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Synthesis, DNA binding and cleavage activities of the copper (II) complexes of estrogen-macrocyclic polyamine conjugates

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Abstract—A series of the copper (II) complexes **5a–d** of estrogen-macrocyclic polyamine conjugates were synthesized and characterized. The interactions of complexes **5a–d** with DNA were studied by fluorescence spectroscopy and gel electrophoresis under physiological conditions. The results indicate that the conjugated estrogens have increased the cleavage efficiency of Cu[cyclen]$^{2+}$ while the conjugates display poor binding affinities. The functional groups of D-ring of estrogens may play a key role in deciding binding and cleavage extent of the complexes to DNA.

1. Introduction

Estrogens have been evoked widespread interest in the clinical and endocrinological applications recently.[1–3] Available evidence indicated that estrogens have also been found to be carcinogenic in humans.[4–6] People have paid special interest in metabolic carcinogenic mechanisms of estrogens. The metabolism of estrogens leading to tumor initiation includes formation of 4-catechol estrogens, and which are further oxidized to the corresponding catechol estrogen quinines. The catechol quinines react with DNA to form depurinating adducts which impair the DNA repair system and induce the accumulation of lesions in the genome.[7–12] Therefore, to further understand the mechanisms of the tumorigenesis induced by estrogen, it was important to investigate structure of estrogens correlating with the relative efficiency of estrogens to bind and cleavage DNA. However, it is difficult to directly study estrogens interaction with DNA due to the poor solubility under physiological conditions. As a result, modification of estrogens was needed for the study of the interaction between estrogens and DNA.

Recently, many macrocyclic polyamine and their derivatives were studied because of their special properties. As a typical compound, 1,4,7,10-tetraazacyclododecane (cyclen) was studied most widely for its good solubility under physiological conditions and strong coordination ability toward metal ion.[13–16] Many cyclen metal complexes were used as chemical nucleases in the DNA recognition and cleavage processes.[17–20] In this paper, we used chloracetyl chloride as a bridge to link up cyclen moiety and different estrogens. As a result, four novel ligands **4a–d** and subsequent copper complexes **5a–d** were prepared. The interactions between **5a–d** and DNA were studied by means of fluorescence spectroscopy and gel electrophoresis. The results showed that these complexes exhibited different DNA binding and cleavage ability under physiological conditions.

2. Results and discussion

2.1. Synthesis of cyclen-estrogen copper complexes

The synthetic route of target complexes **5a–d** from different estrogens is shown in Scheme 1. The reaction between tri-Boc-protected cyclen and chloracetyl chloride

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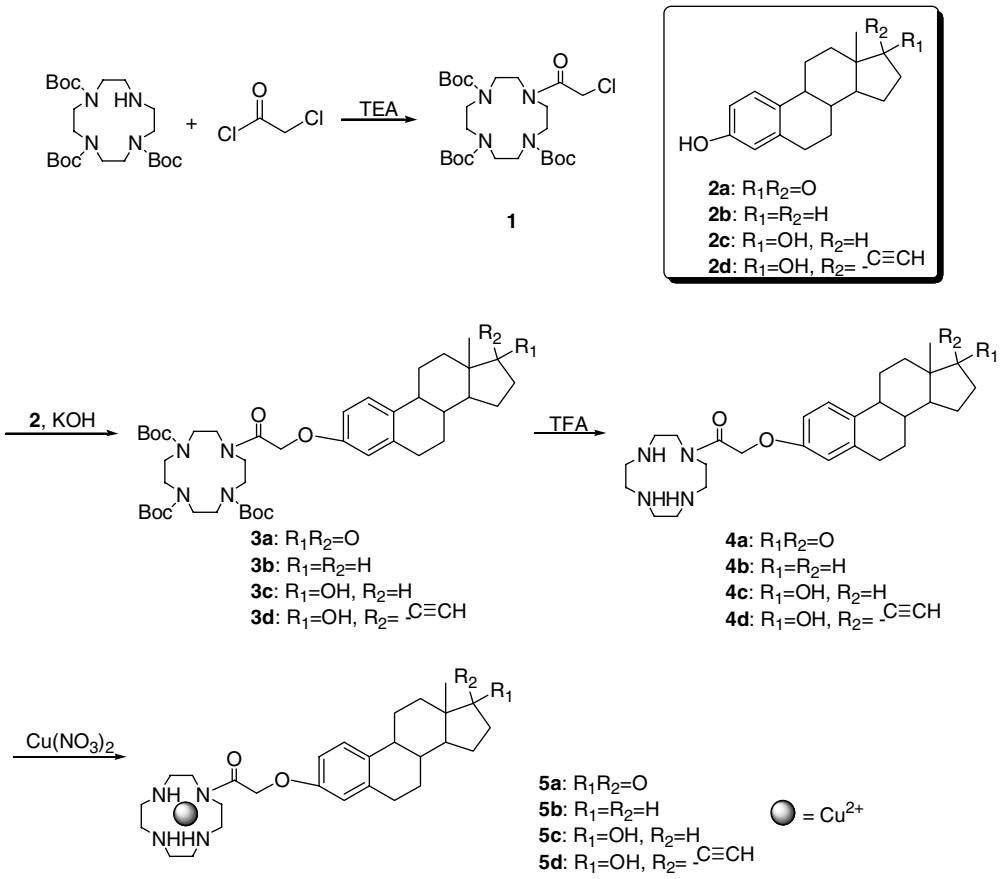

Scheme 1. Synthetic route of the copper (II) complexes of estrogen–cyclen conjugates.

afforded the desired product **1**. Compounds **3a–d** were obtained by the reaction between **1** and **2a–d** in the presence of KOH. The compounds **3a–d** were deprotected by trifluoroacetic acid (TFA) and then basified with NaOH to give the free ligands **4a–d**. These ligands were allowed to react with $Cu(NO_3)_2$ in EtOH overnight to give the target copper complexes **5a–d** as blue solid. The structures of these compounds were confirmed by IR, ^1H NMR, ESI-MS, and HRMS.

2.2. Interaction between the copper complexes and DNA

2.2.1. Fluorescence spectroscopic studies. Ethidium bromide (EB) has weak fluorescence, but its emission intensity in the presence of DNA could be greatly enhanced because of its strong intercalation between the adjacent DNA base pairs. It was previously reported that this enhanced fluorescence could be quenched, at least partly by the addition of a second molecule.[21,22] The emission spectra of EB bound to CT-DNA in the absence and presence of complexes **5a–d** with different concentrations are given in Figure 1. The control experiments were also performed by using $Cu[cyclen]^{2+}$. The addition of these complexes to DNA pretreated with EB caused appreciable decrease in the emission intensity, which indicated that these complexes compete with EB in binding to DNA. According to the classical Stern-Volmer Eq. 1[23]:

$$F_0/F = 1 + K_{SV}[Q] \quad (1)$$

where F_0 and F are the fluorescence intensities in the absence and presence of complexes, respectively, K is the linear Stern-Volmer quenching constant, $[Q]$ is the concentration of the complexes. The fluorescence quenching curves of EB bound to DNA by these complexes are shown in Figure 2. The quenching plot illustrates that the quenching of EB bound to DNA by these complexes are in good agreement with the linear Stern-Volmer equation, which also proves that these complexes bind to CT-DNA. To compare relative binding abilities of these complexes to CT-DNA, the apparent binding constants were calculated from Eq. 2[24]:

$$K_{app} = K_{(EB)} \cdot C_{(EB)}/C_{50} \quad (2)$$

where $K_{(EB)}$ is the binding constant of EB to CT-DNA (1.0×10^7 L/mol), $C_{(EB)}$ is the concentration of EB in buffer solution, C_{50} is the concentration necessary to reduce fluorescence intensity to 50% of the initial value. The K_{app} values are estimated as 1.57×10^7, 1.53×10^7, 0.93×10^4, and 1.23×10^7 for the complexes **5a**, **5b**, **5c**, **5d**, respectively. The binding constant values are lower than $Cu[cyclen]^{2+}$ (2.75×10^7 L/mol). So it is likely that binding abilities of the complexes **5a–d** are mainly attributed to cyclen moiety due to the formation of several hydrogen bonds between –NH– groups in the cyclen and the base pairs in DNA.[25] The introduction of side chain containing estrogens to cyclen could affect binding ability to DNA. The order of binding abilities of the complexes is **5a > 5b > 5d > 5c**. The differences in binding abilities

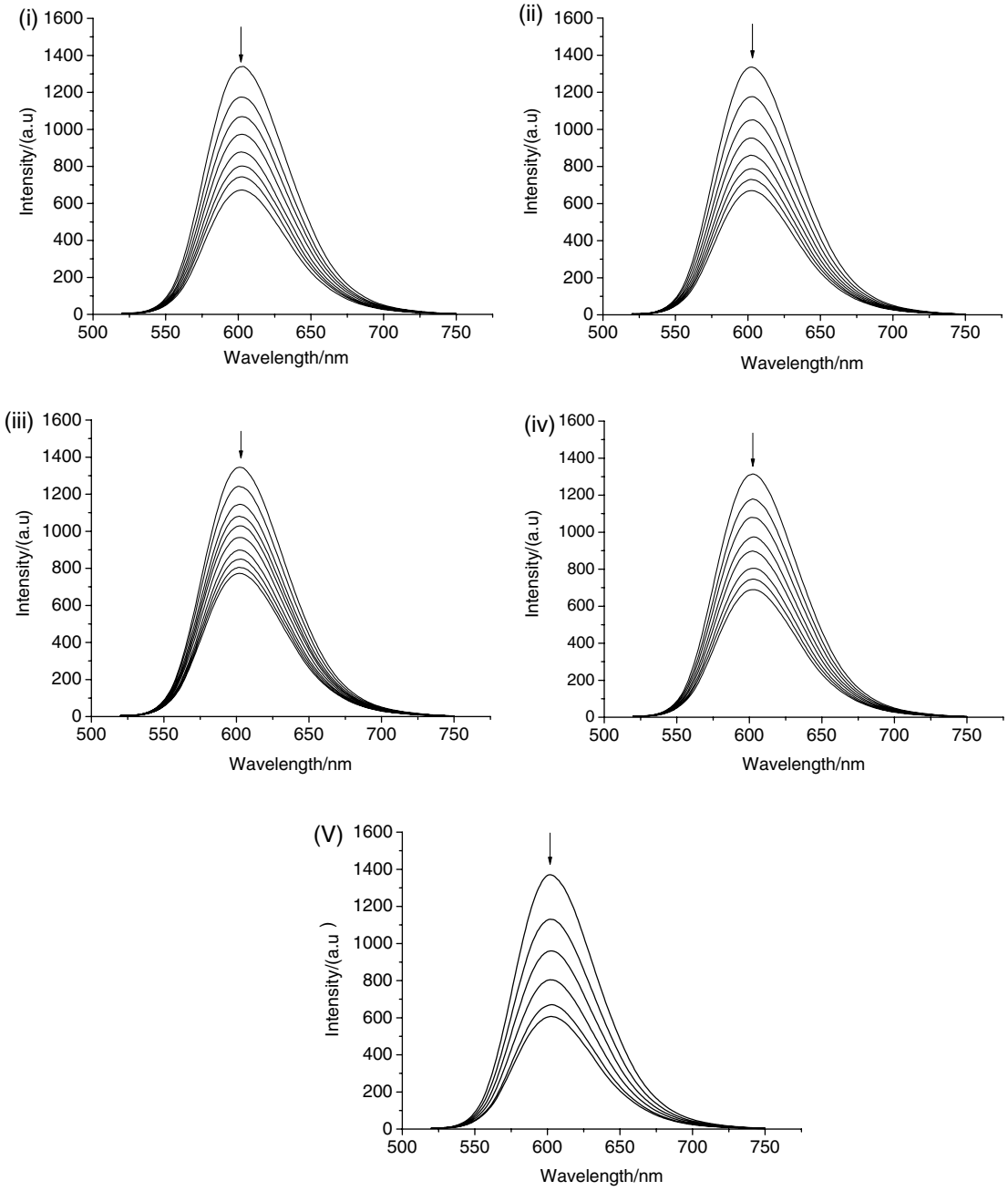

Figure 1. Fluorescence spectra of EB bound to CT-DNA in the presence of complex (i) **5a**, (ii) **5b**, (iii) **5c**, (iv) **5d**, and (v) Cu[cyclen]$^{2+}$ with different concentrations. [EB] = 50 μM, [DNA] = 100 μg/mL, [complexes **5a**, **5 b**, **5d**] =0, 6.0, 12.0, 18.0, 24.0, 30.0, 36.0, 42.0 μM, respectively; [complex **5c**] = 0, 6.0, 12.0, 18.0, 24.0, 30.0, 36.0, 42.0 48.0, 54.0 μM; [Cu[cyclen]$^{2+}$] = 0, 6.0, 12.0, 18.0, 24.0, 30.0 μM. λ_{ex} = 497 nm. The arrow shows the intensity changes on increasing the complex concentration.

of the four complexes to DNA may be explained by different structures of D-ring of estrogens.

2.2.2. Chemical nuclease activity of complexes 5a–d. The cleavage of DNA in the presence of complexes **5a**–**d** was also studied by using plasmid DNA (pUC 19) as substrate. The extent of DNA cleavage was monitored by gel electrophoresis. Figure 3A shows the results of gel electrophoresis carried out with pUC 19 in the presence of complexes **5a**–**d** with different concentrations. Lane 1 in the figure shows the control DNA without any additives. Incubation of DNA with complexes **5a**–**d** led to obvious cleavage of supercoiled circular DNA (Form I) and formation of nicked DNA (Form II). Increasing the concentration of complexes resulted in the increase of Form II. Figure 3B shows the relative cleavage efficiency of complexes **5a**–**d**. The experimental results showed that these complexes could cleavage DNA, and **5a** showed the strongest cleavage ability to DNA. The functional groups of D-ring of estrogens may play a key role in deciding cleavage extent of the complexes to DNA.

In order to investigate the role of estrogen moiety to DNA cleavage, the control experiments were also performed by using Cu[cyclen]$^{2+}$ and complexes **5a**–**d**.

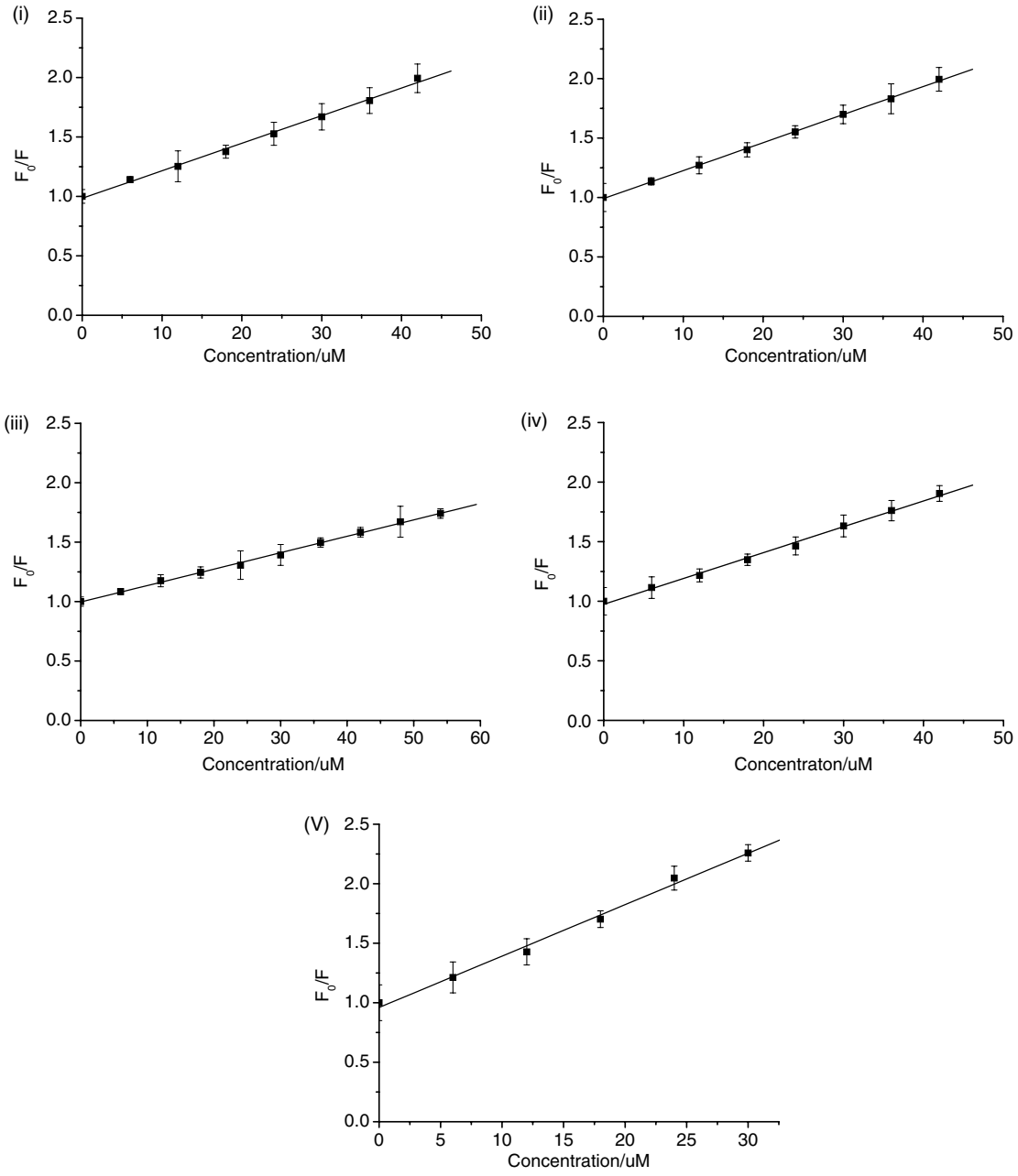

Figure 2. Fluorescence quenching curves of EB bound to CT-DNA in the presence of complex (i) **5a**, (ii) **5b**, (iii) **5c**, (iv) **5d**, and (v) Cu[cyclen]$^{2+}$ with different concentrations.

The experimental results are given in Figure 4. This result showed that DNA cleavage efficiency of Cu[cyclen]$^{2+}$ was lower than complexes **5a**–**d** in the same conditions. It is likely that introduction of side chain containing estrogens to cyclen could result in great increase of DNA cleavage activity because lipophilicity of estrogens should facilitate the transport of cyclen into supercoiled circular DNA.

3. Conclusion

A series of copper complexes **5a**–**d** of estrogen-macrocyclic polyamine conjugates were synthesized and characterized. The interactions of complexes **5a**–**d** with DNA were studied by fluorescence spectroscopy and gel electrophoresis under physiological conditions. The results indicate that the conjugated estrogens have increased the cleavage efficiency of Cu[cyclen]$^{2+}$ while the conjugates display poor binding affinities. This might provide a successful strategy for the direct studies of the interaction between estrogens and DNA under physiological conditions. The results also revealed that the structure difference on the estrogen might lead to obvious difference of DNA binding and cleavage abilities of the complexes. The functional groups of D-ring of estrogens might play a key role in the DNA recognition process. In the next step, we will continue to clarify the binding

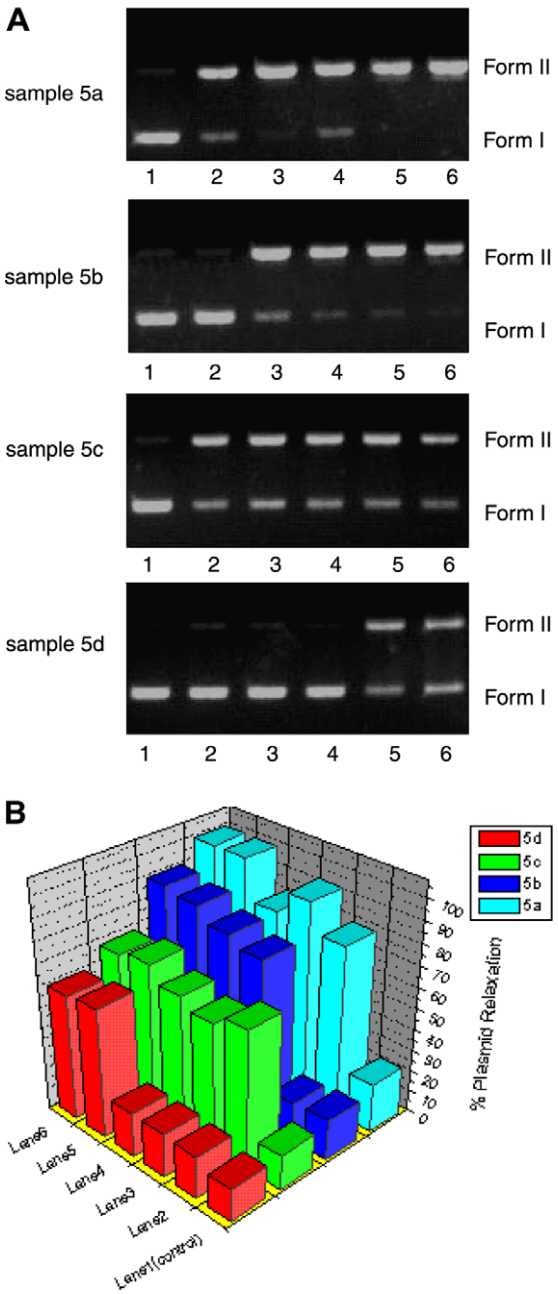

Figure 3. Effect of concentration of complexes **5a**–**d** in a Tris–HCl buffer (100 mM, pH 7.4) at 37 °C for 24 h. (A) Agarose gel electrophoresis diagram: lane 1, DNA control; lanes 2–6, [**5a**–**d**] = 0.28, 0.56, 0.84, 1.12 and 1.43 mM. (B) Quantitation of % plasmid relaxation (Form II%) relative to plasmid DNA per lane.

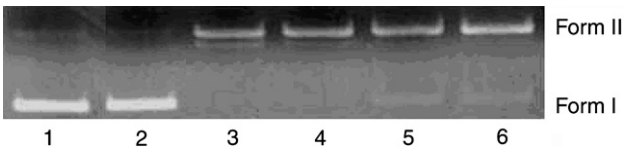

Figure 4. The control experiments with all compounds (1.43 mM) in a Tris–HCl buffer (100 mM, pH 7.4) at 37 °C for 30 h. Agarose gel electrophoresis diagram: Lane 1, DNA control; lane 2, Cu[cyclen]$^{2+}$; lane 3, complex **5a**; lane 4, complex **5b**; lane 5, complex **5c**; lane 6, complex **5d**.

and cleavage mechanisms and to understand the role of the different functional groups of D-ring of estrogens to bind and cleavage DNA.

4. Experimental

4.1. General

All reagents were purchased from commercial sources and used without further purification. Electrophoresis grade agarose and plasmid DNA (pUC 19) were purchased from Takara Biotechnology Company. MS (ESI) data were recorded on a Finnigan LCQ^{DECA} mass spectrometer. HRMS spectral data were recorded on Bruker Daltonics Bio TOF. ^{1}H NMR spectral data were measured on a Varian INOVA-400 spectrometer and chemical shifts in ppm are reported relative to internal Me_4Si. IR spectra were recorded on a Shimadzu FTIR-4200 spectrometer as KBr pellets in the range of 4000–400 cm^{-1}. Fluorescence spectra were recorded on a LS55 spectrofluorometer.

4.2. General procedure for the preparation of compound 1

To a solution of chloracetyl chloride (0.24 mL, 3.0 mmol) in dry CH_2Cl_2 (50 mL) was slowly added a solution of 3Boc-cyclen (0.94 g, 2.0 mmol) and Et_3N (0.84 mL, 2.0 mmol) in dry CH_2Cl_2 (15 mL) at 0 °C. The cooling bath was removed after 0.5 h and the reaction mixture was stirred at room temperature for 3 h. Then, the mixture was quenched with water (10 mL). After phase separation, the aqueous phase was extracted with CH_2Cl_2 (3 × 30 mL). The combined organic phases were subsequently washed with brine and dried. The solvent was removed in vacuo and the residue was purified by column chromatography (petroleum ether/ethyl acetate, 1:1) to give the product **1** as a white solid. Yield: 65%. ^{1}H NMR (400 MHz, $CDCl_3$TMS) δ (ppm): 1.46–1.49 (m, 27H, Boc-H), 3.38–3.56 (m, 16H, –CH_2), 4.06 (s, 2H) MS (ESI) (*m*/*z*): 548 (M^+).

4.3. General procedure for the preparation of compound 3

A THF solution of **1** (0.274 g, 0.5 mmol), estrogen **2a**–**d** (0.5 mmol), and KOH (0.084 g, 1.5 mmol) was stirred at 60 °C for 10 h. After cooling, the reaction mixture was filtered, and the filtrate was concentrated under reduced pressure. The residue was purified by column chromatography (petroleum ether/ethyl acetate, 1:1) to give desired products **3a**–**d** as a white solid.

Compound **3a**, Yield: 97%. IR (KBr, cm^{-1}): 3422, 2973, 2930, 2871, 1740, 1694, 1647, 1606, 1469, 1408, 1365, 1249, 1164, 1100, 969, 858, 777; ^{1}H NMR (400 MHz, $CDCl_3$, TMS) δ (ppm): 0.90 (s, 3H), 1.44–1.97 (m, 37H), 2.01–2.26 (m, 2H), 2.31–2.54 (m, 1H), 2.86–2.88 (m, 2H), 3.39–3.56 (m, 16H, –CH_2), 4.63 (s, 2H), 6.69 (s,1H), 6.74 (d, 1H, J = 10.8 Hz), 7.17(d, 1H, J = 8.8 Hz); HRMS-ESI: *m*/*z* calcd for $C_{43}H_{67}N_4O_9$ $[M+H]^+$: 783.4908, found 783.5000.

Compound **3b**, Yield: 81%. IR (KBr, cm^{-1}): 3380, 2928, 2864, 1693, 1610, 1469, 1367, 1249, 1163, 1052, 969, 859, 777; ^1H NMR (400 MHz, $CDCl_3$, TMS) δ (ppm): 0.73 (s, 3H), 1.44–1.96 (m, 41H), 2.22–2.31 (m, 1H), 2.80–2.82 (m, 2H), 3.39–3.53 (m, 16H, $-CH_2$), 4.61 (s, 2H), 6.66 (s, 1H), 6.73 (d, 1H, $J = 12.0$ Hz), 7.18(d, 1H, $J = 8.8$ Hz); HRMS-ESI: *m/z* calcd for $C_{43}H_{69}N_4O_8$ $[M+H]^+$: 769.5115, found 769.5233.

Compound **3c**, Yield: 71%. IR (KBr, cm^{-1}): 3478, 2974, 2927, 2864, 1695, 1606, 1470, 1410, 1366, 1249, 1163, 1055, 969, 857, 778; ^1H NMR (400 MHz, $CDCl_3$, TMS) δ (ppm): 0.77 (s, 3H), 1.44–1.97 (m, 39H), 2.31–2.54 (m, 1H), 2.86–2.88 (m, 2H), 3.39–3.56 (m, 16H, $-CH_2$), 3.73–3.75 (m, 1H), 4.11 (s, 1H) 4.63 (s, 2H), 6.67 (s, 1H), 6.74 (d, 1H, $J = 10.8$ Hz), 7.17 (d, 1H, $J = 8.8$ Hz); HRMS-ESI: *m/z* calcd for $C_{43}H_{69}N_4O_9$ $[M+H]^+$: 785.5065, found 785.5135.

Compound **3d**, Yield: 86%. IR (KBr, cm^{-1}): 3451, 3305, 2975, 2931, 2871, 1694, 1606, 1470, 1410, 1367, 1249, 1164, 1057, 966, 857, 778; ^1H NMR (400 MHz, $CDCl_3$, TMS) δ (ppm): 0.87 (s, 3H), 1.44–1.94 (m, 39H), 2.31–2.54 (m, 1H), 2.60 (s, 1H), 2.86–2.88 (m, 2H), 3.39–3.56 (m, 16H, -CH_2), 4.13 (s, 1H) 4.62 (s, 2H), 6.67 (s, 1H), 6.74 (d, 1H, $J = 10.8$ Hz), 7.17 (d, 1H, $J = 8.8$ Hz); HRMS-ESI: *m/z* calcd for $C_{45}H_{69}N_4O_9$ $[M+H]^+$: 809.7065, found 809.7062.

4.4. General procedure for the preparation of compound 4

To a stirred solution of **3a–d** (0.3 mmol) in CH_2Cl_2 (5 mL) at room temperature was slowly added trifluoroacetic acid (2 mL), and the solution was stirred for 4 h under N_2. Then, the reaction mixture was concentrated under reduced pressure to give crude product. And the resulting yellow oil liquid was crystallized in anhydrous ether and washed three times with anhydrous ether (5 mL). The trifluoroacetic acid salts of ligand were dissolved in 5 mL water and adjusted the aqueous solution to alkaline (pH $\geqslant$ 12) with 2 mol/L aqueous NaOH. The solutions were extracted with CH_2Cl_2 (4×10 mL). The organic layer was dried over anhydrous Na_2SO_4 and the solutions were concentrated under reduced pressure to give colorless solid **4**.

Compound **4a**, Yield: 93%. IR (KBr, cm^{-1}): 3364, 2933, 2864, 1737, 1674, 1606, 1498, 1369, 1201, 1068, 938, 794; ^1H NMR (400 MHz, D_2O, TMS) δ (ppm): 0.85 (s, 3H), 1.16–1.99 (m, 13H), 2.01–2.26 (m, 2H), 2.31–2.54 (m, 1H), 2.86–2.88 (m, 2H), 3.25–3.71 (m, 16H, $-CH_2$), 4.83 (s, 2H), 6.69 (s, 1H), 6.78 (d, 1H, $J = 10.8$ Hz), 7.28 (d, 1H, $J = 8.8$ Hz); HRMS-ESI: *m/z* calcd for $C_{28}H_{43}N_4O_3$ $[M+H]^+$: 483.3335, found 483.3334.

Compound **4b**, Yield: 85%. IR (KBr, cm^{-1}): 3260, 2932, 2870, 1677, 1497, 1427, 1377, 1201, 1125, 1054, 929, 832, 798, 720; ^1H NMR (400 MHz, $CDCl_3$, TMS) δ (ppm): 0.71 (s, 3H), 1.10–1.87 (m, 16H), 2.17–2.35 (m, 1H), 2.80–2.82 (m, 2H), 3.02–3.63 (m, 16H, $-CH_2$), 4.65 (s, 2H), 6.58 (s, 1H), 6.65 (d, 1H, $J = 12.0$ Hz), 7.18 (d, 1H, $J = 8.8$ Hz); HRMS-ESI: *m/z* calcd for $C_{28}H_{45}N_4O_2$ $[M+H]^+$: 469.3543, found 469.3542.

Compound **4c**, Yield: 88 %. IR (KBr, cm^{-1}): 3429, 3266, 2929, 2864, 1676 1498, 1410, 1382, 1202, 1132, 1059, 834, 799, 721; ^1H NMR (400 MHz, $CDCl_3$, TMS) δ (ppm): 0.87 (s, 3H), 1.11–1.97 (m, 15H), 2.31–2.54 (m, 1H), 2.86–2.88 (m, 2H), 3.07–3.31 (m, 16H, $-CH_2$), 3.70–3.75 (m, 1H), 4.70 (s, 2H), 4.87 (s, 1H), 6.61 (s, 1H), 6.66 (d, 1H, $J = 10.8$ Hz), 7.17(d, 1H, $J = 8.8$ Hz); HRMS-ESI: *m/z* calcd for $C_{28}H_{45}N_4O_3[M+H]^+$: 485.3492, found 485.3491.

Compound **4d**, Yield: 91%. IR (KBr, cm^{-1}): 3423, 3299, 2931, 2860, 1676, 1498, 1427, 1380, 1201, 1131, 1063, 833, 799, 721; ^1H NMR (400 MHz, $CDCl_3$, TMS) δ (ppm): 0.87 (s, 3H), 1.24–1.94 (m, 15H), 2.31–2.36 (m, 1H), 2.60 (s, 1H), 2.86–2.88 (m, 2H), 3.39–3.55 (m, 16H, $-CH_2$), 4.13 (s, 1H) 4.62 (s, 2H), 6.67 (s, 1H), 6.73 (d, 1H, $J = 10.8$ Hz), 7.17(d, 1H, $J = 8.8$ Hz); HRMS-ESI: *m/z* calcd for $C_{30}H_{45}N_4O_3$ $[M+H]^+$: 509.3492, found 509.3491.

4.5. General procedure for the preparation of complex 5

The ethanol solutions (5 mL) of **4a–d** were added, respectively, to excessive $Cu(NO_3)_2$ in the 10 mL ethanol and reflux for 2 h. Then the mixture was stirred overnight under room temperature and then concentrated under reduced pressure. The residue was purified with centrifugal device, and the solids were washed with ethanol (2×2 mL).

Compound **5a**, Yield: 72%. IR (KBr, cm^{-1}): 3443, 3225, 2928, 1732, 1666, 1609, 1492, 1380, 1283, 1160, 1075, 1010, 812; HRMS-ESI: *m/z* calcd for $C_{28}H_{43}CuN_4O_4$ $[M+Cu+OH]^+$: 562.2680, found 562.3165.

Compound **5b**, Yield: 66%. IR (KBr, cm^{-1}): 3439, 3214, 2927, 2860, 1665, 1609, 1494, 1380, 1291, 1158, 1079, 1011, 806; HRMS-ESI: *m/z* calcd for $C_{28}H_{45}CuN_6O_8$ $[M+Cu+2NO_3+H]^+$: 656.2695, found 656.2594.

Compound **5c**, Yield: 62%. IR (KBr, cm^{-1}): 3433, 3237, 2932, 2873, 1672, 1610, 1492, 1382, 1225, 1163, 1063, 1015, 810; HRMS-ESI: *m/z* calcd for $C_{28}H_{43}CuN_4O_3$ $[M+Cu-H]^+$: 546.4695, found 546.4392.

Compound **5d**, Yield: 70%. IR (KBr, cm^{-1}): 3422, 3264, 2931, 2866, 1651, 1610, 1492, 1384, 1291, 1155, 1059, 1012, 811; HRMS-ESI: *m/z* calcd for $C_{30}H_{43}CuN_4O_3[M+Cu-H]^+$: 570.3731, found 570.3843.

4.6. Fluorescence quenching experiments

Fluorescence quenching experiments were carried out by adding the solution of complexes **5a–d** to the samples containing 50 μM of EB and 100 μg/mL of DNA at different concentrations (0–42 μM). All the samples were excited at 497 nm. Ethidium bromide displacement assay was recorded on a Hitachi FL4500 spectrofluorimeter. Excitation of the sample was carried out on 3 mL quartz cuvette with 497 nm excitation light, and emission was measured at 620 nm. The buffer designated 0.01 M Tris–HCl (pH 7.4). Ethidium bromide was dissolved in the buffer to reach the concentration of 1 mg/mL. 10 μL of highly

polymerized calf thymus type I DNA (1 mg/mL of nucleotide concentration in the above buffer) was then added to reach a concentration of 100 μg/mL, and the fluorescence increased to measurement maxima. The test agent in aqueous solution (depending on the compound) was added in microliter portions to reduce the fluorescence of DNA–EB complex.

4.7. Plasmid DNA cleavage experiments

Plasmid DNA (pUC 19) cleavage activity of the complexes **5a**–**d** was monitored by using agarose gel electrophoresis. In a typical experiment, supercoiled DNA (pUC 19) (25 μg/mL, 5 μL) in Tris–HCl (100 mM, pH 7.4) was treated with different concentrations of complexes **5a**–**d**, followed by dilution with the Tris–HCl buffer to a total volume of 17.5 μL. The samples were then incubated at 37 °C for different times, and loaded on a 0.7% agarose gel containing 1.0 μg/mL of ethidium bromide. Electrophoresis was carried out at 40 V for 30 min in TAE buffer and run in duplicate. Bands were visualized by UV light and photographed, then the intensity of the DNA bands was estimated by a Gel Documentation System.

Acknowledgments

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