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A potent antitumor Zn^{2+} tetraazamcrocycle complex targeting DNA: the fluorescent recognition, interaction and apoptosis studies

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Selected bond lengths (Å) and angles (º) for 2: Zn-N1 2.065 (3), Zn-N3 2.319 (3), Zn-N4 2.297 (3), Zn-N5 2.337 (3), Zn-O1 2.757 (3), Zn-O3 2.365 (3), Zn-Cl1 2.2617 (11); N1-Zn-Cl1 171.90 (9), N1-Zn-N4 87.69 (12), Cl1-Zn-N4 100.39 (8), N1-Zn-N3 77.28 (12), Cl1-Zn-N3 104.39 (8), N4-Zn-N3 81.33 (10), N1-Zn-N5 77.19 (11), Cl1-Zn-N5 103.64 (8), N4-Zn-N5 78.48 (10), N3-Zn-N5 147.88 (10), N1-Zn-O3 82.04 (12), Cl1-Zn-O3 90.14 (8), N4-Zn-O3 157.86 (10), N3-Zn-O3 115.05 (10), N5-Zn-O3 80.13 (10).

Experimental

General: All the starting materials were of reagent quality and were obtained from commercial

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sources without further purification. Calf thymus DNA (CT-DNA) and supercoiled pUC 19 plasmid DNA, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Sigma, and Dulbecco's Modified Eagle's Medium (DMEM) was bought from Gibco. Fetal bovine serum (FBS) was bought from Hangzhou Sijiqing Biological Engineering Materials Co., Ltd. Hoechst 33258 was bought from Keygen. All the UV-vis spectra were recorded by a Shimadzu UV-3100 spectrophotometer. The emission spectra were obtained using a PerkinElmer LS 55 fluorescence spectrometer. Circular dichroism (CD) spectra were recorded on a Jasco J-810 spectropolarimeter in a 1 cm path length cylindrical quartz cell at room temperature. The pH values of sample solutions were monitored by a PHS-3 system. The electrospray ionization mass spectra were determined by a LCQ Fleet ThermoFisher mass spectrometer. ^{1}H NMR spectra were determined by a Bruker DRX-500 spectrometer at 25±1 °C. Fluorescence imaging was performed with a Gel Doc XR (BioRad), and quantification analysis was performed with Quantity One software (version 4.6.2). 1,4,7-Tritosyl-1,4,7-triazaheptane was prepared according to a reported procedure[1] in 62% yield after crystallization. 2,6-Bis(bromomethyl)pyridine and the mother ring 3,6,9,15-tetraazabicyclo[9.3.1] pentadeca-1(15),11,13-triene (**L**) were synthesized following the procedure described by the references[2] in the yield of 49% and 65%, respectively.

X-Ray crystallographic studies: Crystal structure determination for **2** by X-ray diffraction was performed on a Bruker SMART APEX CCD diffractometer using graphite-monochromated Mo Kα radiation (λ= 0.71073 Å) at room temperature through the ω-scan technique. Lorentz polarization and absorption corrections were applied. The structures were solved by direct methods and refined with the full-matrix least-squares technique using the SHELXL-97 software

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package.[3] Anisotropic displacement parameters were assigned to all non-hydrogen atoms. Analytical expressions of neutral-atom scattering factors were employed, and anomalous dispersion corrections were incorporated. CCDC reference number: 806621.

Syntheses:

Synthesis of 3, 6, 9, 15-tetraazabicyclo [9.3.1] pentadeca-1 (15), 11, 13-triene-3, 6, 9-trimethyl naphthalene (1): 1-chloromethyl naphthalene (530 mg, 3 mmol), **L** (206 mg, 1 mmol) and Na_2CO_3 (318 mg, 3 mmol) were dissolved in CH_3CN (50 mL). The resulting solution was stirred vigorously and then refluxed for 24 h. After cooling to room temperature, the obtained solution was filtered and concentrated by evaporation. The residue was redissolved in $CHCl_3$ (200 mL) and washed with distilled water (100 mL×3). Then the organic layer was dried over Na_2SO_4 and evaporated to yield a dark yellow solid. The crude product was purified by silica gel column chromatography with CH_2Cl_2/EtOH/$NH_3 \cdot H_2O$ (12:1:0.1 v:v:v; R_f = 0.35), affording **1** as a yellow solid (380 mg, 61%). ^{1}H NMR (500MHz, $CDCl_3$): δH 7.81 (d, *J*=10 Hz, 3H), 7.73 (t, *J*=10 Hz, 3H), 7.55-7.61 (m, 3H), 7.48 (t, *J*=7.5 Hz, 3H), 7.42 (t, *J*=7.5 Hz, 3H), 7.38 (t, *J*=7.5 Hz, 3H), 7.26 (t, *J*=10 Hz, 1H), 7.16-7.20 (m, 3H), 6.91 (d, *J*=10Hz, 2H), 3.81 (s, 4H), 3.68 (s, 2H), 3.09 (s, 4H), 2.64 (br s, 8H); Mass: ES-MS (CH_3CN), m/z (%): 627.67 [**1**+H]$^+$.

Synthesis of $C_{44}H_{42}N_5O_3ZnCl$ (2): $Zn(NO_3)_2 \cdot 6H_2O$ (149 mg, 0.5 mmol) and **1** (313 mg, 0.5 mmol) were stirred and refluxed for 1 h in EtOH-H_2O mixed solution (20 mL, v:v = 1:1), and diluted HCl aqueous solution was added to adjust the pH to about 7.4. Then the solution was cooled to room temperature and filtered, and a yellow powder was dried in vacuum and obtained (288 mg, 73%). ^{1}H NMR (500MHz, $CDCl_3$): 7.97 (t, J=10 Hz, 2H), 7.88 (t, J=10 Hz, 6H), 7.61-7.74 (m, 8H), 7.50 (t, J=7.5 Hz, 4H), 7.43 (d, J=10 Hz, 2H), 7.33 (t, J=7.5 Hz, 2H), 4.92 (s,

Supporting Information

2H), 4.49 (s, 4H), 4.38 (s, 2H), 4.07 (s, 2H), 3.20 (br s, 4H), 3.06 (br s, 2H), 2.85 (br s, 2H); Mass: ES-MS (50 mM Tris-HCl buffer, pH 7.4; CH_3CN/H_2O, v/v 1/999), m/z (%): 727.33 [**1**+Zn+Cl]$^+$. Yellow single crystals suitable for X-ray diffraction were isolated by slow evaporation of the above filtrate. Elemental analysis calcd (%) for $C_{44}H_{42}N_5O_3ZnCl$: C 66.86, H 5.32, N 8.86; found: C 67.02, H 5.54, N 8.65.

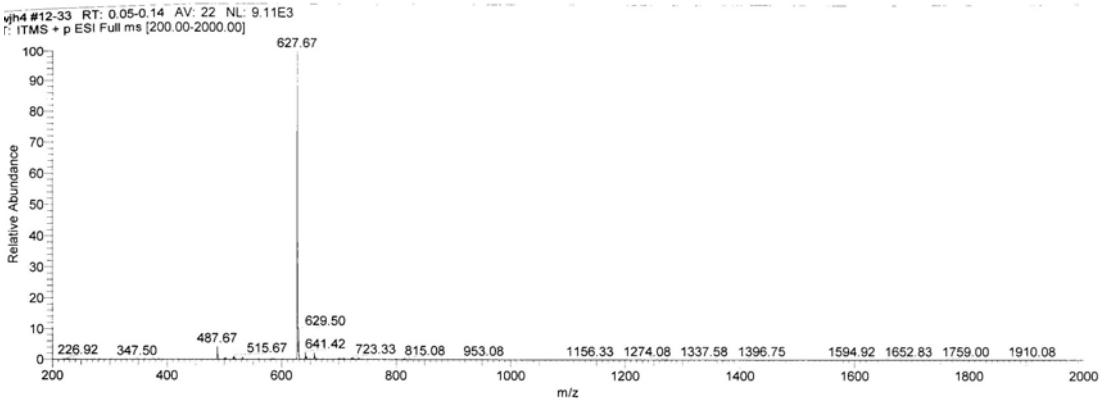

Fig. S1 ESI-MS spectrum of **1** in CH_3CN.

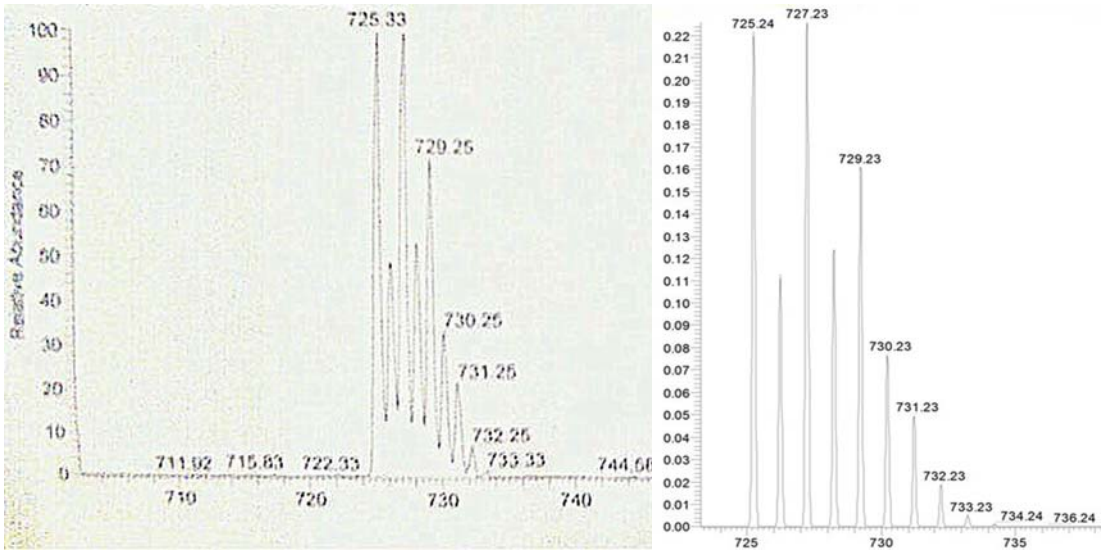

Fig. S2 ESI-MS spectrum of **2** in the buffer (50 mM Tris-HCl, pH 7.4; CH_3CN/H_2O, v/v 1/999). The intensive peak at 727 was assigned to [**1**+Zn+Cl]$^+$ (left). The right part is the simulated isotopic distribution pattern (right).

DNA titration of 1 and 2 determined by fluorescence: The molar concentration of DNA base

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pairs was calculated by taking 660 Da as the average base pair molecular weight. The fluorescent titrations were carried out by first adding DNA solution to 2 mL buffer (50 mM Tris-HCl, pH 7.4; CH_3CN/H_2O, v/v 5/995) containing 10 μM **1** or **2**. All of the spectra were recorded after the fluorescence became steady. The excitation wavelength was 280 nm.

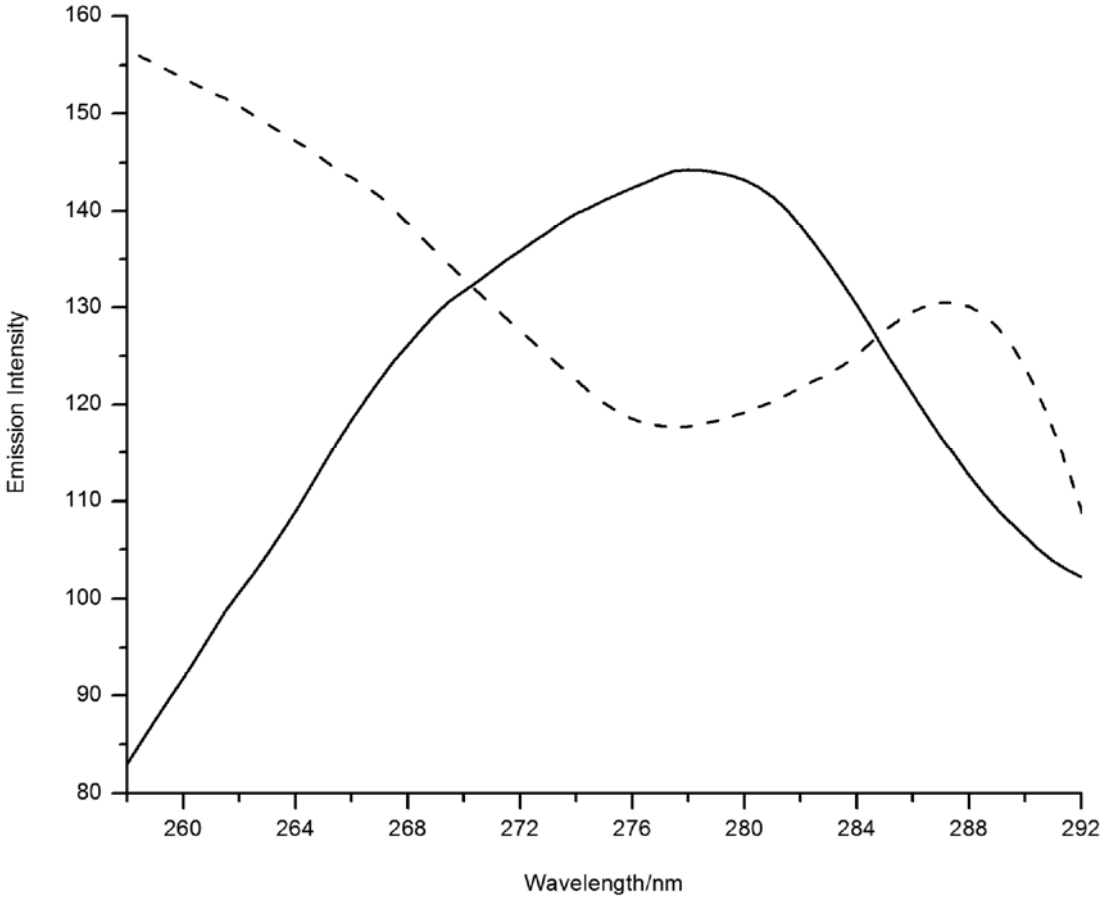

Fig. S3 Fluorescence excitation spectra of 10 μM **2** in the buffer (50 mM Tris-HCl, pH 7.4; CH_3CN/H_2O, v/v 5/995). The solid line and the dashed line correspond to the excitation spectra monitored at the monomer emission (λ_{em} = 335 nm) and the excimer emission (λ_{em} = 390 nm), respectively.

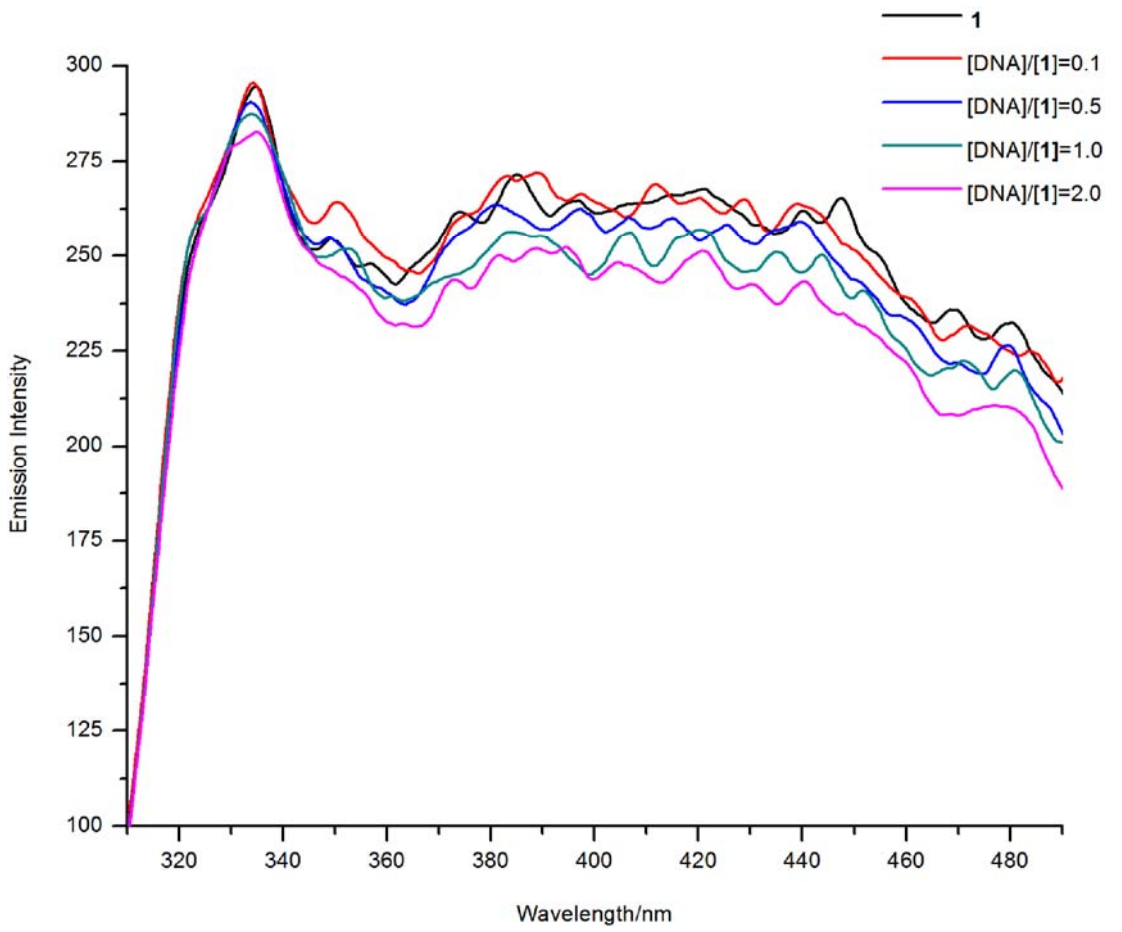

Fig. S4 Fluorescent changes of 10 μM **1** upon the addition of CT-DNA in the buffer (50 mM Tris-HCl, pH 7.4; CH_3CN/H_2O, v/v 5/995).

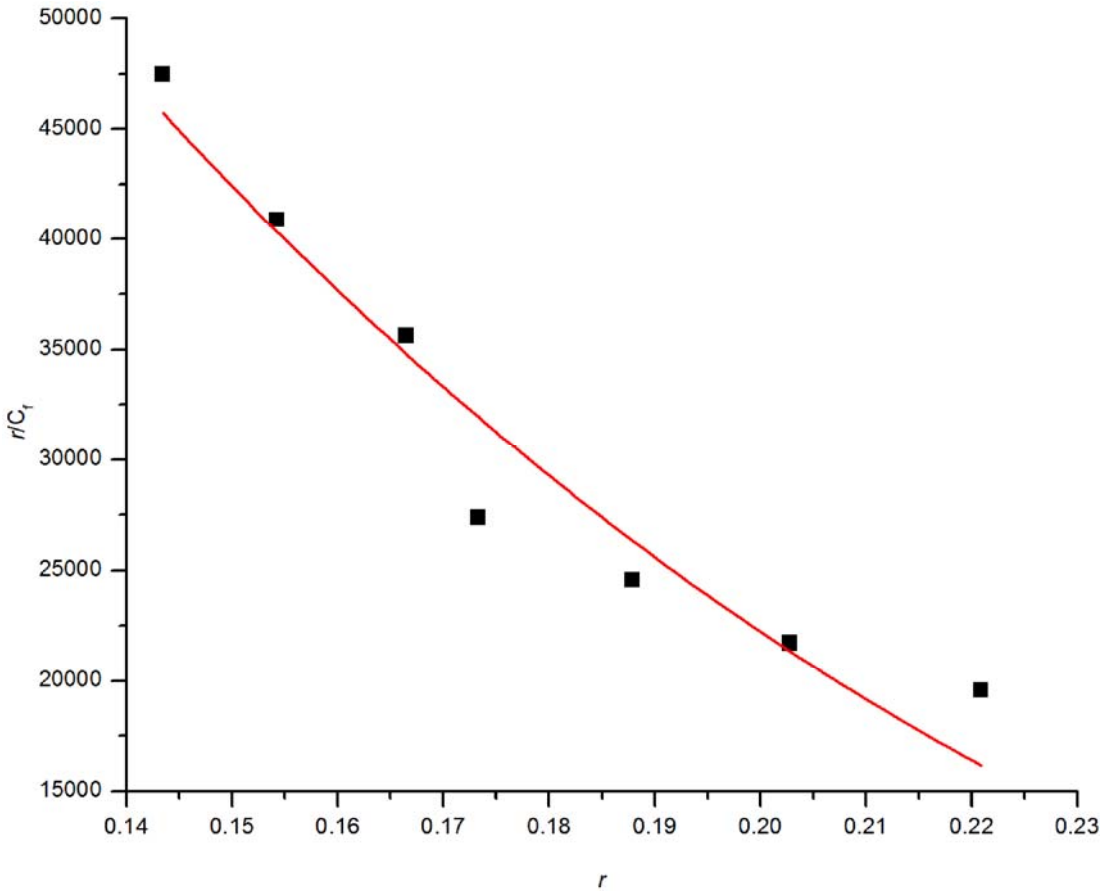

Fig. S5 Scatchard plots utilizing McGhee and von Hippel model for determining the intrinsic binding constant of **2** and DNA. The binding constant was obtained by nonlinear fitting to the data, and the red line is the resulted fitting line.

Preparation of CT-DNA solution: The stock solution of CT-DNA was prepared with the buffer (50 mM Tris-HCl, pH 7.4) and stored at 4 °C. The concentration per nucleotide (or phosphate) of CT-DNA was determined by UV absorbance at 260 nm after proper dilution with water, taking 6600 M^{-1} cm^{-1} as the molar absorption coefficient.[4] The ratio of the UV absorbance at 260 and 280 nm (A_{260}/A_{280}) was ca 1.8, indicating that the DNA solution was sufficiently free of protein.

Circular dichorism study: CT-DNA was incubated alone or with compound **1** or **2** in the buffer (50 mM Tris-HCl, pH 7.4; CH_3CN/H_2O, v/v 5/995) at 37 °C for 30 min. CD spectra were recorded at room temperature in the wavelength range of 220-320 nm. Three scans were performed for each

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spectrum.

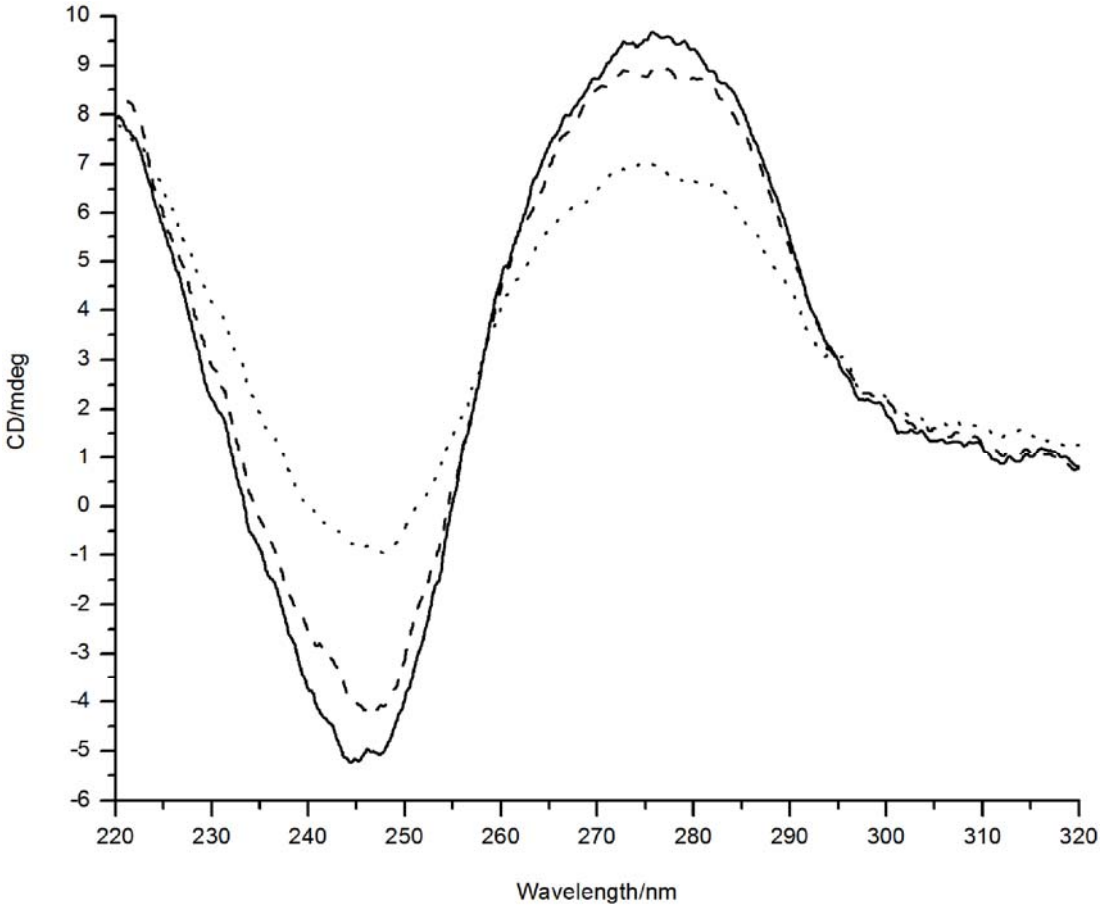

Fig. S6 CD spectra of 100 μM CT-DNA (the solid line) and the interaction with **1** (the dashed line), **2** (the dotted line) with [compound]/[DNA]=0.5. All the spectra were recorded in the buffer (50 mM Tris-HCl, pH 7.4; CH_3CN/H_2O, v/v 5/995).

DNA cleavage studies: The DNA cleavage ability of the compounds was tested by treating supercoiled pUC19 DNA (200 ng) with gradient concentrations of **1** or **2** in the buffer (50 mM Tris-HCl, pH 7.4; CH_3CN/H_2O, v/v 1/999) until the total volume reached 20 μL. The mixtures were incubated at 37 °C for 5 h, and the reactions were quenched by 4 μL of loading buffer (30 mM EDTA, 36% glycerol, 0.05% xylene cyanol FF, and 0.05% bromophenol blue). The resulting solutions were loaded onto the agarose gel (1%) and subjected to electrophoresis in a TAE buffer (40 mM Tris acetate and 1 mM EDTA). DNA bands were stained by EB, visualized under UV

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light, and photographed. Supercoiled pUC 19 DNA values were corrected by a factor of 1.3 on the basis of average literature estimates of the lowered binding of EB to this structure.[5] The fraction of each DNA form was defined as the ratio of the individual band intensity to total band intensity in a specific lane. The reported results are the mean value of the triplicate experiments. HPNP hydrolysis was conducted in the buffer (50 mM Tris-HCl, pH 7.4; CH_3CN/H_2O, v/v 1/999) utilizing 2-hydroxypropyl p-nitrophenyl phosphate (50 μM) as the substrate, different concentrations of **2** were then added to the solution. All the ingredients were mixed, and the visual spectrum was recorded at 400 nm.

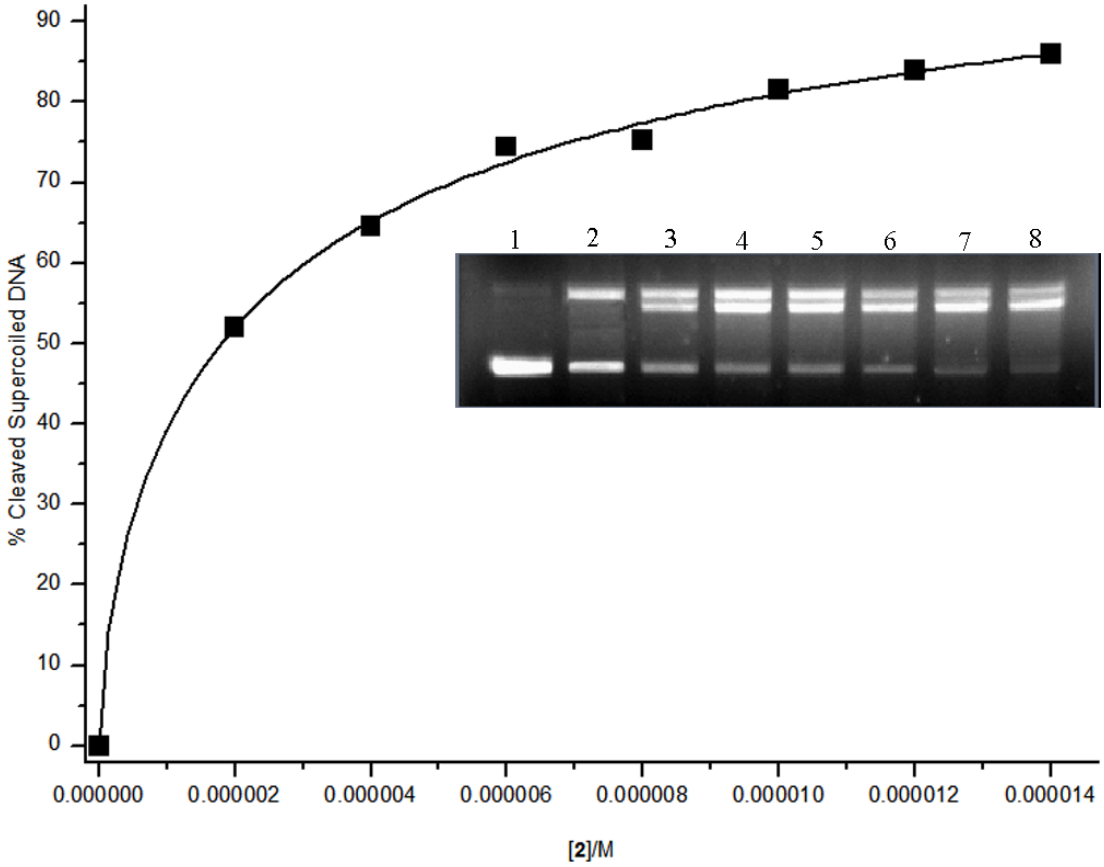

Fig. S7 Percentages of cleaved DNA vs. concentrations of **2**. Inset: Agarose gel (1%) electrophoretograms of pUC 19 DNA (200 ng) incubated for 5 h at 37 °C with different concentrations of **2** in the buffer (50 mM Tris-HCl, pH 7.4; CH_3CN/H_2O, v/v 1/999) Lanes 1-8, 0, 2, 4, 6, 8, 10, 12, 14 μM, respectively.

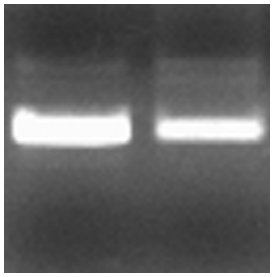

Fig. S8 Agarose gel (1%) electrophoretograms of pUC 19 DNA (200 ng) incubated for 5 h at 37 °C without (left) and with 10 μM **1** (right) in the buffer (50 mM Tris-HCl, pH 7.4; CH_3CN/H_2O, v/v 1/999).

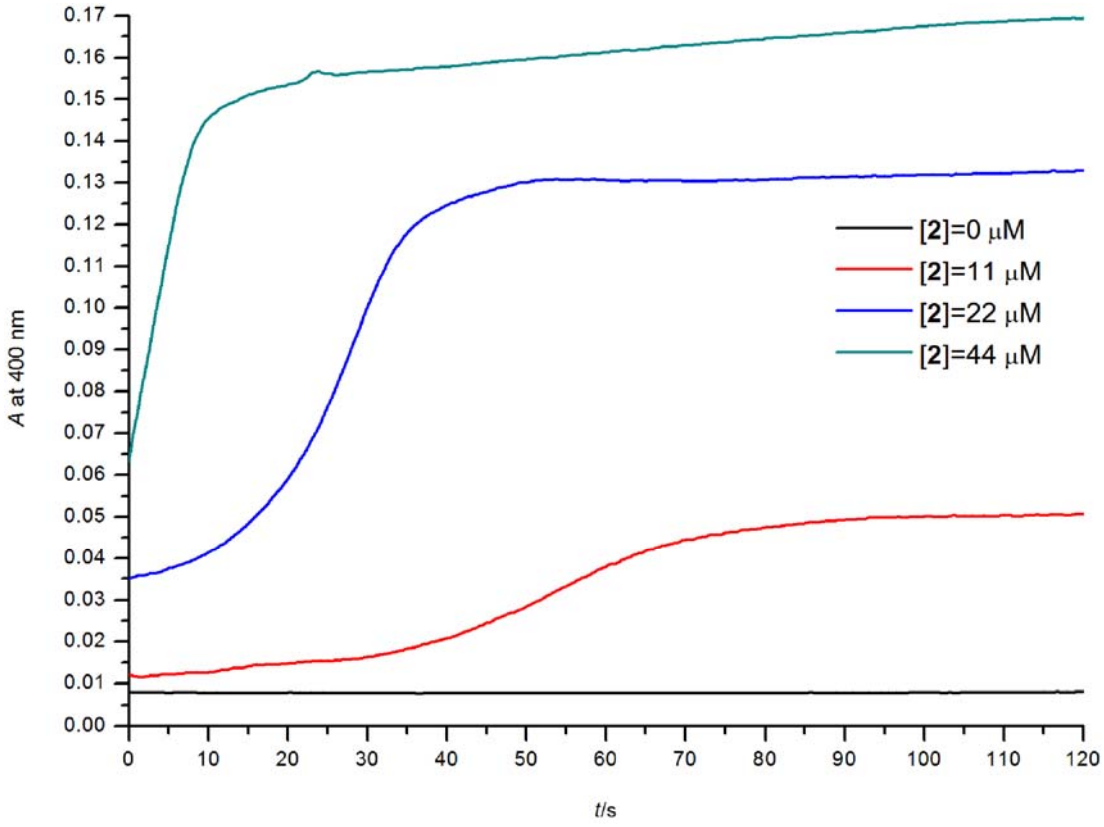

Fig. S9 Hydrolysis of 2-hydroxypropyl-p-nitrophenyl phosphate (HPNP) promoted by different concentrations of **2**.

Cell lines, culture conditions and cytotoxicity assay (MTT assay): Cells were grown in DMEM medium supplemented with 10% freshly inactivated FBS and were seeded equivalently into 96-well plates. The plates were kept at 37 °C in a humidified atmosphere of 5% CO_2 and incubated for 8-10 h. Cells were cultured in 96-well plates for 24 and 48 h with various

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concentrations of **1**, **2** and solely blank CH_3CN-H_2O solution. Cell viability was evaluated with a modified MTT assay. Briefly, 20 μL of 5 mg mL^{-1} MTT in medium (PBS) was added for a further 4 h incubation. After the supernatant had been removed, 150 μL of DMSO was added to dissolve the formazan crystals. The absorbance was read on Thermo Scientific Varioskan Flash at 570 nm. The relative survival rate was determined in relation to that of untreated cells, which was set as 100%.

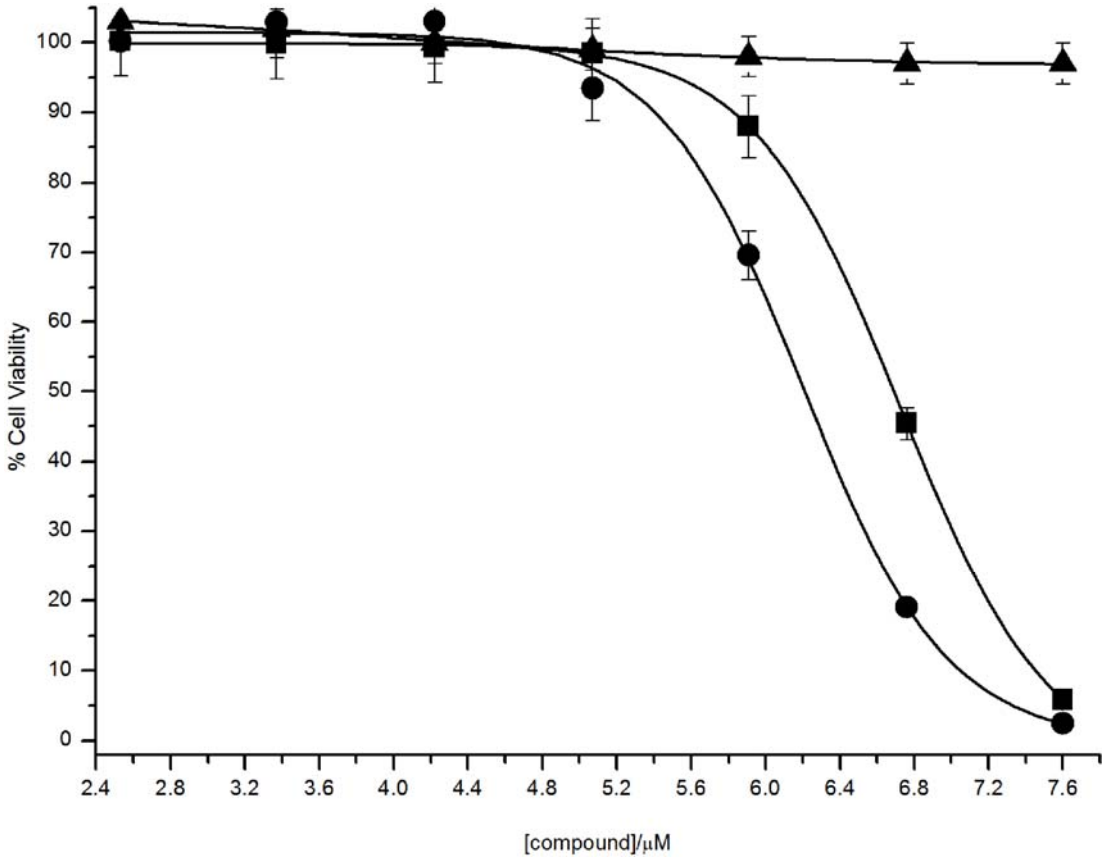

Fig. S10 Inhibition effect of **2** (■) on the proliferation of HeLa cells for 24 h, **2** (●) and **1** (▲) for 48 h. The relative survival rate was determined in relation to that of untreated control cells, which was set to 100%. Data are means ± the standard deviation of three experiments, and each experiment included triplicate wells.

Apoptosis assessment by Hoechst 33258 staining: HeLa cells of exponential growth were inoculated in 6-well plates and cultured for 10 h before treatment. After treatment with 7.6 μM **2**

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for 24 h, Hela cells were fixed with 4% Paraformaldehyde at 4 °C for 10 min and exposed to Hoechst 33258 staining solution (5 μg/mL) in the dark at room temperature for 10 min. Samples were observed under a fluorescence microscope (Olympus DP72, Japan).

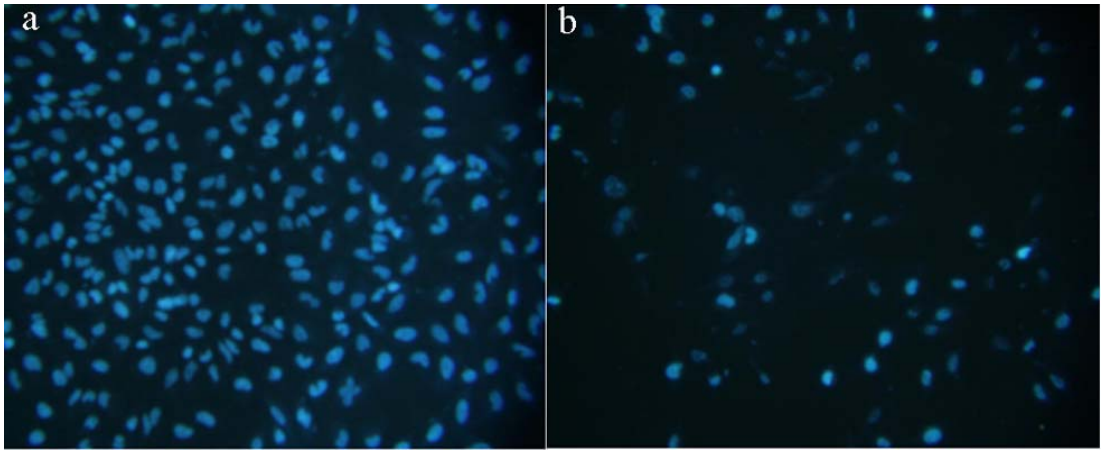

Fig. S11 The morphological changes of Hoechst 33258-stained HeLa cells after treatment with **2** as observed under a fluorescence microscopy. Cells were treated without (a) or with 7.6 μM **2** (b) for 24 h.

Quantitative apoptosis assay by flow cytometry: The ability of **2** to induce apoptosis is evaluated in HeLa cell line using Annexin V conjugated with FITC and propium iodide (PI) counterstaining by flow cytometry. HeLa cells of exponential growth were inoculated in 6-well plates and cultured for 10 h before **2** was added to give the indicated final concentrations. After 48 h incubation, cells were harvested, washed twice in phosphate-buffered saline (PBS), and resuspended in 500 μL binding buffer (including 140 mmol/L NaCl, 2.5 mmol/L $CaCl_2$ and 10 mmol/L HEPES/NaOH, pH 7.4) at a concentration of 1×10^6 cells/mL. Then cells were incubated with 5 μL of Annexin V- FITC (in buffer including 10mmol/L NaCl, 1% bovine serum albumin, 0.02% NaN_3 and 50 mmol/L Tris, pH 7.4) and 5 μL PI (20 μg/mL) for 15 min at room temperature in the dark. Cells were kept shielded from light before being analyzed by flow cytometry using a Becton-Dickinson FACSCalibur.

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Determination of caspase-3 activity by Western blot: After treated with drugs for 24 h, collected 3-5×10^6 HeLa cells, washed twice with PBS collected. Resuspended with 50 μL Lysis buffer containing 0.5 μL DTT. Lysised 30 min on the ice, then centrifuged for 1 min in a microcentrifuge (12,000rpm), the supernatants (cytosolic extract) were transferred to a fresh tube and put on ice to assay the protein concentration. Each supernatants were mixed with sample buffer (60 mM Tris-HCl, pH 6.8, 2% SDS,10% glycerol, 140 mM mercaptoethanol and 0.002% bromophenol blue), boiled for 5 min and resolved on an SDS–polyacrylamide gel (100 mg protein/lane). Proteins were electrotransferred onto PVDF membrane (90 min) and immunoblotted with antipro-caspase-3 (1:1,000 dilution) or anti-bcl-2 (1:1,000 dilution) antibodies. Detection was performed with appropriate HRP-conjugated secondary antibodies (1:5,000 dilution) (Jackson ImmunoResearch, PA) and an enhanced chemiluminescence reagent (Pierce. Denmark).

EB-DNA system and UV study: CT-DNA was incubated alone or with compound **1** or **2** in the buffer (50 mM Tris-HCl, pH 7.4; CH_3CN/H_2O, v/v 5/995) at 37 °C for 30 min. The fluorescent spectra studies were performed by measuring the emission intensity of ethidium bromide (EB). The experiments were carried out by adding 0-45 μM **1** or **2** into the EB (10 μM)-bound CT-DNA (10 μM) solution in the buffer, and the fluorescence of EB-DNA was measured. UV-vis absorption titration was carried out by adding different concentrations of the CT-DNA solution to the compound solutions with constant concentration. The changes of the absorption intensity with increasing concentrations of CT-DNA were recorded after equilibrium.

Supporting Information

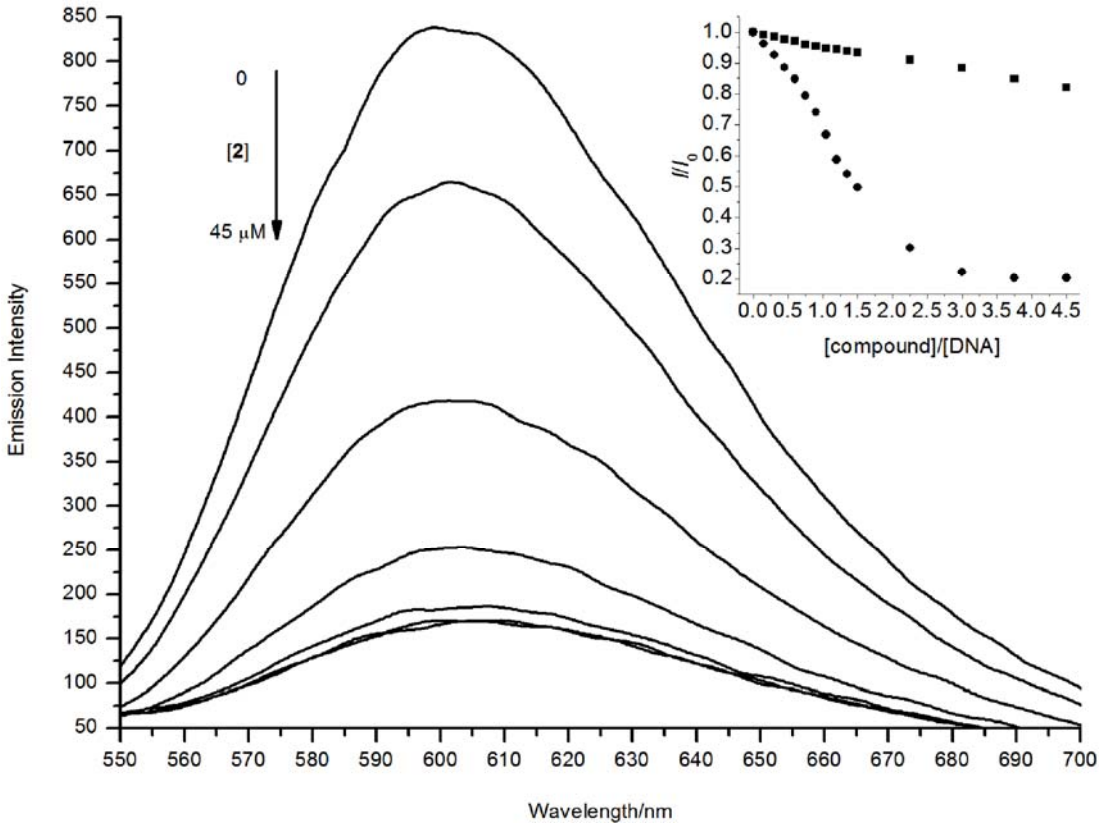

Fig. S12 Emission spectra of EB bound to CT-DNA in the presence of ([EB] = [DNA] = 10 μM, [**2**] = 0-45 μM, from top to bottom). Inset: The plots of emission intensity I/I_0 vs. [compound]/[DNA], **1** (■) and **2** (●). All the spectra were recorded in the buffer (50 mM Tris-HCl, pH 7.4; CH_3CN/H_2O, v/v 5/995). λ_{ex}=520 nm.

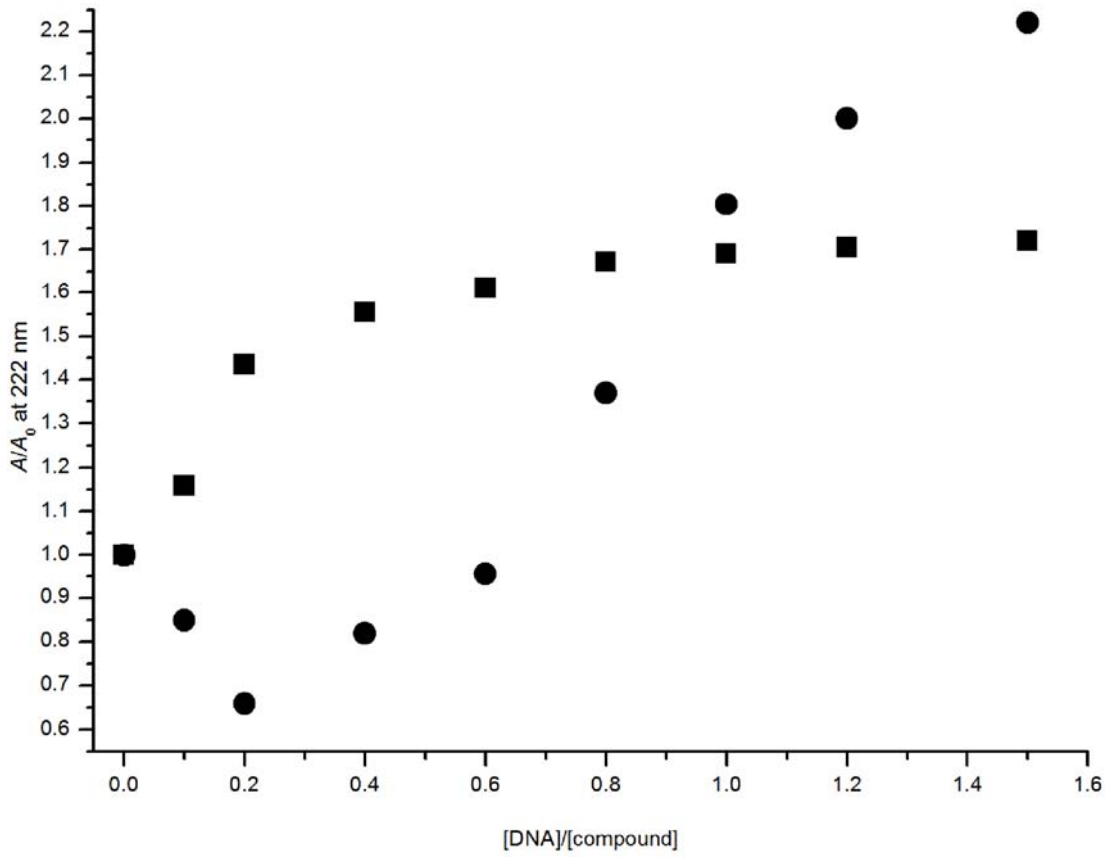

Fig. S13 Effect of addition of CT-DNA on the absorption intensity of 10 μM **1** (■) and **2** (●) at 222 nm in the buffer (50 mM Tris-HCl, pH 7.4; CH_3CN/H_2O, v/v 5/995).

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