Extended ethidium bromide analogue as a triple helix intercalator: synthesis, photophysical properties and nucleic acids binding†

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Ethidium bromide has been extended by fusing an additional aromatic ring resulting in a larger intercalator with increased affinity for poly r(A)·r(U), poly d(A)·d(T) and triple helices when compared to the parent heterocycle.

Intercalation represents an important mode of nucleic acid small molecule interactions.[1] Polarizable polycyclic heteroaromatic systems, such as ethidium and acridinium derivatives, represent classical examples of nucleic acid intercalators.[2] In general, such aromatic molecules bind between adjacent base pairs and tend to display limited selectivity between DNA and RNA as well as inadequate discrimination between different sequences and folding patterns.[3] In recent years, structural modifications have been employed to alter the selectivity of intercalators and improve their nucleic acids recognition characteristics. In particular, attempts to amplify the differentiation between DNA and RNA, between double and triple stranded domains, as well as between matched and mismatched or bulged sites have been reported.[4] Here we present a phenanthridinium analogue **2**, where the aromatic surface of the parent intercalator ethidium **1** is extended (Fig. 1). The new large-surface intercalator, while displaying modestly improved RNA over DNA selectivity, shows significantly improved selectivity toward triple stranded oligonucleotides, when compared to ethidium bromide.

In designing the extended ethidium analogue, we have considered the electronic properties of the parent heterocycle.[5] In particular, we have maintained the electronic communication between the exocyclic amine at the 3 position and the quaternary nitrogen by not perturbing this side of the molecule. In addition, the exocyclic amine on the new aromatic ring electronically communicates with the core structure in the same fashion the 8-amine does in ethidium. Synthetically, our strategy for the formation of the extended phenanthridinium framework involved two key steps: (a) assembly of the phenyl-naphthalene core using a Suzuki cross-coupling reaction of a suitably functionalized naphthalene precursor with a highly functionalized benzene precursor carrying a benzamide unit, and (b) a Morgan–Walls ring-closure to assemble the bridging heterocyclic ring.

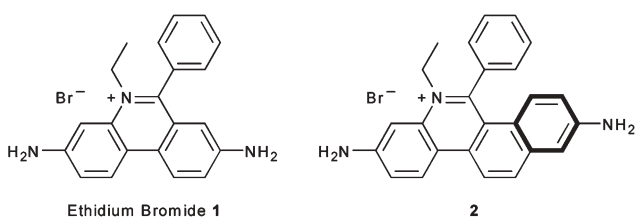

Fig. 1 Structure of ethidium bromide **1** and its extended analogue **2**.

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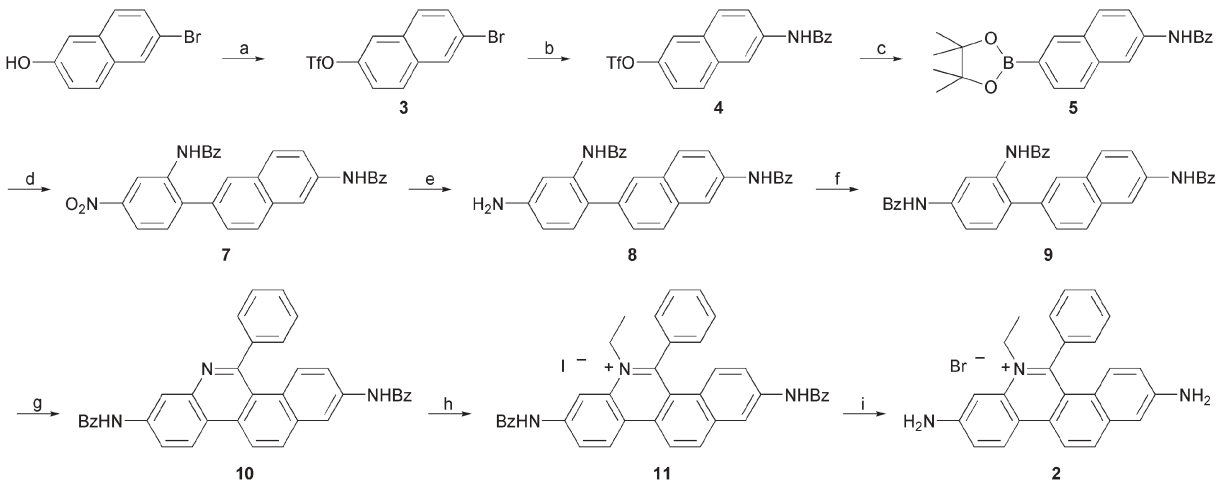
† Electronic supplementary information (ESI) available: synthetic procedures & analytical data, absorption and fluorescence spectra, nucleic acid binding isotherms and additional thermal melting profiles. See DOI: 10.1039/b604281c

The synthesis of the extended ethidium analogue **2** commenced with triflation of commercially available 6-bromo-2-naphthol to afford **3** in high yield (Scheme 1). Selective amidation at the 6-position was accomplished following a protocol developed by Buchwald to cross-couple a benzamide group to the naphthalene core **3** thereby installing one of the extranuclear amines.[6] Palladium-mediated borylation between the triflate **4** and 4,4,5,5-tetramethyl-[1,3,2]dioxaborolane afforded the boronic ester **5** in modest yield.[7] Completion of the aromatic scaffold was accomplished through a Miyaura–Suzuki coupling of **5** and 2′-bromo-5′-nitrobenzanilide **6** to afford **7**.[8] Reduction of the aromatic nitro functionality necessitated not only catalytic hydrogenation conditions but the addition of hydrazine as well. To prevent any possible oxidation or degradation of the free aromatic amine, **8** was immediately reacted with benzoyl chloride under basic conditions to form the tribenzamide **9**. Treatment with phosphorus oxychloride to complete the extended phenanthridine core afforded **10** in high yield. Alkylation of the phenanthridine nitrogen required reaction with iodoethane in refluxing DMF over 20 h to afford **11** as a purple solid. Removal of the benzoyl groups in refluxing 48% HBr over 12 h yielded the desired extended ethidium analogue **2**.[9]

Similarly to ethidium bromide **1**, the extended analogue **2** displays intense absorption in the UV range and a weaker charge transfer band around 500 nm (Fig. 2A). Solvent polarity influences both molecules in a similar fashion. Both chromophores experience a hypsochromic shift with increasing solvent polarity that is mostly apparent with the visible absorption bands (Fig. 2A). Excitation of the major absorption bands leads to emission of both molecules at *ca.* 600 nm. As with the ground state absorption bands, the relatively broad emission band of both chromophores is sensitive to its microenvironment. Increasing solvent polarity results in a modest red shift of the emission maxima (Fig. 2B). Taken together, these observations are consistent with stabilization of the charged ground state upon increasing solvent polarity and concomitant modest destabilization of the more delocalized excited state.

Both ethidium bromide and its extended analogue **2** are much less emissive in water when compared to their emission in apolar organic solvents. As a result, addition of nucleic acids to an aqueous solution of the intercalators results in significant enhancement of emission, which is accompanied by a slight hypsochromic shift (Fig. 2C). While the extended analogue is inherently less emissive than the parent intercalator,[9] it is almost



Scheme 1 Synthesis of extended analogue **2**.[9] *Reagents and conditions*: (a) Tf_2O, pyr., CH_2Cl_2, 0 °C to rt, 3 h, 98%; (b) *N,N′*-dimethylethylenediamine, benzamide, CuI, K_2CO_3, toluene, 100 °C, 48 h, 96%; (c) 4,4,5,5-tetramethyl-[1,3,2]dioxaborolane, Pd(dppf)Cl_2·CH_2Cl_2, Et_3N, dioxane, 95 °C, 18 h, 65%; (d) **6**, Pd(dppf)Cl_2·CH_2Cl_2, 1 M Na_2CO_3, DMF, 80 °C, 16 h, 90%; (e) 80% N_2H_4, Pd/C (10%), H_2(1 atm), 65 °C, 4 h; (f) BzCl, Et_3N, rt, 12 h, 85% over 2 steps; (g) $POCl_3$, 100 °C, 18 h, 90%; (h) EtI, DMF, reflux, 20 h, 71%; (i) 48% HBr, reflux, 12 h, 80%.

completely quenched in water. This results in greater enhancement of emission upon nucleic acids binding for **2**, when compared to the emission intensities of free and bound ethidium (Fig. 2C).

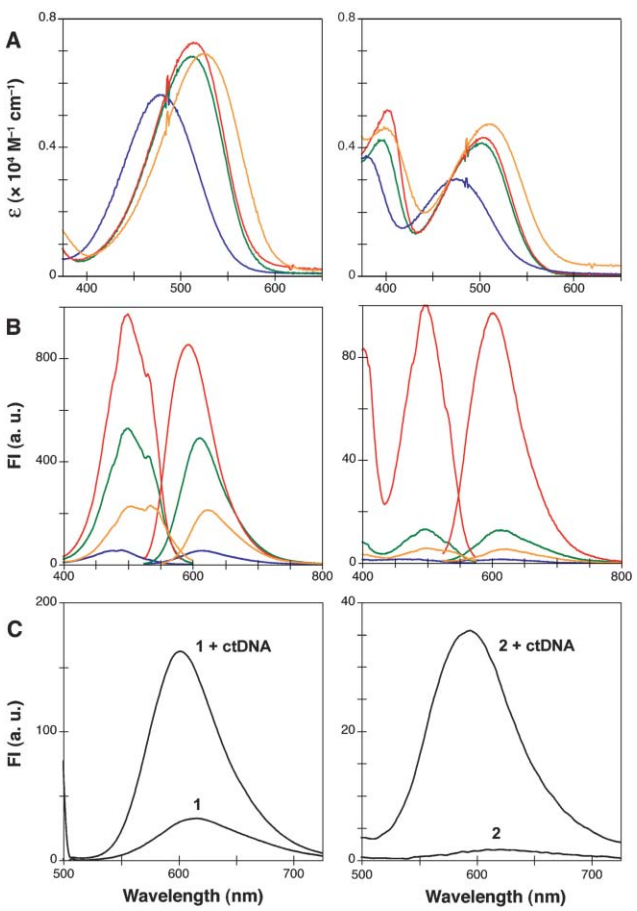


Fig. 2 (A) UV-Vis absorbance spectra of **1** (left) and **2** (right). (B) Excitation and emission spectra of **1** (left) and **2** (right). (C) **1** and **2** in the presence and absence of ctDNA in pH 7.4 buffer.[9] Solvents in (A) and (B): H_2O (blue), MeOH (yellow), CH_3CN (green), CH_2Cl_2 (red).

Nucleic acid affinity studies with calf thymus DNA (ctDNA) revealed no discrimination between the extended ethidium analogue or the parent compound (Table 1). When evaluating the affinity to poly r(A)·r(U), however, **2** displayed a slightly higher affinity for A-form RNA than ethidium bromide. Since A-form RNA has a larger helical diameter compared to B-form DNA (*ca.* 26 to 20 Å, respectively),[10] it provides a larger intercalating surface for the extended ligand, which may explain the enhanced affinity compared to ethidium. To further explore this hypothesis, the ability of **2** to bind triple helical oligonucleotides was investigated. Fluorescence binding affinity studies reveal that **2** has substantially higher affinity for triple helices compared to ethidium bromide (Table 1). This is likely due to more favorable stacking of the extended analogue **2** with the larger intercalating surface provided by the base triplets, as has been observed with other intercalating polyaromatic molecules.[11]

Thermal denaturation experiments with the homo-oligomers dA_{19} and dT_{19} were employed to determine the amount of stabilization imparted by **1** and **2** to double and triple helical DNA oligonucleotides (Fig. 3).[9] While minimal stabilization was observed for the dT_{19}·dA_{19} duplex with both intercalators, dramatic stabilization of the dT_{19}·dA_{19}·dT_{19} triple helix was observed with the extended ethidium analogue **2** (Table 2). As shown in Fig. 3, under low salt conditions, the triple helical dT_{19}·dA_{19}·dT_{19} begins to melt below 10 °C. While addition of ethidium bromide slightly enhances the stability of the triple stranded oligonucleotide (T_m = 24 °C),[12] a dramatic stabilization is

Table 1 Nucleic acid affinities (K_d) of **1** and **2**[a]

Nucleic acid polymers	**1** (μM)	**2** (μM)
calf thymus DNA	15.1 ± 4.1	11.2 ± 1.6
poly r(A)·r(U)	5.0 ± 0.9	1.6 ± 0.2
poly d(A)·d(T)	30.8 ± 10.8	7.9 ± 1.3
poly d(T)·d(A)·d(T)	9.5 ± 5.2	1.0 ± 0.3
poly r(U)·r(A)·r(U)[b]	16.1 ± 2.6	1.7 ± 0.3

[a] Fluorescence titrations performed with 1.0 μM of **1** or **2** as small volumes of increasing concentrations of nucleic acid were added.[9] [b] See ESI (Section S5) for additional information.

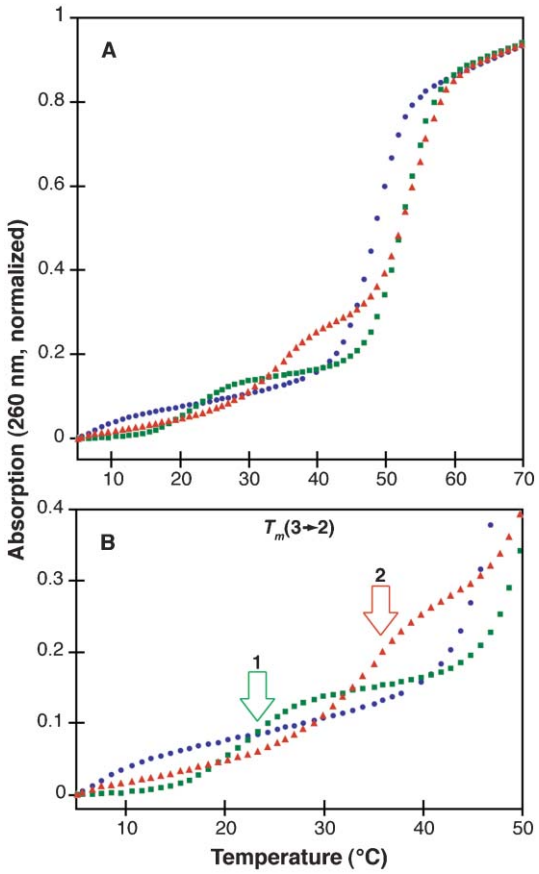


Fig. 3 Melting profiles at 260 nm for (A) $dT_{19}{\cdot}dA_{19}{\cdot}dT_{19}$ with no ligand (blue circles), ethidium bromide **1** (green squares), and the extended analogue **2** (red triangles). (B) is an expansion of the triple helix melting profile.

Table 2 Thermal melting points of triple helix $dT_{19}{\cdot}dA_{19}{\cdot}dT_{19}$[a]

	T_m(2→1)	T_m(3→2)
no ligand	49 ± 0.1 °C	<10 °C
1	53 ± 0.6 °C	24 ± 0.1 °C
2	54 ± 0.6 °C	37 ± 1.0 °C

[a] Thermal melting experiments were performed in a buffer containing 2.0×10^{-2} M PIPES (pH 7.0), 2.0×10^{-2} M NaCl, 1.0×10^{-3} M EDTA. 10.0 μM of **1** or **2** were used for 1.0 μM of triple helix concentration.[9]

apparent with the extended analogue (T_m = 37 °C). These results correlate well with affinities determined by the fluorescence-based titrations with polymeric oligonucleotides as listed in Table 1.

Triple helix DNA is of interest as a therapeutic target and altering its stability could have potential biotechnological applications.[13] Significant efforts in recent years have yielded a handful of new heterocycles with diverse triple-helical selectivity traits, none, however, were based on ethidium.[11] Our observations suggest that extending ethidium by fusing an additional aromatic ring yields an analogue that can be viewed as a new triplex-selective motif. Further structural modification can potentially fine tune the nucleic acids affinity and selectivity of this extended heterocycle.

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