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PAPER

Synthesis and enhanced DNA cleavage activities of bis-tacnorthoamide derivatives†

Li Wei,[a] Ying Shao,*[b] Mi Zhou,[b] Hong-Wen Hu[a] and Guo-Yuan Lu*[a]

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A new metal-free DNA cleaving reagent, bis-tacnorthoamide derivative **1** with two tacnorthoamide (tacnoa) units linked by a spacer containing anthraquinone, has been synthesized from triazatricyclo-[5.2.1.0^{4,10}]decane and characterized by NMR and mass spectrometry. For comparison, the corresponding compounds mono-tacnorthoamide derivative **2** with one tacnorthoamide unit and **6** with two tacnorthoamide units linked by an alkyl (1,6-hexamethylene) spacer without anthraquinone have also been synthesized. The DNA-binding property investigated *via* fluorescence and CD spectroscopy suggests that compounds **1** and **2** have an intercalating DNA binding mode, and the apparent binding constants of **1**, **2** and **6** are 1.3×10^7 M^{-1}, 0.8×10^7 M^{-1} and 8×10^5 M^{-1}, respectively. Agarose gel electrophoresis was used to assess plasmid pUC19 DNA cleavage activity promoted by **1**, **2**, **6** and parent tacnoa under physiological conditions, which gives rate constants k_{obs} of 0.2126 ± 0.0055 h^{-1}, 0.0620 ± 0.0024 h^{-1}, 0.040 ± 0.0007 h^{-1} and 0.0043 ± 0.0002 h^{-1}, respectively. The 50-fold and 15-fold rate acceleration over parent tacnoa is because of the anthraquinone moiety of compound **1** or **2** intercalating into DNA base pairs *via* a stacking interaction. Moreover, DNA cleavage reactions promoted by compound **1** give 5.3-fold rate acceleration over compound **6**, which further demonstrates that the introduction of anthraquinone results in a large enhancement of DNA cleavage activity. In particular, DNA cleavage activity promoted by **1** bearing two tacnoa units is 3.3 times more effective than **2** bearing one tacnoa unit and the DNA cleavage by compound **1** was achieved effectively at a relatively low concentration (0.03 mM). This dramatic rate acceleration suggests the cooperative catalysis of the two positively charged tacnoa units in compound **1**. The radical scavenger inhibition study and ESI-MS analysis of bis(2,4-dinitrophenyl) phosphate (BDNPP) and adenylyl(3′-5′)phosphoadenine (APA) cleavage in the presence of compound **1** suggest the cleavage mechanism would be *via* a hydrolysis pathway by cleaving the phosphodiester bond of DNA.

Introduction

DNA cleaving reagents have attracted continuous and extensive interest due to their potential applications in the fields of molecular biological technology and drug development.[1] Among them, DNA cleavage promoted by transition metal complexes has been widely investigated in the last decade.[2] In particular, transition metal dinuclear complexes are found to be quite efficient due to synergistic catalysis of the two metal centers,[3] but the clinical pharmic use of some metal complexes, such as Cu complexes, is hampered by concerns over the lability and toxicity in the treatment of cancer due to free radical generation *via* a redox pathway.[4] Recently, metal-free cleaving reagents have been put forward by Göbel and co-workers.[5] In fact, DNA cleavage is considered to be safer for therapy in the absence of transition metal ions. Some organic molecules, such as guanidinium derivatives,[6] macrocyclic polyamines,[7] pseudorotaxane composed of cucurbituril[8] and peptides,[9] have been studied as DNA cleaving reagents in recent years. In particular, Yu and co-workers[10] described a metal-free peptide nucleic acid (PNA)–cyclen conjugate as a DNA-cutting agent with efficient and site-selective DNA hydrolysis activity. Yavin's group[11] successfully designed a cyclic peptide scaffold conjugated to anthraquinone as a metal-free DNA nuclease. DNA cleavage was promoted at micromolar concentration under physiological conditions. We have also synthesized a metal-free DNA nuclease containing guanidinoethyl and hydroxyethyl side arms, and DNA cleavage

[a]*Department of Chemistry, State Key Laboratory of Coordination Chemistry, Nanjing University, Nanjing 210093, Jiangsu, P.R. China. E-mail: lugyuan@nju.edu.cn; Fax: +86-25-83317761; Tel: +86-25-83685613*
[b]*Key Laboratory of Fine Petrochemical Engineering, Changzhou University, Changzhou 213164, Jiangsu, P. R. China*

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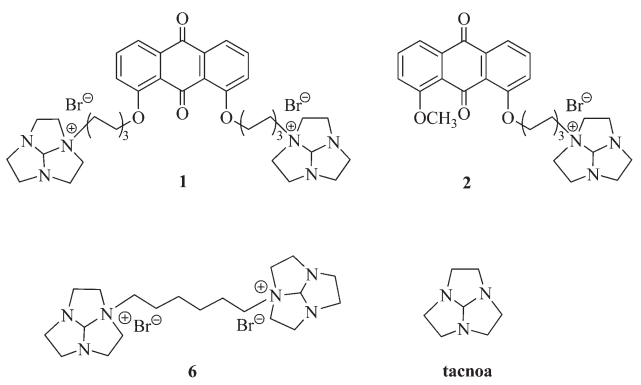
promoted by the compound without metal ions was achieved effectively at a relative low concentration (0.1 mM), giving 10^7-fold rate acceleration over uncatalyzed double-stranded DNA.[12] However, the DNA phosphodiester bond is very stable under physiological conditions with a half-life hydrolysis rate of about 200 million years, which is primarily due to the repulsion between the negatively charged backbone and potential nucleophiles.[13] In nature, the cleavage rate of DNA *via* a hydrolytic pathway is about 10^{15}–10^{16}-fold accelerated by a natural nuclease such as staphylococcal nuclease (SNase).[14] Therefore, the cleaving efficiency of artificial nuclease is still far from that of natural nuclease. Thus, to develop highly efficient metal-free DNA cleaving reagents *via* a non-oxidative pathway is not only necessary for practical value but also is a challenging subject.

It is well known that an organic positively charged group such as ammonium or guanidinium is the key functional group at the active site in a natural nuclease such as staphylococcal nuclease (SNase) and bovine pancreatic ribonuclease (RNase A).[15] Some compounds with a positively charged group as nuclease mimics for cleavage of phosphodiester have been reported, a few of them were identified as efficient cleavers of RNA.[16] The tacnorthoamide is a rigid tribridge circular structure containing both an organic positively charged ammonium group and protonated or deprotonated tertiary amino groups.[17] Therefore, the tacnoa moiety is expected to be a cleaving group, which has not been reported so far. In addition, anthraquinone derivatives are well-known effective DNA intercalators.[18] The introduction of anthraquinone would enhance the binding affinity of a compound to the substrate (DNA). Thus we report here, for the first time, the design and synthesis of a novel metal-free DNA cleaving reagent bis-tacnorthoamide derivative **1**, in which two tacnoa units and anthraquinone are linked by two alkyl (1,6-hexamethylene) spacers (Scheme 1). Compound **1** has two side arms bearing cationic tacnorthoamide like a pair of long-chain drop earrings, a similar model to dinuclear synergetic metallonuclease. It would be expected to catalyze the cleavage of DNA cooperatively *via* a hydrolytic mechanism in the absence of metal ions. For comparison, the corresponding compounds mono-tacnorthoamide derivative **2** with one tacnorthoamide unit and **6** with two tacnorthoamide units linked by an alkyl (1,6-hexamethylene) spacer without anthraquinone have also been synthesized (Scheme 1). Binding behaviors of these compounds with calf thymus DNA (CT-DNA) were investigated by fluorescence and circular dichroism (CD) spectroscopy, and plasmid pUC19 DNA cleaving behavior was assessed *via* the agarose gel electrophoresis. The DNA cleavage activity of the parent compound tacnoa was studied as well. The mechanism of the cleavage process was suggested on the basis of a radical scavenger inhibition study and ESI-MS analysis of bis(2,4-dinitrophenyl) phosphate (BDNPP) and adenylyl(3′–5′)phosphoadenine (APA) cleavage in the presence of compound **1**.

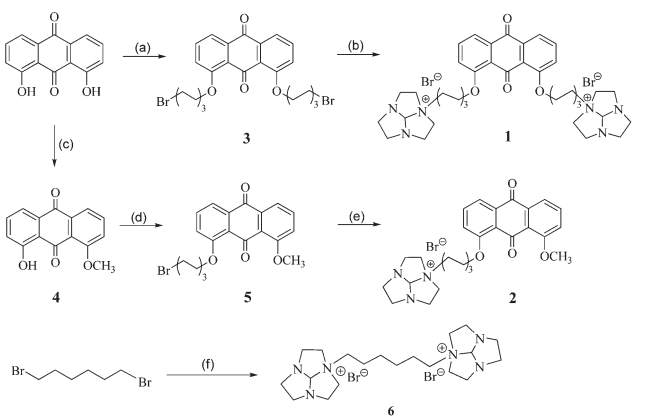


Scheme 1 Chemical structure of compounds **1**, **2**, **6** and **tacnoa**.

Results and discussion

Synthesis of compounds 1, 2 and 6

Syntheses of compounds **1**, **2** and **6** were achieved *via* a series of nucleophilic substitution reactions from 1,8-dihydroxyanthraquinone and 1,6-dibromohexane (Scheme 2). Triazatricyclo [5.2.1.$0^{4,10}$]decane (tacnoa)[3a] and compound **3**[19] were prepared according to the literature method. All the target compounds are ammonium salts, so they gradually precipitated from anhydrous tetrahydrofuran, and then were filtered off. The purified compound was obtained after washing with THF and ether. ^{1}H NMR, ^{13}C NMR, ESI-MS spectra (ESI,† p2–9) and elemental analysis data of compounds **1**, **2** and **6** are in accord with the assigned structure. ^{1}H NMR spectra of compounds **1**, **2** and **6** show the methine single peak at δ 5.4. In the ^{13}C NMR spectrum of compound **1**, the peaks at δ 116.7, 118.3, 120.0, 122.3, 133.3, 134.5, 158.1 are assigned to the central carbon atom of tacnoa and aryl carbon atoms; the peaks at δ 183.2, 183.8 are assigned to the signals of carbonyl carbon atoms in anthraquinone. In the ^{13}C NMR spectrum of compound **2**, the peaks at δ 118.2, 118.9, 118.9, 119.0, 119.2, 119.5, 123.3, 124.0, 124.1, 134.0, 134.8, 158.1, 159.2 and 182.7, 183.9 are ascribed to signals of the tacnoa central carbon atom, aryl carbon atoms and carbonyl carbon atoms in anthraquinone, respectively. In the ^{13}C NMR spectrum of compound **6**, the peak at 116.5 is assigned to the central carbon atom of tacnoa. In the ESI-MS spectra, the signal at *m*/*z* 342.42 is assigned as $[\mathbf{1} - 2Br^-]^{2+}$ (calcd 342.22), *m*/*z*



Scheme 2 Synthesis of compounds **1**, **2** and **6**. Reagents and conditions: (a) 1,6-dibromohexane, K_2CO_3, N_2, 110 °C, 72 h, 55.3%; (b) tacnorthoamide, NaI, THF, N_2, rt, 18 h, 77.4%; (c) iodomethane, K_2CO_3, N_2, 55 °C, 24 h, 75.6%; (d) the same as (a), 75.0%; (e) the same as (b), 79.0%; (f) tacnorthoamide, NaI, THF, N_2, rt, 24 h, 86.9%.

476.58 corresponds to $[\mathbf{2} - Br^-]^+$ (calcd 476.25) and *m*/*z* 181.15 corresponds to $[\mathbf{6} - 2Br^-]^{2+}$ (calcd 181.25), respectively.

DNA binding assays

DNA binding is an essential step for DNA cleavage in most cases. Therefore, the binding behaviors of **1**, **2** and **6** to calf thymus DNA (CT-DNA) have been studied by fluorescence and CD spectroscopy.

Fluorescence spectroscopic studies

The fluorescence spectral method is one of the convenient tools for examining the interaction between small molecules and nucleic acids. The binding of the compounds to CT-DNA was studied by evaluating the fluorescence emission intensity of the ethidium bromide (EB)–DNA system with the addition of these compounds. The emission intensity of ethidium bromide (EB) is used as a spectral probe as EB shows a significant enhancement of the intensity when bound to DNA. Binding of the compounds to DNA decreases the emission intensity and the extent of the reduction of the emission intensity gives a measure of the DNA binding propensity of the investigated compounds. The fluorescence quenching effect of EB bound to DNA induced by **1**, **2** and **6** is shown in Fig. S1† (ESI, p10–11), in which the fluorescence intensity is at 604 nm (λ_{ex} = 530 nm) for EB in bound form. Addition of compounds caused a reduction in the emission intensity. The relative binding propensity of compound to CT-DNA was determined from the classical Stern–Volmer equation $I_0/I = 1 + Kr$, where I_0 and I are the fluorescence intensities in the absence and presence of the quencher; K is the linear Stern–Volmer quenching constant dependent on the ratio of r_{bE} (the ratio of the bound concentration of EB to the concentration of DNA); r is the ratio of total concentration of quencher to that of DNA.[20] Fig. 1 is the plot of I_0/I *versus* [*quencher*]/[*DNA*]; the quenching constant K is given by the gradient of the slope. The quenching constant K values obtained for **1**, **2** and **6** are 1.3 ± 0.02, 0.8 ± 0.01 and 0.08 ± 0.001, respectively.

The apparent binding constant (K_{app}) is also calculated from the equation K_{EB}[EB] = K_{app}[compound], where the compound concentration is the value at a 50% reduction of the fluorescence intensity of EB and $K_{EB} = 1.0 \times 10^7$ M^{-1} ([EB] = 3.9 μM).[21] The K_{app} values for **1**, **2** and **6** are 1.3×10^7 M^{-1}, 0.8×10^7 M^{-1} and 8×10^5 M^{-1}, respectively. The calculated value illustrates that both compounds **1** and **2** have stronger affinity to CT-DNA than that of compound **6** as a result of the presence of anthraquinone in their chemical structure which facilitates DNA binding propensity. The DNA-binding mode could be evaluated from the magnitude of the binding constants: a value above 10^6 M^{-1} is an indication of classical intercalation (ethidium and daunomycin bind DNA with an affinity over 10^6 M^{-1}), while values in the range of 10^4–10^5 M^{-1} imply a groove binding mode or electrostatic interaction.[22] Therefore, we concluded that the binding of compounds **1** and **2** to CT-DNA could be accomplished *via* the intercalation binding mode, while compound **6** to DNA is *via* groove or electrostatic interaction. In fact, the anthraquinone ring is very common as a DNA intercalating group and potential antitumour agent.[23] In addition, there was no obvious difference between **1** and **2** in their quenching constant K and the apparent binding constant K_{app}, only that **1** was slightly higher than **2**. This result illustrates that the anthraquinone group, present in both their structures, plays a dominant role in the process of binding with DNA.

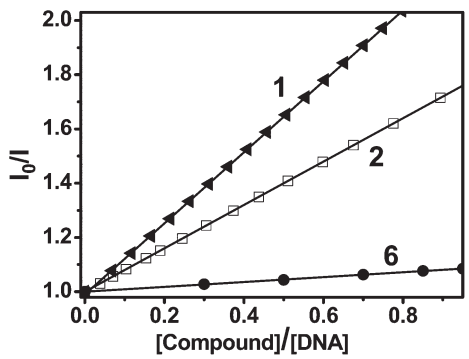


Fig. 1 Stern–Volmer quenching plots of EB bound to DNA by **1** (▲), **2** (□) and **6** (●), which give the quenching constants K. Experiments were conducted by adding 0–3.6 μM **1**, **2** or **6** to the EB-bound CT-DNA solution in 5 mM Tris-HCl buffer (pH 7.0) at room temperature.

Circular dichroism studies

Circular dichroism is a very sensitive, powerful technique for diagnosing changes in DNA morphology during drug–DNA interactions, as the positive band due to base stacking (275 nm) and the negative band due to right-handed helicity (248 nm) are quite sensitive to the mode of DNA interaction with small molecules.[24] The change in CD signal of DNA observed on interaction with a compound may often be assigned to the corresponding change in DNA structure.[25] Thus, simple groove binding and electrostatic interaction of small molecules show little or no perturbation on the base-stacking and helicity bands, while intercalation enhances the intensities of both the bands stabilizing the right-handed **B** conformation of CT-DNA as observed for the classical intercalator methylene blue.[26] Fig. 2 displays CD spectra of CT-DNA treated with **1**, **2** and **6** with the ratio of 0.4 ([compound]/[DNA]), the positive band (~275 nm)

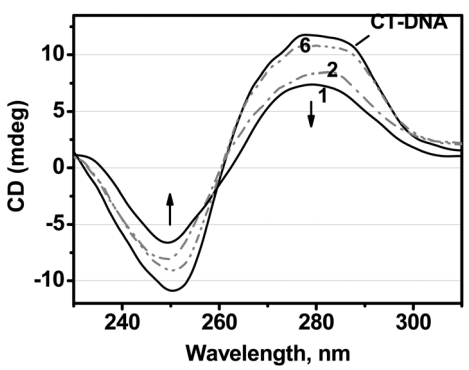


Fig. 2 CD spectra of CT-DNA (0.129 mM) in alone and its interaction with **1**, **2** and **6** at the ratio of [compound]/[DNA] = 0.4. All the spectra were recorded in 5 mM Tris-HCl buffer (pH 7.0) at room temperature.

of CT-DNA decreases in intensity with the addition of the compound, while the negative band (~247 nm) undergoes the obvious reduction. The results suggest that compound **6** shows a slight perturbation of DNA while compounds **1** and **2** unwind the DNA helix and lead to the loss of helicity possibly due to the partial intercalation of **1** and **2** into the DNA base pairs.[27]

From the results of these fluorescence and CD spectroscopic studies, it is concluded that the binding interaction ability of the compounds to CT-DNA follows the order **1** > **2** ≫ **6**, and the DNA binding constants of **1** and **2** indicate that both of the compounds might bind strongly with DNA *via* the intercalation binding mode.

DNA cleavage activity

pH Dependence of DNA cleavage promoted by 1 and 2. Incubation of supercoiled pUC19 DNA with compound **1** or **2** for 16 h at 37 °C results in a different extent of cleavage of DNA depending on the corresponding pH value of the buffer. Fig. 3 shows the bell-shaped pH-dependent profiles for DNA cleavage which indicate that pH 7.25 is the optimal pH for DNA cleavage in the presence of compound **1** or **2**. Therefore, pH 7.25 was selected for all of the following DNA cleavage reactions. The two compounds have almost the same change of cleavage activity at various pH values at 37 °C. According to the structure of the compounds, the pH value of the buffer affects the protonation or deprotonation of the nitrogen atom on the tacnoa moiety which may result in nucleophilic attack of compounds to substrate, and then determines cleavage efficiency of DNA. Consequently, we infer that function of tacnoa moiety in compounds is as the cleavage part. In order to verify this conclusion, we conducted a control experiment: incubation of supercoiled pUC19 DNA with compound **3**, which had no tacnoa in its structure, for 16 h at 37 °C. The experimental result is shown in Fig. S3† (ESI, p12) and indicates that compound **3** has almost no cleavage activity on DNA, which confirms the inference. The impact of ionic strength on cleavage activity for compound **1** was also investigated (Fig. S4,† ESI, p13). The results showed that the cleavage activity decreases with an increase in the ionic strength, and therefore a relatively low NaCl concentration (5 mM) was used for controlling ionic strength in all experiments.

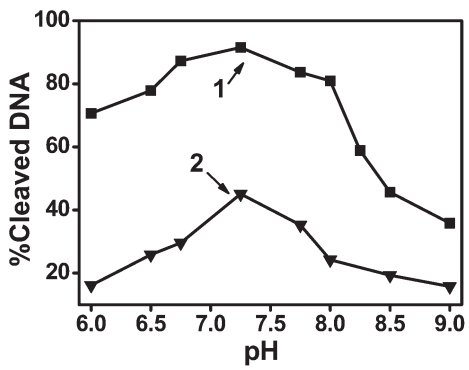


Fig. 3 pH-Dependent profile for pUC19 DNA (0.025 mM bp) cleavage promoted by 0.033 mM **1** (■) and **2** (▲) in Tris-HCl buffer of different pH values. (The agarose gel (Fig. S2,† ESI, p12) of pUC19 DNA (0.025 mM bp) was incubated for 16 h in buffer of different pH values (50 mM Tris-HCl) at 37 °C.)

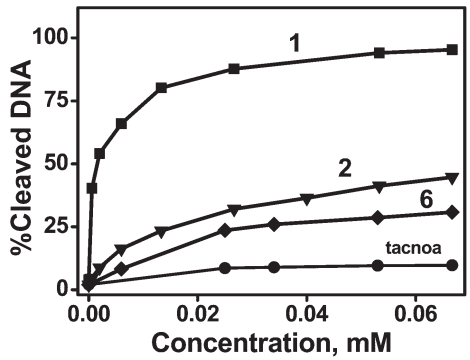


Fig. 4 Plots of % cleaved DNA *vs.* different concentrations of **1**, **2**, **6** and tacnoa. (The agarose gel of pUC19 DNA (0.025 mM bp) was incubated with different concentrations of **1**, **2**, **6** and tacnoa for 16.0 h at 37 °C in pH 7.25 buffer (50 mM Tris-HCl–5 mM NaCl) (Fig. S5,† ESI, p13–14)).

Concentration dependence assay of DNA cleavage promoted by 1, 2, 6 and tacnoa

Cleavage reactions that create relaxed circular DNA from supercoiled DNA over various concentrations of **1**, **2**, **6** and tacnoa (0.0006–0.067 mM) and constant DNA concentration (25 μM, bp) were carried out for 16.0 h at 37 °C in pH 7.25 buffer (50 mM Tris-HCl–5 mM NaCl). Fig. 4 displays the plots of % cleaved DNA *vs.* different concentrations, which shows that cleavage activities on DNA of these compounds increase with the increase of their concentrations. Under the same concentration condition, DNA cleavage abilities of these compounds are in the following order: **1** > **2** > **6** > tacnoa. However, a further increase concentration of **1** (larger than 0.067 mM) results in DNA becoming stuck in the well of the gel (the agarose gel electrophoretogram is given in Fig. S6,† ESI, p13–14). This phenomenon may due to the formation of relatively high molecular weight DNA-**1** complexes from the strong binding between DNA and the anthraquinone group.

Kinetics assays

The kinetics of pUC19 DNA degradation have been studied. Fig. 5 shows the time course of the supercoiled plasmid DNA cleavage promoted by **1** (0.033 mM) into nicked form in Tris-HCl buffer (pH 7.25) at 37 °C. The rate of conversion from form I to form II increases with increased reaction time. The graph shows that the extension of supercoiled DNA cleavage varies exponentially with the reaction time giving pseudo first-order kinetics with an apparent initial first-order rate constant (k_{obs}) of 0.183 ± 0.0104 h^{-1}. The apparent initial first-order rate constants of DNA cleavage reactions promoted by a series of various concentrations of **1**, **2** and **6** under the same conditions as described above are summarized in Table S1† (ESI, p15). The kinetics profiles of the supercoiled DNA cleavage at various concentrations of **1**, **2** and **6** are presented in Fig. 6. In order to clarify the impact of introduction of anthraquinone and the load of positive charge on DNA cleavage efficiency, the DNA-cleaving behavior of parent tacnoa was also investigated under the same conditions in 50 mM Tris-HCl–5 mM NaCl buffer (pH 7.25).

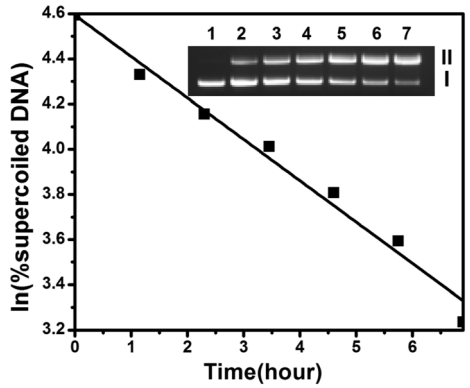


Fig. 5 Time course of pUC19 DNA (0.025 mM bp) cleavage promoted by **1** (0.033 mM). Inset is the agarose gel (1%) of the time-variable reaction products. lanes 1–7, reaction times of 0, 1.15, 2.30, 3.45, 4.60, 5.75 and 6.90 h, respectively. The reactions were carried out at 37 °C in 50 mM Tris-HCl–5 mM NaCl buffer (pH 7.25).

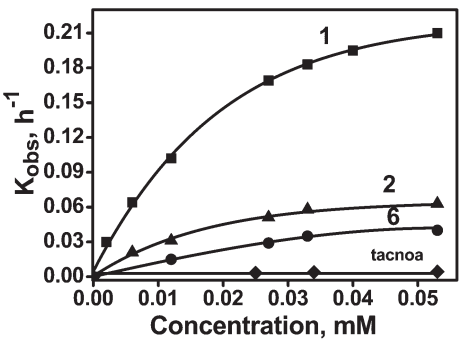


Fig. 6 Kinetics plot of k_{obs} *versus* various concentrations of compounds **1**, **2**, **6** and tacnoa. Every reaction was carried out at 37 °C in 50 mM Tris-HCl–5 mM NaCl buffer (pH 7.25).

The apparent initial first-order rate constants k_{obs} of DNA cleavage reactions promoted by **1**, **2**, **6** and tacnoa are 0.2126 ± 0.0055 h^{-1}, 0.062 ± 0.0024 h^{-1}, 0.040 ± 0.0007 h^{-1} and 0.0043 ± 0.0002 h^{-1}, respectively. The 50-fold and 15-fold rate acceleration over parent tacnoa is because of the anthraquinone moiety of compound **1** or **2** intercalating into DNA base pairs *via* a stacking interaction. This indicates that the intercalating subunit – anthraquinone – evidently increases binding ability to the substrate (DNA) and then increases DNA cleavage activity effectively. Moreover, DNA cleavage reactions promoted by compound **1** give 5.3-fold rate acceleration over that of compound **6** despite both of them have two tacnoa units. This further demonstrates that the introduction of anthraquinone in compound **1** results in a large enhancement of DNA cleavage activity. In particular, the DNA cleavage activity promoted by **1** bearing two tacnoa units is 3.33 times more effective than that of **2** bearing one tacnoa unit. And the binding affinity and mode of the two compounds to DNA are almost the same from the study of fluorescence and circular dichroism spectroscopy. Therefore, the rate acceleration of DNA cleavage promoted by **1** over **2** indicates that the two positively charged tacnoa units in compound **1** catalyze cleavage of DNA cooperatively.

Studies on the mechanism of DNA cleavage

The anthraquinone moiety usually undergoes a redox pathway, which could directly produce a cytotoxic effect.[28] To confirm if reactive oxygen species (ROS) are, at least partly, responsible for the cleavage of DNA promoted by compound **1**, reactions were carried out in the presence of typical scavengers[29] for singlet oxygen (NaN_3), for superoxide (KI), and for hydroxyl radical (DMSO and *t*-BuOH) (Fig. 7; Fig. S7 and Table S3† of the ESI, p31–32). Obviously, there is no significant inhibition effect on DNA cleavage in the presence of any of the scavengers (NaN_3, KI, DMSO and *t*-BuOH), which rules out the involvement of these reactive oxygen species, at least in a free and diffusible form, and implies a non-oxidative pathway.

Generally, the oxidative DNA cleavage process is due to the oxidation of the ribose or base group of DNA by the reactive oxygen species.[30] Thus, the DNA samples containing **1** were incubated in the presence of four nucleosides (adenosine, uridine, guanosine and cytidine, respectively) with the ratio of [nucleoside]/[DNA bp] = 1 : 1, followed by electrophoresis and quantitation (Fig. S8,† ESI, p32). No inhibition in the DNA cleavage was detected after the treatment with **1** in the presence of each of the four nucleosides. This suggests a non-oxidative process (Table 1). On the other hand, in the DNA cleavage

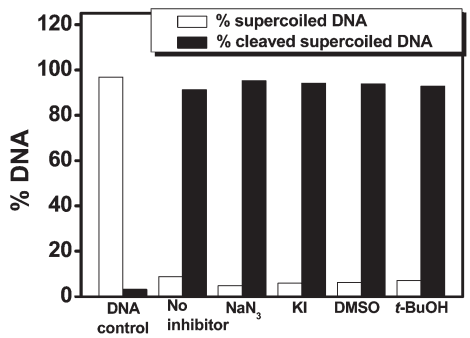


Fig. 7 Histogram representing cleavage of pUC19 plasmid DNA (0.025 mM bp) by **1** (0.04 mM) in the presence of standard radical scavengers for singlet oxygen (NaN_3, 10 mM), for superoxide (KI, 10 mM), and for hydroxyl radical (1 mM DMSO and 1 mM *t*-BuOH), incubated for 16 h at 37 °C in pH 7.25 buffer (50 mM Tris-HCl/5 mM NaCl).

Table 1 DNA cleavage promoted by **1** in the presence of nucleoside monophosphate or BDNPP[a] (Fig. S8, ESI, p32)

	DNA %	
Added compounds	Form I	Form II
DNA control	97.35	2.65
1 only	19.29	80.71
1 + Adenosine	19.70	80.30
1 + Uridine	20.31	79.69
1 + Guanosine	24.23	75.77
1 + Cytidine	26.22	73.78
1 + BDNPP (0.10 mM)	58.40	41.60
1 + BDNPP (0.20 mM)	76.88	23.12

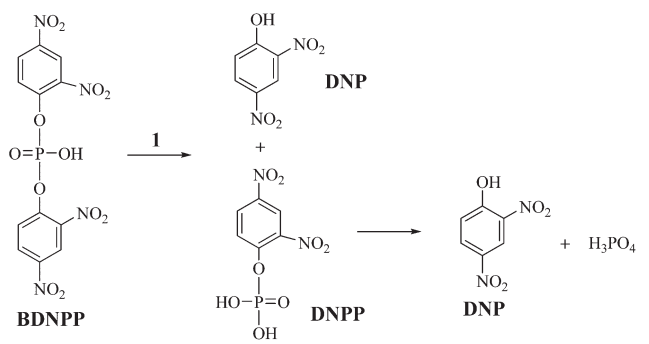
[a] Cleavage reactions were carried out in pH 7.25 Tris-HCl buffer for 10 h at 37 °C.

reactions promoted by **1**, when the active phosphates bis(2,4-dinitrophenyl) phosphate (BDNPP) without the pentose and the pyrimidine or purine base were added, the reactions of DNA cleavage were partially inhibited (Table 1) (Fig. S8,† ESI, p32). The inhibition can be ascribed to the preferential hydrolyzed phosphodiester of BDNPP. It implies that the hydrolysis pathway for the DNA cleavage process is possible.

To study the cleavage mechanism, a small dinucleotide model system, adenylyl(3′–5′)phosphoadenine (APA) was used as the nucleic acid mimic. ApA (0.10 mM) and **1** (0.05 mM) were dissolved in deionized water (1 : 1), and after reaction for 16 h at 37 °C, ESI-MS analysis was carried out. In the ESI-MS spectrum (Fig. S9(1),† ESI, p33), besides the peak at *m/z* 342.42 indicating the signal of species $[\mathbf{1} - 2Br^-]^{2+}$ (calcd *m/z* 342.42), the signals at *m/z* 268.17 and 346.00 show the presence of ApA cleavage products adenosine (A) ($[A + H]^+$, calcd *m/z* 268.10) and adenosine monophosphate (AMP) ($[AMP - H]^-$, calcd *m/z* 346.06); no sign of ApA was found. An ESI-MS analysis of ApA alone was also carried out as a control experiment under the same conditions (Fig. S9(2),† ESI, p34), showing only the signal of ApA and no signs of A and AMP. The generation of adenosine and AMP indicates that the possible cleavage mechanisms of ApA promoted by compound **1** would be *via* an intramolecular transphosphorylation to form cAMP, and then hydrolysis, or for the phosphodiester bond of ApA hydrolyse directly.

To further study the cleavage mechanism, bis(2,4-dinitrophenyl) phosphate (BDNPP) was used as a DNA mimic (Scheme 3). BDNPP and **1** were dissolved in DMF–deionized water (v : v = 1 : 1) at 37 °C. ESI-MS analysis was carried out. At the beginning, the peaks of ESI-MS spectra at *m/z* 342.35 and 428.90 (Fig. S10(1),† ESI, p35) show the signals of $[\mathbf{1} - 2Br^-]^{2+}$ (calcd 342.22) and $[BDNPP - H^+]^-$ (calcd 429.18), respectively. After 3 h, besides the peaks at *m/z* 342.35 ($[\mathbf{1} - 2Br^-]^{2+}$) and 428.90 ($[BDNPP - H^+]^-$), the signals at *m/z* 182.95 and 263.05 (Fig. S10(2),† ESI, p36) show the presence of BDNPP cleavage products ($[DNP - H^+]^-$, calcd *m/z* 183.11) and ($[DNPP - H^+]^-$, calcd *m/z* 263.09), respectively. After 5 h, the peak at 428.90 ($[BDNPP - H^+]^-$) disappears, ESI-MS spectra (Fig. S10(3),† ESI, p36) show only peaks (*m/z* 182.95 and 263.05) of BDNPP cleavage products. An ESI-MS analysis of BDNPP alone was also carried out as a control experiment under the same conditions, showing only the signal of BDNPP and no signs of new peaks. The generation of DNPP and DNP indicates that a possible cleavage mechanism of BDNPP promoted by compound **1** is *via* a hydrolysis pathway of the phosphodiester bond. Thus, similar to BDNPP, the hydrolysis of the phosphodiester bond is a possible mechanism for the DNA cleavage promoted by **1**.

In addition, mechanistic profiles of DNA cleavage by **1** were also evaluated in the presence of excess EDTA to scavenge adventitious transition metal ions. (Fig. S11 and Table S4,† ESI, p37). There was almost no inhibition of DNA cleavage, which rules out the involvement of adventitious transition metal ions.



Scheme 3 The cleavage reaction of BDNPP in the presence of compound **1**.

Experimental

Materials

Plasmid pUC19 DNA was purchased from TaKaRa Biotechnology Co. Ltd., and the purity was checked by agarose gel electrophoresis and the concentration was determined by UV spectroscopy using the extinction coefficient appropriate for double-stranded DNA (1.0 OD260 = 50 μg mL^{-1}). Agarose was from Oxoid Limited of Basingstoke, ethidium bromide (EB) was from Amresco. Inc., and tris(hydroxymethyl)amino-methane (Tris-Base) was from Robiot Co. Ltd. Dinucleotide (APA) and bis(2,4-dinitrophenyl) phosphate (BDNPP) were purchased from Sigma Aldrich. Bromophenol blue, glycerol and ethyldiaminetetraacetic acid (EDTA) were commercially available. Deionized water was obtained by ionized column from double distilled water. All reagents and chemicals were of analytical grade and used without further purification. All solvents were purified by standard procedures.

The stock solution of CT-DNA (stored at 4 °C and used for not more than 2 d) was prepared in 5 mM Tris-HCl in water, pH 7.0. The concentration of CT-DNA was determined according to its absorption intensity at 260 nm with a known molar extinction coefficient value of 6600 M^{-1} cm^{-1}. The ratio of the UV absorbance at 260 and 280 nm, A_{260}/A_{280} = 1.8–1.9, indicated that DNA was sufficiently free of protein.[31]

Apparatus

^1H NMR and ^{13}C NMR data were recorded on a Brucker AM 300 spectrometer (Germany). Mass spectra were obtained on an electrospray mass spectrometer (LCQ, Finnigan). Elemental analyses were carried out using Perkin Elmer 240C. The fluorescent spectra and CD spectra were carried out using AMINCO Bowman Series 2 luminescence spectrometer and Jasco J-810 automatic recording spectropolarimeter, respectively. The pH value was confirmed by ORION868 pH meter with an Ag/AgCl electrode as the reference electrode in saturated KCl solution at room temperature. The agarose gel electrophoresis was conducted by DYY-5 electrophoresis apparatus. Bands were visualized by UV light and photographed using DigiDoc–ItTM gel imaging and documentation system (version 1.1.23, UVP, Inc. Unpland, CA). The intensity of the DNA bands was estimated by TotalLab image analysis software (version 2.01).

Fluorescence measurements. The fluorescent spectral studies were performed by the measurement of the emission intensity of ethidium bromide (EB) on an AMINCO Bowman Series 2 luminescence spectrometer. The experiments were done by

adding 0–3.6 μM 1 or 2 into the EB-bound CT-DNA (3.9 μM) solution in 5 mM Tris-HCl buffer (pH 7.0), and the fluorescence was measured and normalized to 100% relative fluorescence.

Circular dichroism measurements. All CD spectroscopic studies were carried out with a continuous flow of nitrogen purging the polarimeter, and the measurements were performed at room temperature with 1 cm pathway cells. The CD spectra were run from 320–220 nm at a speed of 20 nm min^{-1} and the buffer background was automatically subtracted. Data were recorded at an interval of 0.1 nm. The CD spectrum of CT-DNA alone (129 μM) was recorded as control experiment.

General procedures for the synthesis of compounds

Triazatricyclo[5.2.1.$0^{4,10}$]decane (tacnoa). Triazatricyclo [5.2.1.$0^{4,10}$]decane was prepared according to the literature method.[3a] ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.67–2.74 (m, 6H, $3CH_2$), 2.92–3.00 (m, 6H, $3CH_2$), 4.93 (s, 1H, CH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 51.8 (CH_2), 104.0 (CH).

1-Hydroxy-8-methoxy-9,10-anthraquinone (4). The compound **4** was synthesized according to the literature procedure with some modification.[32] 1,8-Dihydroxyanthraquinone (1.5 g, 6.2 mmol), anhydrous potassium carbonate (2.76 g, 20 mmol) and iodomethane (0.3 mL, 4.65 mmol) were added to 60 mL acetone and the mixture stirred at 55 °C under an atmosphere of dry N_2. The reaction was monitored by TLC. After 24 h, the reaction mixture was cooled and filtered, and the residue was washed with acetone (3 × 20 mL). All of the organic layers were merged and the solvent was removed under vacuum. Column chromatography (silica gel, petrol ether–ethyl acetate, 4 : 1, v/v) afforded 1-hydroxy-8-methoxy-9,10-anthraquinone (1.2 g, 4.72 mmol) as an orange solid. Yield 75.6%; mp 95–96 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.08 (s, 3H, OCH_3), 7.27–7.31 (dd, J = 8.2 Hz, 0.9 Hz, 1H, ArH), 7.35–7.39 (dd, J = 8.2 Hz, 0.9 Hz, 1H, ArH), 7.62 (t, J = 8.1 Hz, 1H, ArH), 7.73–7.79 (m, 2H, 2 × ArH), 7.95–7.99 (m, 1H, ArH).

1-Methoxy-8-(6-bromohexyloxy)anthraquinone (5). 1-Hydroxy-8-methoxy-9,10-anthraquinone (**4**) (1.2 g, 4.72 mmol) and anhydrous potassium carbonate (3.0 g, 22 mmol) were added to 1,6-dibromohexane (20 mL, 130 mmol) and the mixture stirred at 110 °C under an atmosphere of dry N_2. A color change from orange to brown was observed. After 72 h, the reaction mixture was cooled and filtered, and the residue was washed with $CHCl_3$ (3 × 10 mL). All of the organic layers were merged and the solvent was removed under vacuum. Column chromatography (silica gel, petrol ether–dichloromethane, 1 : 1, v/v) followed by recrystallization from EtOAc afforded **5** (1.35 g, 2.4 mmol) as orange solid. Yield 75.0%; mp 96–97 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.55–1.67 (m, 4H, 2 × CH_2), 1.92–2.00 (m, 4H, 2 × CH_2), 3.39 (t, J = 7.0 Hz, 2H, CH_2Br), 3.90 (s, 3H, OCH_3), 4.07 (t, J = 6.5 Hz, 2H, CH_2O), 7.18–7.24 (m, 2H, 2 × ArH), 7.49–7.56 (m, 2H, 2 × ArH), 7.71–7.75 (m, 2H, 2 × ArH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 25.1 (CH_2), 27.8 (CH_2), 28.8 (CH_2), 32.6 (CH_2), 33.9 (CH_2Br), 56.6 (OCH_3), 69.4 (OCH_2), 118.0 (Ar C), 118.8 (Ar C), 119.4 (Ar C), 124.1 (Ar C), 124.2 (Ar C), 133.7 (Ar C), 134.7 (Ar C), 158.8 (Ar C), 159.3 (Ar C), 182.3 (C═O), 184.0 (C═O); ESI-MS m/z [M (^{79}Br) + Na^+] calcd 439.06, found 439.08; [M(^{81}Br) + Na^+] calcd 441.06, found 441.08.

1,8-Bis(1-dihexyloxy-1-azonia-4,7-diazatricyclo[5.2.1.$0^{4,10}$]-decane)anthracene-9,10-dione dibromide (1). Triazatricyclo [5.2.1.$0^{4,10}$]decane (tacnoa) (0.74 g, 5.32 mmol) and NaI (1.2 mg, 0.008 mmol) were added to 5 mL anhydrous THF. An anhydrous THF (20 mL) solution of **3** (0.30 g, 0.53 mmol) was then added dropwise with stirring at room temperature under an atmosphere of dry N_2. The mixture was refluxed for 18 h and then cooled to 30 °C, stirred for another 48 h. A yellow solid precipitated, and was filtered off, washed with THF (2 × 1 mL) and ether (2 × 2 mL), and then taken to dryness under reduced pressure. A yellow solid (0.35 g, 0.41 mmol) was obtained. Yield 77.4%; ^{1}H NMR (300 MHz, D_2O) δ 1.44–1.76 (m, 8H, 4 × CH_2), 1.91–2.04 (m, 8H, 4 × CH_2), 3.21–3.33 (m, 8H, 4 × NCH_2), 3.55–3.65 (m, 8H, 4 × NCH_2), 3.77–3.84 (m, 4H, 2 × NCH_2), 3.91–4.06 (m, 8H, 4 × NCH_2), 4.12–4.16 (m, 4H, 2 × OCH_2), 5.47 (s, 2H, 2 × methine H), 7.27–7.30 (d, J = 8.5 Hz, 2H, 2 × ArH), 7.58–7.64 (m, 2H, 2 × ArH), 7.79–7.81 (d, J = 7.0 Hz, 2H, 2 × ArH); ^{13}C NMR (75 MHz, D_2O) δ 24.0 (CH_2), 24.8 (CH_2), 25.6 (CH_2), 28.0 (CH_2), 51.0 (NCH_2), 51.7 (NCH_2), 53.3 (NCH_2), 57.9 (NCH_2), 60.0 (NCH_2), 69.2 (OCH_2), 116.7, 118.3, 120.0, 122.3, 133.3, 134.5, 158.1 (tacnoa central C and Ar C), 183.2 (C═O), 183.8 (C═O); ESI-MS m/z [M − $2Br^-$]$^{2+}$ calcd 342.22, found 342.42; Anal. Calcd for $C_{40}H_{56}Br_2N_6O_4$: C, 56.87; H, 6.68; N, 9.95; Found: C, 56.79; H, 6.55; N, 9.73%.

1-Methoxy-8-(1-hexyloxy-1-azonia-4,7-diazatricyclo[5.2.1.$0^{4,10}$]-decane)anthracene-9,10-dione bromide (2). Compound **2** was synthesized in a similar procedure to compound **1** using 1,4,7-triazatricyclo[5.2.1.$0^{4,10}$]decane (tacnoa) (0.57 g, 4 mmol), NaI (1.2 mg, 0.008 mmol) and 1-methoxy-8-(6-bromohexyloxy)-anthraquinone (**5**) (0.22 g, 0.53 mmol). A yellow solid (0.23 g, 0.42 mmol) was obtained. Yield 79.0%; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.27–2.00 (m, 8H, 4 × CH_2), 3.03–3.04 (m, 2H, NCH_2), 3.23–3.26 (m, 2H, NCH_2), 3.41–3.45 (m, 2H, NCH_2), 3.47–3.50 (m, 2H, NCH_2), 3.67–3.74 (m, 2H, NCH_2), 3.75–3.78 (m, 2H, NCH_2), 4.01 (s, 3H, OCH_3), 4.15–4.17 (m, 2H, NCH_2), 4.25–4.30 (m, 2H, OCH_2), 5.47 (s, 1H, methine H), 7.30–7.33 (m, 2H, 2 × ArH), 7.60–7.67 (m, 2H, 2 × ArH), 7.78–7.85 (m, 2H, 2 × ArH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 24.5 (CH_2), 25.3 (CH_2), 25.7 (CH_2), 28.1 (CH_2), 52.2 (NCH_2), 52.6 (NCH_2), 56.0 (NCH_2), 56.8 (NCH_2), 57.7 (NCH_2), 58.9 (OCH_2), 69.1 (OCH_3), 118.2, 118.9, 118.9, 119.0, 119.2, 119.5, 123.3, 124.0, 124.1, 134.0, 134.8, 158.1, 159.2 (tacnoa central C and Ar C), 182.7 (C═O), 183.9 (C═O); ESI-MS m/z [M − Br^-]$^+$ calcd 476.25, found 476.58; Anal. Calcd for $C_{28}H_{34}BrN_3O_4$: C, 60.43; H, 6.16; N, 7.55; Found: C, 60.38; H, 6.04; N, 7.34%.

1,1′-(Hexamethylene)bis(1-azonia-4,7-diazatricyclo[5.2.1.$0^{4,10}$]-decane) dibromide (6). A solution of (0.61 g, 2.5 mmol) 1,6-dibromohexane in anhydrous tetrahydrofuran (5 mL) was added to a stirred solution of 1,4,7-triazatricyclo[5.2.1.$0^{4,10}$]-decane (0.696 g, 5 mmol) and NaI (1.2 mg, 0.008 mmol) in anhydrous tetrahydrofuran (25 mL) under an atmosphere of dry N_2, resulting in a pale yellow precipitate. After stirring for 24 h, the precipitate was filtered off; washed with tetrahydrofuran (25 mL), and then ether (25 mL), dried in a vacuum desiccator. A off-white solid (1.13 g, 2.17 mmol) was obtained. Yield

86.9%; ^{1}H NMR (300 MHz, D_2O) δ 1.37–1.38 (m, 4H, 2 × CH_2), 1.79–1.81 (m, 4H, 2 × CH_2), 3.08–3.15 (m, 8H, 4 × NCH_2), 3.36–3.55 (m, 16H, 8 × NCH_2), 3.70–3.74 (m, 4H, 2 × NCH_2), 5.44 (s, 2H, 2 × methine H); ^{13}C NMR (75 MHz, D_2O) δ 23.9 (CH_2), 25.4 (CH_2), 51.1 (NCH_2), 53.2 (NCH_2), 58.0 (NCH_2), 60.0 (NCH_2), 116.5 (tacnoa central C); ESI-MS m/z $[M - 2Br^-]^{2+}$ calcd 181.25, found 181.15; Anal. Calcd for $C_{20}H_{38}Br_2N_6$: C, 45.99; H, 7.33; N, 16.09; Found: C, 45.79; H, 7.42; N, 15.97%.

Agarose gel electrophoresis assays

The plasmid DNA cleavage experiments were performed using pUC19 DNA in Tris-HCl buffer. Reactions were carried out by incubating DNA (0.025 mM bp) at 37 °C in 50 mM Tris-HCl–5 mM NaCl buffer with a total volume of 15 μL in the dark for the indicated time. All reactions were quenched by loading buffer (3.5 μL) (30 mM EDTA, 0.05% (w/v) glycerol, 36% (v/v) bromophenol blue). Agarose gel electrophoresis was carried out on a 1% agarose gel in 0.5 × TAE (Tris-acetate-EDTA) buffer containing 1.0 μg mL^{-1} EB at 80 V for 1.5 h. The resolved bands were visualized with a UV transilluminator and quantified using TotalLab 2.01 software. The supercoiled plasmid DNA values were corrected by a factor of 1.3 on the basis of average literature estimates of lowered binding of EB to this structure.[33]

Conclusions

In conclusion, 1,8-bis(1-dihexyloxy-1-azonia-4,7-diazatricyclo-[5.2.1.0^{4,10}]decane)anthracene-9,10-dione dibromide **1**, 1-methoxy-8-(1-hexyloxy-1-azonia-4,7-diazatricyclo[5.2.1.0^{4,10}]decane)-anthracene-9,10-dione bromide **2** and 1,1′-(hexamethylene)bis-(1-azonia-4,7-diazatricyclo[5.2.1.0^{4,10}]decane) dibromide **6** were prepared as metal free artificial nucleases. The interaction of **1**, **2** and **6** with calf thymus DNA was studied by spectroscopic techniques (fluorescence and CD spectroscopy). The results indicate that compounds **1** and **2** have strong DNA binding affinity. The binding constants of **1**, **2** and **6** are 1.3 × 10^7 M^{-1}, 0.8 × 10^7 M^{-1} and 8 × 10^5 M^{-1}, respectively. The DNA cleavage promoted by **1**, **2**, **6** and parent tacnoa under physiological conditions was studied by agarose gel electrophoresis, which gives the observed rate constants k_{obs} of 0.2126 ± 0.0055 h^{-1}, 0.0620 ± 0.0024 h^{-1}, 0.040 ± 0.0007 h^{-1} and 0.0043 ± 0.0002 h^{-1}, respectively. The 50-fold and 15-fold rate acceleration over parent tacnoa because of the anthraquinone moiety of compound **1** or **2** intercalating into DNA base pairs *via* stacking interaction. Moreover, DNA cleavage reactions promoted by compound **1** give 5.3-fold rate acceleration over that of compound **6** despite the fact that both of them have two tacnoa units. This further demonstrates that the introduction of anthraquinone in compound **1** can result in a large enhancement of DNA cleavage activity. Compared with **2**, compound **1** exhibits higher DNA cleavage activity due to the cooperative catalysis of the two positively charged tacnoa units. The radical scavenger inhibition study and ESI-MS analysis of APA and BDNPP cleavage in the presence of compound **1** suggest the cleavage mechanism would be *via* a hydrolysis pathway by cleaving phosphodiester bond of DNA.

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