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Published in:
Chemistry and Biodiversity

DOI:
10.1002/cbdv.200900386

Published: 01/12/2010

Document Version:
Peer reviewed version

Synthesis and DNA-binding Affinities of Protoberberine-based Multivalent Agents

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Abstract: Three novel berberine derivatives bearing two, three and four primary amino groups at the 9-position, respectively, were synthesized and characterized with NMR (^{1}H and ^{13}C), MS and HRMS. Their non-covalent binding with calf thymus (CT) DNA was investigated by means of spectrophotometric titration and ethidium bromide (EB) displacement experiments. The results indicated that these multivalent berberine derivatives exhibited up to 130-fold enhanced binding affinities relative to berberine, thus may be exploitable as potent DNA-binding agents.

Keywords: Protoberberine; polyamine; synthesis; multivalency; DNA-binder.

1. Introduction. - Over the past two decades, there has been a growing interest in the synthesis and biomedical application of multivalent molecules that are capable of targeting biomacromolecules, such as DNA [1]. These efforts have been spurred primarily by the biological significance of the ubiquitous multivalent interactions that play crucial roles in the function of biological systems. Thus, multivalent molecules may serve as effective

probes to elucidate the mechanism of action of some biological processes. On the other hand, multivalent binding discloses the molecular basis for the high DNA-binding affinities and sequence selectivities of many naturally-occurring antitumor antibiotics that exert their biological activities through specific and non-covalent interaction with DNA [1]. Therefore, the concept of multivalent design can be used as an effective strategy, for example, in designing drugs that influence biological systems potently and selectively.

We have keenly become interested in the development of multivalent DNA-binding molecules based on naturally-occurring protoberberines as lead compounds [2-3]. One of our goals is aimed at improving the binding affinities of protoberberines by using multivalency principle [4-8]. Thus, we have shown in our previous study that protoberberine derivatives bearing totally two, four and six amino groups at the 3- and 9-positions, respectively, exhibit remarkably enhanced DNA-binding affinities [4]. This result has demonstrated the great potential of amination in improving the DNA-binding affinities of protoberberines. This potential, however, is largely dependent on the number of amino groups that are present, as revealed by the limited enhancement of the DNA-binding affinity we observed in mono-amino berberine [5]. On the other hand, recent

computer-aided modeling studies of the complex of berberine (**1**, Chart 1) with double-stranded DNA suggest that berberine binds from its C_5-C_6-N^+-C_8 side [9]. This result, together with the report that the 9-position in berberine analogs is an important determinant of DNA topoisomerase II inhibition [11-12], makes us reason that modification at the 9-position may have more significant impact on the interaction with DNA [10]. The primary aim of the work reported herein was, therefore, to examine the consequences on the DNA binding affinity of the *multiple* amination of berberine at the 9-position. Specifically, we described the synthesis of berberine derivatives **2-4** (Chart 1) bearing two, three and four primary amino groups at the 9-position, respectively, and their binding affinities toward double-stranded DNA.

Insert here Chart 1

2. Results and Discussion. - 2.1. *Synthesis of berberine derivatives 2-4.* The synthetic approach that was used for the synthesis of compounds **2-4** is outlined in Scheme 1. Acylation (51%) of 9-*O*-(2'-aminoethyl)berberine **5** [4] with $N_\alpha N_\varepsilon$-di-Boc-*L*-lysine hydroxylsuccinimide ester (Boc-*L*-Lys(Boc)-OSu), followed by the deprotection (91%) of Boc groups with trifluoroacetic acid (TFA), afforded **2**. Condensation (42%) of **5** with Boc-protected N^6-*L*-lysyl-*L*-lysine **6** [13] that was activated with HOBt, and

subsequent deprotection (87%) of Boc groups gave compound **3**. Compound **4** was obtained from the acylation (38%) of **2** with Boc-*L*-Lys(Boc)-OSu and subsequent deprotection (93%). Compounds **2-4** were fully characterized with NMR (^{1}H and ^{13}C), MS and HRMS. They afforded MS spectra with the *m/z* values corresponding to $[M-Cl]^{+}$. Their NMR spectra were also in full agreement with the given structures (see experimental section). The purity of each compound was judged from ^{1}H NMR and TLC.

Insert here Scheme 1

2.2. Spectrophotometric titration. The interactions of compounds **2-4** with calf thymus (CT) DNA were firstly monitored by absorption spectrometry. The interaction of DNA-binding agents with DNA usually accompanies spectral changes in which wavelength shifts, absorbance changes and/or isosbestic points provide useful information for understanding of the binding strength and modes [14]. In this case, we observed that the addition of CT DNA to the solutions of compounds **2-4** resulted in large hypochromicities (28~58%) and bathochromic shifts (3.0~5.0 nm) (Figure **1** and Table 1). These spectroscopic variations unambiguously indicate that compounds **2-4** are capable of forming stable complexes with CT DNA. Their larger hypochromicities relative to that of berberine, indicates that they bind to CT DNA more strongly than berberine [15-16]. On the other hand, during the titrations with CT DNA, three well-resolved isosbestic

points were observed, revealing the existence of one preferential, almost exclusive binding mode between compounds **2-4** and CT DNA [14]. The hypochromicities and bathochromic shifts suggest that these compounds may interact with CT DNA *via* an intercalation mode, that is, the protoberberine subunit serves as an anchor for DNA intercalation, whereas the polyamino groups impart enhanced binding affinities through multivalent bonding (i.e. hydrogen bonding and electrostatic interactions) with DNA.

Insert here Figure 1.

Insert here Table 1.

2.3. Ethidium bromide displacement. The binding affinities of compounds **2-4** toward CT DNA were then evaluated by means of ethidium bromide (EB) displacement experiments. EB strongly fluoresces upon intercalation into DNA duplexes and the displacement from its DNA complex leads to a decrease in the fluorescence intensity. This fluorescence-based competition technique is commonly used to study the binding of polyaminonium compounds to DNA [17], and can afford the relative DNA-binding affinity.

Insert here Figure 2.

Figure **2** shows the plots of the relative fluorescence intensity (FI, I/I_0) of EB against the concentrations of compounds **2-4** that were added to the solution of EB and CT DNA. It is clear that the fluorescence intensity of EB

decreases upon the addition of **2-4**, indicating that they can replace EB bound to CT DNA. The relative binding affinities of compounds **2-4** were then evaluated from their apparent binding constants (K_{app}'s, Table 1) that were calculated from equation $K_{app} = K_{EB} \times [EB]/[B]_{50}$ in which K_{EB} is the association constant of EB with CT DNA, [EB] is the initial concentration of EB and $[B]_{50}$ is defined as the concentration of the compound (i.e. **2-4**) that is added to generate a 50% decrease in the initial fluorescence of the EB-CT DNA complex [18].

Berberine **1** binds to CT DNA so weakly that it cannot induce 50% decrease in the fluorescence intensity under our measuring conditions [5]. This may be because it has only one positively charged center and thus is incapable of competing with other DNA-binding cations. Compound **2** displaces EB from DNA much more efficiently than berberine, with the DNA binding affinity being about 16-fold higher. This indicates that attaching only two amino groups can largely increase the DNA binding ability. Compounds **3** and **4** bearing three and four amino groups, respectively, exhibit further stronger DNA binding. Their affinities are up to 130-fold greater than that of berberine **1**. Thus, compounds **2-4** act as very effective DNA binders. These results, together with our previous result on mono-aminoberberine **5** [5], indicate that the DNA binding affinities of polyamino protoberberines

increase with the increase of the number of amino groups that are present. This result may be accounted for if their structures are taken into consideration. According to the report that ε-amino groups in *L*-Lysine and poly(*L*-Lysine) have pKa values in the range from 9.80 to 11.32 [19], there should be no doubt that all the ε-amino groups in compounds **2-4** were protonated under our measuring conditions (pH 6.35). To determine to what extent the α-amino groups were protonated, we measured the pKa values of all the α-amino groups in compounds **2-4**. As a result, we obtained pKa values of 8.29 for compound **2** and of 6.81 and 8.15 for compound **4**. However, for compound **3**, we could not obtain two separated pKa values but one consolidated value of 8.24. From these pKa values, we can see that most of the primary amino groups existed in protonated forms, thus compounds **2-4** could interact with CT DNA, *via* multiple hydrogen bonding and enhanced electrostatic forces. That is, *multivalent cooperative interactions* were optimized.

In addition, it may be interesting to compare the increments in the binding affinity of compounds **2-4** with CT DNA. As shown in Table 1, the values of the increments are about 16 for compound **2** having one α-NH_2 and one ε-NH_2 groups, 30 for compound **3** having two α-NH_2 and one ε-NH_2 groups and 138 for compound **4** having two α-NH_2 and two ε-NH_2 groups,

respectively. These results, together with our previous report that compound **5** having one primary amino group showed about 7-fold greater affinity than berberine [5], enable one to deduce that, under our measuring conditions, one α-NH_2 group can double the binding affinity, whereas one ε-NH_2 group can increase the affinity by 5-7 times. The different contributions of α-NH_2 and ε-NH_2 groups may be attributed to the spatial orientation of these two kinds of amino groups, as described in our previous study [4].

2.3. Salt effect. To provide further insight into the interaction of compounds **2-4** with DNA, their apparent binding constants were measured under the condition of physiological salt concentration (i.e. 150 mM NaCl), by means of EB displacement experiments. As shown in Table 1, even under high salt conditions, these amino compounds, especially compounds **3** and **4**, demonstrate their strong DNA-binding ability. This result indicates that organizing of several protonated amino groups onto protoberberines is capable of amplifying the DNA-binding activity, that is, the *multivalency principle* is in operation. It should be noted that, however, the binding affinities of compounds **2-4** are affected by the increase of salt concentration, suggesting that they are not fully capable of competing with inorganic cations (e.g. Na^+) for binding sites on the surface of the DNA helix.

3. Concluding remarks. - Three berberine derivatives bearing two, three and four primary amino groups at the 9-position, respectively, have been successfully synthesized and characterized with HRMS and NMR. The results obtained from spectrophotometric titration and EB displacement experiments have shown that these compounds have very high DNA-binding affinities, thus are exploitable as effective DNA-binding agents. However, the decrease in binding affinity under high salt concentrations suggests that more cationic groups that are organized in a well-defined multivalent array may be needed to achieve salt-independent binding. Further efforts aiming at their potential application are continuing in our labs.

Acknowledgment. - This work was financially supported by Southern Medical University, Department of Education of Guangdong Province, China (WHC), and Research Grants Council, University Grants Committee of Hong Kong (ZHJ).

Experimental Part

General. ^{1}H and ^{13}C NMR spectra were recorded in CD_3OD using a Varian unity INOVA-300 and 500 spectrometers, respectively, and TMS as an internal reference. ESI, HR-ESI and HR-MALDI mass spectra were measured on Thremo LCQ DECA XP, Thremo MAT95XP and LTQ

Orbitrap XL, respectively. α-Cyano-4-hydroxycinnamic acid (α-CHCA) was used as the matrix for the MALDI-TOF mass spectra. Absorption spectra were measured on Shimadzu UV-3150 UV-Vis spectrometer. Fluorescence measurements were made on a Shimadzu RF-5301 PC. Thin-layer chromatography (TLC) was performed on an aluminum plate precoated with silica gel and a fluorescence indicator (Merck). Detection on TLC was made by use of iodine and UV (254 nm). CT DNA was purchased from Pharmacia (Uppsala, Sweden), and its concentration (in base pair) was determined spectrophotometrically using the molar extinction coefficient of 13,200 $M^{-1}cm^{-1}$ per base pair. All other reagents and chemicals were obtained from commercial sources and used as received unless otherwise stated.

Synthesis of compound 2. To a solution of 9-*O*-(2'-aminoethyl)berberine **5** (80 mg, 0.2 mmol) in anhydrous DMF (3 mL) were added at 0°C Boc-*L*-Lys(Boc)-OSu (134 mg, 0.3 mmol) and Et_3N (1.0 mL). The reaction mixture was stirred at 0°C for 24 h and concentrated under reduced pressure. The resulting residue was purified by chromatography on a neutral Al_2O_3 column ($CHCl_3/CH_3OH/ NH_3{\cdot}H_2O$, 60/1/1% by volume) to afford Boc-protected **2** (77 mg, 51%) as a red solid. Then to the solution of Boc-protected **2** (20 mg, 0.027 mmol) in CH_2Cl_2 (1.0 mL) was added TFA (1.0 mL). The reaction mixture was stirred at room temperature for 3 h and then concentrated under reduced pressure. The obtained residue was washed

with ether (5.0 mL×3) and CH_2Cl_2 (5.0 mL×3) to give **2** (13 mg, 91%) as a red solid having ^1H NMR (CD_3OD, 300 MHz) δ 1.50~1.57 (m, CH_2 (6')), 1.60~1.73 (m, CH_2 (5')), 1.89~1.99 (m, CH_2 (4')), 2.89~2.97 (m, CH_2 (7')), 3.25 (t, J = 7.0 Hz, CH_2 (5)), 3.87 (t, J = 6.3 Hz, CH_2 (1')), 4.06 (s, CH_3 (b)), 4.09 (t, J = 6.3 Hz, CH_2 (2')), 4.44 ~ 4.53 (m, H-C (3')), 4.82~4.90 (m, CH_2 (6) overlapped), 6.09 (s, CH_2 (a)), 6.94 (s, H-C (4)), 7.62 (s, H-C (1)), 7.72 (d, J = 8.7 Hz, H-C (12)), 7.94 (d, J = 8.7 Hz, H-C (11)), 8.56 (s, H-C (13)), 9.75 (s, H-C (8)); ^{13}C NMR (CD_3OD, 125 MHz) δ 170.3 (C=O), 151.6 (C(10)), 149.8 (C(3)), 149.5 (C(2)), 149.5 (C(9)), 146.6 (C(8)), 137.9 (C(14)), 136.9 (C(17)), 134.9 (C(16)), 131.3 (C(13)), 125.3 (C(12)), 121.6 (C(15)), 121.1 (C(11)), 119.9 (C(18)), 109.1 (C(4)), 106.0 (C(1)), 103.3 (C(a)), 64.4 (C(1')), 54.6 (C(6)), 54.1 (C(b)), 50.9 (C(3')), 37.3 (C(7')), 34.1 (C(2')), 28.0 (C(4')), 25.4 (C(6')), 23.3 (C(5)), 20.2 (C(5')); ESI-MS *m/z* 494 ($[M-Cl]^+$) and HR-ESI-MS for $C_{27}H_{33}N_4O_5^+$ ($[M-Cl]^+$) calcd: 493.2451, found: 493.2443.

Synthesis of compound ***3***. To a mixture of Boc-protected N^6-*L*-lysyl-*L*-lysine **6** [13] (95 mg, 0.16 mmol), DCC (33 mg, 0.16 mmol) and HOBt (22 mg, 0.16 mmol) in CH_2Cl_2 (4.0 mL) were added Et_3N (1.0 mL) and *N*-methylmorphone (1.0 mL). The mixture was stirred at 0°C for 2 h, and then 9-*O*-(2'-aminoethyl)berberine **5** (55 mg, 0.14 mmol) was added. After 24 h, the reaction mixture was concentrated and subjected to purify by chromatography on a neutral Al_2O_3 column ($CHCl_3/CH_3OH/NH_3{\cdot}H_2O$,

60/1/1% by volume) to afford Boc-protected **3** as a red solid (58 mg, 42%). The solid (30 mg, 0.033 mmol) was dissolved in CH_2Cl_2 (1.0 mL) and then TFA (1.0 mL) was added. The reaction mixture was stirred at room temperature for 2 h and then concentrated under reduced pressure. The obtained residue was washed with diethyl ether (5.0 mL×3) and CH_2Cl_2 (5.0 mL×3) to give **3** (18 mg, 87%) as a red solid having ^{1}H NMR (CD_3OD, 300 MHz) δ 1.44~1.56 (m, CH_2 (11', 10', 5')), 1.58~1.73 (m, CH_2 (6')), 1.84~1.92 (m, CH_2 (4', 9')), 2.94 (t, J = 8.3 Hz, CH_2 (12')), 3.10~3.18 (m, CH_2 (7)), 3.25 (t, J = 7.0 Hz, CH_2 (5)), 3.81~3.89 (m, CH_2 (1', 2')), 4.06 (s, CH_3 (b)), 4.46~4.51 (m, H-C (3', 8')), 4.85~4.89 (m, CH_2 (6)), 6.09 (s, CH_2 (a)), 6.94 (s, H-C (4)), 7.62 (s, H-C (1)), 7.69 (d, J = 8.7 Hz, H-C (12)), 7.93 (d, J = 8.7 Hz, H-C (11)), 8.56 (s, H-C (13)), 9.76 (s, H-C (8)); ^{13}C NMR (CD_3OD, 125 MHz) δ 170.7 (C=O), 170.1 (C=O), 152.0 (C(10)), 150.3 (C(3)), 149.9 (C(2)), 149.9 (C(9)), 147.0 (C(8)), 138.8 (C(14)), 137.7 (C(17)), 135.3 (C(16)), 131.7 (C(13)), 125.5 (C(12)), 121.7 (C(15)), 121.5 (C(11)), 120.1 (C(18)), 109.4 (C(4)), 106.4 (C(1)), 103.6 (C(a)), 67.5 (C(1')), 57.6 (C(6)), 57.1 (C(b)), 56.3 (C(3')), 54.3 (C(8')), 42.6 (C(12')), 40.3 (C(7')), 32.1 (C(2')), 29.8 (C(9')), 29.8 (C(4')), 29.7 (C(11')), 28.1 (C(6')), 26.1 (C(5)), 23.5 (C(5')), 23.4 (C(10')); ESI-MS *m/z* 622 ($[M\text{-}Cl]^+$) and HR-MALDI-TOF-MS for $C_{33}H_{45}N_6O_6^+$ ($[M\text{-}Cl]^+$) calcd: 621.3401, found: 621.3469.

Synthesis of compound 4. The solution of compound **2** (60 mg, 0.11 mmol),

Boc-*L*-Lys(Boc)-OSu (270 mg, 0.6 mmol), Et_3N (0.5 mL) and *N*-methylmorphone (0.5 mL) in DMF (4.0 mL) was stirred at room temperature for 12 h. The reaction mixture was concentrated and subjected to purify by chromatography on a neutral Al_2O_3 column ($CHCl_3$/CH_3OH/ $NH_3{\cdot}H_2O$, 60/1/1% by volume) to afford Boc-protected **4** as a red solid (53 mg, 38%). The solid (15 mg, 0.013 mmol) was dissolved in CH_2Cl_2 (1.0 mL) and then TFA (1.0 mL) was added. The reaction mixture was stirred at room temperature for 3 h and then concentrated under reduced pressure. The obtained residue was washed with diethyl ether (5.0 mL×3) and CH_2Cl_2 (5.0 mL×3) to give **4** (9 mg, 93%) as a red solid having ^{1}H NMR (CD_3OD/ $CDCl_3$, 1/1 by volume, 300 MHz) δ 1.34~1.53 (m, CH_2 (10', 11', 14', 15', 16')), 1.61~1.79 (m, CH_2 (6', 9')), 1.82~1.90 (m, CH_2 (4', 5')), 2.85~2.92 (m, CH_2 (17', 5)), 3.06~3.21 (m, CH_2 (7', 12')), 3.77~3.85 (m, H-C (3', 8', 13')), 4.01 (s, CH_3 (b)), 4.03~4.01 (m, CH_2 (2')), 4.30~4.32 (m, CH_2 (1')), 4.81~4.84 (m, CH_2 (6)), 6.07 (s, CH_2 (a)), 6.83 (s, H-C (4)), 7.46 (s, H-C (1)), 7.58 (d, J = 8.7 Hz, H-C (12)), 7.78 (d, J = 8.7 Hz, H-C (11)), 8.29 (s, H-C (13)), 9.81 (s, H-C (8)); ^{13}C NMR (CD_3OD, 125 MHz) δ 173.3 (C=O), 170.6 (C=O), 170.0 (C=O), 152.0 (C(10)), 150.5 (C(3)), 149.9 (C(2)), 149.9 (C(9)), 146.9 (C(8)), 138.3 (C(14)), 137.3 (C(17)), 135.3 (C(16)), 131.6 (C(13)), 125.5 (C(12)), 121.9 (C(15)), 121.5 (C(11)), 120.4 (C(18)), 109.4 (C(4)), 106.4 (C(1)), 103.6 (C(a)), 66.5 (C(1')), 57.6 (C(6)), 57.2 (C(b)), 54.3 (C(3')), 54.1 (C(8')), 54.0 (C(13')), 42.9 (C(17'), C(12')), 40.3 (C(7')),

37.0 (C(2')), 32.1 (C(14')), 32.1 (C(9')), 32.1 (C(4')), 31.6 (C(16')), 29.8 (C(11')), 28.4 (C(6')), 28.1 (C(5)), 24.4 (C(5')), 23.0 (C(10')), 22.6 (C(15')); ESI-MS *m/z* 750 ($[M-Cl]^+$) and HR-MALDI-TOF-MS for $C_{39}H_{57}N_8O_7^+$ ($[M-Cl]^+$) calcd: 749.4350, found: 749.4435.

Spectrophotometric titration. Absorption spectra were recorded at room temperature using conventional quartz cells of 1 cm path. Spectrophotometric titrations were performed by fixing the concentrations of compounds **2-4** while gradually increasing the concentration of CT DNA. Typically, to a solution of **2** (3.75×10^{-5} M) in 50 mM Tris-HCl buffer (pH 6.35, 0.05 mM EDTA) were added aliquots of CT DNA (1.75×10^{-3} M) solution containing **2** (3.75×10^{-5} M) in 50 mM Tris-HCl buffer (pH 6.35, 0.05 mM EDTA). This operation ensured the gradual increase of the concentration of CT DNA from 0 to 5.09×10^{-5} M, while keeping the concentration of compound **2** constant. After the mixture was equilibrated, the corresponding absorption spectra were measured at room temperature. This operation was repeated until saturation reached. The spectrophotometric titrations of compounds **3** and **4** were conducted in a similar way.

EB displacement. Competitive EB displacement experiments were performed in matched quartz cells of 1 cm path. To a solution (1500 μL) of CT DNA (3.98×10^{-6} M) and EB (5.04×10^{-6} M) in 50 mM Tris-HCl buffer (pH 6.35, 0.05 mM EDTA) were added aliquots of the solutions of compounds **2-4** ($2.07\sim9.90\times10^{-4}$ M) containing CT DNA and EB of the

same concentrations in 50 mM Tris-HCl buffer (pH 6.35, 0.05 mM EDTA). This operation ensured that the concentrations of drugs increased gradually from 0 to 6.50×10^{-5} M, while the concentrations of CT DNA and EB were kept constant. The resulting solution was stirred gently and allowed to stand for 30 seconds for equilibrium before the fluorescence spectra were measured (ex 490 nm, ex/em 5 nm/5 nm). Apparent binding constants (K_{app}'s) were calculated from the equation $K_{app} = K_{EB}\times[EB]/[B]_{50}$. The experiments for salt effect were conducted in a similar way, except under the condition of physiological salt concentration (i.e. 150 mM NaCl).

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Table 1. Apparent association constants (K_{app}'s, M^{-1}) and photophysical properties of **1-4** bound to CT DNA[a,b])

[NaCl]	Compound	Binding affinity		Red Shift	Hypochro-	Isosbestic Point
		K_{app} [c])	RA [d])	(nm) [e])	micity (%) [e])	(nm)
0 mM	**1**	1.12×10^4	1	1.0	5.0	360, 425, 475
	2	1.78×10^5	16	3.0	27.8	373, 419, 493
	3	3.39×10^5	30	4.0	57.8	377, 430, 501
	4	1.55×10^6	138	5.0	45.2	381, 421, 482
150	**2**	1.01×10^4				
mM	**3**	1.30×10^5				
	4	2.00×10^5				

[a]) Measured in 50 mM Tris-HCl buffer (pH 6.35, 0.05 mM EDTA) at r.t..

b) The data for compound **1** were from reference 5.

c) Calculated from the equation $K_{app} = K_{EB}\times[EB]/[B]_{50}$. K_{EB} obtained from spectro-fluorimetric titration, was 6.26×10^5 M^{-1} and 9.75×10^4 M^{-1} in 50 mM Tris-HCl buffer (pH 6.35, 0.05 mM EDTA) in the absence and presence of 150 mM NaCl, respectively.

d) RA denotes relative affinity.

e) Obtained at 345 nm. The concentrations of **1-4** were 1.25×10^{-5} M, 3.71×10^{-5} M, 2.18×10^{-5} M and 3.67×10^{-5} M, respectively.

Legends

Chart 1

Scheme 1. Synthetic route for compounds **2-4**.

Figure 1. Spectrophotometric titrations of **2** (3.71×10^{-5} M) with CT DNA of increasing concentrations ($0\sim5.09\times10^{-5}$ M) in 50 mM Tris buffer (pH 6.35, 0.05 mM EDTA) at room temperature. The dash-dot arrows indicate the decreasing absorption bands during the course of titration; solid arrows indicate the isosbestic points.

Figure 2. Fluorescence decrease of EB (5.04×10^{-6} M) induced by the competitive binding of compounds **2** (■), **3** (●) and **4** (▼) to CT DNA (3.98×10^{-6} M) in 50 mM Tris-HCl buffer (pH 6.35, 0.05 mM EDTA) at room temperature, ex 490 nm, em 594 nm.

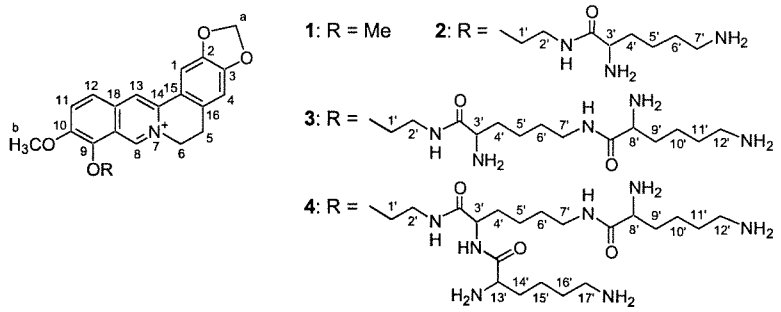
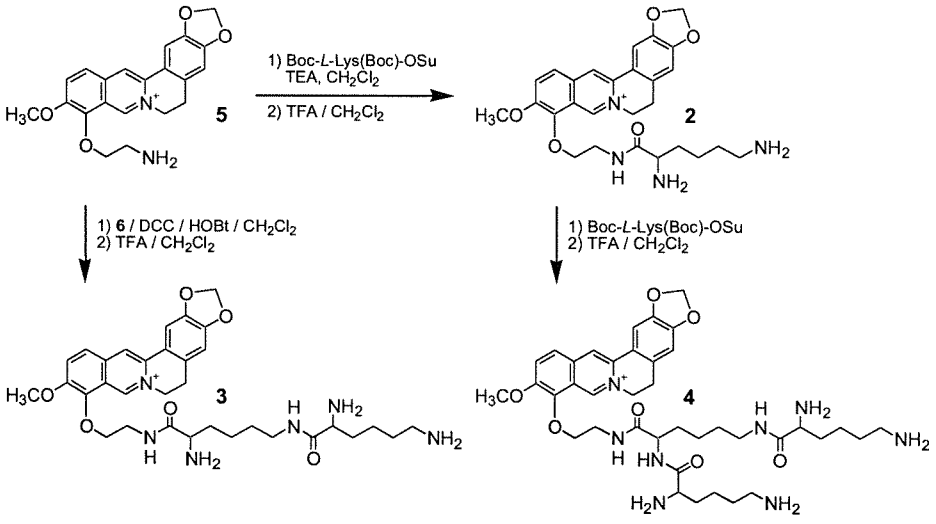


Chart 1



Scheme 1

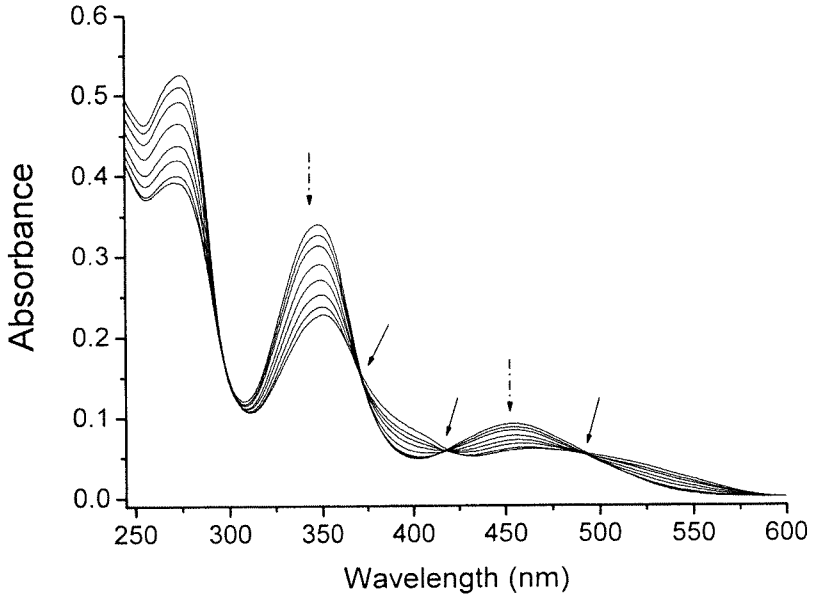


Figure 1.

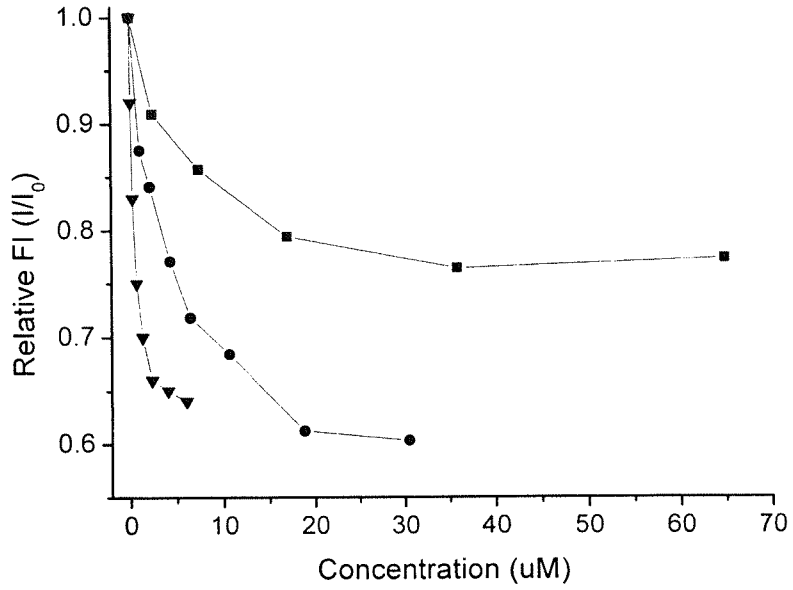


Figure 2.