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Facile synthesis of a polyether-tethered dimeric berberine as a highly effective DNA-cleaving agent in the presence of Cu(II) ion†

Yong-Min Wang,‡[a] Chun-Qiong Zhou,‡[a] Jin-Xiang Chen,[a] Yan-Ling Lin,[a] Wei Zeng,[b] Bi-Cheng Kuang,[a] Wei-Long Fu[a] and Wen-Hua Chen*[a]

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A polyether-tethered dimeric berberine was synthesized and found to be capable of efficiently converting pBR322 DNA into open circular and linear forms in the presence of Cu(II) ions under physiological conditions, most probably *via* an oxidative mechanism. Michaelis–Menten analysis afforded *ca.* 3.5×10^9-fold rate acceleration over uncatalyzed cleavage of supercoiled DNA. The probable mode of action is also briefly discussed.

Multivalent interactions are ubiquitous in nature and play a crucial role in the function of biological systems. Therefore, identification of multivalent molecules that are capable of targeting biomacromolecules such as DNA, has been attracting increasing attention from chemical biologists.[1] Such molecules may serve as effective probes to elucidate the mechanisms of action of some biological processes, and to disclose 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 interaction with DNA.[2]

By using the multivalency principle and naturally occurring protoberberines as lead compounds, we have designed a series of multivalent DNA binders.[3–8] Thus, in previous studies we have shown that dimerization of berberine through alkyl linkers (for example, to give compound **I**, Chart 1) leads to up to 10^2-fold enhanced DNA-binding affinities.[6–8] More significantly, these alkyl-linked berberine dimers show high inhibitory activities towards DNA topoisomerase I, probably by blocking the enzyme's access to plasmid DNA and thereby inhibiting its relaxation.[9] These results have highlighted the promising potential of these dimeric berberines as candidates in the discovery of anticancer drugs. However, the alkyl linkers have little contribution to the interaction with DNA, and also can not serve as functional groups for further structural modification to exploit the potential applications of these dimers. Given the fact that a large class of DNA targeting molecules, such as the naturally occurring antitumor antibiotic bleomycin, are bifunctional in nature, combining a chemically reactive moiety with a DNA binding unit,[10] we modified berberine with a carboxylic group that possesses strong metal-complexing ability, to afford compound **II** (Chart 1). Complexation with metal ions, such as Cu(II) ion, leads to a dimeric structure in which the berberine subunits cooperatively bind to DNA, whereas the Cu(II) ion-containing spacer acts as a highly cleavage-active moiety for pBR322 DNA.[11] However, the dimeric structure relies on the coordination of the carboxylate ion to metal ions, and thus has low stability in aqueous media.

With this background in mind and as our sustained interest in creating of potent DNA cleavers that are active under physiological conditions,[11–15] we designed a new berberine derivative **1** (Chart 1) by replacing the alkyl chain in compound **I** with a tetraethylene glycol tether in a bichromophoric fashion. While acting as an anchor for DNA binding, the two berberine subunits are expected to self-assemble to assist the polyether chain to form a pseudomacrocycle that is capable, in principle, of forming complexes with metal ions.[16] Such strategies have also been utilized to create, for example, a sequence specific DNA cleaving molecule and calcium ion sensitive sensor.[17,18] Herein we describe the synthesis of compound **1** and its remarkable cleaving activity toward plasmid pBR322 DNA in the presence of Cu(II) ions under physiological conditions. In addition, polyether-appended monomeric berberine **2** was chosen as a control compound. Thus, comparison of compounds **1** and **2** provides a means for evaluating the role of an additional berberine subunit in the DNA binding and cleaving processes.

Compounds **1** and **2** were synthesized according to the approach shown in Scheme 1. Thus, reaction of berberrubine **3** with 1,11-ditosyl-3,6,9-trioxaundecane **4** and subsequent

[a]*School of Pharmaceutical Sciences, Southern Medical University, Guangzhou 510515, P. R. China. E-mail: whchen@smu.edu.cn*

[b]*School of Chemistry and Chemical Engineering, South China University of Technology, Guangzhou 510640, P. R. China*

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‡ YMW and CQZ contributed equally to this work.

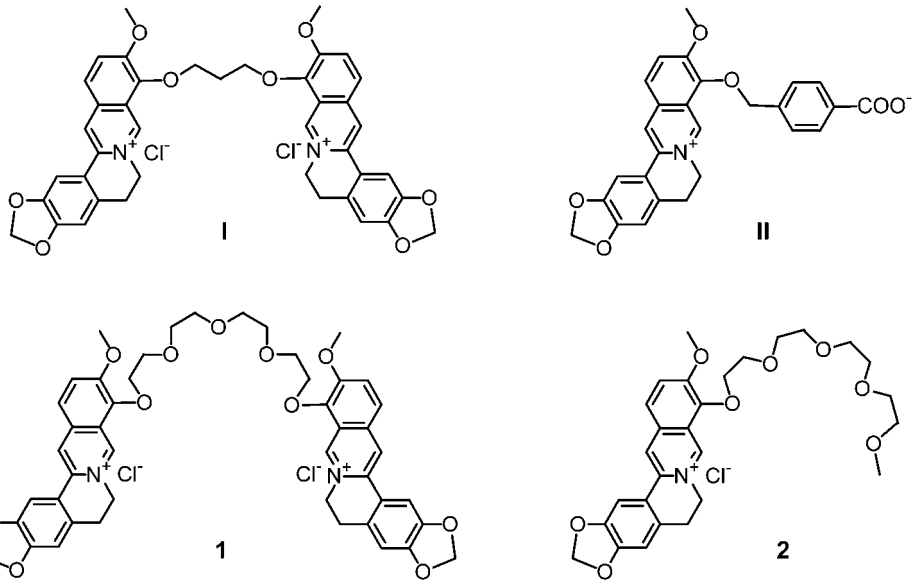
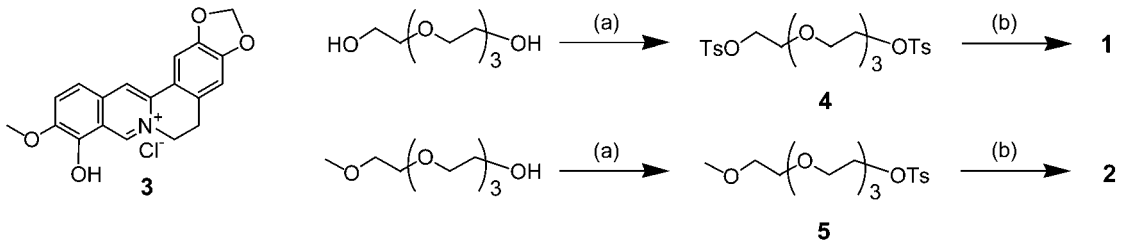


Chart 1 Structures of berberine derivatives **I–II** and **1–2**.



Scheme 1 Synthesis of compounds **1** and **2**. *Reagents and conditions*: (a) TsCl, KOH, CH_2Cl_2; (b) **3**, CH_3CN, reflux.

anion-exchange into chloride form afforded compound **1** in 86% yield. Compound **2** was synthesized in 79% yield in an analogous fashion from the reaction of berberrubine **3** with tetraethylene glycol monomethyl ether tosylate **5**. Compounds **4** and **5** were prepared according to reported protocols.[19] Compounds **1** and **2** were fully characterized on the basis of ^{1}H NMR, ESI-MS and HRMS data (also see ESI†).[20] Compound **1** gave a mass spectrum with the *m*/*z* value corresponding to $[M - 2Cl^-]^{2+}$, and only one set of NMR signals was observed, indicative of its symmetric structure. Compound **2** gave a mass spectrum with the *m*/*z* value corresponding to $[M - Cl^-]^+$. Its ^{1}H NMR spectrum was also in full agreement with the given structure.

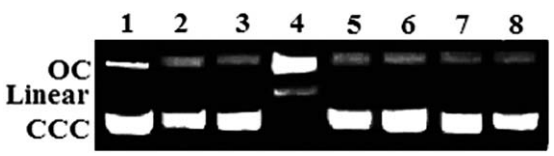


Fig. 1 Agarose GE patterns for the cleavage of pBR322 DNA in 5 mM Tris–HCl buffer (5 mM NaCl, 5% DMF, pH 7.0) and at 37 °C for 5 h, by compound **1** (0.5 mM) in the presence of an equivalent amount of Co^{2+} (Lane 2), Mg^{2+} (Lane 3), Cu^{2+} (Lane 4), Zn^{2+} (Lane 5), Mn^{2+} (Lane 6) and Ni^{2+} (Lane 7), respectively; Lanes 1 and 8, DNA in the absence and presence of compound **1**, respectively.

The cleaving activity toward pBR322 DNA of compound **1** in the presence of six biologically important metal ions, including Co^{2+}, Mg^{2+}, Cu^{2+}, Zn^{2+}, Mn^{2+} and Ni^{2+}, was screened by means of agarose gel electrophoresis (GE) (Fig. 1).[11–14] It can be seen that at the concentration of 0.5 mM, compound **1** was inactive in the presence of an equivalent amount of Co^{2+} (Lane 2), Mg^{2+} (Lane 3), Zn^{2+} (Lane 5), Mn^{2+} (Lane 6) and Ni^{2+} (Lane 7) ions. However, it was capable of completely converting the supercoiled form of DNA (CCC, Form I) into open circular (OC, Form II) and linear forms in the presence of Cu^{2+} ion (Lane 4).

As compound **1** was very active in cleaving pBR322 DNA in the presence of Cu(II) ions, we investigated the probable structure of compound **1** in the presence of $CuCl_2$, by means of single crystal X-ray crystallography and ^{1}H NMR. Firstly, a mixture of compound **1** and slight excess of $CuCl_2 \cdot 2H_2O$ in MeOH–EtOH (8/1, v/v) was allowed to stand at room temperature to afford a crystal of $[\mathbf{1}\text{-}2Cl^-]^{2+} \cdot [Cu_2Cl_6]^{2-}$, the structure of which is shown in Fig. 2 (see Table S1† for crystallographic data). It can be seen that the Cu(II) ion does not coordinate to the polyether tether of compound **1** but exists as an independent $[Cu_2Cl_6]^{2-}$ dianion. In other words, compound **1** forms an ion pair with $CuCl_2$ rather than a coordinated complex in alcoholic media. Secondly, we carried out NMR titration of compound **1** with $CuCl_2$ of varying equivalents. The finding that no obvious chemical shift change

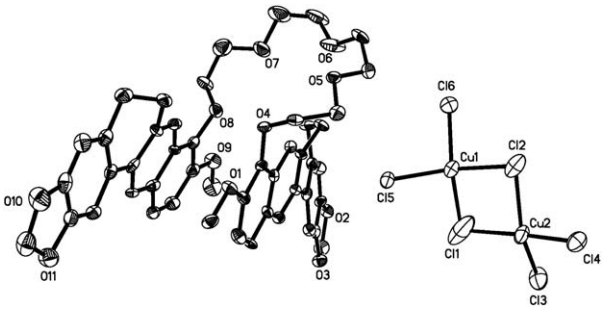


Fig. 2 X-ray structure of $[\mathbf{1}\text{-}2Cl^{-}]^{2+}\cdot[Cu_2Cl_6]^{2-}$.

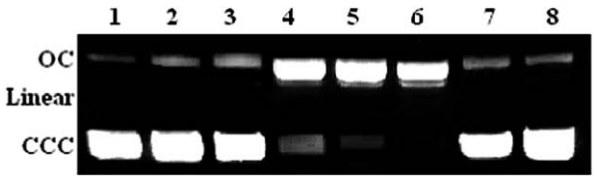


Fig. 4 Agarose GE patterns for the cleavage of pBR322 DNA by **1**@Cu^{2+} of varying concentrations in 5 mM Tris–HCl buffer (5 mM NaCl, 5% DMF, pH 7.0) and at 37 °C for 5 h. Lane 1, DNA alone; Lanes 2–6, DNA + **1**@Cu^{2+} at the concentrations of 0.01, 0.02, 0.04, 0.06, and 0.08 mM, respectively; Lane 7: DNA + **1** (0.08 mM) and Lane 8: DNA + $CuCl_2$ (0.08 mM).

($\Delta\delta \leq 0.05$ ppm) of the tetraethylene glycol tether in the ^{1}H NMR spectrum (1 : 1 DMSO-d_6-D_2O, v/v) was observed, suggests that the interaction between compound **1** and Cu(II) ion was very weak. These results indicate that compound **1** did not form a typical complex with Cu(II) ion. We assume that this is a likely consequence of the low affinity of the oxygen atoms of the polyether chain toward Cu(II) ions as well as the electrostatic repulsion of the two positively charged berberine subunits.

Though there is no obvious interaction between compound **1** and Cu(II) ion, the high DNA-cleaving activity observed in compound **1** in the presence of Cu(II) ions still stimulated us to carry out a detailed investigation. Because we did not detect $[Cu_2Cl_6]^{2-}$ dianion in the negative ESI mass spectrum, we firstly measured the DNA-cleaving activity of compound **1** in the presence of Cu(II) ions of varying equivalents, by means of agarose GE, to optimize the conditions for the cleavage reaction (Fig. 3). It can be seen that the cleaving activity increased with the concentration ratios of Cu(II) ion to compound **1** until the ratio was about 1.0. When the ratio was above 1.0, there was no significant enhancement in the cleaving activity. Therefore, the 1 : 1 mixture of compound **1** with $CuCl_2$ (**1**@Cu^{2+} hereafter) was used in the subsequent cleavage experiments.

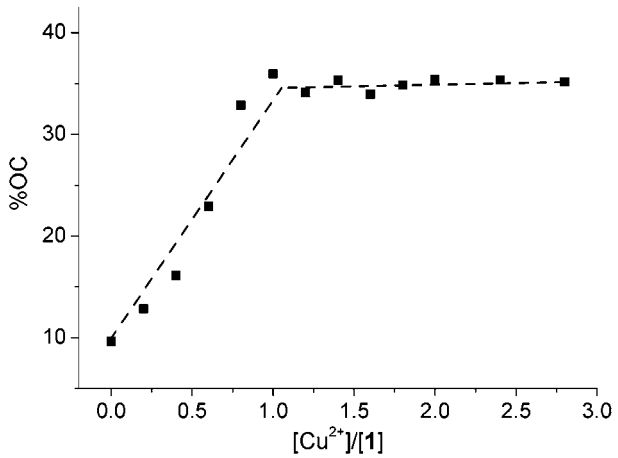


Fig. 3 Plot of the DNA-cleaving activity in terms of OC percentages *vs.* the [Cu(II)]/[compound **1**] ratios varying from 0 to 2.8 in 5 mM Tris–HCl buffer (5 mM NaCl, 5% DMF, pH 7.0) and at 37 °C for 5 h. The concentration of compound **1** was 0.05 mM. These and all the subsequently mentioned GE experiments were performed in triplicate, and the mean values were taken.

Secondly, we conducted the concentration dependence experiments (Fig. 4). It can be seen that the cleaving efficiency had a strong dependence on the concentration of **1**@Cu^{2+} (Lanes 2–6). As discussed elsewhere,[11–14] such a dependence has lent strong support to it being **1**@Cu^{2+} that catalyzed the cleavage. It should be noted that neither compound **1** (Lane 7) nor $CuCl_2$ (Lane 8) showed any significant activity.[21]

To gain further insight into the cleaving activity of **1**@Cu^{2+}, we measured the kinetics of pBR322 DNA degradation.[11–14] As shown in Fig. 5a, the cleavage of supercoiled DNA into the OC and linear forms by **1**@Cu^{2+} obeyed *pseudo*-first-order kinetics. Michaelis–Menten analysis of the pseudo-first-order rate constants (k_{obsd}) against the concentrations of **1**@Cu^{2+} (Fig. 5b) afforded the maximal catalytic rate constant k_{max} of 124.64 h^{-1} and Michaelis constant K_M of 0.25 mM, respectively.[22] This gives a rate acceleration of *ca.* 3.5×10^9 over uncatalyzed cleavage of supercoiled DNA,[23] which represents one of the highest rate enhancements in DNA cleavage to date.[24] In addition, it is noteworthy that the overall catalytic activity (k_{max}/K_M) of **1**@Cu^{2+} is *ca.* 550-fold higher than that of **II**@Cu^{2+} ($k_{max} = 2.41$ h^{-1} and $K_M = 2.64$ mM).[11]

It is known that nucleic acids can be cleaved through an oxidative or hydrolytic pathway.[25,26] Therefore, to gain insight into the probable mechanism of action, we monitored the pBR322 DNA cleavage in the presence of singlet oxygen scavenger 2,2,6,6-tetramethyl-4-piperidone (TMP), superoxide scavenger KI, and hydroxyl radical scavengers DMSO and *t*-BuOH (Fig. 6). As a result, the addition of TMP (Lane 3), DMSO (Lane 5) or *t*-BuOH (Lane 6) did not inhibit the cleavage, ruling out the involvement of singlet oxygen or hydroxyl radical in the cleavage reaction. Significant inhibition was observed in the presence of KI (Lane 4), suggesting that superoxide radicals may participate in the DNA cleavage by **1**@Cu^{2+}.

Generally, a metal complex-based DNA-cleaving agent functions in a fashion in which a metal ion firstly coordinates to its ligand, and then the metal complex formed binds to and cleaves DNA.[27] It appears unlikely from the results obtained from ^{1}H NMR and the solid structure of compound **1** with $CuCl_2$, that the cleavage by **1**@Cu^{2+} proceeded *via* this typical DNA-cleaving process. To gain further insight into this, we firstly investigated the DNA-cleaving activity of compound **2** in the presence of Cu(II) ions. As a result, no DNA-cleaving activity was observed for compound **2** in the presence of Cu(II) ion, suggesting that an additional berberine subunit is essential to exert the cleaving

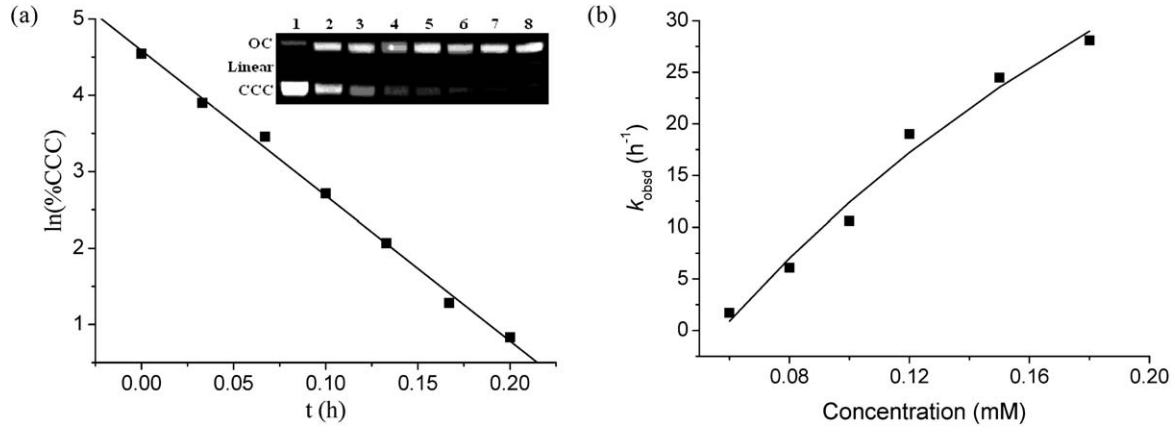


Fig. 5 (a) Time course of pBR322 DNA cleavage by **1**@Cu^{2+} (0.12 mM) in 5 mM Tris–HCl buffer (5 mM NaCl, 5% DMF, pH 7.0) and at 37 °C. Inset: agarose GE patterns of the time-variable reaction products. Reaction time for Lanes 1–8 was 0, 2, 4, 6, 8, 10, 12 and 15 min, respectively. (b) Plot of k_{obsd} *versus* the concentrations of **1**@Cu^{2+}.

Fig. 6 Agarose GE patterns for the cleavage of pBR322 DNA by **1**@Cu^{2+} (0.06 mM) in 5 mM Tris–HCl buffer (5 mM NaCl, 5% DMF, pH 7.0) and at 37 °C for 5 h, in the presence of TMP (0.2 mM, Lane 3), KI (10 mM, Lane 4), DMSO (1.0 mM, Lane 5) and *t*-BuOH (1.0 M, Lane 6). Lanes 1 and 2 were DNA in the absence and presence of **1**@Cu^{2+}, respectively.

activity. Secondly, we investigated the DNA-cleaving activity of compound **I** in the presence of Cu(II) ions. If compound **1** did not cooperate with Cu(II) ion during the cleavage process, it is reasonable to deduce that compound **I**, because of its much higher DNA-binding affinity,[6,22,27] should show similar or even higher cleaving activity in the presence of Cu(II) ions. The finding that compound **I** exhibited no detectable cleaving activity in the presence of Cu(II) ions, under similar conditions, revealed that the polyether chain is indispensable in the cleavage process. These results, in combination with our previous studies that alkyl-tethered dimeric berberines are a class of DNA bisintercalators,[6–8] strongly suggest that the cleavage by **1**@Cu^{2+} might proceed *via* a different mode of action. Specifically, the two berberine subunits of compound **1** bisintercalate into the adjacent base pairs of pBR322 DNA, which may impel the polyether tether to form a *pseudo*-macrocycle podand that is capable of forming a cleavage-active complex with Cu(II) ions.[17]

In summary, a polyether-tethered dimeric berberine has been synthesized, and fully characterized by ^{1}H NMR, ESI MS, and HRMS. This compound was found, in the presence of Cu^{2+} ions, to be capable of efficiently cleaving plasmid pBR322 DNA, most probably through an oxidative pathway. The fact that its corresponding polyether-appended monomeric analog and propyl chain-tethered dimeric berberine showed no cleaving activity supported a mode of action in which the two covalently-linked berberine subunits bisintercalate into the adjacent base pairs to compel the polyether chain to form a podand that is capable of forming a catalytically active complex with Cu(II) ion. Efforts aimed at clarifying the structure–activity correlations of polyether-tethered dimeric berberines and creating more active biocompatible DNA cleavers of this type are currently in progress with a view toward the design of new potential chemotherapeutic agents, for example, for cancers.

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19 C. A. Traina, R. C. Bakus II and G. C. Bazan, *J. Am. Chem. Soc.*, 2011, **133**, 12600–12607.
20 Synthetic procedures for compounds **1** and **2**: to a solution of berberrubine **3** (320 mg, 1.0 mmol) in MeCN (20 mL) was added 1,11-ditosyl-3,6,9-trioxaundecane **4** (243 mg, 0.48 mmol). The resulting mixture was refluxed for 6 days, and then concentrated under reduced pressure. The obtained residue was subjected to anion exchange into chloride form, and subsequently purified by chromatography on a reverse-phase column, eluting with a gradient of methanol in water (0–10%), to give compound **1** (360 mg, 86%) as a yellow powder having ^{1}H-NMR (400 MHz, DMSO-d_6) δ 9.66 (s, 2H), 8.85 (s, 2H), 8.05 (d, J = 9.6 Hz, 2H), 7.88 (d, J = 9.2 Hz, 2H), 7.69 (s, 2H), 7.05 (s, 2H), 6.15 (s, 4H), 4.95–4.92 (m, 4H), 4.28–4.26 (m, 4H), 3.89 (s, 6H), 3.78–3.76 (m, 4H), 3.65 (s, 8H), 3.20 (m, 4H); ESI MS m/z: 401.9 ($[M - 2Cl^-]^{2+}$) and HRMS for $C_{46}H_{46}N_2O_{11}$ ($[M - 2Cl]^{2+}$) calc.: 401.1545, found: 401.1538. Compound **2** (173 mg, 79%) was synthesized in a similar way from the reaction of berberrubine **3** (134 mg, 0.4 mmol) with tetraethylene glycol monomethyl ether tosylate **5** (148 mg, 0.4 mmol), as a yellow powder having ^{1}H-NMR (400 MHz, DMSO-d_6) δ 9.78 (s, 1H), 8.96 (s, 1H), 8.20 (d, J = 9.2 Hz, 1H), 8.02 (d, J = 9.2 Hz, 1H), 7.81 (s, 1H), 7.10 (s, 1H), 6.18 (s, 2H), 4.94 (t, J = 6.2 Hz, 2H), 4.43–4.41 (m, 2H), 4.07 (s, 3H), 3.82–3.80 (m, 2H), 3.61–3.58 (m, 2H), 3.54–3.51 (m, 2H), 3.47–3.42 (m, 6H), 3.36–3.35 (m, 2H), 3.22 (t, J = 6.2 Hz, 2H), 3.16 (s, 3H); ESI MS m/z: 512.9 ($[M - Cl^-]^+$) and HRMS for $C_{28}H_{34}NO_8$ ($[M - Cl^-]^+$) calc.: 512.2284, found: 512.2279.
21 As stated in the text, $Cu_2Cl_6^{2-}$ dianion was not detected in the negative ESI mass spectrum. Therefore, $CuCl_2$ rather than the salts of $Cu_2Cl_6^{2-}$ was used as a control.
22 Under the condition of 5 mM Tris–HCl buffer (5 mM NaCl, pH 7.0), the binding constants with calf-thymus DNA that were obtained by means of spectrofluorimetric titrations were $(8.99 \pm 0.04) \times 10^3$ M^{-1} for berberine, $(1.15 \pm 0.18) \times 10^5$ M^{-1} for compound **I**, $(1.51 \pm 0.15) \times 10^4$ M^{-1} for compound **1**, $(2.38 \pm 0.11) \times 10^4$ M^{-1} for **1**@Cu^{2+} and $(1.46 \pm 0.01) \times 10^3$ M^{-1} for compound **2**, respectively.
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