Efficient DNA Cleavage by Acridine Conjugates of Mono- and Dinuclear Cu(II) Complexes

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There have been reported suitably designed, simple mono- and dinuclear metal complexes which are efficient in hydrolyzing phosphate esters including DNA.[1] We have previously reported the dinuclear $Cu(II)_2L1$ complex (**L1**: 1,3-*bis*(1,4,7-triaza-1-cyclononyl)propane) efficiently hydrolyzed DNA, producing nicked and linearized DNA over the mononuclear CuL2 complex (**L2**: 1,4,7-triazacyclononane).[2] We were interested in nuclease activity of the Cu complexes having DNA binding groups since it would provide valuable information in developing sequence specific artificial nucleases. Several acridine conjugates of mononuclear Cu, Zn complexes had been reported, where strong binding through an intercalation of acridine moiety to DNA was the main factor for the nuclease activity, yet the cleavage mechanisms were not well established.[3]

In this study, we synthesized new acridine conjugates, **L3** and **L4** (Figure 1). Instead of using repeating alkyl chain as used in most of model studies,[3] polyethylene glycol unit was chosen for the linker since it allowed a significant increase in the length of the linker with relatively small change in the hydrophobicity of the molecule.[4] Determination of the association constants for the binding and the cleavage rate measurements would allow for a detailed dissection of the factors responsible for the rate enhancement in cleaving DNA by the acridine conjugates of the Cu complexes.

Experimental Section

Materials. Amberlyst 21 resin was purchased from Alpha. All other chemicals were from Aldrich and used without further purification. Supercoiled pCMV-Myc DNA was purchased from Clonetech. Labs (Mountain View, CA). Calf tymus-DNA was purchased from Sigma and dialyzed against water.

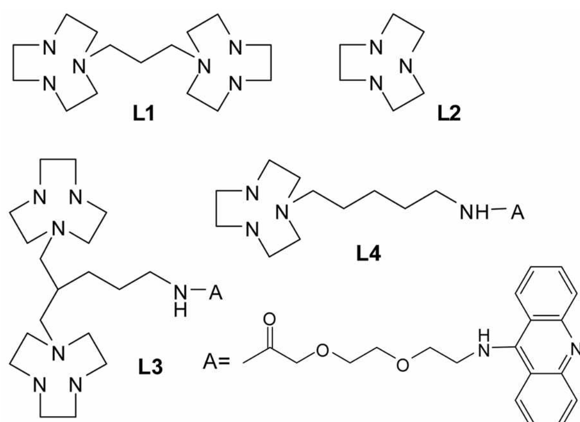


Figure 1. Chemical structures of the ligands.

Synthetic procedure. ^1H and ^{13}C NMR spectrum were obtained from VARIAN UNITY-INOVA 300 MHz spectrometer. Mass spectra were recorded on a Thermo Finnigan AQA Lc-Mass. The ligands, **L3** and **L4** were synthesized by modified literature methods.[5] Cu(II) complexes of **L1-L4** were prepared according to the known method by mixing ethanolic solution of the ligand and 1.0-2.0 equivalents of $Cu(NO_3)_2$, respectively.[6]

7-(5-{2-[2-(2-Azido-ethoxy)-ethoxy]-acetylamino}-pentyl)-[1,4,7]-triazonane-1,4-dicarboxylic acid di-*tert*-butyl ester (1): 7-(5-Amino-pentyl)-[1,4,7]-triazonane-1,4-dicarboxylic acid di-*tert*-butyl ester **R1** (1.14 g, 2.75 mmol)[7] and [2-(2-azido-ethoxy)-ethoxy]-acetic acid[8] (520 mg, 2.75 mmol) were dissolved in THF. DCC (680 mg, 3.3 mmol) was added slowly at 0 °C, followed by addition of catalytic amount of DMAP. The solution was stirred overnight at room temperature, filtered and evaporated to dryness. After column chromatography (CH_2Cl_2: MeOH), 1.6 g of **1** was obtained as a syrup (99%). ^1H NMR ($CDCl_3$): 6.82 (bs, 1H), 3.97 (s, 2H), 3.70-3.66 (m, 6H), 3.46-3.38 (m, 6H), 3.30-3.16 (m, 6H), 2.60 (bt, 4H), 2.45 (bt, 2H), 1.6-1.28 (m, 24H), ESI MS (−): m/z 584.8 $[M]^-$.

2-[2-(2-Azido-ethoxy)-ethoxy]-*N*-[5-(4,7-di-*tert*-Boc-[1,4,7]-triazonan-1-yl)-4-(4,7-di-*tert*-Boc-[1,4,7]-triazonan-1-ylmethyl)-pentyl]-acetamide (2): **R2** (1 g, 1.32 mmol) and [2-(2-azido-ethoxy)-ethoxy]-acetic acid (250 mg, 1.32 mmol) gave **2** (1.1 g, 1.19 mmol) as a syrup (90%). ^1H NMR ($CDCl_3$): 6.90 (m, 1H), 3.97 (s, 2H), 3.70-3.67 (m, 6H), 3.60-3.08 (m, 18H), 2.69-2.5 (m, 8H), 2.5-2.37 (m, 2H), 2.37-2.21 (m, 2H), 1.62-1.32 (m, 40H), ESI MS: m/z 928.1 $[MH]^+$.

7-(5-{2-[2-(2-Amino-ethoxy)-ethoxy]-acetylamino}-pentyl)-[1,4,7]-triazonane-1,4-dicarboxylic acid di-*tert*-butyl ester (3): To **1** (1.3 g, 2.22 mmol) in 50 mL EtOH was added 130 mg of 10% Pd/C. The solution was stirred underHydrogen pressure (50 psi) until no more starting material left. The mixture was filtered on celite and the filtrate was evaporated to dryness. After short column chromatography (CH_2Cl_2:MeOH = 5:1), **3** (660 mg, 1.18 mmol) was obtained as a syrup (53%). ESI MS: m/z 560.5 $[MH]^+$.

**2-[2-(2-Amino-ethoxy)-ethoxy]-*N*-[5-(4,7-di-*tert*-Boc-[1,4,

7]-triazonan-1-yl)-4-(4,7-di-*tert*-Boc-[1,4,7]-triazonan-1-ylmethyl)-pentyl]-acetamide (4): **2** (1.1 g, 1.19 mmol) and 10% Pd/C produced **4** (1 g, 1.11 mmol) in quantitative yield. ESI MS: m/z 901.7 $[MH]^-$.

7-[5-(2-{2-[2-(Acridin-9-ylamino)-ethoxy]-ethoxy}-acetyl-amino)-pentyl]-[1,4,7]-triazonane-1,4-dicarboxylic acid di-*tert*-butyl ester (5): To **3** (0.53 g, 0.95 mmol) in toluene 20 mL was added 9-phenoxyacridine[9] (257 mg, 1 equiv.). The reaction mixture was refluxed for 4 h. The mixture was cooled to room temperature. After column chromatography (CH_2Cl_2:MeOH:Et_3N = 9:1:0.1), **5** (0.42 g, 0.57 mmol) was obtained as a yellowish solid (61%). ^1H NMR (acetone-d_6): 8.40 (d, 2H, J = 8.7), 7.92 (d, 2H, J = 8.7), 7.69 (dd, 2H, J = 8.4, 1.2), 7.39 (dd, 2H, J = 8.4, 1.2), 7.24 (bs, 1H), 4.12 (t, 2H, J = 5.4), 3.95 (s, 2H), 3.90 (t, 2H, J = 5.4), 3.55-3.45 (m, 4H), 3.33-3.18 (m, 4H), 3.18-3.11 (q, 2H, J = 5.1), 2.59 (dd, 4H, J = 4.8), 2.44-2.36 (m, 2H), 1.48-1.23 (m, 24H), 1.25-1.176 (m, 2H), ESI MS: m/z 737.6 $[MH]^-$, 369.6 $[M\cdot 2H]^{2+}$.

2-{2-[2-(Acridin-9-ylamino)-ethoxy]-ethoxy}-*N*-[5-(4,7-di-*tert*-Boc-[1,4,7]-triazonan-1-yl)-4-(4,7-di-*tert*-Boc-[1,4,7]-triazonan-1-ylmethyl)-pentyl]-acetamide (6): 4 (400 mg, 0.44 mmol) and 9-phenoxyacridine (120 mg, 1 equiv.) produced **6** (220 mg, 0.3 mmol) as a yellow foam (68%) after column chromatography (CH_2Cl_2:MeOH:Et_3N = 9:1:0.1). ^1H NMR (acetone-d_6): 8.47 (d, 2H, J = 8.7), 8.0 (d, 2H, J = 8.7), 7.71 (dd,H, J = 8.4, 1.5), 7.36 (dd, 2H, J = 8.4, 1.5), 7.34 (t, 1H, J = 6.3), 4.2 (t, 2H, J = 5.1), 3.99-3.96 (m, 4H), 3.79-3.76 (m, 4H), 3.64-3.14 (m, 18H), 2.64 (m, 8H), 2.47-2.41 (m, 2H), 2.3-2.2 (m, 2H), 1.7-1.27 (m, 40H), ESI MS: m/z 1079.1 $[MH]^-$, 540.3 $[M\cdot 2H]^{2+}$.

2-{2-[2-(Acridin-9-ylamino)-ethoxy]-ethoxy}-*N*-(5-[1,4,7]-triazonan-1-yl-pentyl)-acetamide (L3): 5 (170 mg, 0.16 mmol) was dissolved in 20 mL of CH_2Cl_2:TFA (1:1) and stirred for 3 h at room temperature. After evaporation of solvent, the resulting syrup was washed with diethylether several times. **L3**·TFA salt (175 mg) was dissolved in 6 mL of CH_2Cl_2/MeOH (5:1), treated with Amberlyst 21 resin (10 equiv) for 1 h, filtered, and washed with 5-10 mL of 1:1 CH_2Cl_2/MeOH.[10] The combined filtrates were evaporated to give **L3** (100 mg, 0.147 mmol) as a free base amine. ESI MS: **L3**·TFA: m/z 537.6 $[MH]^+$, 651.6 $[M\cdot TFA]^-$, 269.5 $[M\cdot 2H]^{2-}$, ^1H NMR (acetone-d_6): **L3**: 8.42 (d, 2H, J = 8.7), 7.97 (d, 2H, J = 8.7), 7.76 (dd, 2H, J = 7.6), 7.44 (dd, 2H, J = 8.4), 4.40 (s,H_2O), 4.14 (t, 2H, J = 5.1), 3.98 (s, 2H), 3.90 (t, 2H, J = 4.8), 3.74 (m, 4H), 3.11 (m, 4H), 2.97-2.34 (*m*, 12H), 1.47-1.25 (m, 4H), 1.23-1.09 (m, 2H).

2-{2-[2-(Acridin-9-ylamino)-ethoxy]-ethoxy}-*N*-(5-[1,4,7]-triazonan-1-yl-4-[1,4,7]-triazonan-1-ylmethyl-pentyl)-acetamide (L4): L4 (100 mg, 0.18 mmol) was obtained from **4**·TFA salt (180 mg) as yellowish foam. ESI MS: **L4**·TFA: m/z 678.8 $[MH]^+$, 792.8 $[M\cdot TFA]^-$, 340.1 $[M\cdot 2H]^{2+}$. ^1H NMR (acetone-d_6): **L4**: 8.44 (d, 2H, J = 8.7), 7.99 (d, 2H, J = 8.7), 7.78 (dd, 2H, J = 7.8), 7.45 (dd, 2H, J = 8.7), 4.40 (s,H_2O), 4.16 (t, 2H, J = 5.1), 4.03 (s, 2H), 3.92 (t, 2H, J = 5.4), 3.77 (m, 4H), 3.49 (m, 2H), 3.21-2.27 (m, 28H), 1.75-1.53 (m, 2H), 1.55-1.19 (m, 4H).

L1/L3-$[Cu(II)]_2(NO_3)_4$·xH_2O and L2/L4-Cu(II)$(NO_3)_2$ xH_2O. Cu(II) complexes of L1-L4 were prepared by mixing ethanolic solution of the ligand and 1.1 or 2.1 equivalents of $Cu(NO_3)_2$, respectively. The resulting precipitate was filtered, washed with cold ethanol, diethylether, and dried *in vaccuo*. In general, The solution (5-100 mM) of L·xHCl and $Cu(NO_3)_2$ were prepared separately in an appropriate buffer and mixed just before use. **L1-$[Cu(II)]_2(NO_3)_4$ xH_2O**: ESI MS: 425.0 [M-4(NO_3)], 611.9 [M-(NO_3)]. **L2-Cu(II)$(NO_3)_2$ xH_2O**: ESI MS: m/z 253.9 [M-NO_3)]. **L3-$[Cu(II)]_2(NO_3)_4$ xH_2O**: ESI MS (−): 803.5 [M-4(NO_3)], 864.73 [M-3(NO_3)]. **L4-Cu(II)$(NO_3)_2$ xH_2O**: ESI MS: m/z 599.67 [M-2(NO_3)], 661.4 [M-(NO_3)].

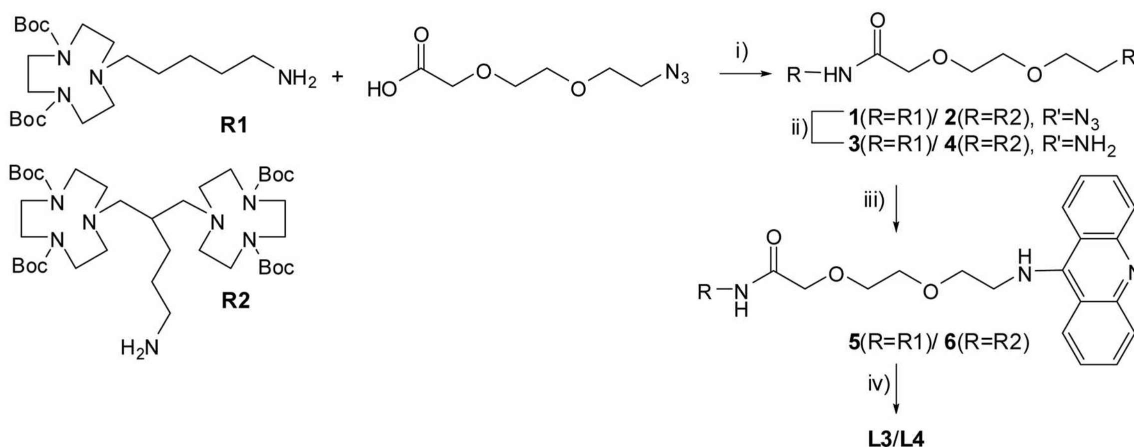
Spectroscopic measurement. Absorption spectra were recorded on SINCO 3100 UV spectrophotometer (Seoul, Korea). Solutions (100 μL) containing 10-20 μM substrate were incubated in cuvett at 25 °C for 10 min. The pH of the solution was maintained with 10 mMHEPES (pH 7.0, I = 10 mM $NaNO_3$). About 1 μL of an aliquot of CT-DNA stock solution (0.2-1 mM bp) was mixed. The DNA concentration was determined using extinction coefficient ε_{258nm} = 6700 $M^{-1}cm^{-1}$. In general, binding of a substrate to DNA produces hypochromism, a broadening of the band and a red-shift in the substrate absorption region. When the binding mode is homogeneous, the ground state association constant for the drug-DNA complex formation may be estimated from the changes in absorbance at a fixed wavelength, using the Benesi-Hildebrand equation.[11]

DNA cleavage. The DNA cleavage experiments were performed by the literature method.[2] The cleavage products were analyzed in 1% agarose gels. The gels were stained in buffer containing 1 μg/mL ethidium bromide and the extent of DNA degradation was determined by using volume quantitation method with KODAK EDAS 290 gel documentation system. The relative amounts of the different forms of DNA were determined by dividing absorbance intensity for a particular band by the total intensities of the each band in the same lane. The correction factor of 1.22 for form I DNA was utilized.

Results and Discussion

The acridine conjugates, **L3** and **L4** were synthesized from di-*tert*-butyl-1,4,7-triazonane-1,4-dicarboxylate *via* several steps including final 9-phenoxyacridine coupling (Scheme 1). Following deprotection of fully N-Boc-protected **L3** and **L4** by TFA/CH_2Cl_2 gave the ligand·TFA salts and treatment with ion exchange resin produced free bases **L3** and **L4**, respectively. Synthetic order should be kept as shown in Scheme since coupling **R1** or **R2** with the linker-acridine conjugate gave the desired product in very low yield. Characterization of the intermediates was based on Mass data. ^1H and ^{13}C NMR spectra were complex since there existed several conformers of N-Boc-protected tacn moieties.

Figure 2 showed UV/Vis absorption titration spectrum of the Cu_2L3 and CuL4 complexes with calf-thymus (CT) DNA at pH 7 and 25 °C. In the absence of DNA, 9-amino-



Scheme 1. Synthesis of ligands, L3 and L4. i) DCC, DMAP, CH_2Cl_2, RT. ii) H_2/Pd, EtOH. iii) 9-phenoxyacridine, toluene, 60 °C. iv) TFA/ CH_2Cl_2, amberlyst 21.

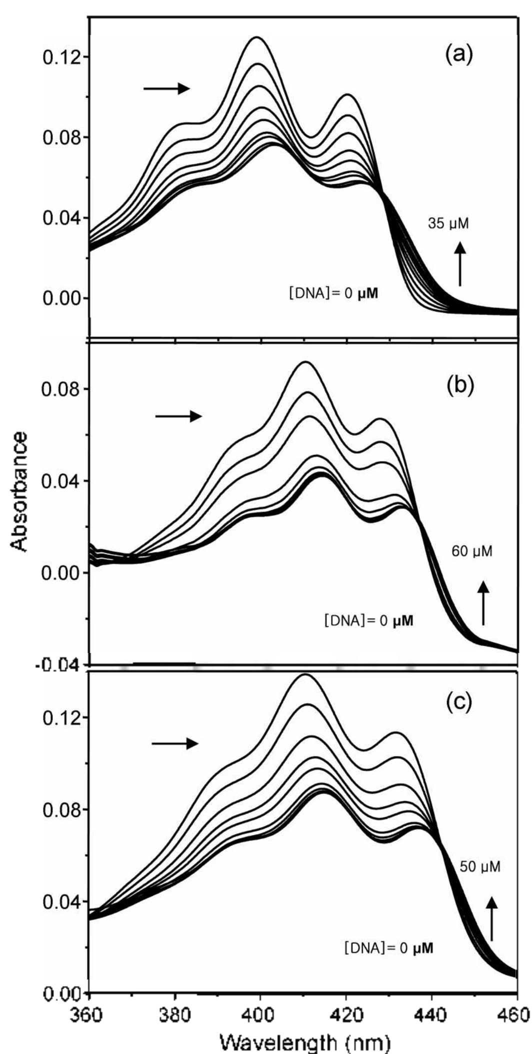


Figure 2. Absorption titration of CT-DNA by 9-aminiacridine (a), the Cu_2L3 (b) and CuL4, (c) complexes at pH 7.0 (10 mM HEPES), I = 10 mM $NaNO_3$, and 25 °C: [M] = 20 μM, [DNA] = 0-60 μM to the arrow direction.

acridine showed its maximum absorbance at 382 nm, 399 nm, and 420 nm and both Cu_2L3 and CuL4 complexes at

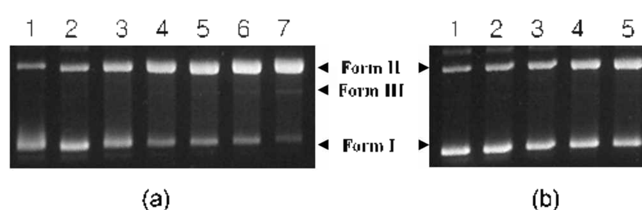


Figure 3. DNA (pCMV-Myc: 76 μM) cleavage at pH 7.3 (10 mMHEPES), I = 10 mM $NaNO_3$, and 30 °C: a) + Cu_2L3 (2 μM), lane 1-7: t = 0.1, 1, 3, 5.5, 8, 11, 24H, respectively. b) CuL4 (3.6 μM), lane 1-5: t = 1, 6, 11, 18, 24H, respectively.

392 nm, 411 nm, and 433 nm. Attachment of Cu_2L1 and CuL2 to 9-aminoacridine resulted in 10-13 nm red-shift suggesting interactions between the Cu and acridine moieties of the Cu_2L3 and CuL4 complexes. As the concentration of DNA increased, a 4-7 nm red shift for all absorption bands and *ca* 40-42% hypochromism for 9-aminoacridine, Cu_2L3 and CuL4 complexes were observed. The extent of spectral changes for all three compounds indicated that they all associated with DNA through a similar binding mode, intercalation.[12] The Benesi-Hildebrand plot was constructed from the change in absorbance at 435 nm for 9-aminoaridine, and at 450 nm for Cu_2L3 and CuL4 complexes.[11] Association constants were calculated to be 3.4×10^4 M^{-1}, 1.9×10^5 M^{-1} and 3.0×10^5 M^{-1}, for 9-aminoacridine, the Cu_2L3 and CuL4 complexes, respectively. Association constants for the acridine conjugates of the Cu complexes were not influenced significantly by the total charge on the catalytic group, rather depended on the length between the catalytic group and the acridine moiety as also shown in the previous experiment.[7]

The cleavage reaction of supercoiled DNA by the Cu complexes was followed by monitoring conversion of plasmid DNA to relaxed circular (form II) and linear (form III) forms, if any (Figure 3). The decrease of form I to form II was plotted against time and it was fitted well with first order exponential decay curve (Figure 4). The extents of DNA cleavage by the Cu_2L1 and Cu_2L3 complexes were proportional to the concentration up to 10-30 μM and

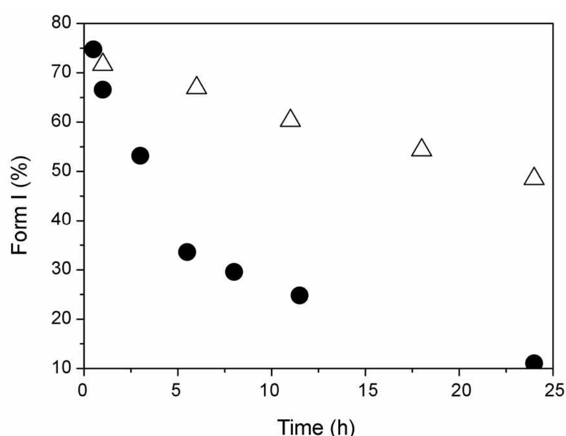


Figure 4. Plot of % degradation of DNA (pCMV-Myc: 76 μM) form I to form II by the Cu complexes at pH 7.3 and 30 °C: ●: Cu_2L3 (2 μM), △: CuL4 (3.6 μM).

decreased thereafter (data not shown). The ionic strength dependencies on the cleavage rates by both complexes indicated strong electrostatic interactions with DNA.[13]

From Figure 4, the second order rate constants for degradation of form I to form II were obtained as 20.0 $M^{-1}s^{-1}$ and 1.3 $M^{-1}s^{-1}$ by the Cu_2L3 and CuL4 complexes, respectively.[14] Since association constants of the Cu_2L3 and CuL4 complexes to DNA were about the same, the 20 fold rate enhancement observed could be ascribed to the cooperative role of two Cu ions in the Cu_2L3 complex. The similar mechanism had been suggested in Cu_2L1/CuL2 complexes mediated DNA hydrolysis reactions.[2] At the moment, however, the coordination modes of the acridine conjugates of the Cu complexes to DNA and the induced conformational changes are yet to be investigated.

It is important to determine the association constants of the metal complexes to DNA and measure the rate constants for the DNA cleavage, since each step could be a key step for the simple metal complexes in hydrolyzing phosphates. In our study, the rate constants for the mono and dinuclear Cu(II) complexes having the same binding moieties to DNA were directly compared for the first time. With the similar binding constants to DNA, the dinuclear Cu_2L3 complex was far more efficient than the corresponding mononuclear CuL4 complex in cleaving DNA, suggesting that cleavage of phosphate diester backbone of DNA was the key step in hydrolyzing DNA.

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14. Instead of the first order rate constant (k) obtained from Figure 4, the second order rate constant (k/[Cu]) was used for the direct comparison of efficiency since working concentrations of the catalysts were not the same.