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Synthesis and DNA-binding affinities of monomodified berberines

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Abstract—Four new monomodified berberines have been synthesized in moderate to good yields starting from berberine and fully characterized by HRMS and ^{1}H NMR. Spectrometric titration and ethidium bromide displacement experiments indicate that these berberine derivatives, especially the one having primary amino group, strongly bind with calf-thymus DNA, presumably via an intercalation mechanism.

1. Introduction

A large number of molecules can bind noncovalently and specifically to DNA. These associative interactions lead to significant modification of the structures of the DNA, and therefore may have an important impact on the physiological function of the DNA, namely gene expression. Therefore, there is increasing interest in the development of DNA-binding agents that can probe the structure and function of DNA. To date, several classes of DNA-binding molecules have been established and studied in detail.

In these aspects, berberine (**1**, Chart 1), a representative protoberberine alkaloid, is attractive as one of the promising lead structure for the development of functional DNA-binding drugs. This is, in part, because this naturally occurring compound exhibits a broad range of pharmacological activities,[1] such as antimicrobial, antimalarial, antiinflammtory, and antifungal, as well as anticancer activities with significantly low toxicity. Another reason lies in the fact that berberine has been proven to be a DNA-binder, by means of several analytical techniques, including absorption, fluorescence, NMR, electrospray ionization mass (ESI-MS), and CD spectrometries.[2,3] Meanwhile, it is reported that berberine has the ability to induce apoptosis in promyelocytic leukemia HL-60 cells.[4] These biological

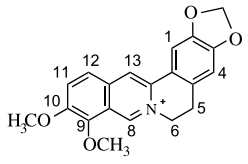

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Chart 1.

properties are thought to result from the binding of berberine to DNA,[5] and spur various efforts to clarify the nature of their interactions.[2,3]

Though a few issues need to be further addressed in the interaction of berberine with DNA, computer-aided modeling studies of berberine–DNA complex suggest that berberine binds to DNA from its C5–C6–N^+–C8 side (Chart 1).[2b] Furthermore, it has been shown that the 9-position in berberine analogs is an important determinant of DNA topoisomerase II inhibition.[6] Thus, the interaction with DNA of berberine derivatives with structural perturbation at the 9-position may provide more insight into the different aspects of association process of berberine with DNA. In this paper, we describe the synthesis and DNA-binding affinities of four new berberine derivatives, **4a–d** (Scheme 1), having hydroxyethoxy, aminoethoxy, imidazolylethoxy, and *N*-methylpiperazinylethoxy groups at the 9-position, with the aim to assess the effect of the modification at this position on the DNA-binding affinity and mode. These functional substituents, frequently used in the design of DNA-binders, are expected to increase binding affinities through hydrogen bonding and/or electrostatic interaction with DNA.

Keywords: Berberine; Synthesis; DNA-binder; Spectrometry.
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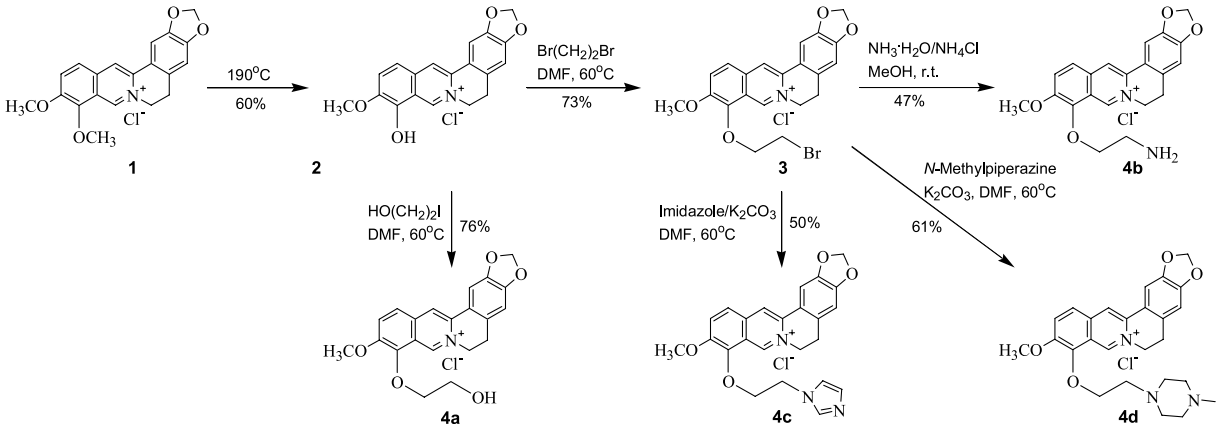

Scheme 1. Synthetic route for compounds **4a-d.**

2. Results and discussion

2.1. Synthesis of monomodified berberines 4a–d

The synthetic route of monomodified berberines **4a**–**d** is shown in Scheme 1. Partial demethylation of berberine **1** at 190 °C under vacuum for 15 min, gave berberrubine **2**[7] in 60% yield. Alkylations of **2** with 1,2-dibromoethane and 2-iodoethanol in DMF, followed by exchanging the counter anions into chloride, afforded **3** and **4a** in 73% and 76%, respectively. Ammonolysis of **3** with 28% ammonia solution–NH_4Cl, imidazole, and *N*-methylpiperazine gave compounds **4b**–**d** in 47–61% yields, respectively. Additional alkaline catalyst (i.e., K_2CO_3) was necessary in the synthesis of **4c**–**d**. Compounds **4a**–**d** were fully characterized by HRMS and ^{1}H NMR. All these compounds afforded correct HR ESI-MS spectra with the *m*/*z* values corresponding to $[M–Cl]^+$. Their ^{1}H NMR spectra, for example, **4b** in Figure 1, were also consistent with the given structures.

2.2. DNA-binding interaction

The interactions of compounds **4a**–**d** with DNA were investigated by means of spectrometric titration and ethidium bromide (EB) displacement experiments to evaluate their binding affinities and modes.[8] Figure 2 exemplifies the spectrofluorimetric titration of **4a** with calf-thymus (CT) DNA. It is observed that the addition of CT DNA leads to large increase in the fluorescence intensities of these compounds except **4b**, strongly indicating their interactions with CT DNA. These significant changes ensured the availability of the association constants (K_a's) of **1**, **4a**, and **4c**–**d** with CT DNA (Table 1), from the analyses of the relationship between the fluorescence intensities and the DNA concentrations by nonlinear curve fitting methods.[9] It should be noted, however, that the complexation of **4b** with CT DNA did not induce significant change in the fluorescence intensity. Therefore, its association constant with CT DNA was obtained from EB displacement experiment (vide infra).

It can be seen from Table 1 that the relative binding affinities of berberine **1** and **4a**–**d** with CT DNA are in the order of **4d > 4c > 4a ⩾ 1** from spectrofluorimetric titration, and **4b > 4d > 4c > 4a** from EB displacement assay. Thus, the overall order of relative binding affinities is established as **4b > 4d > 4c > 4a ⩾ 1**. Consequently compounds **4a**–**d**, compared with berberine **1**, show comparable or significantly higher binding affinities toward CT DNA. This result indicates that the modification at the 9-position can enhance the DNA-binding ability of berberine, which was further supported by the following spectrophotometric titration and competitive EB displacement experiments. The most efficient binding was observed in the primary amino group-modified derivative **4b** whose binding ability is over sevenfold higher than that of **4a** (and **1**).[10] This substantial increase may be rationalized by taking into account the structural characteristics inherent in **4b**: its less hindered protonated amino group, compared with protonated imidazolyl and methylpiperazinyl groups of **4c**–**d**, can interact more strongly with CT DNA through hydrogen bonding and enhanced electrostatic interactions in the present measuring conditions (pH 6.35).

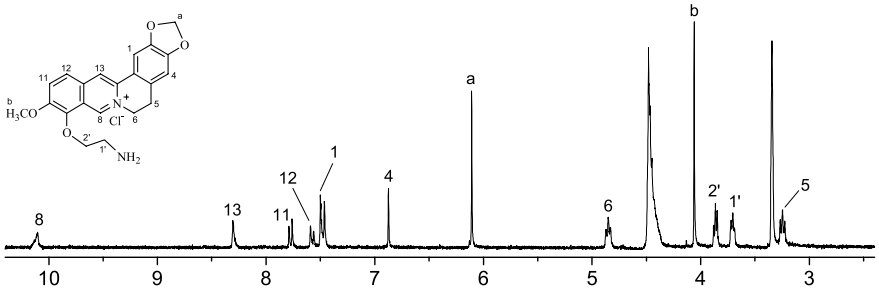

Figure 1. ^{1}H NMR spectrum (300 MHz) of **4b** in $CDCl_3/CD_3OD$ (1/1).

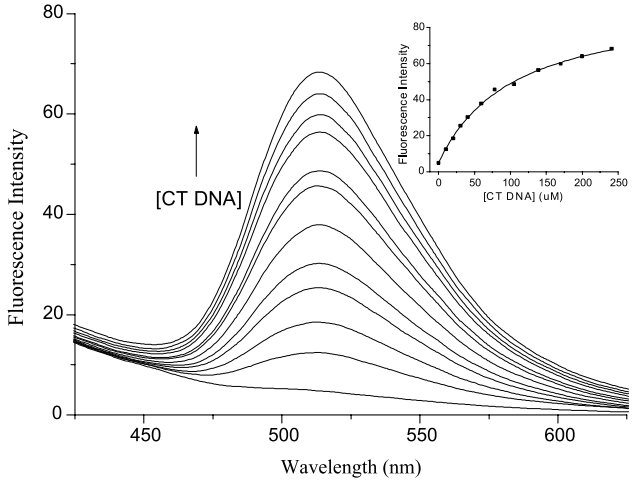

Figure 2. Spectrofluorimetric titration of **4a** (2.26×10^{-6} M) with CT DNA of increasing concentration ($0 - 2.41 \times 10^{-4}$ M) in 50 mM Tris–HCl buffer (pH 6.35) at room temperature, ex 355 nm. The inset indicates the relationship between the fluorescence intensity (at em 513 nm) and the concentration of CT DNA.

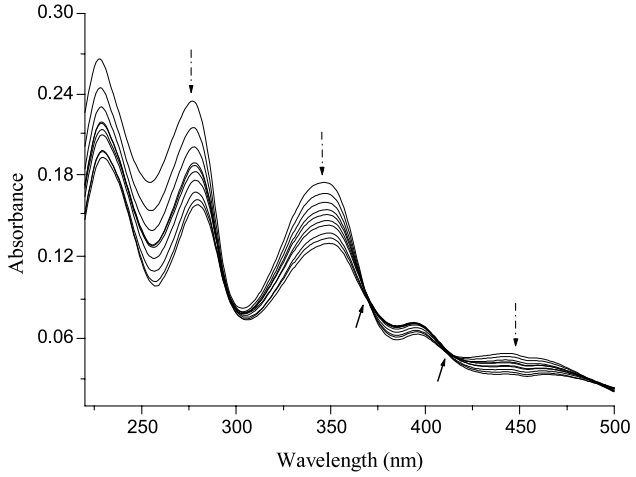

Figure 3. Spectrophotometric titration of **4b** (8.3×10^{-6} M) with CT DNA ($0 - 8.5 \times 10^{-5}$ M) in 50 mM Tris–HCl buffer (pH 6.35) at room temperature. The figure contains the absorption spectrum of free **4b**, intermediate spectra, and final spectrum of **4b**–CT DNA complexes. The dash–dot arrows indicate the decreasing absorption bands during the course of titration; solid arrows indicate the isosbestic points.

Table 1. Association constants (K_a's, M^{-1}) and photo physical properties of **1** and **4a–d** with CT DNA[a]

Compound	K_a ($\times 10^{-4}$)	Red shift (nm)[d]	Hypochromicity[d] (%)
1	1.12 ± 0.04[b]	1	5
4a	1.18 ± 0.07[b]	7	14
	4.64 ± 0.25[c]		
4b	33.65 ± 1.73[c]	2	26
4c	3.56 ± 0.35[b]	9	23
	5.10 ± 0.69[c]		
4d	6.08 ± 0.55[b]	8	19
	8.24 ± 1.45[c]		

[a] In 50 mM Tris–HCl buffer (pH 6.35) at room temperature.
[b] Obtained from spectrofluorimetric titration experiment.
[c] Obtained from EB displacement experiment.
[d] Measured at the absorption maximum at 340 nm.

This result may provide some guidances for future rational design of modified berberines having potentially high DNA-binding affinities.

The interactions of **4a–d** with CT DNA were also studied by absorption spectrometry. A representative spectrophotometric titration is shown in Figure 3 for **4b** with CT DNA. It is observed that the addition of CT DNA, at pH 6.35, to the solutions of **4a–d** at the DNA/berberine molar ratios varying from 0 to 10 induced large bathochromic shifts (2 – 9 nm) and hypochromicities (14 – 26%) (Fig. 3 and Table 1). These spectroscopic variations are strongly indicative of the interaction of **4a–d** with CT DNA. The extent of spectral change, especially hypochromicity is related to the strength of binding.[11] Thus, the observed larger red shifts and much greater hypochromicity values of compounds **4a–d** with CT DNA than compound **1** suggest that **4a–d** bind to CT DNA more strongly than **1**. On the other hand, during the course of titration, well-resolved isosbestic points at 370 and 410 nm were observed, revealing the existence of, mainly, one particular binding mode between **4a–d** and CT DNA.[12] The magnitudes of these spectral perturbations provide prima facie evidence for intercalative binding because they establish that π systems of **4a–d** are intimate contacts with the DNA bases.[13]

The DNA-binding modes of **4a–d** were further monitored by a fluorescent EB displacement assay.[14,15] It is well known that EB can intercalate nonspecifically into DNA and strongly fluoresces upon complexation. Competitive binding of other drugs to DNA with EB will result in the displacement of bound EB and a decrease in the fluorescence intensity. This fluorescence-based competition technique can provide indirect evidences for the DNA-binding mode. Figure 4 shows the plots of the relative fluorescence intensity (FI; I/I_0) of EB against the

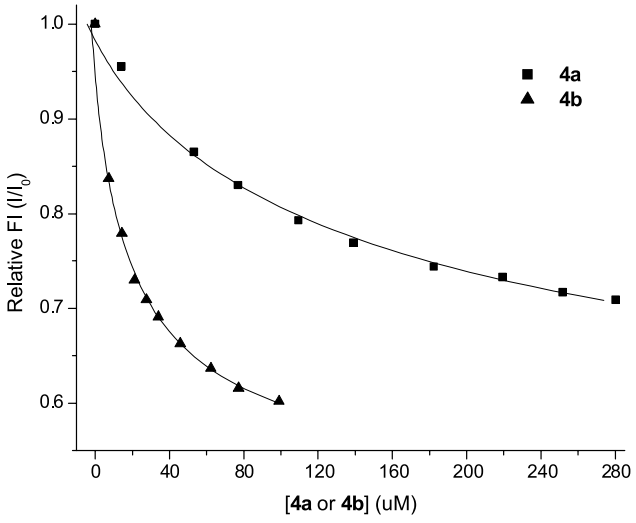

Figure 4. Fluorescence decrease of EB (3.03×10^{-6} M) induced by the competitive binding of **4a** and **4b** to CT DNA (2.40×10^{-6} M) in 50 mM Tris–HCl buffer (pH 6.35) at room temperature, excitation 490 nm, and emission 594 nm.

concentrations of added **4a**–**b**. It is clear that the fluorescence intensity of EB decreases upon the addition of **4a** or **4b**, indicating that **4a**–**b** can substitute EB bound to CT DNA. Similar phenomena were observed for **4c**–**d**. These results, together with the bathochromic shifts, hypochromicities, and isosbestic points observed in the spectrophotometric titration, imply that **4a**–**d** may bind to CT DNA via the same binding mechanism (i.e., intercalation) with EB. Thus, the DNA-binding mode of berberine has been reserved during modification at the 9-position.[2c] On the other hand, analyses of I/I_0 as a function of the concentrations of the added competitive **4a**–**d** by nonlinear curve fitting methods[16] can afford their association constants with CT DNA (Table 1).

3. Concluding remarks

Four new monomodified berberines have been successfully synthesized in moderate to good yields. Spectrometric titration and EB displacement experiments indicate that these derivatives, especially the one having primary amino group, strongly bind with CT DNA, presumably via an intercalation mechanism. The present results suggest that the modification at the 9-position of berberine can significantly improve its DNA-binding affinity while reserving its binding mode as an intercalator. The efforts aiming at exploiting the practical potential of berberine derivatives having diverse substituents at the 9-position are actively continuing in our laboratories.

4. Experimental

Fluorescence measurements were made on a Perkin-Elmer Luminescence Spectrometer LS55. Absorption spectra were recorded on a Jasco UV-530 ultraviolet–visible spectrophotometer. NMR spectra were recorded at Varian Unity INOVA-300 in CD_3OD–$CDCl_3$, and TMS was used as internal reference. HR MS spectra were measured on Perkin-Elmer Sciex Api Qstar Pulsar i LCMS.

CT DNA was purchased from Pharmacia (Uppsala, Sweden). Its concentration was determined spectrophotometrically using the molar extinction coefficient of 6600 M^{-1} cm^{-1} at 260 nm. Berberine chloride was isolated from Chinese herbal medicine ('Huang-Lian') and recrystallized from hot water. All other synthetic reagents were of analytical grade. Berberrubine **2** was prepared according to the reported methods.[7]

4.1. Synthesis of 9-*O*-2-bromoethylberberine 3

A solution of **2** (0.7 g, 2.0 mmol) and 1,2-dibromoethane (3.7 g, 20 mmol) in dry DMF (5 mL) was heated at 60 °C for 6 h and then Et_2O was added. The resulting solid was filtered and then subject to anion-exchange into chloride form. The crude product was chromatographed on a silica gel column, eluted with $CHCl_3$/MeOH (10/1, v/v) to give a light yellow solid of **3** (0.68 g, 73%). ^1H NMR (1/1 CD_3OD/$CDCl_3$, 300 MHz): δ 3.29 (t, 2H, J = 7.0 Hz), 3.85 (t, 2H, J = 6.0 Hz), 4.14 (s, 3H), 4.80 (t, 2H, J = 6.0 Hz), 4.94 (t, 2H, J = 7.0 Hz), 6.13 (s, 2H), 6.92 (s, 1H), 7.57 (s, 1H), 8.02 (d, 1H, J = 10.0 Hz), 8.73 (d, 1H, J = 10.0 Hz), 8.61 (s, 1H), 9.87 (s, 1H); ESI-MS: m/z 428 ($[M–Cl]^+$).

4.2. Synthesis of 9-*O*-2-hydroxyethylberberine 4a

A solution of **2** (0.7 g, 2.0 mmol) and 2-iodoethanol (3.4 g, 20 mmol) in dry DMF (5 mL) was heated at 60 °C for 6 h and then Et_2O was added. The resulting solid was filtered and then subject to anion-exchanged into chloride form. The crude product was chromatographed on a silica gel column, eluted with $CHCl_3$/MeOH (8/1, v/v) to give a light yellow solid of **4a** (0.53 g, 76%). ^1H NMR (1/1 CD_3OD/$CDCl_3$, 300 MHz): δ 3.20 (t, 2H, J = 6.2 Hz), 3.80 (t, 2H, J = 5.6 Hz), 4.04 (s, 3H), 4.35 (t, 2H, J = 5.6 Hz), 4.87 (t, 2H, J = 6.0 Hz), 6.15 (s, 2H), 7.07 (s, 1H), 7.77 (s, 1H), 7.94 (d, 1H, J = 10.0 Hz), 8.15 (d, 1H, J = 10.0 Hz), 8.90 (s, 1H), 9.85 (s, 1H); HRMS for $C_{20}H_{21}NO_5$ ($[M–Cl]^+$) Calcd: 366.1342. Found: 366.1338.

4.3. Synthesis of 9-*O*-2-aminoethylberberine 4b

A solution of **3** (92 mg, 0.2 mmol), 28% ammonia solution (3 mL) and NH_4Cl (50 mg) in MeOH (5 mL) was stirred at rt for 5 h, and then extracted with CH_2Cl_2 (15 mL × 3). The organic layer was washed with brine (5 mL × 3). After the solvent was evaporated in vacuum, the obtained crude product was chromatographed on an Al_2O_3 column, eluted with $CHCl_3$/MeOH (8/1, v/v) and then anion-exchanged with AgCl into chloride form. A red solid of **4b** (37 mg, 47%) was obtained. ^1H NMR (1/1 CD_3OD/$CDCl_3$, 300 MHz): δ 3.24 (t, 2H, J = 7.0 Hz), 3.69 (t, 2H, J = 5.6 Hz), 3.86 (t, 2H, J = 5.6 Hz), 4.06 (s, 3H), 4.85 (t, 2H, J = 7.0 Hz), 6.10 (s, 2H), 6.87 (s, 1H), 7.46 (s, 1H), 7.56 (d, 1H, J = 9.3 Hz), 7.76 (d, 1H, J = 9.6 Hz), 8.30 (s, 1H), 10.10 (s, 1H); HRMS for $C_{21}H_{21}N_2O_4$ ($[M–Cl]^+$) Calcd: 35.1501. Found: 366.1523.

4.4. Synthesis of 9-*O*-2-(1′-imidazolylethyl)berberine 4c and 9-*O*-2-(4′-ethylpiperazinylethyl)berberine 4d

4.4.1. General procedures. A solution of **3** (0.2 mmol), imidazole or 4-methylpiperazine (2.0 mmol) and anhydrous K_2CO_3 (2.0 mmol) in dry DMF (5 mL) was heated at 60 °C for 10 h and then Et_2O was added. The resulting solid was filtered and subject to anion-exchange into chloride form. The crude product was chromatographed on an Al_2O_3 column, eluted with $CHCl_3$/MeOH (10/1, v/v) to give **4c–d**.

*Compound **4c***. A light yellow solid, yield 50%. ^1H NMR (1/1 CD_3OD/$CDCl_3$, 300 MHz): δ 3.24 (t, 2H, J = 6.3 Hz), 4.08 (s, 3H), 4.56 (t, 2H, J = 5.3 Hz), 4.68 (t, 2H, J = 5.3 Hz), 4.78 (t, 2H, J = 6.6 Hz), 6.11 (s, 2H), 6.88 (s, 1H), 7.09 (s, 1H), 7.22 (s, 1H), 7.47 (s, 1H), 7.48 (s, 1H), 7.82 (s, 1H), 7.96 (s, 1H), 8.48 (s, 1H), 8.71 (s, 1H); HRMS for $C_{24}H_{22}N_3O_4$ ($[M–Cl]^+$) Calcd: 416.1610. Found: 416.1643.

*Compound **4d***. A light yellow solid, yield 61%. ^1H NMR (1/1 CD_3OD/$CDCl_3$, 300 MHz): δ 2.32 (s, 3H),

2.47–2.58 (m, 4H), 2.60 – 2.72 (m, 4H), 2.92 (t, 2H, $J = 6.0$ Hz), 3.26 (t, 2H, $J = 7.0$ Hz), 4.10 (s, 3H), 4.53 (t, 2H, $J = 6.0$ Hz), 4.93 (t, 2H, $J = 7.0$ Hz), 6.10 (s, 2H), 6.92 (s, 1H), 7.59 (s, 1H), 7.96 (d, 1H, $J = 10.0$ Hz), 8.04 (d, 1H, $J = 10.0$ Hz), 8.63 (s, 1H), 9.83 (s, 1H); HRMS for $C_{26}H_{30}N_3O_4$ ($[M–Cl]^+$) Calcd: 448.2236. Found: 448.2263.

4.5. Spectrofluorimetric titration experiments

Fluorescence spectra were measured at rt using quartz cells of 1 cm path. To the solutions of the drugs (2.1×10^{-6} M) in 50 mM Tris–HCl buffer (pH 6.35) were added aliquots of CT DNA (1.39×10^{-3} M) solution containing drugs (2.1×10^{-6} M) in 50 mM Tris–HCl buffer (pH 6.35). This operation ensured that the concentration of CT DNA increased gradually from 0 to 2.6×10^{-4} M, while the concentrations of drugs were kept constant. The mixing was achieved by stirring for 10 min. Then, the corresponding fluorescence spectra were measured (ex 355 nm, ex/em 15 nm/15 nm). Association constants (K_a's) were derived from nonlinear curve fitting, using the equation $I = I_0 + ((I_\infty - I_0)/2[B]_0)\{([DNA]_0+[B]_0+1/K_a) - (([DNA]_0+[B]_0+1/K_a)^2 - 4[DNA]_0[B]_0)^{1/2}\}$,[9] wherein $[DNA]_0$ and $[B]_0$ are the initial analytical concentrations of DNA and drugs, respectively; I, I_0, and I_∞ represent the fluorescence intensities (at 520 nm) of the sample, drugs (i.e., **1**,**4a**, and **4c**–**d**) alone, and the intensity when drugs are totally bound, respectively.

4.6. Spectrophotometric titration experiments

Absorption spectra were recorded at rt using conventional quartz cells of 1 cm path. Spectrophotometric titrations were performed with fixed concentrations of the drugs (i.e., **1** and **4a**–**d**) while gradually increasing concentration of CT DNA. Typically, to a solution of **4a** (8.0×10^{-6} M) in 50 mM Tris–HCl buffer (pH 6.35) were added aliquots of CT DNA (8.0×10^{-4} M) solution containing **4a** (8.0×10^{-6} M) in 50 mM Tris–HCl buffer (pH 6.35). The mixing was achieved by stirring for 10 min. Then, the corresponding absorption spectra were measured. The spectrophotometric titrations of **1** and **4b**–**d** were conducted in a similar way.

4.7. EB displacement experiments

Competitive EB displacement experiments were performed in matched quartz cells of 1 cm path. To a solution of CT DNA (2.40×10^{-6} M) and EB (3.03×10^{-6} M) in 50 mM Tris–HCl buffer (pH 6.35) were added aliquots of the drug (3.74×10^{-4} M) solutions containing CT DNA (2.40×10^{-6} M) and EB (3.03×10^{-6} M) in 50 mM Tris–HCl buffer (pH 6.35). This operation ensured that the concentration of drugs increased gradually from 0 to 9.9×10^{-5} M, while the concentrations of CT DNA and EB were kept constant. Then, the corresponding fluorescence spectra were measured (ex 490 nm, ex/em 15 nm/15 nm). Association constants were derived from nonlinear curve fitting, using the equation: $[B]_0 = ((I_\infty - I)/((I - I_0) \times K_d \times K_a) + 1) \times ([DNA]_0 - (I - I_0) \times K_d/(I_\infty - I) - [EB]_0 \times (I - I_0)/(I_\infty - I_0))$,[16] wherein $[DNA]_0$, $[EB]_0$, and $[B]_0$ are the initial analytical concentrations of CT DNA, EB, and **4a**–**d**, respectively. K_d ($= 2.40 \times 10^{-6}$ M) is the disassociation constant between CT DNA and EB.

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