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Synthesis and testing of a triaza-cyclopenta[*b*]phenanthrene scaffold as a DNA binding agent

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Abstract—A novel DNA binding agent based upon a triaza-cyclopenta[*b*]phenanthrene scaffold, compound **1**, has been synthesized. dsDNA binding analysis of this compound using the ethidium bromide displacement assay indicated a preference for GC-rich sequences. However, equilibrium dialysis experiments against a variety of nucleic acids showed that the target compound bound about 20-fold tighter to G-quartet DNA than to dsDNA under physiological salt concentrations. The binding of **1** to G-quartet DNA was verified by the ability of the compound to promote the formation of the quartet and to compete with TmPyP4 for binding to the quadruplex. Given the importance of G-quartet binding agents in the treatment of cancer and in the understanding of drug–DNA interactions, **1** and its related analogs should find utility as a new class of G-quartet specific agents.

1. Introduction

Gene expression is one of the most fundamental processes in biology, and as such, exerts tremendous influence on the health of the cell and organism. Errors in gene expression, either by falsely expressing a gene or by expressing too much or too little of a particular gene, can often lead to the formation of disease.[1–5] Perhaps, the best known example is the expression of oncogenes, where transcription of a single gene can result in transformation to a cancerous state.[6,7] However, transcriptional analysis has shown that other ailments such as heart disease,[8,9] chronic obstructive pulmonary disorder,[10] Alzheimer's disease,[11–13] and diabetes[14,15] also display aberrant gene expression. In addition to causing disease, altered gene expression also plays a secondary role in the outcome and severity of human disease. For example, expression of HER2 in breast cancer is associated with higher mortality and ErbB2 overexpression in tumors confers resistance to treatment and increases metastasis.[16,17] Given the strong correlation between gene expression and disease development, severity and treatment, methods to pharmacologically correct aberrant gene expression are critical. Medicinal agents can affect gene expression by facilitating, mimicking or inhibiting any one of the properties that exists in typical transcriptional systems.[2,18–21] For example, the seminal work by Dervan, Dickerson, and Lown on minor groove DNA binding agents has resulted in agents that inhibit gene expression by preventing the binding of transcription factors to DNA.[2,22–30] Recent efforts have focused on designed zinc-finger proteins[19] and synthetic molecules as artificial transcription regulators,[20] peptide nucleic acids as antigene agents,[31,32] and RNAi that result in mRNA degradation.[33–35]

Recently, our laboratory has become interested in the role that promoter architecture plays in gene regulation. As part of a program aimed at constructing medicinal agents to alter promoter architecture, we designed the triaza-cyclopenta[*b*]phenanthrene scaffold, **1**, as a DNA recognition molecule. In this report, we describe the design, synthesis, and evaluation of this scaffold as a DNA binding agent.

2. Results and discussion

2.1. Design of a pyridone-based DNA binding agent

The triaza-cyclopenta[*b*]phenanthrene scaffold, **1**, was designed to mimic two methylpyrrole heterocycles, which are commonly found in minor groove binding polya-

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mides such as distamycin and netropsin (Fig. 1). A key design feature was the ability of **1** to present two hydrogen bond donors; one via the amine of an amide bond and the other five bonds away. Such an arrangement is seen in existing minor groove binding agents. While one of these hydrogen bonds could be accomplished by an amide bond, the second required the presence of a 4-pyridone unit to provide the necessary hydrogen bond donor within the cyclic system. The 4-pyridone unit can exist in one of two tautomers, either the 4-keto or the 4-enol. In the 4-keto tautomer, we hypothesized that a bifurcated hydrogen bond could form between the $N^{17}H$ and either the O^2 of thymine or the N^3 of adenine in AT-rich sequences (Fig. 1). A similar binding mechanism has been observed for distamycin and Hoechst 33258.[26,36] In the 4-enol tautomer, we hypothesized that a hydrogen bond could form between the pyridine nitrogen and the hydrogen on the exocyclic amine of guanine, in a manner analogous to the imidazole containing polyamides. Since previous research has shown that 4-pyridones, particularly those conjugated to an aromatic group, exist predominately in the keto conformation in aqueous solution, we hypothesized that **1** would bind preferentially to AT-rich DNA.[37–39]

Compound **1** contains three additional features. First, the compound is crescent shaped, which allows the molecule to follow the contour of the DNA in the minor groove. Second, **1** contains a dimethylaminopropylamine group, which will be positively charged at physiological pH, to provide an electrostatic interaction with the negatively charged phosphate groups on DNA. Previous researchers have used this cationic group in the design of minor groove binding agents.[40] Finally, we chose a nitro group at C^{13} since it would serve as a masked amine which we could unmask in future studies to allow for the addition of other ring systems to the scaffold.

2.2. Synthesis of 1

The synthesis of **1** is outlined in Scheme 1. Synthesis of the desired chloronitroquinoline,[41] **2**, was accomplished according to literature procedures and reacted with the known aminopyrrole[40] (**3**) to yield the desired **4** in modest yields. Selective deprotection of the ethyl ester in the presence of the methyl ester was accomplished using sodium hydroxide as previously reported.[42] Cyclization of the free carboxylic acid was accomplished by refluxing in freshly distilled phosphorus oxychloride to yield **6**.[43] The final step was the addition of dimethylaminopropylamine. The methyl ester was saponified to the corresponding carboxylic acid, **7**, in excellent yields. However, addition of dimethylaminopropylamine proved difficult under a variety of standard conditions (i.e., DMAP, EDCI, HBTU, etc.). We found that we could produce the desired compound in low yields using HOBt/DCC. The low yield is attributed to the extremely polar nature of **1** making isolation and purification of this compound difficult to achieve.

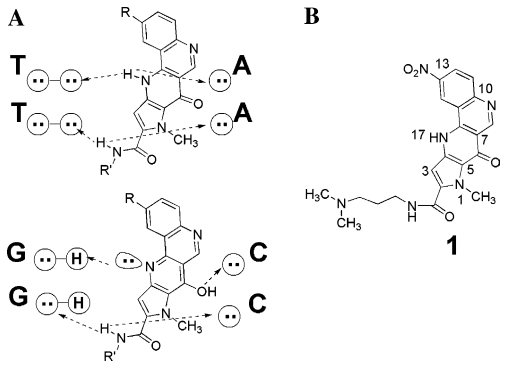
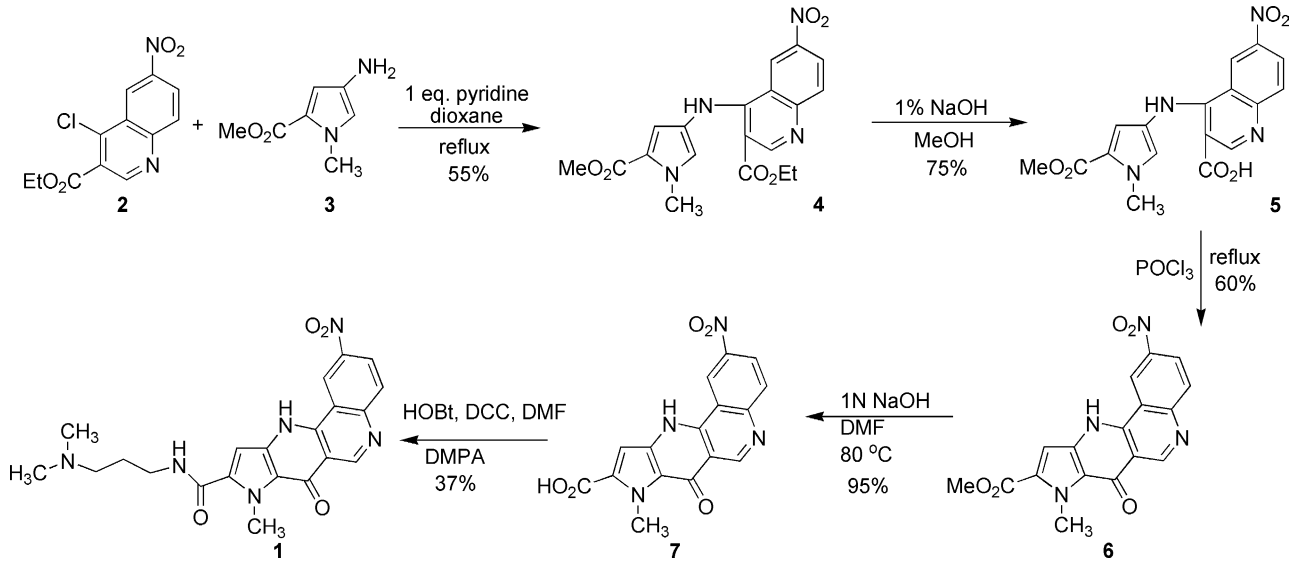


Figure 1. (A) Theoretical binding mechanisms to either AT- or GC-rich sequences depend upon the tautomeric form of the 4-pyridone unit in the triaza-cyclopenta[*b*]phenanthrene scaffold. The circles with the two dots represent the lone pair of electrons on either the O^2 of pyrimidines (T, C) or the N^3 of purines (A, G). The circle containing the H represents the hydrogen on the 2-amino group of guanine. (B) Structure of molecule **1**.

2.3. Ethidium bromide displacement assay indicates that 1 binds to GC-rich sequences

Initial investigations of the DNA binding potential of **1** to double stranded DNA were attempted using changes in the UV–vis or fluorescence spectrum of **1** upon addition of various nucleic acids. However, neither of these methods proved satisfactory. Therefore, we utilized the fluorescence intercalation displacement assay as recently described by Boger.[44,45] In this assay, binding of **1** to dsDNA is measured by a decrease in fluorescence as bound ethidium bromide is displaced from the nucleic acid.

Two hairpin oligonucleotides, one containing an AT-rich sequence and the other containing a GC-rich sequence, were chosen for the initial DNA binding investigations (Fig. 2). These hairpins have previously been utilized by the Boger group to determine the sequence specificity and binding affinity of minor groove binding agents.[44,45] To estimate the binding constant of **1** to the hairpin oligonucleotides, we used a simple competitive binding model for **1**. Using this model, the binding constant can be estimated from the equation $K = K_{EB}[E]/[\text{agent}]$, where K is the binding constant, K_{EB} is the binding constant for ethidium bromide, $[E]$ is the total concentration of ethidium bromide, and [agent] is the concentration of drug that reduces fluorescence by 50%.[44–47] Previous work has determined the K_{EB} for the AT hairpin as $0.27 \times 10^6\ M^{-1}$ and $10 \times 10^6\ M^{-1}$ for GC-rich nucleic acids.[44,45] Using these values, the binding constants were estimated to be $0.018 \times 10^6\ M^{-1}$ and $0.22 \times 10^6\ M^{-1}$ to AT-rich and GC-rich sequences, respectively, suggesting that **1** has a preference for GC-rich sequences. GC-specific minor groove binding agents are rare due to the difficulty in overcoming the steric influence of the exocyclic amine on guanosine. One potential explanation for the sequence specificity of **1** is that this compound exists in the 4-enol tautomer instead of the 4-keto tautomer as anticipated. As shown in Figure 1, the 4-enol tautomer could potentially form a hydrogen bond to the exocyclic amine of guanine, which would lead to a preference for GC-rich sequences. However, previous work on tautomerization of 4-pyridones suggests that the 4-keto is



Scheme 1. Synthesis of **1**.

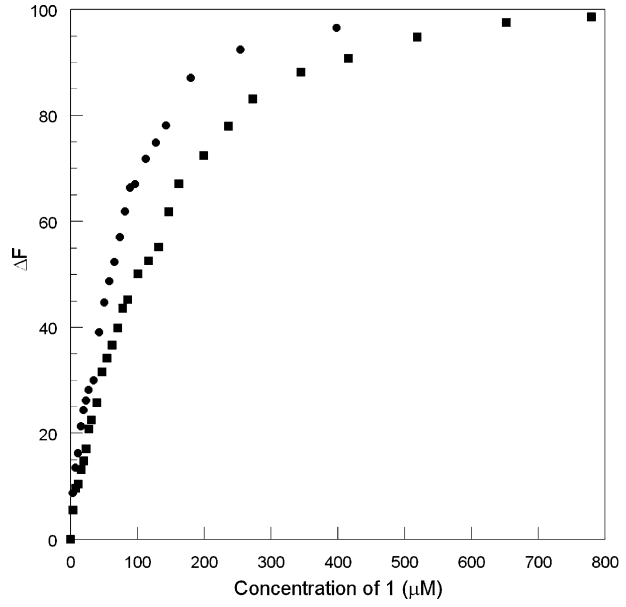


Figure 2. Ethidium bromide displacement assay of **1** with GC and AT hairpin oligonucleotides. Various concentrations of **1** were added to a solution containing 4.0 μM ethidium bromide and 8.0 μM (bp) of either the AT (●) or GC (■) hairpin oligonucleotide. The percent change in fluorescence was calculated by estimating the post-saturation value and setting this value to 100%.

the predominate tautomer in aqueous solution.[37–39] Thus, we set out to investigate the tautomeric preference for **1** in solution.

2.4. Tautomeric form of 1

To examine the tautomeric preference of **1**, the enol and keto forms of an analog, **7**, were constructed in GaussView 3.0 and calculations were carried out with the Gaussian 2003 program. Each structure was energy optimized using the semi-empirical PM3 method in vacuo. Previous studies on the tautomeric equilibrium of other 4-pyridone systems found that studies in vacuo did not yield the correct total free energy but did yield a correct relative prediction between the two tautomeric forms of the molecule.[37–39] Frequency calculations were employed to determine the free energy of each of the tautomers. The keto form was 7.7 kcal/mol ($K = 4.5 \times 10^5$) more stable than the enol form, clearly indicating that the keto form is the predominate form of **7**. While different solvent conditions could change the magnitude of the equilibrium constant, experimental and computational studies on the tautomerization of other 4-pyridiones and related 4-hydroxyquinolines have shown that the keto form predominates in more polar solvents such as water.[37–39,48] Thus, we would predict that the equilibrium constant would favor the keto tautomer even more in an aqueous environment.

To verify the tautomeric state of **1**, we relied upon previous work on the tautomerization of 4-pyridones which utilized UV–vis spectroscopy as a method to determine the predominate tautomeric form in solution.[48] UV–vis spectroscopy of 4-methoxyquinoline (trapped in the enol form) at pH 7 revealed a single peak at 300 nm, while the *N*-methyl-4-oxoquinolone (trapped in the keto form) contained two peaks separated by 15 nm at 320 and 335 nm.[48] UV–vis spectroscopy of **1** indicates the presence of two peaks at 360 and 375 nm, respectively, and these peaks are also separated by 15 nm (data not shown). The shift to a higher wavelength is due to the extended conjugation present in molecule **1** when compared to the simple 4-hydroxyquinoline system.

We utilized Gaussian 2003 to predict the IR spectra for the two tautomeric forms of **6** and thus **1**. We used **6** instead of **1** since it contained fewer atoms in the calculations and quality of the IR spectra was better. Theoretical spectra for both the enol- and keto forms of compound **6** were generated and compared to the experimentally determined spectra. The intense peak at 1519–1524 cm^{-1} due to the nitro group was used to align the predicted and experimental spectra. Subtrac-

tion of the predicted IR spectra of the enol tautomer from the predicted IR spectra of the keto generated a difference plot where positive peaks indicate spectral features that are indicative of the keto tautomer and negative peaks indicate features representative of the enol tautomer. The difference spectra specify that IR bands at frequencies of 1627, 1589, 1570, 1456, and 1247 cm^{-1} are indicative of the keto tautomer. The IR spectra of **6** contains bands at frequencies of 1618, 1590, 1563, 1445, and 1252 cm^{-1}, close to the peaks that are characteristic of the keto tautomer. The spectra of **6** have only one peak (1380 cm^{-1}) that is similar to IR peaks that are indicative of the enol tautomer (1399 cm^{-1}) and the difference between the observed peak in **6** and the predicted peak is 19 cm^{-1}, far greater than the average difference of 6.6 cm^{-1} observed for the keto tautomer. Based upon these results, we conclude that **6** and thus **1** exists as the keto tautomer in solution.

These results do not take into account the influence that the DNA may play on the tautomerization of **1** upon binding and we have no evidence that the keto form is present when **1** binds to DNA. However, given the fact that the predicted equilibrium greatly favors the keto form and the fact that the keto form has previously been demonstrated to be the major tautomeric form in all solvents, we feel that **1** probably binds to DNA in the keto form. A definitive answer to this question will have to await further structural experiments.

2.5. Compound 1 does not unwind supercoiled DNA

Another potential explanation for the sequence specificity of **1** is that this compound is not a groove binding agent but rather an intercalator. Previous research has shown that intercalators display a preference for the electron-rich GC base pair.[49] We investigated the ability of **1** to intercalate using the helical unwinding assay.[50,51] In this assay, intercalation into supercoiled DNA results in an unwinding of the helix leading to a change in the migration of the plasmid DNA in an agarose gel.[50,51] Compound **1** was incubated with supercoiled pBR322 up to a 20:1 molar ratio with no observed change in the migration of the supercoiled plasmid (Fig. 3). Furthermore, **1** does not display any altered UV–vis or fluorescence properties upon binding to DNA, a trait which is common among intercalating agents. While these results are negative and previous researchers have suggested caution in ruling out intercalation as a mechanism of action for DNA binding agents,[52] our results suggest that **1** binds to one of the grooves in DNA.

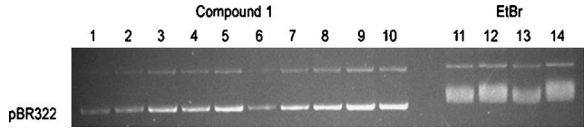


Figure 3. Unwinding assay. Various concentrations of **1** (0–200 nM, lanes 1–10) were incubated with 10 nM pBR322 for 1 h, electrophoresed and stained with ethidium bromide. No change in the migration of supercoiled DNA was observed when compared with four different concentrations of the known intercalator, ethidium bromide (0.01–1.0 nM, lanes 11–14).

2.6. Equilibrium dialysis, stability, and competition assays indicate that 1 binds to G-quadruplexes

We utilized the equilibrium dialysis method to verify the preference of **1** for GC-rich sequences.[49] In this approach, dialysis tubes containing various nucleic acids are placed into a solution containing the agent of interest. After incubation, the amount of agent bound to each nucleic acid is determined by UV–vis spectroscopy. Incubation of various nucleic acids with **1** was conducted under conditions in which the concentrations of the nucleic acids were 15 times greater than that of **1** (Fig. 4). This ratio was increased from the literature value due to difficulties in accurately measuring **1** at the lower ratio.[49,53] When this method was applied to **1**, we were surprised to see that **1** displayed a significant preference for G-quartet structures, with lesser binding to triplex and double stranded GC or AT rich sequences (Fig. 4). Compound **1** shows a selectivity ratio of G-quartet to poly(dA–dT) of 5.1, which is greater than the ratios of known G-quartet binding agents such as TmPyP4 (selectivity ratio 1.0), ethidium bromide (ratio 0.1), and BOQ1 (ratio 3.3) determined using the same method.[49,54] Equilibrium dialysis data can be used to estimate the binding affinity of **1** for G-quartet DNA using the equation $K_{app} = C_b/C_f * (S - C_b)$, where C_b is the amount of **1** bound to DNA, C_f is the amount of free **1** in solution, and S is the concentration of the G-quartet.[49,53] Using this equation, **1** has a K_{app} of 2.7×10^4 M^{-1}, comparable to that of the known G-quartet binding agent TmPyP4 ($K_{app} = 4.2 \times 10^4$ M^{-1}).[49,53]

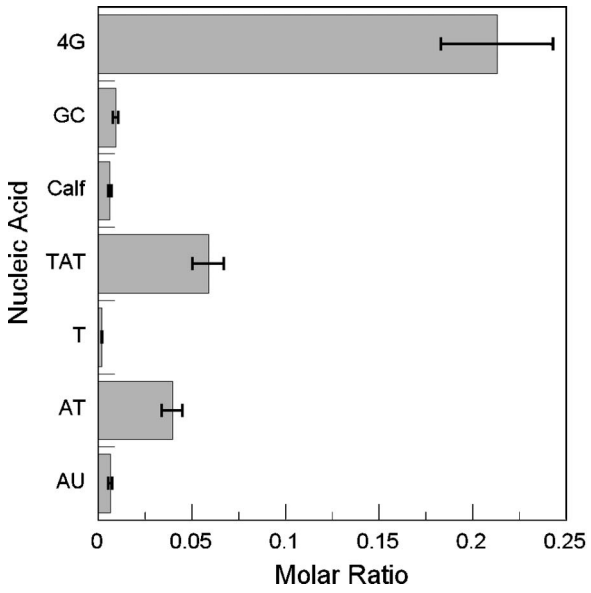


Figure 4. Equilibrium dialysis. All measurements were performed according to the literature, with the exception that the concentration of **1** was 5 μM.[49,53] The nucleic acids used are shown on the right and information regarding the sequences of these nucleic acids can be found in Section 4. Data are given as the molar ratio of drug bound versus the nucleic acid as determined by UV–vis spectroscopy. Error bars are calculated based upon an estimated 10% error in the measurement of the concentrations of the drug and nucleic acid, respectively.[49,53]

DNA binding analysis using the ethidium bromide displacement assay indicates that **1** had a binding constant of $0.22 \times 10^6\ M^{-1}$ to a GC-rich sequence, yet displayed very poor binding to a similar sequence in the equilibrium dialysis assay. The apparent difference in binding affinity of **1** to GC-rich sequences is most likely due to the higher salt concentration (180 vs 25 mM) used in the dialysis assay. Previous work on other DNA binding agents has shown at least a 10-fold decrease in binding affinity when binding was measured using the equilibrium dialysis assay compared to other methods.[49,53] The equilibrium dialysis assay also indicated that, in contrast to our previous results, **1** bound better to AT-rich sequences than GC-rich sequences. Differences in salt and nucleic acid concentrations, coupled with sequence differences in the nucleic acids used for each assay, could account for this result, but this has not been examined in detail. However, regardless of the exact reason for this difference, the results of the equilibrium dialysis experiment clearly indicate that **1** displays a strong preference for G-quadruplex DNA over duplex DNA.

We sought to verify the ability of **1** to bind to G-quartets using two additional assays. The ability of **1** to stabilize and increase the concentration of G-quartets under conditions that are unfavorable for their formation was investigated using native gel electrophoresis.[54,55] Increasing concentrations of **1** were incubated at room temperature with $T_2G_{20}T_2$ in buffer containing 100 mM KCl, which has previously been shown not to favor G-quartet formation.[54] Electrophoresis of the resulting complexes indicated a dose-dependent increase in the concentration of G-quartet, verifying that **1** binds to G-quartet structures (Fig. 5).

We next examined whether **1** could compete with the known G-quartet binding agent, TmPyP4, for binding to the G-quadruplex. TmPyP4 is fluorescent when bound to quadruplex DNA and increasing concentrations of **1** should result in a decrease in the fluorescent signal due to TmPyP4 if **1** were able to compete for binding to the quadruplex.[56] As shown in Figure 6A,

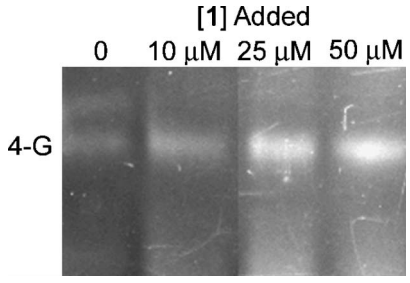


Figure 5. Drug-induced formation of G-quartet. Compound **1** was added at the indicated concentrations to an 8.0 μM solution $T_2G_{20}T_2$. Equal molar amounts of each mixture were analyzed using a native 12% polyacrylamide gel containing 20 mM KCl followed by staining using SYBR Gold. The location of the G-quartet (4-G), shown on the right, was determined using stabilized G-quartet (data not shown). Single-stranded DNA is located at the bottom of the gel and stained poorly. The image was created using Photoshop in which the first two lanes (0 and 10 μM) were placed next to the last two lanes (25 and 50 μM).

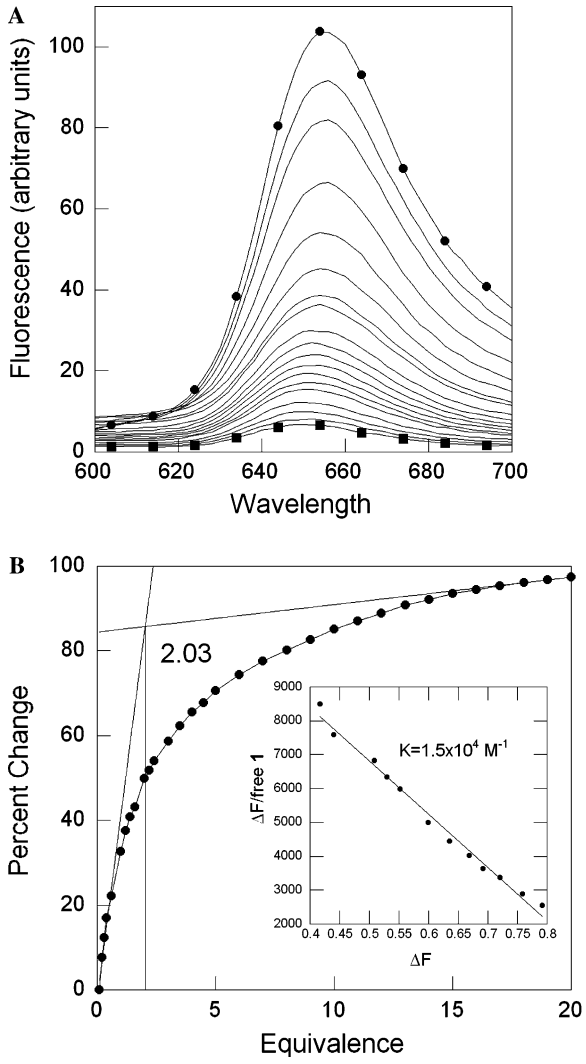


Figure 6. Competition assay between TmPyP4 and **1**. (A) Addition of **1** to a solution containing TmPyP4 and $T_2G_{20}T_2$ results in a decrease in fluorescence due to displacement of TmPyP4 from the quadruplex. (●) TmPyP4 and quadruplex alone. (■) TmPyP4, quadruplex, and 1.0 mM **1**. For clarity, not all scans are shown. (B) Percent change in TmPyP4 fluorescence versus equivalence of **1** added to the quadruplex. The intersection between the pre- and post-saturation regions of the curve indicates the stoichiometry of binding. Inset: Scatchard analysis of **1** binding to quadruplex DNA.

addition of **1** results in a dose-dependent decrease in the fluorescence of TmPyP4. Since TmPyP4 has been shown to bind to the ends of quadruplex DNA, the ability of **1** to compete with TmPyP4 suggests that **1** also binds to the terminal quartet(s).[57]

The stoichiometry of binding was determined from a plot of the change in fluorescence versus equivalence of **1** (Fig. 6B).[44,45] The plot indicated that **1** binds to quadruplex DNA with a 2:1 stoichiometry. There are two potential binding mechanisms that are consistent with this stoichiometry, namely, binding of one molecule of **1** to each of the two ends of the quadruplex or two molecules of **1** binding to a single terminal quartet. In our studies, TmPyP4 was present in a concentration that was 10-fold lower than the amount of available

quadruplex terminal tetrads. Thus, it is likely that only one molecule of TmPyP4 was bound to a single quadruplex, which suggests that two molecules of **1** bind to a single terminal tetrad. Further studies are needed to verify this binding mechanism.

Scatchard analysis was performed to determine the binding constant.[44,45] This analysis indicates that **1** has a binding constant of $1.5 \times 10^4\ M^{-1}$ for parallel quadruplex DNA (Fig. 6B, inset). This value is in agreement with the binding constant calculated using the equilibrium dialysis method and is also similar to those of other known quartet binding agents.

3. Conclusions

In summary, we have reported the synthesis and DNA binding properties of a triazaphenanthrene scaffold. Although this compound was designed to be a minor groove binding agent, our results show that the compound has only modest affinity for double stranded DNA. Instead, the compound displays considerable selectivity for G-quartet structures. The discovery of a new and selective G-quartet binding compound provides an additional example of agents that target these important nucleic acid structures. G-quartets have been suggested to play a role in the inhibition of telomerase and in the inhibition of c-myc transcription.[58,59] Given the biological importance of G-quartet structures, the triaza-cyclopenta[*b*]phenanthrene scaffold described here may find utility as an anticancer agent. Additional experiments aimed at exploring the anticancer activity of **1** and in the structure-activity relationships of this new agent are currently underway and will be reported in due course.

4. Experimental

All chemicals unless otherwise specified were purchased from Acros and utilized without further purification. Dry solvents were purchased from Acros. Thin layer chromatography was performed using aluminum backed silica plates containing a fluorescent indicator. Silica gel used for flash chromatography was purchased from Fisher Scientific and Sorbent Technologies. All solvents used, unless otherwise noted, were of HPLC grade. Melting points were determined with a Mel-Temp II and are uncorrected. ^{1}H NMR spectra were recorded on a Bruker WH-300 MHz spectrometer and chemical shifts were determined based upon standard solvent shifts. Infrared spectra were determined in potassium bromide using a Perkin-Elmer Infrared spectrophotometer. Ultraviolet–visible spectra were determined using a Cary 2.0 spectrophotometer using 1-cm pathlength cuvettes in pH 7.0 Tris buffer. Fluorescence spectra were determined using a Perkin-Elmer LS55 spectrophotometer with 1-cm pathlength cuvettes. Elemental analyses were preformed by Atlantic Microlabs Inc., Norcross, GA. Molecular calculations were done using Gaussian 2003 (Gaussian, Pittsburgh, PA). Poly(dA–dT), poly(dG–dC), poly(A–U), poly(dT), and calf thymus were purchased from Sigma and prepared according to the literature.[49,53] G-quartet (4-G) and triplex (poly (dA–dT–dT)) were prepared according to literature procedures.[49,53] Oligonucleotides 5′-$T_2G_{20}T_2$, 5′-CGG GCGGCAAAAAGCCGCCCG (GC-hairpin), and 5′-CGAAAAACAAAAAGTTTTTCG (AT-hairpin) were purchased purified from TriLink Biotechnologies (San Diego, CA) and prepared for use according to literature procedures.[44,46,49,53]

4.1. 3-(Ethoxycarbonyl)-6-nitro-4-[(2-methoxycarbonyl-4-nitro-1-methylpyrrole)-amino]quinoline (4)

A solution of chloroquinoline **2** (200 mg, 0.713 mmol)[41] and 1-methyl-2-carboxymethyl-4-aminohydrochloride pyrrole **3** (182 mg, 0.8 mmol)[40] in dioxane (4 mL) was added to a round-bottomed flask under argon. Pyridine (160 μL) was added to the reaction and the mixture was refluxed for 2 h. The reddish solid, which was formed, was removed by filtration of the hot suspension. The solid residue was taken up in CH_2Cl_2 (10 mL) and was washed with NaOH (2 M, 2 × 3 mL). The CH_2Cl_2 solution was dried over Na_2SO_4 and concentrated in vacuo. The resulting solid was purified by column chromatography using EtOAc/hexanes (1:3) as an eluent to yield **4** (218 mg, 77%). Mp: 172 °C. ^{1}H NMR (DMSO-d_6): 10.27 (s, 1H), 9.17 (s, 1H), 9.01 (s, 1H), 8.40 (d, 2H), 8.02 (d, 2H), 7.22 (s, 1H), 6.73 (s, 1H), 4.17 (q, 4H), 3.81 (s, 3H), 3.73 (s, 3H), 1.26 (t, 3H). ^{13}C NMR ($CDCl_3$): 14.0, 36.8, 51.0, 61.2, 104.0, 113.4, 117.2, 122.3, 123.2, 130.8, 142.5, 153.4, 154.1, 154.8, 160.7, 168.0. Anal. calcd for $C_{19}H_{18}N_4O_6$: C, 57.29; H, 4.55; N, 14.06. Found: C, 57.46; H, 4.41; N, 14.02.

4.2. 4-(5-Methoxycarbonyl-1-methyl-1*H*-pyrrol-3-ylamino)-6-nitro-quinoline-3-carboxylic acid (5)

To a hot (50 °C) stirred solution of **4** (150 mg, 0.375 mmol) in methanol (5 mL), a solution of 1% NaOH was added dropwise. The solution was heated at 100 °C for 1 h, cooled, filtered, and then acidified to pH 2.0 with 6 N HCl. The resulting orange precipitate was collected, washed with distilled water, and air-dried (97 mg, 70%). Mp: 260 °C. ^{1}H NMR (DMSO-d_6): 9.09 (s, 1H), 8.86 (s, 1H), 8.39 (d, 2H), 8.01 (d, 2H), 7.31 (s, 1H), 6.81 (s, 1H), 3.90 (s, 3H), 3.81 (s, 3H). Anal. calcd for $C_{17}H_{14}N_4O_6 \cdot 1$ HCl: C, 50.19; H, 3.94; N, 13.77. Found: C, 50.19; H, 3.94; N, 13.52.

4.3. 8-Methyl-2-nitro-7-oxo-8,11-dihydro-7*H*-5,8,11-triaza-cyclopenta[*b*]phenanthrene-9-carboxylic acid methyl ester (6)

A solution of **5** (25 mg, 0.0678 mmol) in excess $POCl_3$ (3 mL) was refluxed for 3 h. $POCl_3$ was removed under vacuum and the residue was partitioned between $CHCl_3$ (5 mL) and water (2 mL). The aqueous layer was separated, basified with NaOH (1 M, 2 mL), and extracted with $CHCl_3$ (3× 5 mL). The combined organic extracts were dried over Na_2SO_4 and concentrated in vacuo. The resulting mixture was purified by column chromatography using $CHCl_3$ as an eluent to yield **6** (14 mg, 60%). Mp: 264 °C. ^{1}H NMR ($CDCl_3$): 10.09 (s, 1H),

9.95 (s, 1H), 8.57 (d, 2H), 8.32 (d, 2H), 7.65 (s, 1H), 7.25 (s, 1H), 4.63 (s, 3H), 4.05 (s, 3H). Anal. calcd for $C_{17}H_{12}N_4O_5 \cdot 1\ Na^+$: C, 54.55; H, 2.96; N, 14.96. Found: C, 54.98; H, 3.06; N, 14.85.

4.4. 8-Methyl-2-nitro-7-oxo-8,11-dihydro-7*H*-5,8,11-triaza-cyclopenta[*b*]phenanthrene-9-carboxylic acid (7)

To a solution of compound **6** (105 mg, 0.3 mmol) in DMF (8 mL), 1 N NaOH (6 mL) was added dropwise and the reaction mixture was heated to 90 °C for 48 h. The reaction mixture was cooled to room temperature and acidified to pH 2.0 by dropwise addition of 6 N HCl. The red precipitate was collected, washed with distilled water, and air-dried to give the carboxylic acid 7 (95 mg, 95%). Mp: 268 °C. ^{1}H NMR (DMSO-d_6): 13.4 (s, 1H), 9.75 (s, 1H), 9.52 (s, 1H), 8.6 (d, 2H), 8.24 (d, 2H), 6.9 (s, 1H), 4.37 (s, 3H). Anal. calcd. for $C_{16}H_{10}N_4O_5 \cdot 1$ HCl·0.5 H_2O: C, 50.08; H, 3.15; N, 14.60. Found: C, 49.93; H, 3.38; N, 14.65.

4.5. 8-Methyl-2-nitro-7-oxo-8,11-dihydro-7*H*-5,8,11-triaza-cyclopenta[*b*]phenanthrene-9-carboxylic acid (2-dimethylamino-ethyl)-amide (1)

To a chilled (0 °C) solution of carboxylic acid **7** (50 mg, 0.148 mmol) and HOBt (20 mg, 0.148 mmol) in anhydrous DMF (3 mL), a solution of DCC (32 mg, 0.1554 mmol) in anhydrous DMF was added. The mixture was stirred at 0 °C for 1 h followed by the addition of 3-dimethylaminopropylamine (19 μl, 0.148 mmol) The reaction mixture was stirred for an additional 1 h at 0 °C, followed by 12 h at 25 °C. The reaction mixture was poured into ethyl acetate and the solution was washed several times with saturated solution of sodium bicarbonate. The organic layer was separated from the aqueous layer, dried over Na_2SO_4, and evaporated to dryness. The residue was purified by column chromatography using $CHCl_3$/MeOH (85:15) to yield 22 mg of **8** (37%). Mp: 205 °C. λ_{max} (aqueous buffer, pH 7.0): 260 (41,620 $M^{-1}\,cm^{-1}$), 285 (42,213 $M^{-1}\,cm^{-1}$), 360 (19,110 $M^{-1}\,cm^{-1}$), 375 (18,600 $M^{-1}\,cm^{-1}$), 430 (13,500 $M^{-1}\,cm^{-1}$) ^{1}H NMR ($CDCl_3$): 10.12 (s, 1H), 9.72 (s, 1H), 9.19 (s, 1H), 8.54 (d, 2H), 8.23 (d, 2H), 7.09 (s, 1H), 4.83 (s, 3H), 3.63 (m, 2H), 3.33 (s, 6H), 2.60 (t, 2H), 1.84 (m, 2H). ^{13}C NMR ($CDCl_3$): 24.7, 33.8, 40.8, 45.5, 59.6, 104.2, 117.9, 120.8, 122.8, 126.5, 128.8, 130.2, 143.2, 144.2, 145.8, 147.8, 151.2, 153.9, 161.2. Anal. calcd for $C_{21}H_{22}N_6O_4 \cdot$DCU: C, 63.14; H, 7.17; N, 17.32. Found: C, 63.09; H, 6.88; N, 17.74.

4.6. Ethidium bromide displacement assay of 1

To a 1.0 mL quartz cell containing Tris buffer (0.1 M Tris–HCl, 25 mM NaCl, pH 7) and ethidium bromide (4.0 μM), either the AT or GC hairpin oligonucleotide (8.0 μM bp) was added. The fluorescence was determined using an excitation wavelength of 545 nm and an emission wavelength of 595 nm. Various concentrations of **1** (in DMSO) were added, the solution mixed, and the change in fluorescence at 595 nm was recorded after a 5-min incubation period. The data were corrected for differences in volume due to the addition of drug and the binding constants were determined as stated in the text.

4.7. Unwinding assay of supercoiled plasmid DNA

To a solution of supercoiled plasmid DNA pBR322 (10 nM, New England Biolabs) in TAE buffer, various concentrations of compound **1** were added up to 200 nM. The mixtures were incubated at room temperature for 1 h followed by electrophoresis on a 0.8% agarose gel using TAE buffer at 75 V. After electrophoresis, the gel was stained for 30 min with ethidium bromide and washed with water for 20 min. The gel was then analyzed under UV light using a Kodak Image station running Kodak 1D Image software.

4.8. Equilibrium dialysis assay of 1

Concentrations of nucleic acids were determined using the published extinction coefficients.[49,53] A dialysis solution composed of 300 mL of a buffer containing 6 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1 mM Na_2EDTA, and 185 mM NaCl (pH 7.0), and compound **1** (5.0 μM final) was prepared. Individual solutions (100 μL, 75 μM bp) of nucleic acids were pipetted into Slide-A-Lyzer Mini dialysis units (Pierce). The dialysis units were placed into a beaker containing the dialysis solution, covered, and equilibrated with continuous stirring at room temperature for 24 h. At the end of the equilibration, the nucleic acid samples were removed from the dialysis units, placed into a microcentrifuge tube, and treated with SDS to a final concentration of 1%. The tubes were spun to remove any precipitate and the concentration of compound **1** was determined by UV–vis spectroscopy.

4.9. Drug-induced formation of G-quartet by 1

For each reaction, oligonucleotide 5′-$T_2G_{20}T_2$ (8.0 μM) was heated to 95 °C for 5 min in TE buffer containing 0.1 M KCl. The mixture was slowly cooled to room temperature and compound **1** (0, 10, 25, and 50 μM) was added. The mixtures were incubated at room temperature for 1 h and then loaded into a 12% polyacrylamide gel prepared with 0.5× TBE supplemented with 20 mM KCl. The gel was run at 4 °C for approximately 6 h and then stained with SYBR Gold. The gel was analyzed under UV light using a Kodak Image Station running Kodak 1D Image software. The location of G-quartet was determined by running a sample of known 5′-$T_2G_{20}T_2$ G-quartet on the same gel.

4.10. Competition with TmPyP4

Quartet DNA (5′-$T_2G_{20}T_2$, 50 μM) and TmPyP4 (10 μM, Calbiochem) were added to a 1.0 mL quartz cell containing 6 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1 mM Na_2EDTA, and 185 mM NaCl (pH 7.0) buffer. The fluorescence was determined using an excitation wavelength of 429 nm and the emission wavelength was scanned from 600 to 700 nm.[56] Various concentrations of **1** (in DMSO) were added, the solution mixed, and the change in fluorescence was determined after a five min incubation period. The data were corrected for differences in

volume due to the addition of drug and binding constants were determined according to the method outlined by Boger.[44,45]

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