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Synthesis, characterization and potent DNA-cleaving activity of copper(II)-complexed berberine carboxylate

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ABSTRACT

9-*O*-(4-carboxybenzyl)berberine (CBB) **1** was synthesized from the reaction of berberrubine with methyl 4-(bromomethyl)benzoate and subsequent hydrolysis. Its Cu(II) complex **2** was prepared from the reaction with $Cu(NO_3)_2{\cdot}3H_2O$, and established as $[Cu(CBB)_2](NO_3)_2{\cdot}2H_2O$ by means of ^{1}H NMR, UV, IR, elemental analysis and TGA measurements. Agarose gel electrophoresis study on the cleavage of plasmid pBR322 DNA indicated that complex **2** was capable of efficiently cleaving DNA under physiological conditions, most probably via an oxidative mechanistic pathway involving the formation of singlet oxygen as the reactive species. Kinetic assay afforded the maximal first-order rate constant k_{max} of 2.41 h^{-1} and Michaelis constant K_M of 2.64 mM, respectively, representing ca. 10^8-fold acceleration in the cleavage. This catalytic efficacy is attributed to the Cu(II)-assisted formation of dimeric species, in which the two berberine subunits cooperatively bind to DNA, whereas the carboxylate-coordinated Cu(II) moiety functions as the cleavage-active center.

During the past decades, considerable interest has been attracted in transition metal complexes that are capable of binding and cleaving DNA under physiological conditions. This is spurred by their high potentials as structural probes for nucleic acids and as metal-based anticancer agents.[1] Among them, copper-based complexes have been receiving particular attentions because copper is a bio-essential element on which many enzymes rely to exert their activities. Accordingly, many copper complexes have been synthesized and shown to be able to induce hydrolytic or oxidative cleavage of DNA.[2]

Most of the DNA cleaving molecules are created by linking DNA-recognizing units with cleavage active moieties, such as metal complexed macrocyclic polyamines[3] and azacrowns.[4] Since DNA binding is widely recognized as a critical step in the process of DNA cleavage in most cases,[5] the selection of DNA-binding units is crucial to the construction of potent cleaving agents. Among the building motifs that are frequently used, natural products that exert their activities through specific interactions with DNA with established affinities and modes are attractive.[6] This is motivated by the biocompatibility of natural products as well as by the hypothesis that their structural modification may lead to significantly enhanced activities.[7]

As our sustained interest in creating potent DNA-cleaving agents that are active under physiological conditions,[8–10] we designed a berberine derivative bearing a carboxylic group, that is, compound **1** (Scheme 1). It is known that carboxylic group has strong metal-complexing ability, and naturally-occurring berberine and its derivatives are capable of forming stable complexes with DNA,[11] including poly(A) sequences, quadruplex and triplex structures.[12] Thus, attachment of a metal-complexed carboxylic group to berberine could lead to a *biocompatible* DNA-cleaving agent. Herein we describe the synthesis of compound **1** and its Cu(II) complex **2**, and the potent cleaving activity of complex **2** toward plasmid pBR322 DNA under physiological conditions.

The synthetic route of compound **1** and complex **2** is shown in Scheme 1. Thus, reaction of berberrubine **3** with methyl (4-bromomethyl)benzoate **4** gave compound **5**, which upon hydrolysis afforded compound **1**. Complex **2** was prepared in 30% yield from the reaction of **1** with $Cu(NO_3)_2{\cdot}3H_2O$ in methanol.

The structure of compound **1** was firstly confirmed by ^{1}H NMR, ESI MS, elemental analysis and IR (see Supplementary data). It gave a mass spectrum with the *m/z* value corresponding to $[M+H]^+$. Its ^{1}H NMR was also in full agreement with the given structure. The unambiguous evidence was obtained from the single-crystal X-ray crystallography (CCDC-882128, Fig. S4).

Efforts to crystallize complex **2** proved unsuccessful. Therefore, we established its structure by means of spectral, elemental and thermogravimetric analyses (TGA). Firstly, the coordination of

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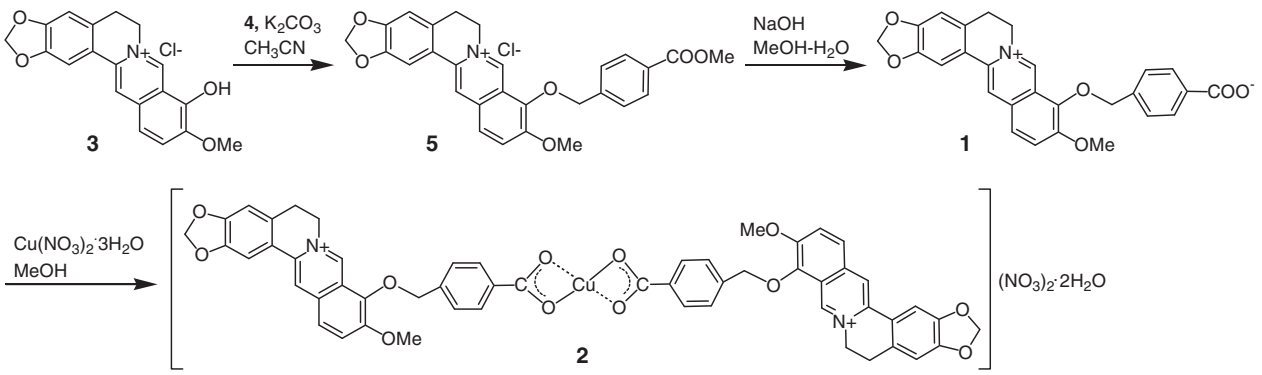

Scheme 1. Synthesis of compound **1** and complex **2**.

Cu(II) ion to the carboxylic group was convincingly established on basis of ^{1}H NMR (Fig. 1). It can be seen that compound **1** and complex **2** exhibited well resolved signals. In complex **2**, the H1′ and H4′ protons became very broad and exhibited a remarkable downfield shift ($\Delta\delta$ = 0.31 ppm), indicating the coordination between the carboxylic group and Cu(II) ion. In addition, the H8 proton received a significant deshielding effect from the coordinated benzoate group, showing a large downfield shift ($\Delta\delta$ = 0.26 ppm). The shielding and/or deshielding effects became very weak for all the other protons.

The deprotonation and the coordination mode of the carboxylic group in complex **2** were confirmed by IR spectroscopy. Both compound **1** and complex **2** exhibited broad bands in the range of 3000–3500 cm^{-1} that are assigned to the $\nu(H_2O)$ vibration of water molecules. The absence of strong bands around 1700 cm^{-1} indicated that the carboxylic groups of both compound **1** and complex **2** were deprotonated. Moreover, the bands at 1597 and 1396 cm^{-1} that were assigned to the asymmetric and symmetric stretching vibrations ν_{asym}(C=O) and ν_{sym}(C=O) of the carboxylate moiety (–COO) of compound **1**, respectively, were shifted to 1601 and 1359 cm^{-1} for complex **2**, respectively. Their difference $\Delta\nu$ [= ν_{asym}(C=O) – ν_{sym}(C=O)], a useful characteristic parameter for determining the coordination modes of carboxylate ligands, reached 242 cm^{-1} for complex **2**, suggesting that the carboxylate ions coordinated to the Cu(II) ion predominantly in a monodentate coordination mode.[13] The non-coordinated nitrate group in complex **2** was ascertained by the presence of characteristic $\nu(NO_3^-)$ frequency at 1384 cm^{-1}.[14]

Finally, the number and the coordination mode of H_2O molecules in complex **2** were determined by TGA (Fig. S5). The total weight loss was 3.37% below 105 °C and ignorable between 105 and 242 °C. This result indicated that there were two solvated H_2O molecules in complex **2**, which was consistent with the value (3.18%) from elemental analysis. It is unusual for Cu(II) ion to exhibit linear two-coordination geometry. We speculate that the Cu(II) ion may have weak interactions with the other two O atoms of the carboxylate ions of two molecules of compound **1** in the solid state to fulfil its coordination sphere.

To confirm the solution structure of complex **2**, we measured the stoichiometry of compound **1** with Cu(II) ion, by means of absorption spectrometry. As shown in Figure 2, the absorbance at 345 nm decreased with the increase in the concentration ratio of Cu(II) to compound **1** until the ratio was about 0.5. When the ratio was above 0.5, there was no change in the absorbance. This result indicated the strong interaction of compound **1** with Cu(II) ion and supported the formation of their 2:1 complex. In addition, the molar conductivity Λ_M of complex **2** in MeOH was measured to be 196 S cm^2 mol^{-1}. Such value has been suggested for 1:2 electrolytes.[15] Taken together, the structure of complex **2** was established as shown in Scheme 1.

The DNA-cleaving activity of complex **2** toward pBR322 DNA was studied under physiological conditions (37 °C and pH 7.0).

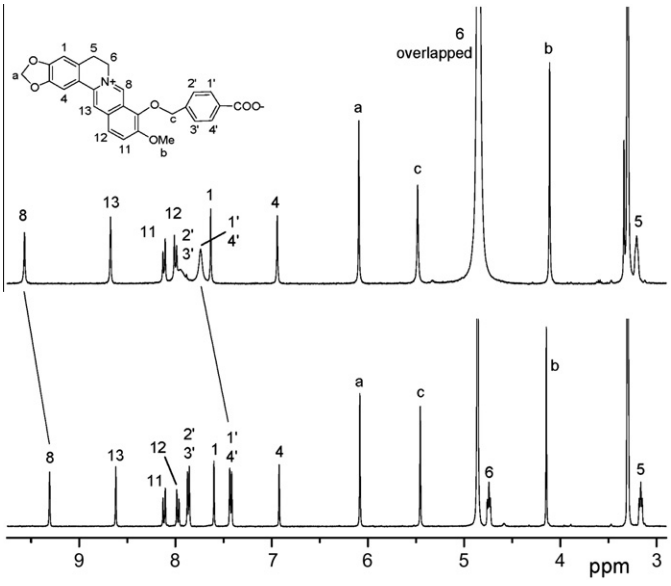

Figure 1. ^{1}H NMR spectra of compound **1** (bottom) and of complex **2** (top) in CD_3OD.

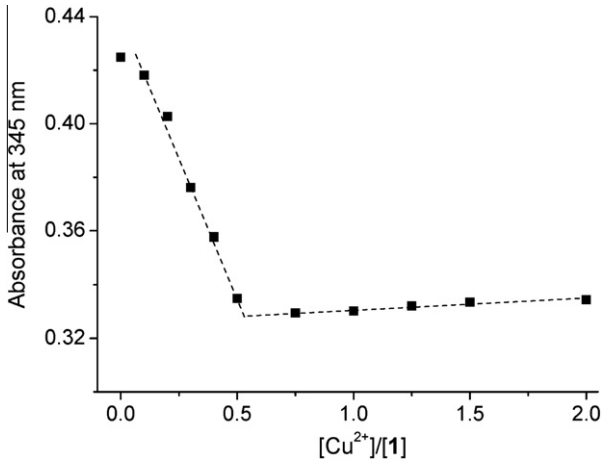

Figure 2. Plot of the absorbance at 345 nm versus the [Cu(II)]/[compound **1**] ratios varying from 0 to 2 in 5 mM Tris–HCl buffer (5 mM NaCl, pH 7.0).

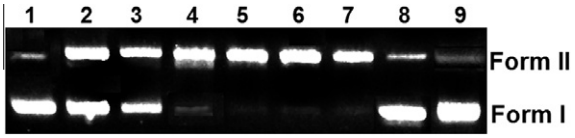

Figure 3. Agarose GE patterns for the cleavage of pBR322 DNA by complex **2** of increasing concentrations at pH 7.0 and 37 °C (5 h). Lane 1: DNA alone; Lanes 2–7: DNA + complex **2** at the concentrations of 0.05, 0.25, 0.5, 1.0, 1.5, and 1.9 mM, respectively; Lane 8, DNA + **1** (3.8 mM); and Lane 9, DNA + $Cu(NO_3)_2{\cdot}3H_2O$ (1.9 mM).

The extent of cleavage of supercoiled pBR322 DNA was determined by use of agarose gel electrophoresis (GE). Figure 3 shows the GE patterns for the cleavage of pBR322 DNA by complex **2** of varying concentrations. It can be seen that complex **2** was capable of converting pBR322 DNA into open circular form (OC, Form II), and that the cleaving activity showed a strong dependence on the concentration of complex **2** (Lanes 2–7). When the concentration of complex **2** was about 0.5 mM, almost all the CCC form (Form I) was converted to the OC form. Such a dependence has lent strong support that it was complex **2** that catalyzed the cleavage. It should be noted that neither compound **1** (Lane 8) nor $Cu(NO_3)_2{\cdot}3H_2O$ (Lane 9) showed any significant cleaving activity.

To gain further insight into the cleaving activity of complex **2**, the kinetics of pBR322 DNA degradation was studied. Figure 4a shows that the extent of supercoiled DNA cleavage into the nicked form varied exponentially with the reaction time, giving *pseudo* first-order kinetics with an apparent rate constant (k_{obs}) of $0.38 \pm 0.01\ h^{-1}$. Michaelis–Menten analysis of the k_{obs} values against the concentrations of complex **2** gave the maximal first-order rate constant k_{max} of $2.41 \pm 0.39\ h^{-1}$ and Michaelis constant K_M of 2.64 ± 0.76 mM, respectively (Fig. 4b).[4] Thus, complex **2** can catalyze the cleavage with a rate acceleration of ca. 10^8-fold over uncatalyzed cleavage of supercoiled DNA.[16] This enhancement is thought to be due to the Cu(II)-assisted formation of dimeric species, in which the two berberine subunits cooperatively bind to DNA with enhanced affinity,[11f] whereas the carboxylate ion-coordinated Cu(II) part functions as the cleavage-active moiety. To test this hypothesis, we measured the relative binding affinity of complex **2** toward calf-thymus (CT) DNA, by means of ethidium bromide (EB) displacement experiment. It was estimated to be $(2.19 \pm 0.10) \times 10^5\ M^{-1}$, ca. 20-fold higher than that of berberine under similar conditions.[17]

It is known that nucleic acid can be cleaved through either an oxidative or hydrolytic pathway. Therefore, to gain insight into the probable mechanism of action of complex **2**, we conducted the cleavage reactions in the presence of hydroxyl radical scavengers DMSO and MeOH, singlet oxygen scavenger NaN_3 and 2,2,6,6-tetramethyl-4-piperidone (TMP), and metal ion-chelating agent EDTA (Fig. 5).[14] As a result, EDTA (Lane 7) efficiently inhibited DNA cleavage, indicating that complex **2** was obligatory in DNA cleavage reaction. When DMSO (Lane 3) and MeOH (Lane 4) were added, no inhibition was observed, ruling out the possibility that DNA was cleaved by hydroxyl radical. In the presence of NaN_3 (Lane 5) and TMP (Lane 6), the DNA cleavage was significantly inhibited, suggesting that singlet oxygen was likely to be the reactive species responsible for the nuclease activity.[18] In addition, we monitored the hydrolysis of bis(4-nitrophenyl) phosphate (BNPP) in the presence of excess complex **2** at physiological pH and 25 °C. If BNPP can be hydrolyzed by complex **2**, this is detected by an increase of the absorbance at 400 nm because of the release of 4-nitrophenoxide. As a result, no change in the absorbance was observed over 30 h (Fig. S17), ruling out the possibility that complex **2** acted as a hydrolytic agent.[19] Taken together, these results strongly suggest that DNA cleavage by complex **2** preceded, most probably via an oxidative mechanism arising from singlet oxygen $^1O_2$.[14]

In summary, 9-*O*-(4-carboxybenzyl)berberine and its Cu(II) complex have been synthesized and fully characterized. The Cu(II) complex was capable of efficiently cleaving plasmid pBR322 DNA under physiological conditions, most probably through an oxidative mechanistic pathway involving the formation of singlet oxygen as the reactive species. Kinetic assays indicated that the Cu(II) complex was capable of catalyzing the cleavage of pBR322 DNA at a rate acceleration of ca. 10^8-fold over uncatalyzed cleavage of supercoiled DNA, suggesting a synergistic effect from the enhanced binding of the two berberine subunits and the potent

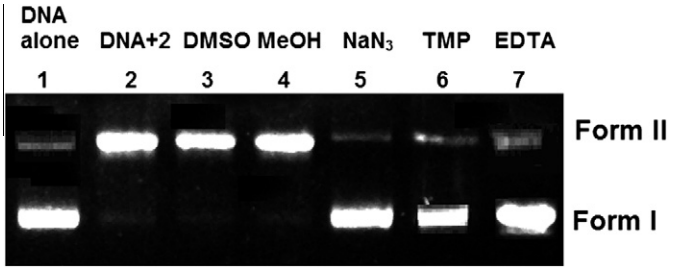

Figure 5. Agarose GE patterns for the cleavage of pBR322 DNA by complex **2** (1 mM) at pH 7.0 and 37 °C, in the presence of DMSO (1 mM, Lane 3), MeOH (1 M, Lane 4), NaN_3 (0.2 M, Lane 5), TMP (0.2 M, Lane 6) and EDTA (0.1 M, Lane 7). Lanes 1 and 2 were DNA alone and DNA + complex **2**, respectively.

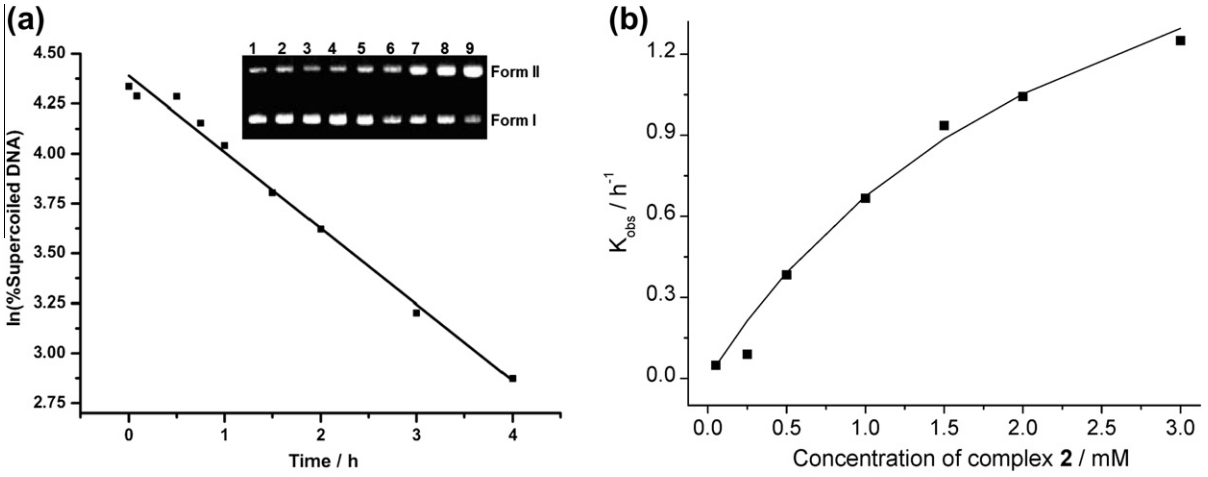

Figure 4. (a) Time course of pBR322 DNA cleavage promoted by complex **2** (0.5 mM) at pH 7.0 and 37 °C. Inset: agarose GE patterns of the time-variable reaction products. Lanes 1–9, reaction time was 0, 0.083, 0.5, 0.75, 1.0, 1.5, 2.0, 3.0 and 4.0 h, respectively. (b) Saturation kinetics plot of k_{obs} versus the concentrations of complex **2**.

cleavage of carboxylate ion-coordinated copper(II) ion. The present results highlight the fact that simple modification of a natural product, such as berberine, by a metal-coordinating carboxylic group can afford a bifunctional derivative with promising properties. From the standpoint of new drug discovery, such strategies may provide a platform for the study of other metal ion-complexed natural products targeting biomacromolecules, which is currently under investigation in our laboratories.

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Supplementary data

Supplementary data (synthesis and characterization of compound **1** and complex **2**; and experimental procedures for the measurement of their DNA-binding and cleaving activities) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.09.087.

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