mg, 74 μmol) and methanol (10 mL) were mixed and brought to −78 °C whereupon approximately 2 mL of ammonia was added (by bubbling in ammonia gas). The pressure tube was sealed and allowed to warm to rt. The reaction was then heated at 80 °C for 1 h and cooled back to −78 °C. The tube was opened and the ammonia was out-gassed by passing argon into the solution as it slowly warmed to rt. All volatiles were then removed under reduced pressure. The solid product was washed with diethyl ether (2×20 mL) then dissolved in 20% acetonitrile/water and treated with AG1-X4 (Cl^-) exchange resin (1 g, 3.5 mmol, 47 mequiv) for 5 min at rt. The resin was removed by filtration, and the solution lyophilized to yield 32 mg (99%) of a yellow solid. ^{1}H NMR (300 MHz, DMSO-d_6, 20 °C): δ 9.76 (s, 1H), δ 9.34 (s, 1H), δ 8.97 (d, J = 9.3 Hz, 1H), δ 8.92 (d, J = 9.3 Hz, 1H), δ 8.85 (d, J = 1.2 Hz, 1H), δ 8.30 (dd J_1 = 9.3 Hz, J_2 = 2.4 Hz, 1H), δ 7.93 (dd J_1 = 9.3 Hz, J_2 = 1.2 Hz, 1H), δ 7.74–7.78 (m, 5H), δ 7.55 (d, J = 2.1 Hz, 1H), δ 6.36 (s, 2H), 6.05 (s, 2H), δ 4.58 (q, J = 7.8 Hz, 2H), δ 1.48 (t, J = 6.9 Hz, 3H). ESI MS calcd for $C_{23}H_{22}N_5O_2$: 400.2, found 400.3 $[M]^+$. UV–vis (50 mM sodium phosphate pH 7.5): λ_{max} (nm) and ε (cm^{-1} M^{-1}): 280 (4.3×10^4), 434 (3.6×10^3).

3,8-Bis-urea-pyrrolidine-ethidium · TFAc (16). 3,8-Bis-phenoxycarbamate-ethidium · H_2PO_4 (**14**) (12 mg, 19 μmol), DMSO (1 mL) and pyrrolidine (40 μL, 460 μmol, 27 equiv) were combined and heated for 5 min (90 °C). The reaction was then diluted into water (9 mL, 0.1% TFA) and loaded onto an activated Water's 'Sep-pack' C-18 reversed phase column (activated with 10 mL acetonitrile, 10 mL water). The column was washed with water (10 mL, 0.1% TFA), then 10% acetonitrile (10 mL, in water with 0.1% TFA). The product was then eluted with 35% acetonitrile (10 mL, in water with 0.1% TFA) and lyophilized to yield a yellow solid (12 mg, 100%). ^{1}H NMR (400 MHz, DMSO-d_6, 21 °C): δ 9.01 (d, J = 9.6 Hz, 1H), δ 8.98 (s, 1H), δ 8.94 (d, J = 9.6 Hz, 1H), δ 8.89 (d, J = 2.0 Hz, 1H), δ 8.72 (s, 1H), δ 8.40 (dd J_1 = 9.2 Hz, J_2 = 2.4 Hz, 1H), δ 8.26 (dd J_1 = 9.2 Hz, J_2 = 1.6 Hz, 1H), δ 7.34–7.80 (m, 6H), δ 4.56 (q, J = 6.4 Hz, 2H), δ 3.46 (m, 4H), δ 3.32 (m, 4H), δ 1.91 (m, 4H), δ 1.81 (m, 4H), δ 1.48 (t, J = 7.2 Hz, 3H). ESI MS calcd for $C_{31}H_{34}N_5O_2$: 508, found 508 $[M]^+$. UV–vis (50 mM sodium phosphate pH 7.5): λ_{max} (nm) and ε ($cm^{-1}M^{-1}$): 288 (4.7×10^4), 438 (4.5×10^3).

3,8-Bis-urea-arginine-ethidium · $TFAc_3$ (17). 3,8-Bis-phenoxycarbamate-ethidium · H_2PO_4 (**14**) (10 mg, 15.3 μmol), DMSO (400 μL), water (100 μL), L-Arg · HCl (50 mg, 237 μmol, 15.5 equiv) and 2,4,6-collidine (84 μL, 711 μmol, 46 equiv) were heated for at 90 °C for 1 h then cooled to rt and quenched with 500 mM sodium phosphate pH 6.5 (0.6 mL). The reaction was then diluted into 5 mL water (0.1% TFA) and loaded onto an activated Water's 'Sep-pack' C-18 reversed-phase

column (activated with 10 mL acetonitrile, 10 mL water). The column was washed with water/0.1% TFA (5 mL), the product eluted with 25% acetonitrile/water (0.1% TFA) and was lyophilized. The product was further purified using a reversed phase C-18 semi-prep HPLC column using 15% acetonitrile/water (0.1% TFA) (R_T = 6.3 min) to yield 4.5 mg (31%) of a yellow solid. ^{1}H NMR (400 MHz, D_2O, 20 °C): δ 8.37 (d, J = 8.8 Hz, 1H), δ 8.31 (d, J = 8.8 Hz, 1H), δ 8.30 (d, J = 8.8 Hz, 1H), δ 7.67–7.76 (m, 4H), δ 7.53 (d, J = 9.2 Hz, 1H), δ 7.39–7.45 (m, 3H), δ 4.59 (q, J = 6.8 Hz, 2H), δ 4.20 (t, J = 5.6 Hz, 1H), δ 4.08 (t, J = 5.2 Hz, 1H), δ 3.15 (t, J = 6.6 Hz, 2H), δ 3.10 (t, J = 6.8 Hz, 2H), δ 1.52–1.83 (m, 8H), δ 1.33 (t, J = 7.0 Hz, 3H). ESI MS calcd for $C_{35}H_{44}N_{11}O_6$: 714, found 715 $[M+H]^+$, UV–vis (50 mM sodium phosphate pH 7.5): λ_{max} (nm) and ε (cm^{-1} M^{-1}): 216 (3.8×10^4), 288 (6.8×10^4), 444 (5.7×10^3).

3,8-Bis-urea-ethylenediamine-ethidium · TFA_3 (18). 3,8-Bis-phenoxycarbamate ethidium · H_2PO_4 (**14**) (9 mg, 13.8 μmol), DMSO (300 μL), and ethylene diamine (100 μL) were heated at 85 °C for 30 min then cooled to rt. 500 mM sodium phosphate pH 6.5 (0.6 mL) was added and the reaction was diluted into water (5 mL, 0.1% TFA) and loaded onto an activated Water's 'Sep-pack' C-18 reversed-phase column (activated with 10 mL acetonitrile, 10 mL water). The column was washed with water/0.1% TFA (5 mL), the product eluted with 25% acetonitrile/water (0.1% TFA) and lyophilized to yield 10.5 mg (91%) of a yellow solid. ^{1}H NMR (300 MHz, D_2O, 20 °C): δ 8.58 (d, J = 8.4 Hz, 1H), δ 8.50 (d, J = 8.4 Hz, 1H), δ 8.43 (s, 1H), δ 7.76 (d, J = 9.0 Hz, 1H), δ 7.60–7.69 (m, 4H), δ 7.50 (s, 1H), δ 7.40–7.43 (m, 2H), δ 4.60 (q, J = 7.5 Hz, 2H), δ 3.40 (t, J = 6.0 Hz, 2H), δ 3.26 (t, J = 5.7 Hz, 2H), δ 3.03 (t, J = 5.7 Hz, 2H), δ 2.92 (t, J = 5.7 Hz, 2H), δ 1.37 (t, J = 7.2 Hz, 3H). ESI MS calcd for $C_{27}H_{32}N_7O_2$: 486, found 486 $[M]^+$. UV–vis (50 mM sodium phosphate pH 7.5): λ_{max} (nm) and ε (cm^{-1} M^{-1}): 216 (3.8×10^4), 286 (6.6×10^4), 444 (6.5×10^3).

3,8-Bis-urea-2-DOS ethidium · TFA_3 (19). 3,8-Bis-phenoxycarbamate-ethidium · H_2PO_4 (**14**) (20 mg, 31 μmol), DMSO (1.5 mL), phenol (1.5 g), Na_2CO_3 (50 mg, 472 μmol, 15 equiv), 2-deoxystreptamine · 2HCl (110 mg, 277 μmol, 8.9 equiv) pre-dissolved in water (0.7 mL), were heated at 85 °C for 45 min. The reaction was diluted into water (80 mL) and washed with CH_2Cl_2 (2×40 mL), $CHCl_3$ (2×40 mL), and ethyl acetate (40 mL). The aqueous phase was then concentrated to a solid and purified by reversed-phase chromatography (C-18 silica gel 60). The column was conditioned with pure acetonitirile, pure water, and the crude product was loaded in water (0.1% TFA) and an acetonitrile gradient (0–8% acetonitrile/water (0.1% TFA) was used to elute the product. Fractions were collected and lyophilized to yield 8 mg of a yellow solid (25%). ^{1}H NMR (400 MHz, D_2O, 20 °C): δ 8.55 (d, J = 8.4 Hz, 1H), δ 8.47 (d, J = 8.4 Hz, 1H), δ 8.44 (s, 1H), δ 7.62–7.74 (m, 6H), δ 7.50 (s, 1H), δ 7.39 (s, 1H), δ 7.38 (d, J = 8.4 Hz, 1H), δ 4.59 (q, J = 6.4 Hz, 2H), δ 3.70 (m, 1H), δ 3.52 (m, 1H), δ 3.08–3.38 (m, 8H), δ 2.21 (td J_1 = 12.4 Hz, J_2 = 4.0 Hz, 1H), δ 2.08 (td J_1 = 12.4 Hz, J_2 = 4.0 Hz, 1H), δ 1.56 (q, J = 12.4 Hz, 1H), δ 1.44 (q, J = 12.4 Hz, 1H), δ 1.37 (t, J = 6.4 Hz, 3H). ESI MS calcd for $C_{35}H_{44}N_7O_8$: 690, found 690 $[M]^+$, UV–vis (50 mM sodium phosphate pH 7.5): λ_{max} (nm) and ε (cm^{-1} M^{-1}): 216 (3.8×10^4), 288 (6.8×10^4), 444 (5.7×10^3).

3-Pyrrole-ethidium · TFA (20), and 8-pyrrole-ethidium · TFA (21). A mixture (5:1 respectively) of 8-cbz-ethidium · Cl (**3**) and 3-cbz-ethidium · Cl (**2**) (90 mg, 186 μmol) was added to glacial acetic acid (4 mL), heated to 120 °C, and 3 portions (15 min apart) of dimethoxytetrahydrofuran (3×15 μL, 348 μmol total, 1.87 equiv) were added over 30 min. The reaction was kept at 120 °C for an additional 45 min then cooled to rt, diluted into $CHCl_3$ (100 mL), and washed with saturated sodium bicarbonate (3×50 mL), brine (50 mL), dried over sodium sulfate, and concentrated to a solid under reduced pressure. The mixture of cbz-protected products was purified using a neutral alumina column using pure acetone as an eluent and concentrated to a yellow solid. This mixture was carried over, directly to the next step. Deprotection was conducted in a 3:1 mix of methanol/acetic acid (4 mL), with Pd black (30 mg), and rigorously stirring under 1 atm of H_2 for 3 h at rt. The catalyst was removed by centrifugation, and the solution concentrated to an orange solid under reduced pressure. The products separated using a reversed-phase C-18 semi-prep column using 38% acetonitrile/water (0.1% TFA) to yield 14.6 mg (18%) of **3-pyrrole-ethidium · TFA (20)** (R_t = 13.8 min). ^{1}H NMR (400 MHz, D_2O, 20 °C): δ 8.43 (d, J = 9.2 Hz, 1H), δ 8.31 (d, J = 9.2 Hz, 1H), δ 7.87 (d, J = 1.6 Hz, 1H), δ 7.70 (dd J_1 = 9.2 Hz, J_2 = 1.6 Hz, 1H), δ 7.60–7.67 (m, 3H), δ 7.42 (dd J_1 = 9.2 Hz, J_2 = 2.4 Hz, 1H), δ 7.30–7.32 (m, 2H), δ 7.19 (dd, J_1 = J_2 = 2.0 Hz, 2H), δ 6.52 (d, J = 2.4 Hz, 1H), δ 6.29 (dd, J_1 = J_2 = 2.0 Hz, 2H), δ 4.61 (q, J = 7.2 Hz, 2H), δ 1.30 (t, J = 7.0 Hz, 3H). FAB MS calcd for $C_{25}H_{22}N_3$: 364.1814, found 364.1823 $[M]^+$. UV–vis (50 mM sodium phosphate pH 7.5): λ_{max} (nm) and ε (cm^{-1} M^{-1}): 223 (2.8×10^4), 287 (4.7×10^4), 453 (4.0×10^3). **8-pyrrole-ethidium · TFA (21)** (3.3 mg (4%)), (R_T = 18.1 min). ^{1}H NMR (400 MHz, D_2O, 20 °C): δ 8.42 (d, J = 9.2 Hz, 1H), δ 8.40 (d, J = 8.8 Hz, 1H), δ 7.86 (dd J_1 = 9.2 Hz, J_2 = 2.0 Hz, 1H), δ 7.61–7.71 (m, 3H), δ 7.36–7.37 (m, 2H), δ 7.29 (d, J = 1.2 Hz, 1H), δ 7.24 (dd J_1 = 8.8 Hz, J_2 = 1.2 Hz, 1H), δ 6.99 (d, J = 2.4 Hz, 1H), δ 6.84 (dd, J_1 = J_2 = 2.0 Hz, 2H), δ 6.13 (dd, J_1 = J_2 = 2.0 Hz, 2H), δ 4.49 (q, J = 7.6 Hz, 2H), δ 1.30 (t, J = 7.6 Hz, 3H). FAB MS calcd for $C_{25}H_{22}N_3$: 364.1814, found 364.1822 $[M]^+$. UV–vis (50 mM sodium phosphate pH 7.5): λ_{max} (nm) and ε (cm^{-1} M^{-1}): 223 (2.1×10^4), 239 (1.4×10^4), 289 (4.8×10^4), 466 (4.1×10^3).

3,8-Bis-pyrole-ethidium · OAc (22). Ethidium bromide/4% water (264 mg, 670 μmol) was dissolved in glacial acetic acid (10 mL) (by sonication) and brought to 130 °C. Two portions of dimethoxytetrahydrofuran (2×110 μL, 1.65 mmol total, 2.5 equiv) were added 15 min apart. The reaction was kept under reflux (at 130 °C) for an additional 1 h and cooled to rt. All volatiles were then removed under reduced pressure, and the

solid was dissolved in methanol (~40 mL) and filtered over a plug of silica gel (~30 mL). The gel was washed with methanol (~60 mL), and the methanolic fractions combined and concentrated to 280 mg (90%) of a yellow solid under reduced pressure. The product can be further purified using a reversed phase C-18 semi-prep column with a 50–80% acetonitrile/water (0.1% TFA) gradient over 20 min. ^{1}H NMR (400 MHz, acetone-d_6, 20 °C): δ 9.38 (d, J = 9.0 Hz, 1H), δ 9.32 (d, J = 9.0 Hz, 1H), δ 8.73 (d, J = 2.1 Hz, 1H), δ 8.64 (dd J_1 = 9.0 Hz, J_2 = 2.4 Hz, 1H), δ 8.49 (dd J_1 = 9.0 Hz, J_2 = 2.1 Hz, 1H), δ 7.91–8.01 (m, 5H), δ 7.72 (dd, $J_1 = J_2$ = 2.1 Hz, 2H), δ 7.54 (d, J = 2.4 Hz, 1H), δ 7.24 (dd, $J_1 = J_2$ = 2.1 Hz, 2H), δ 6.46 (dd, $J_1 = J_2$ = 2.1 Hz, 2H), δ 6.34 (dd, $J_1 = J_2$ = 2.1 Hz, 2H), δ 5.24 (q, J = 7.2 Hz, 2H), δ 1.58 (t, J = 7.2 Hz, 3H). FAB MS calcd for $C_{29}H_{24}N_3$: 414.1970, found 414.1951 $[M]^+$. UV–vis (50 mM sodium phosphate pH 7.5): λ_{max} (nm) and ε (cm^{-1} M^{-1}): 302 (4.6×10^4), 428 (5.2×10^3).

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