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Synthesis, DNA-binding affinities, and binding mode of berberine dimers

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Abstract—Six novel berberine dimers (**3a**–**f**) were synthesized in 37–84% yield from the reaction of berberrubine (**2**) with dihaloalkanes of varying lengths from two to seven carbons. Their interactions with calf thymus (CT) DNA and three double helical oligodeoxynucleotides, d(AAGAATTCTT)$_2$, d(AAGCATGCTT)$_2$, and d(TAAGAATTCTTA)$_2$, were investigated by means of fluorometric titration and ethidium bromide (EB) displacement experiments. Compared with the monomeric parent berberine (**1**), these dimers' DNA-binding affinities increased up to approximately 100-fold, suggesting a cooperative interaction of the two berberine subunits in the molecules. Furthermore, these dimers linked by different spacers show a prominent structure–activity relationship when bound with oligodeoxynucleotides. The relative binding affinities are in the order of **3b** > **3a** > **3c** > **3d** > **3e** > **3f** with d(AAGAATTCTT)$_2$ and d(TAAGAATTCTTA)$_2$, and **3b** > **3c** > **3a** > **3d** > **3e** > **3f** with d(AAGCATGCTT)$_2$. Dimer **3b**, linked with a propyl chain, exhibits the highest binding affinity. This suggests that a propyl chain may be the most suitable spacer to bridge the two berberine units for DNA binding. Spectrophotometric titration and competitive EB displacement of berberine (**1**) and dimer **3b** indicate that both berberine and its dimers form intercalating complexes with duplex DNA. A larger redshift, a stronger hypochromic effect, and a much higher EB displacement ratio, observed in **3b**, indicate that the dimer is in more intimate contact with DNA than berberine. In addition, no obvious binding of canadine (**4**), a hydrogenated product of berberine, with CT DNA was observed, suggesting critical roles of the quaternary ammonium cation and planar structure in the DNA-binding of berberine.

1. Introduction

Controlling gene expression with small DNA-binding molecules has been a challenge at the interface of medicinal chemistry and biology.[1] To achieve this goal, a number of chemical approaches have been investigated to search for small molecules that can selectively bind to DNA and either activate or inhibit gene expression. This includes the examination of the specific and noncovalent interactions of small organic molecules with DNA and RNA.[2] Efforts have focused on the rational design of ligands capable of binding tightly and specifically to any sequence of double-stranded DNA. These agents would potentially have wide applications in elucidating the mechanism of action of antitumor and antivirus drugs,[3] and developing chemotherapeutic agents.[4] Modification of natural products with established affinities and binding modes with DNA has been demonstrated to be a practicable approach. A typical example is the rational structural modification of the antibiotics netropsin and distamycin leading to the development of lexitropsins.[5]

Alkaloids are well-known to be a group of important natural products in medicinal chemistry due to their extensive biological activities. Especially noteworthy are the isoquinoline alkaloids that are widely distributed in several botanical families having myriad therapeutic applications.[6] Protoberberine alkaloids, such as berberine (**1**) and palmatine, possessing antimicrobial, antileukemic, anticancer, and topoisomerase inhibitory activities,[7] are useful for the development of more efficient DNA-binding agents. Berberine is known as a DNA binder and its binding affinities have been extensively characterized by employing several analytical techniques including absorption, fluorescence, NMR,

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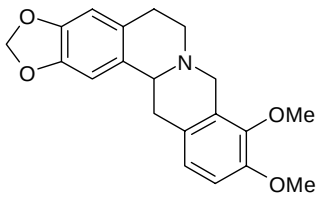


Figure 1. Structure of canadine.

and electrospray ionization mass (ESI-MS) spectrometries.[8] However, its modest binding affinities with duplex DNA necessitate structural modifications to produce novel DNA-binding agents with enhanced affinities.

We have previously reported the synthesis of five dimeric berberines **3a**–**e** linked with C2 ~ C6 alkyl chains and described their greatly enhanced DNA-binding affinities.[9] The results strongly suggest that these dimers **3a**–**e** are exploitable as potentially strong DNA-binders. Accordingly, these observations intensify our further efforts to establish the structure–activity relationship and to clarify their DNA-binding modes, by (i) synthesizing a series of dimeric berberines linked with alkyl chains of varying lengths from two carbons to six carbons, that is **3a**–**e**, plus another dimer **3f** linked by a longer alkyl chain (i.e., heptyl group); and (ii) evaluating the binding affinities of **3a**–**f** toward various DNA sequences, that is, three double-stranded oligodeoxynucleotides, d(AAGAATTCTT)$_2$, d(AAGCATGCTT)$_2$, d(TAAGAATTCTTA)$_2$, and calf thymus (CT) DNA. In this paper, we present a full account of the synthesis, DNA-binding affinities, and binding mode of these dimeric berberines. In addition, to evaluate the effect of the quaternary ammonium cation of berberine on DNA-binding, the interaction of canadine (**4**, namely, tetrahydroberberine, Fig. 1) with CT DNA was examined.

2. Results and discussion

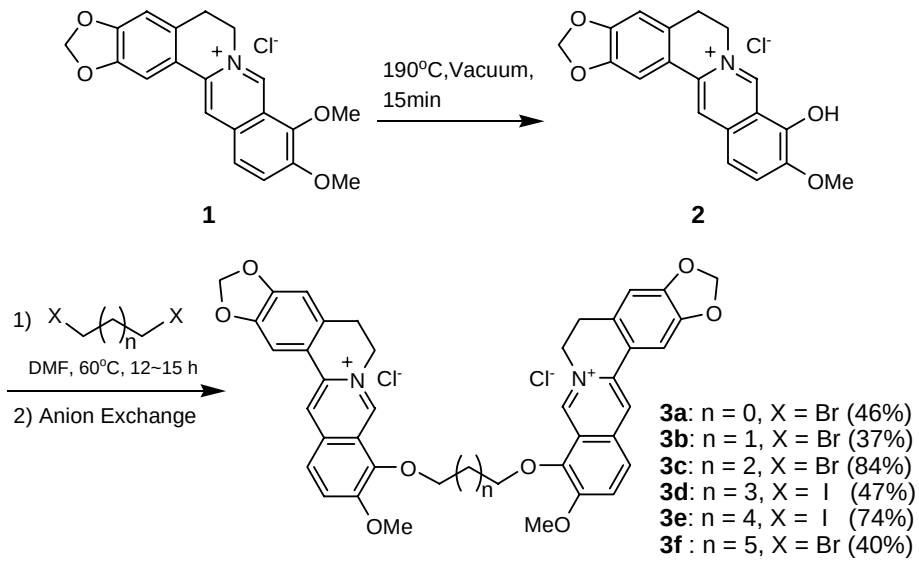
2.1. Synthesis

The route of synthesis of compounds **3a**–**f** is shown in Scheme 1. Partial demethylation of berberine (**1**) at 190 °C under vacuum for 15 min, according to the reported protocols,[10] gave berberrubine (**2**) in 60% yield. Linking two berberrubine molecules with dibromo-(**3a**–**c** and **3f**) or diiodoalkanes (**3d**,**e**) of varying lengths from two to seven carbons, followed by the exchange of all anions into chloride, afforded the corresponding berberine dimers **3a**–**f** in 37–84% yield. Compounds **3a**–**f** were fully characterized by ESI-HRMS and ^{1}H NMR spectral analysis. Each compound afforded two-charged ESI-MS peaks ($[M-2Cl]^{2+}$) in the mass spectrum. The ^{1}H NMR spectra of these compounds showed only one set of berberine moiety, indicating the presence of a symmetric structure in the molecule. The ratios of the integrated areas for the protons of berberine subunits to those for the alkyl chain protons in the ^{1}H NMR spectra were in full accord with the linked berberine dimeric structures.

2.2. DNA-binding affinities of berberine dimers (3a–f)

Various techniques for precise determination of binding constants have been developed and provide information for understanding the nature of the complexes between small molecules and biomacromolecules. Fluorescence spectrometry is a very sensitive analytical technique, which has been widely used in the investigation of noncovalent complexes of small organic molecules with biopolymers, such as DNA.[2a,b] The complex formation is reflected in the change, either enhancement or quenching, of fluorescence intensities.

Berberine and dimeric berberines **3a**–**f** studied in this paper have strong absorbance peaks at around



Scheme 1. Synthetic route for berberine dimers **3a**–**f**.

350 nm, and relatively weak and broad absorbance peaks above 400 nm. They weakly fluoresce at around 520 nm in aqueous solution when excited at 350 or 450 nm.[8c,d,e] Upon complex formation with DNA, their fluorescences are greatly enhanced. These fluorescence spectroscopic changes ensure the spectrofluorimetric determination of the binding activities of berberine and its dimers with DNA, such as CT DNA and self-complementary double-stranded oligodeoxynucleotides, d(AAGAATTCTT)$_2$, d(AAGCATGCTT)$_2$, and d(TAAGAATTCTTA)$_2$, which were described in this paper. Representative spectrofluorimetric titration spectra of **3b** with d(TAAGAATTCTT A)$_2$ are shown in Figure 2. The weak fluorescence of **3b** was dramatically enhanced upon the addition of DNA, suggesting an interaction of **3b** with the added DNA. The 1:1 binding stoichiometry between **3b** and d(TAAGAATTCTTA)$_2$ was determined by molar ratio methods shown as the inset in Figure 2. The binding stoichiometries between other dimeric berberines and duplex oligodeoxynucleotides were determined by the same method.

Analyses of fluorescence intensity against the DNA concentrations by nonlinear curve fitting methods[11] were used to determine the association constants (K_a's) of **3a–f** with CT DNA and three self-complementary double-stranded oligodeoxynucleotides (Table 1). For comparison, the binding constants of **1** with CT DNA and three self-complementary oligodeoxynucleotide duplexes were obtained in a similar way. The binding affinities of **3a–f**, relative to **1** with CT DNA, d(AAGAATTCTT)$_2$, d(AAGCATGCTT)$_2$, and d(TAAGAATTCTTA)$_2$, are illustrated in Figure 3.

It is apparent from Figure 3 and Table 1 that the dimers **3a–f** have higher affinities of binding than their monomeric parent compound **1**. Dimers **3a–f** bind to CT DNA at least 10 times, to d(AAGAATTCTT)$_2$ at least 1.5–20 times, to d(AAGCATGCTT)$_2$ at least 1.5–7 times, and to d(TAAGAATTCTTA)$_2$ at least 1.5–90 times greater than **1**. The binding of **3b** to d(TAAGAATTCTTA)$_2$, with the association constant, is approximately two orders of magnitude higher than **1**. These increases in the binding affinities may be due to

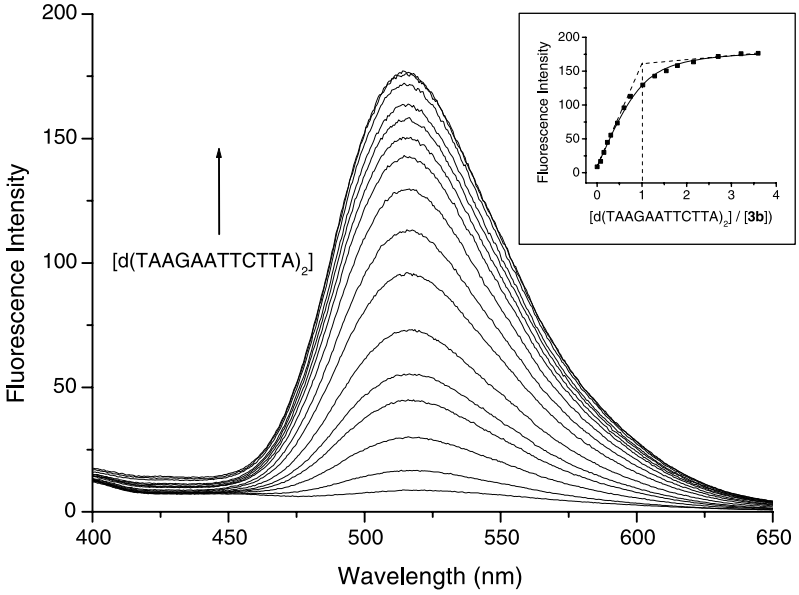


Figure 2. Fluorescence spectra of **3b** (2.47×10^{-6} M) with d(TAAGAATTCTTA)$_2$ of increasing concentrations (0–8.89×10^{-6} M) in 50 mM Tris–HCl (pH 6.35) at room temperature, excitation 355 nm. The inset indicates the relationship between the fluorescence intensities (emission at 516.7 nm) and the molar ratio of d(TAAGAATTCTTA)$_2$ to **3b**.

Table 1. Association constants (K_a's, M^{-1}) of **1** and **3a–f** with CT DNA, d(AAGAATTCTT)$_2$, d(AAGCATGCTT)$_2$, and d(TAAGAATTCTTA)$_2$[a]

Compound	CT DNA		d(AAGAATTCTT)$_2$		d(AAGCATGCTT)$_2$		d(TAAGAATTCTTA)$_2$	
	K_a	RA[b]	K_a	RA[b]	K_a	RA[b]	K_a	RA[b]
1	$(1.12 \pm 0.04) \times 10^4$	1	$(1.24 \pm 0.12) \times 10^4$	1	$(1.85 \pm 0.09) \times 10^4$	1	$(2.93 \pm 0.31) \times 10^4$	1
3a	$(1.45 \pm 0.22) \times 10^5$	12.9	$(1.18 \pm 0.10) \times 10^5$	9.5	$(4.52 \pm 0.33) \times 10^4$	2.4	$(1.62 \pm 0.35) \times 10^6$	54.9
3b	$(1.33 \pm 0.18) \times 10^5$	11.9	$(2.46 \pm 0.07) \times 10^5$	19.8	$(1.30 \pm 0.09) \times 10^5$	7.0	$(2.76 \pm 0.37) \times 10^6$	94.2
3c	$(1.60 \pm 0.05) \times 10^5$	14.3	$(8.78 \pm 0.26) \times 10^4$	7.1	$(8.47 \pm 0.44) \times 10^4$	4.6	$(3.49 \pm 0.60) \times 10^5$	11.9
3d	$(1.64 \pm 0.08) \times 10^5$	14.6	$(5.77 \pm 0.13) \times 10^4$	4.7	$(3.63 \pm 0.23) \times 10^4$	2.0	$(3.21 \pm 0.20) \times 10^5$	11.0
3e	$(1.27 \pm 0.03) \times 10^5$	11.3	$(3.42 \pm 0.13) \times 10^4$	2.8	$(3.60 \pm 0.52) \times 10^4$	1.9	$(2.48 \pm 0.34) \times 10^5$	8.5
3f	$(1.42 \pm 0.39) \times 10^5$	12.7	$(2.10 \pm 0.13) \times 10^4$	1.7	$(2.71 \pm 0.14) \times 10^4$	1.5	$(4.10 \pm 0.37) \times 10^4$	1.4

[a] In 50 mM Tris–HCl (pH 6.35) at room temperature unless specified. These values were derived from the experimental data by nonlinear curve fitting methods using KaleidaGraph (version 3.6).

[b] RA denotes relative affinity.

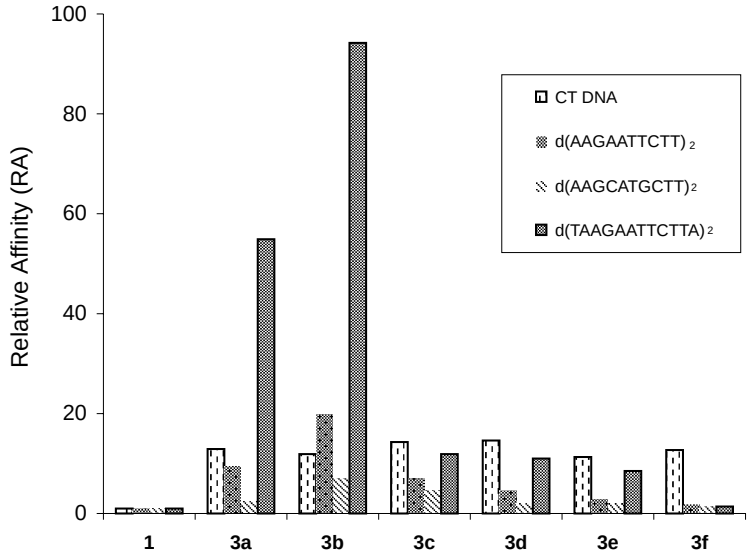


Figure 3. Relative binding affinities of **3a**–**f** with CT DNA, d(AAGAATTCTT)$_2$, d(AAGCATGCTT)$_2$, and d(TAAGAATTCTTA)$_2$ in 50 mM Tris–HCl (pH 6.35) at room temperature.

the cooperative interactions of the two berberine subunits in dimers **3a**–**f**, demonstrating the usefulness of a 'dimeric analog' for refining the rational design process to obtain promising candidates. The great enhancement in binding abilities was demonstrated further by spectrophotometric titration and competitive EB displacement (vide infra). Second, compounds **3a**–**f** show a prominent structure–activity relationship and their binding abilities with three self-complementary duplex DNAs can be modulated by the lengths of the alkyl chains. Compounds **3a**–**f** show binding affinities in the order of **3b > 3a > 3c > 3d > 3e > 3f** with d(AAGAATTCTT)$_2$ and d(TAAGAATTCTTA)$_2$, and **3b > 3c > 3a > 3d > 3e > 3f** with d(AAGCATGCTT)$_2$. In all cases, dimer **3b**, linked by a propyl chain, exhibits the highest binding affinity with all these self-complementary duplex DNAs. This shows that the propyl is the most suitable spacer of two berberine units in these studies and may lead to future rational design efforts. However, no prominent structure–activity relationship is observed with CT DNA binding, perhaps because CT DNA is a mixture of different DNA chains ranging from 200 to 6000 bases. Third, compounds **3a**–**f** show higher binding affinities with the 12-mer d(TAAGAATTCTTA)$_2$ than with the 10-mer d(AAGAATTCTT)$_2$ and d(AAGCATGCTT)$_2$. For example, the binding constants of **3a**,**b** with d(TAAGAATTCTTA)$_2$ are about 5–20 times higher than with d(AAGAATTCTT)$_2$ or d(AAGCATGCTT)$_2$. This result suggests that dimeric berberines occupy a greater number of base pairs,[12] with potentially more stringent sequence recognition compared with the monomeric berberine (**1**).

2.3. DNA-binding mode of berberine dimers 3a–f

Some planar cationic molecules readily bind to nucleic acids and display a variety of interesting biological properties.[13] A particular binding mode depends on considerable factors including the DNA base pair composition, as well as ligand structural features.[14,15] Intercalation and groove binding are the two main binding modes for small organic molecules that bind noncovalently to DNA.[16,17] Binding modes can be convincingly established by high-resolution structural studies, using either X-ray diffraction methods or NMR. In the absence of high resolution data, binding modes may be inferred from the results of solution studies,[18] and evidence for the intercalative mode of binding can be obtained from a UV–vis spectrometric investigation.[19]

The spectrophotometric titration of berberine and **3b** is shown in Figure 4, in which spectra a and b represent their UV–vis spectra before and after the addition of CT DNA, respectively. The addition of CT DNA to the solutions of berberine (**1**) and **3b** of fixed concentrations induces redshifts of 1 nm from 343 to 344 nm for berberine (the redshift of berberine becomes more obvious from 343 to 345 nm when the ratio of DNA-phosphate/berberine reaches 8.6:1, figure not shown),[20] and 5 nm from 342 to 347 nm for **3b**, and hypochromic responses around 5% at 343 nm for berberine, and around 30% at 342 nm for **3b**. These spectral variation effects of berberine and **3b** may be considered as the characteristic features of aromatic chromophore (π–π^*) interactions with the nucleic acid bases.[21]

The bathochromic shifts and hypochromic effects of berberine and **3b** toward CT DNA strongly indicate their interaction with DNA. The magnitudes of these spectral alterations provide evidences for intercalative binding because it was established that the π systems of berberine or **3b** are in intimate contact with those of the DNA bases.[19a,b] The binding of berberine and **3b** is proposed to be through intercalation, which is consistent with the previous studies on berberine and related protoberberine alkaloids.[7c,8h,22]

The binding modes of berberine and **3b** were confirmed further by a competitive EB displacement assay that has been employed to assess the binding affinities of DNA

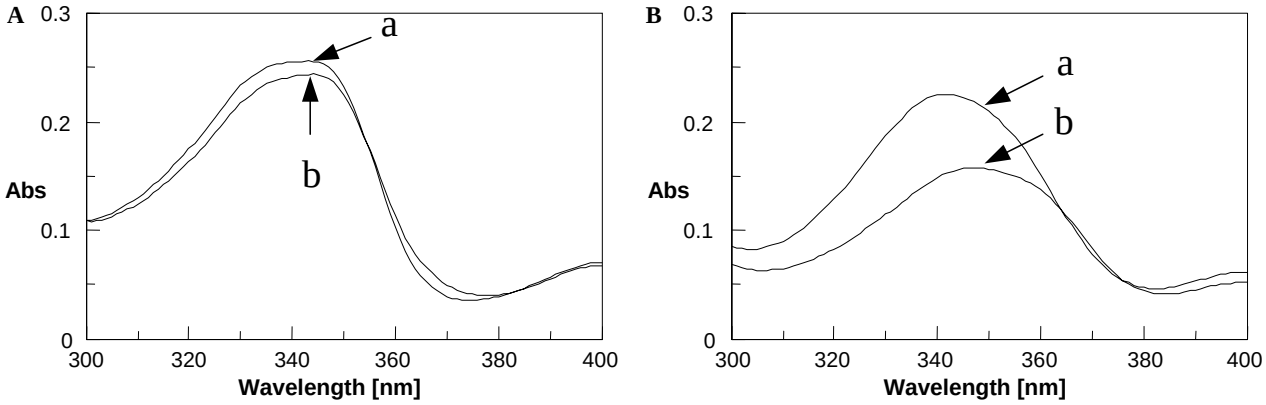


Figure 4. UV-vis absorption spectra of berberine (1.17×10^{-5} M, A) and **3b** (9.26×10^{-6} M, B) in 50 mM Tris–HCl buffer (pH 6.35) before (a) and after (b) the addition of CT DNA. The concentration ratios of DNA-phosphate/berberine and DNA-phosphate/**3b** are 4.4:1 and 5.6:1, respectively.

binders, especially for typical intercalators.[23] Ethidium bromide is a cationic dye that is widely used as a probe for native DNA because the ethidium ion displays a dramatic increase in fluorescence efficiency when it intercalates into DNA. This technique, based on the decrease of fluorescence resulting from the displacement of EB from a DNA sequence, is an efficient and rapid method to confirm the intercalating binding mode. The plots of the relative fluorescence intensity (I/I_0) of EB against the concentrations of added berberine and **3b** are shown in Figure 5. It is obvious that both berberine and **3b** can decrease the fluorescence intensity of the complex of EB and CT DNA, suggesting that berberine and **3b** can replace EB bound to CT DNA. Compound **3b** induces a much larger decrease in fluorescence intensity than berberine, indicating that **3b** is a much stronger DNA intercalator than berberine.

Thus, DNA-binding modes of berberine and dimeric berberine **3b** are evident from these spectrophotometric titration and competitive EB replacement experiments. Under these specific experimental conditions, both berberine and **3b** form intercalating complexes with CT DNA. It should be noted that both complexes possess different spectral characteristics. Compared with berberine, compound **3b** shows a larger redshift and a stronger hypochromic effect. Consistent with this spectrophotoscopic titration study, **3b** shows a displacement ratio higher than that of berberine in the competitive EB displacement experiment, possibly because it is effected through a binding mode of bis-intercalation.

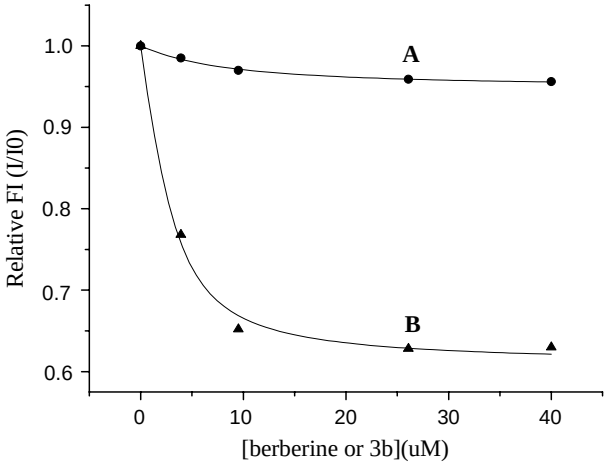


Figure 5. Fluorescence decrease of EB (3.03×10^{-6} M) induced by the competitive binding of (A) berberine and (B) **3b** to CT DNA (2.40×10^{-6} M) in 50 mM Tris–HCl buffer (pH 6.35) at room temperature (excitation 491 nm, emission 592 nm).

To investigate the role of the quaternary ammonium group of berberine in the DNA-binding activity, we examined the interaction of canadine (**4**), a reductive product of berberine (**1**), with CT DNA using spectrofluorimetric titration and competitive EB displacement experimental techniques. In both experiments, no change in the fluorescence intensity was observed after the addition of CT DNA to canadine solution, indicating that no detectable binding occurred between them. Compared to berberine, the most important structural characteristic of canadine is the absence of a quaternary ammonium cation. The loss of positive charge also makes the planar structure of berberine having sp^2 nitrogen transform into a tetrahedral structure of canadine having sp^3 nitrogen. Thus, these experiments indicate that the quaternary ammonium cation and planar structure may be critical factors for the binding of protoberberine alkaloids with double-stranded DNA.[10]

3. Summary

Six novel berberine dimers (**3a**–**f**) have been successfully synthesized in moderate- to high-yield from the reaction of berberrubine with dihaloalkanes of varying lengths. Their structures were fully characterized by ESI-HRMS and ^{1}H NMR.

Spectrofluorometric titration suggests that six dimers show a prominent structure–activity correlation when bound with short double-stranded DNA sequences. They show similar relative binding affinities in the order of **3b > 3a > 3c > 3d > 3e > 3f** with $d(AAGAATTCTT)_2$ and $d(TAAGAATTCTTA)_2$, and **3b > 3c > 3a > 3d > 3e > 3f** with $d(AAGCATGCTT)_2$. In all cases, dimer **3b** linked by a propyl chain exhibits the highest affinity,

indicating that propyl chain may be the most suitable linker to bridge the two berberine units. Spectrophotometric titration and competitive EB replacement assays indicate that both berberine (**1**) and its dimeric derivatives (**3a**–**f**) bind with duplex DNA in an intercalating mode.

Additionally, we clarified that the quaternary ammonium cation and planar structure are important for the binding ability of protoberberine alkaloids with double-stranded DNA.

4. Experimental

4.1. General

^{1}H NMR spectra were recorded on a VARIAN UNITY INOVA-300 spectrometer at 300 MHz and tetramethylsilane was used as an internal standard. FAB-MS was carried on a JEOL JMS-700 mass spectrometer. High-resolution mass spectra were measured on a mass spectrometer API QSTAR PULSAR *i*. Fluorescence spectra were obtained on a Perkin-Elmer LS55 Luminescence Spectrometer equipped with a fluorescence free quartz cuvette of 2 mm path length. UV–vis absorption spectra were measured on a Jasco UV-530 UV/vis spectrophotometer in matched quartz cuvettes of 2 mm path length.

Berberine chloride was obtained from the National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China. Tris(hydroxymethyl) aminomethane (Tris, ultrapure) was from USB Corporation, Cleveland, OH, USA. Silica gel 60 (0.063–0.200 mm, 70–230 mesh) used for flash chromatography was from Merck, and TLC was carried out on silica gel 60 F_{254} (Merck) aluminum-backed commercial plates. Milli-Q water (Millipore Co.) was used in all solutions. All other reagents were purchased from either Aldrich Chemical Company or Sigma Chemical Company and used without further purification unless otherwise stated.

Oligodeoxynucleotides and calf thymus DNA were purchased from Invitrogen Life Technologies and Amersham Pharmacia Biotech, respectively. Oligodeoxynucleotides were purified by open ODS column chromatography before use. The elution solution ranged from 5% to 11% acetonitrile aqueous solution containing 10 mM ammonium acetate. Double-stranded DNA was formed by heating the solutions of single-stranded oligodeoxynucleotides in 50 mM Tris–HCl (pH 6.35) buffer at 95 °C in a water bath for 4 min and then chilling to room temperature slowly over several hours. CT DNA was used without further purification. The concentrations of single-stranded self-complementary oligodeoxynucleotides were determined from UV absorbance at 260 nm, supposing the molar extinction coefficients of A, T, G, and C to be 16,000, 9600, 12,000, and 7000 $mol^{-1}\ dm^3\ cm^{-1}$, respectively, and the molar extinction coefficient for CT DNA was approximated with A_{260} being 13,200 $mol^{-1}\ dm^3\ cm^{-1}$ in base pair.

4.2. Preparation of berberrubine 2

The synthetic procedures of berberrubine have been described previously.[9] ^{1}H NMR (CD_3OD–$CDCl_3$, 300 MHz): δ 3.16 (t, 2H, J = 6.2 Hz, 6-H), 3.93 (s, 3H, OCH_3), 4.62 (t, 2H, J = 6.2 Hz, 5-H), 6.06 (s, 2H, OCH_2O), 6.82 (s, 1H, 4-H), 6.92 (d, 1H, J = 8.4 Hz, 12-H), 7.41 (s, 1H, 1-H), 7.53 (d, 1H, J = 8.4 Hz, 11-H), 8.00 (s, 1H, 13-H), 9.28 (s, 1H, 8-H); FAB MS: m/z 322 ($[M-Cl]^+$). The data of ^{1}H NMR were in accordance with those reported by Iwasa et al.[10]

4.3. Synthesis of dimeric berberines 3a–f

General procedures: to a solution of berberrubine **2** (0.12 mmol) in DMF (4 mL) was added dihaloalkane (0.05 mmol). The reaction was monitored by TLC. After stirring at 60 °C for 12–15 h, the solvent was removed under reduced pressure. The obtained residue was washed by 95% ethanol for several times and subject to anion exchange into chloride form. The solvents were evaporated and the residues were dried in vacuum to afford the target compounds **3a**–**f** as a yellow powder.

4.3.1. Compound 3a. Obtained yield 46%. ^{1}H NMR (DMSO-d_6, 300 MHz): δ 3.17 (s, 4H, 5-H), 4.02 (s, 6H, OCH_3), 4.79 (s, 4H, OCH_2), 4.85 (s, 4H, 6-H), 6.16 (s, 4H, OCH_2O), 7.08 (s, 2H, 4-H), 7.75 (s, 2H, 1-H), 7.98 (d, 2H, J = 9.6 Hz, 12-H), 8.19 (d, 2H, J = 9.6 Hz, 11-H), 8.91 (s, 2H, 13-H), 9.83 (s, 2H, 8-H); HRMS (ESI) for $C_{40}H_{34}N_2O_8$ ($[M-2Cl]^{2+}$) Calcd 335.1152. Found: 335.1129.

4.3.2. Compound 3b. Obtained yield 37%. ^{1}H NMR (CD_3OD–$CDCl_3$, 300 MHz): δ 2.65 (m, 2H, CH_2CH_2O), 3.23 (t, 4H, J = 6.3 Hz, 5-H), 4.09 (s, 6H, OCH_3), 4.76 (t, J = 6.3 Hz, 4H, OCH_2CH_2), 4.92 (t, J = 6.3 Hz, 4H, 6-H), 6.09 (s, 4H, OCH_2O), 6.91 (s, 2H, 4-H), 7.59 (s, 2H, 1-H), 7.98 (d, 2H, J = 9.0 Hz, 12-H), 8.07 (d, 2H, J = 9.0 Hz, 11-H), 8.65 (s, 2H, 13-H), 9.87 (s, 2H, 8-H); HRMS (ESI) for $C_{41}H_{36}N_2O_8$ ($[M-2Cl]^{2+}$) Calcd 342.1231. Found: 342.1213.

4.3.3. Compound 3c. Obtained yield 84%. ^{1}H NMR (DMSO-d_6, 300 MHz): δ 2.14 (s, 4H, CH_2CH_2O), 3.15–3.24 (m, 4H, 5-H), 4.04 (s, 6H, OCH_3), 4.40 (t, 4H, J = 5.6 Hz, OCH_2CH_2), 4.93 (s, 4H, 6-H), 6.17 (s, 4H, OCH_2O), 7.08 (s, 2H, 4-H), 7.78 (s, 2H, 1-H), 7.98 (d, 2H, J = 9.0 Hz, 12-H), 8.19 (d, 2H, J = 9.0 Hz, 11-H), 8.92 (s, 2H, 13-H), 9.78 (s, 2H, 8-H); HRMS (ESI) for $C_{42}H_{38}N_2O_8$ ($[M-2Cl]^{2+}$) Calcd 349.1309. Found: 349.1318.

4.3.4. Compound 3d. Obtained yield 47%. ^{1}H NMR (DMSO-d_6, 300 MHz): δ 1.65–1.80 (m, 2H, $CH_2CH_2CH_2O$), 1.99 (m, 4H, $CH_2CH_2CH_2O$), 3.20 (m, 4H, 5-H), 4.04 (s, 6H, OCH_3), 4.32 (t, 4H, J = 6.3 Hz, OCH_2CH_2), 4.93 (t, 4H, J = 6.4 Hz, 6-H), 6.16 (s, 4H, OCH_2O), 7.07 (s, 2H, 4-H), 7.75 (s, 2H, 1-H), 7.97 (d, 2H, J = 8.7 Hz, 12-H), 8.18 (d, 2H, J = 8.7 Hz, 11-H), 8.90 (s, 2H, 13-H), 9.75 (s, 2H, 8-H); HRMS (ESI) for $C_{43}H_{40}N_2O_8$ ($[M-2Cl]^{2+}$) Calcd 356.1387. Found: 356.1385.

4.3.5. Compound 3e. Obtained yield 74%. ^{1}H NMR (DMSO-d_6, 300 MHz): δ 1.60 (s, 4H, $CH_2CH_2CH_2O$), 1.90–2.00 (m, 4H, $CH_2CH_2CH_2O$), 3.20 (m, 4H, 5-H), 4.03 (s, 6H, OCH_3), 4.29 (t, 4H, J = 5.8 Hz, OCH_2CH_2), 4.92 (t, 4H, J = 6.9 Hz, 6-H), 6.16 (s, 4H, OCH_2O), 7.07 (s, 2H, 4-H), 7.75 (s, 2H, 1-H), 7.97 (d, 2H, J = 9.3 Hz, 12-H), 8.18 (d, 2H, J = 9.3 Hz, 11-H), 8.90 (s, 2H, 13-H), 9.73 (s, 2H, 8-H); HRMS (ESI) for $C_{44}H_{42}N_2O_8$ ($[M-2Cl]^{2+}$) Calcd 363.1471. Found: 363.1504.

4.3.6. Compound 3f. Obtained yield 40%. ^{1}H NMR (CD_3OD–$CDCl_3$, 300 MHz): δ 1.53–1.66 (m, 6H, $CH_2CH_2CH_2CH_2CH_2O$), 1.94–2.03 (m, 4H, CH_2CH_2O), 3.24 (m, 4H, 5-H), 4.07 (s, 6H, OCH_3), 4.42 (t, 4H, J = 5.8 Hz, OCH_2CH_2), 5.00 (t, 4H, J = 6.9 Hz, 6-H), 6.07 (s, 4H, OCH_2O), 6.84 (s, 2H, 4-H), 7.49 (s, 2H, 1-H), 7.92 (d, 2H, J = 9.3 Hz, 12-H), 7.95 (d, 2H, J = 9.3 Hz, 11-H), 8.49 (s, 2H, 13-H), 9.73 (s, 2H, 8-H); HRMS (ESI) for $C_{45}H_{44}N_2O_8$ ($[M-2Cl]^{2+}$) Calcd 370.1549. Found: 370.1521.

4.4. Preparation of canadine (4)

To a solution of berberine (200 mg, 0.54 mmol) in refluxing MeOH (15 mL) was added $NaBH_4$ powder (43 mg, 1.13 mmol). The reaction mixture was stirred for 20 min at 70 °C and then at room temperature for 4 h. The formed light yellow precipitates were collected and recrystallized from MeOH to yield canadine (**4**, 126 mg, 63%). FAB MS: m/z 339 ($[M-Cl]^+$). The data of ^{1}H NMR were essentially identical to those published.[24]

4.5. Spectrofluorimetric titration experiments

To the solutions of alkaloids (2.0×10^{-6} M) in 50 mM Tris–HCl buffer (pH 6.35) were added aliquots of double-stranded DNA (CT DNA, 8.0×10^{-4} M; d(AAGAATTCTT)$_2$ and d(AAGCATGCTT)$_2$, 4.0×10^{-4} M; d(TAAGCATGCTTA)$_2$: 4.0×10^{-5} M) solution containing the same concentration of drugs in 50 mM Tris–HCl buffer (pH 6.35). This operation ensured that the concentration of DNA increased gradually from 0 to 2.0×10^{-4} M (0–2.0×10^{-5} M for d(TAAGCATGCTTA)$_2$), while the concentrations of drugs were kept constant at 2.0×10^{-6} M. The mixing was achieved by turning the cuvette up and down for 5 min. Then, the corresponding fluorescence spectra were measured at room temperature (20 °C) without degassing (ex 355 nm).

The association constants (K_a's) were derived from an analysis of the relationship between relative fluorescence intensity (I/I_0) and the DNA concentrations by a nonlinear curve fitting to the equation $I/I_0 = 1 + ((I_\infty - I_0)/(2I_0[\text{alkaloid}]_0)) \times \{([\text{DNA}]_0 + [\text{alkaloid}]_0 + 1/K_a) - (([\text{DNA}]_0 + [\text{alkaloid}]_0 + 1/K_a)^2 - 4[\text{DNA}]_0[\text{alkaloid}]_0)^{1/2}\}$, wherein I_0, I, and I_∞ represent the fluorescence intensities of alkaloid alone, the sample and alkaloid totally bound, respectively. $[\text{DNA}]_0$ and $[\text{alkaloid}]_0$ are the initial analytical concentrations of DNA and alkaloids, respectively. KaleidaGraph version 3.6 (Synergy Software, USA) was applied for the nonlinear curve fitting.

4.6. Spectrophotometric titration experiments

To the solution of alkaloids (1.17×10^{-5} and 9.26×10^{-6} M for berberine and **3b**, respectively) in 50 mM Tris–HCl buffer (pH 6.35) were added aliquots of CT DNA (1.60×10^{-3} M) solution containing the same concentrations of alkaloids in 50 mM Tris–HCl buffer (pH 6.35). The mixing was achieved by turning the cuvette up and down for 5 min. After the measuring solution was allowed to stand for 1 min, the corresponding absorption spectra in the region from 200 to 500 nm were measured at room temperature.

4.7. EB displacement experiment

Competitive EB displacement experiments were carried out in matched quartz cuvettes of 2 mm path length. Aliquots of the alkaloids' (2.0×10^{-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) were added to the 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). This operation ensured that the concentrations of alkaloids increased gradually from 0 to 4.0×10^{-5} M, while the concentrations of CT DNA and EB were kept constant. Then, the corresponding fluorescence spectra were measured at room temperature (excitation at 491 nm, emission at 592 nm).

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