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Synthetic DNA Ligands Conjugated with Metal Binding Moiety. Regulation of the Interaction with DNA by Metal Ions and the Ligand Effect on Metal Assisted DNA Cleaving

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Various functionalized molecules were synthesized by covalently conjugating DNA intercalators with metal chelators which carry such metal-binding functional groups as ethers, polyamines, and carboxylates. Systematic studies were made on the interaction of these conjugates with DNA in the presence and in the absence of metal ions. DNA binding of the conjugates were strongly affected by coexisting metal ions. This can be accounted for by the change in the net charge and in the conformation of the conjugates through complexation with metal ions. In some of the complexes thus obtained (DNA-intercalator-metal ion ternary complexes), DNA underwent a metal assisted cleavage of the strand, the activities of which were highly dependent on the nature of metal chelating moieties adopted. It is likely that a residual Lewis acidity on the metal in the complex and the strain in the DNA backbone induced by intercalation contribute to facilitate DNA cleavage.

INTRODUCTION

In all living systems, every biological reaction has to be mutually connected and reconciled with each other for attaining their homeostasis. It is well known that many of these homeostasis-related regulatory reactions are achieved at a DNA transcription level, in which the interaction of gene regulatory factors with DNA is controlled directly or indirectly by several means, e.g., dimerization,[1] phosphorylation,[2] binding with the third species such as proteins,[3] ligands including steroid hormones and metabolites,[4] and metal ions,[5] and so on. Among these regulatory means, metalloregulation of DNA-ligand interaction is one of the most promising strategy for the construction of artificial DNA ligands possessing on/off switching ability for DNA-ligand binding, because it is possible as occasions demand to choose an appropriate combination of metal ions and chelating reagents from the enormous library of assets in metal complex chemistry.

The authors have designed systematically the series of DNA ligands that are composed of two functionally different parts: one part carries a DNA intercalating function and the other has an

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ability to bind metal ions through such functional groups as polyether, polyamine, and polycarboxylate. The DNA binding characteristics of these synthetic ligands are expected to be influenced or regulated by metal ions coexisting in solution through metal complexation by the ligands, because the metal complexation by the ligands should lead to the change in the net electric charge as well as the change in the whole conformation of the ligands. It is also reasonable to expect that the DNA-cleaving activity (Lewis acid-base behavior) of metal ions is affected by such interactions. Preliminary studies on the series of conjugated DNA ligands have been already described by us and other research group[6] concerning their DNA binding and DNA cleaving behavior in the presence of metal ions. This paper is intended to present some comprehensive and systematic investigation of the interaction of these DNA ligands with DNA from a view emphasizing synthetic ligand-DNA-metal ternary interactions.

RESULTS AND DISCUSSION

As intercalator-metal chelator conjugate ligands, we synthesized anthraquinone derivatives indicated in Figure 1. Anthraquinone nuclei is well known as a DNA intercalating element, of which the derivatives have been widely studied for their thermodynamics and kinetics[7] in the interaction with DNA. As metal binding moieties, we adopted three types of typical metal chelating elements, i.e., crown ether (15-crown-5), polyamine (en, dien, and tren), and complexane

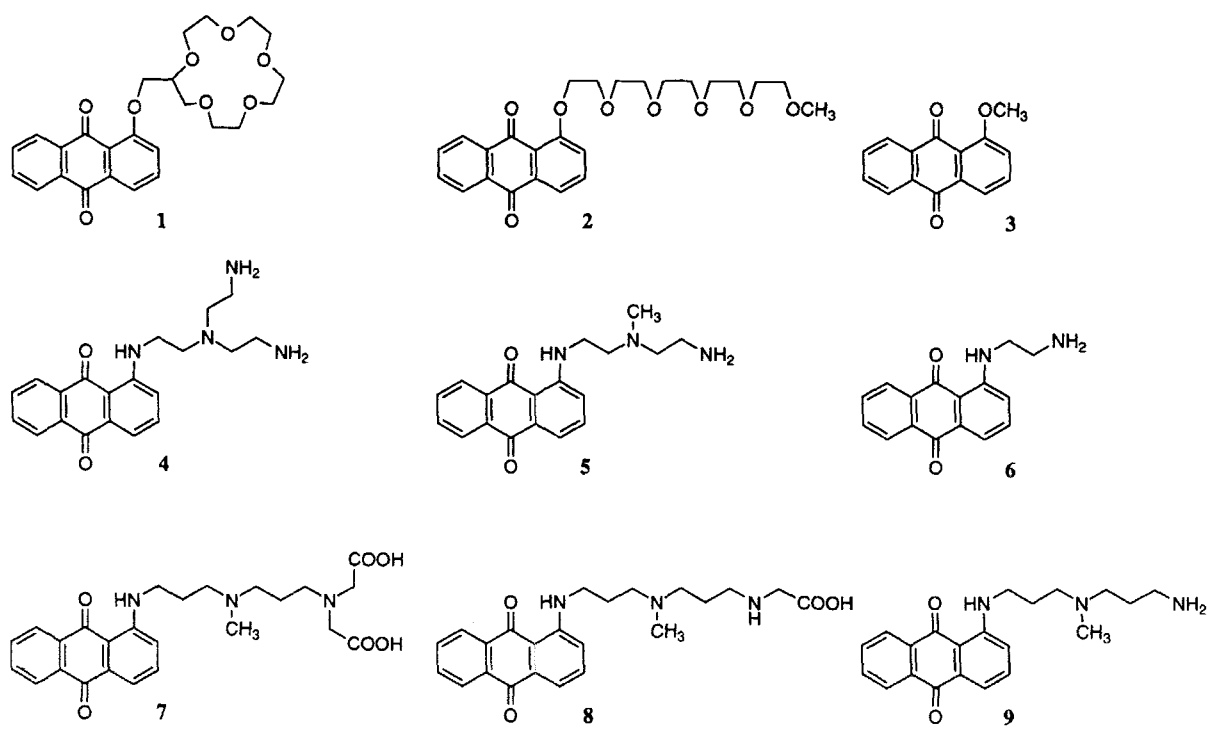


FIGURE 1 Synthetic DNA ligands bearing metal binding moiety and their control compounds.

(aminocarboxylic acids; iminodiacetic acid and glycine).

The spectra of polyamine (**4–6**, **9**)-and complexane (**7**, **8**)-anthraquinone conjugates indicated hypochromism and bathochromic shifts on interaction with calf thymus DNA. These spectral characteristics are attributed to a mode of binding which involves a stacking interaction between the aromatic chromophore and the base pairs of DNA. One example of these spectral changes is indicated in Figure 2 when an increasing amount of DNA is added to a ligand solution. The magnitude of the spectral change observed with **4–9** revealed that all these change reveals that these anthraquinone derivatives interacted with double-stranded DNA in an intercalative mode.[8]

Such clear spectral behaviors, however, were not observed for ether type anthraquinone derivatives (**1–3**) under some standard experimental conditions adopted above. Topoisomerase I assay were therefore carried out for these ligands to confirm the interaction mode with DNA. Intercalator molecules thrust between adjacent base pairs on binding with double stranded DNA. This causes a double helical DNA strand to unwind. Therefore, a helical unwinding of closed-circular DNA induced by ligand binding provides the evidence of intercalative interaction. The helical unwinding induced by noncovalently bound intercalators is able to be detected by the change in superhelical density in a closed-circular plasmid DNA, after relaxation of the plasmid in the presence of

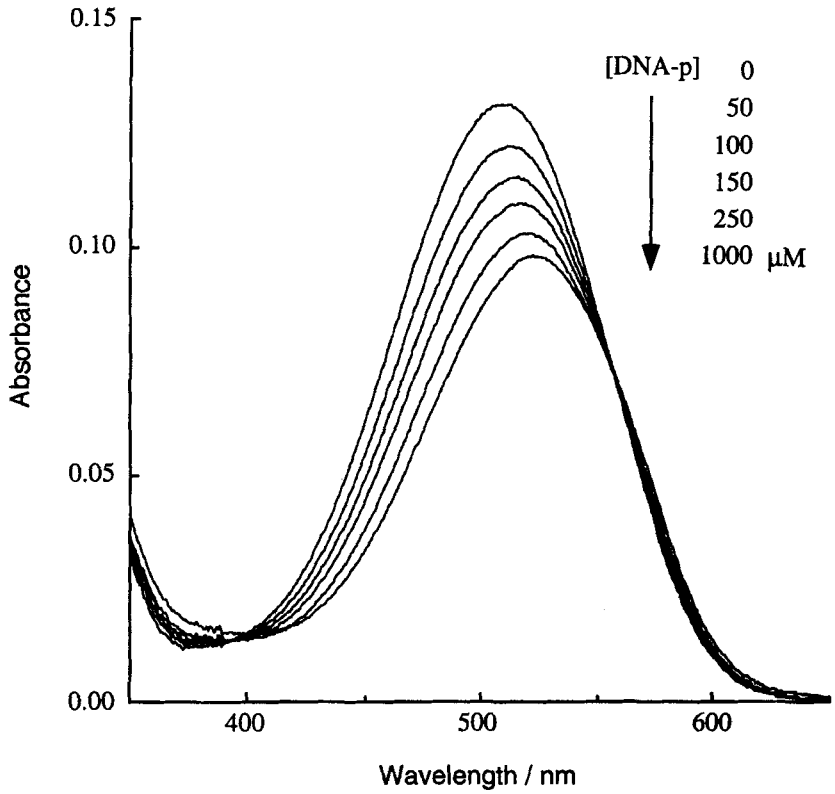


FIGURE 2 Spectral change of **5** on titration with calf thymus DNA. DNA phosphate molarity is (top to bottom curves at 500 nm) 0, 50, 100, 150, 250, and 1000 × 10^{-6}. The titration was conducted for 20 μM **5** in 1-cm cuvette in 0.2 mM HEPES (2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid) buffer (pH 7.0) with 0.1 M NaCl at 25°C by using Hitachi U-3210 spectrophotometer.

bound ligand by topoisomerase I and then removal of the ligand.[9] The result for **1** is indicated in Figure 3. The extent of residual superhelical density is increased with the increase of the concentration of **1**. This is a typical behavior for the ligand binding with DNA in an intercalation mode. Small changes in uv-visual spectra observed for ether-anthraquinone conjugates is quite likely to be attributed to their weak affinity with DNA rather than different type binding mode. In this connection one may recall that ethereal derivatives of anthraquinone (**1–3**) are electrically uncharged while the other derivatives (**4–9**) are net positively charged or at least carry a positive charge close to the intercalating anthraquinone nucleus under neutral pH region. In summation, it is now clearly shown that all the ligands **1–9** bind to DNA with their anthraquinone nuclei intercalated to double stranded DNA.

The Effect of Metal Ions on DNA Binding Behaviors

Binding properties of ether-, polyamine-, and complexane-anthraquinone conjugates with calf thymus DNA were studied in the presence of targeted metal ions. The results are shown in Figures 4, 5, and 6. In all figures, the fraction of the DNA-bound ligands increases with increasing the P/D ratio. Binding affinity of the ligands were enhanced upon the addition of metal ions for **1**, **4**, **6**, **7**, and **8**. For other ligands (**2**, **3**, **5**, and **9**), the effect of metal ion was negligible or, in some cases, even negative. The binding constants with DNA, summarized in Tables I and II, were determined by analyzing the spectral change of the ligand on DNA titration in a standard manner using McGhee and von Hippel equation.[6,7b,10]

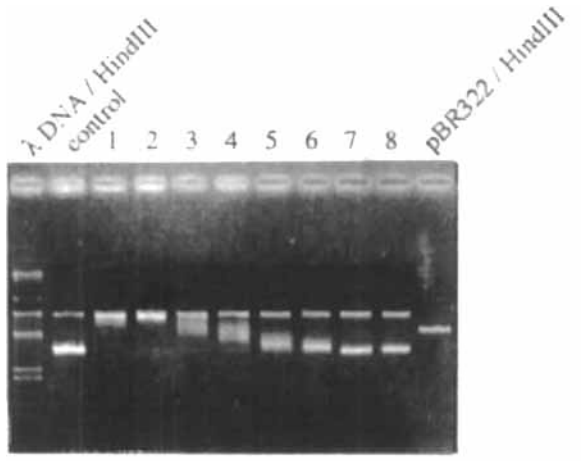


FIGURE 3 Unwinding of pBR322 DNA by **1** after incubation with topoisomerase I in the presence of increasing concentrations of **1**. lane 1, DNA and topoisomerase alone; lane 2–8, DNA, topoisomerase, and increasing **1** concentrations from 20 to 200 μM. Detail condition of topoisomerase I reaction is indicated in Materials and Methods.

The DNA binding behaviors of 15-crown-5-anthraquinone conjugates, **1**, and its control molecules, **2** and **3**, were studied by filter-binding assay due to their poor spectral change (Figure 4). Here we focus on only qualitative discussions for their interaction with DNA because of the difficulty in making quantitative measurements due to their weak DNA binding affinity. The binding of **1** to DNA is strongly influenced by the nature of alkali metal ions in solution as shown in Figure 4 (a). This is not the case at all with **2** and **3**. The DNA binding affinity of **1** increases in the order of $Na^+ \geq K^+ > NH_4^+ > Li^+$, which is similar to the order of metal complex stability of 15-crown-5 ($Na^+ = K^+ > NH_4^+ > Li^+$) in methanol. The binding behavior of **1** in the absence of cation was almost the same with that in the presence of Li^+. These observations are rationalized quite well by considering the picture of metal-assisted intercalation we postulated, in which electrically uncharged DNA ligand, **1**, forms a cationic complex with Na^+ or K^+ at least in the microenvironment around the double-helical DNA, thus acquiring a significant DNA binding activity. Similar behaviors were also observed for other crown-intercalator that have 15-crown-5 and acridine instead of anthraquinone nucleus.[11]

Similarly, Table I and II shows that DNA binding of polyamine-and complexane-anthraquinone conjugates are appreciably affected by coexisting Cu^{2+}. In order to obtain insight

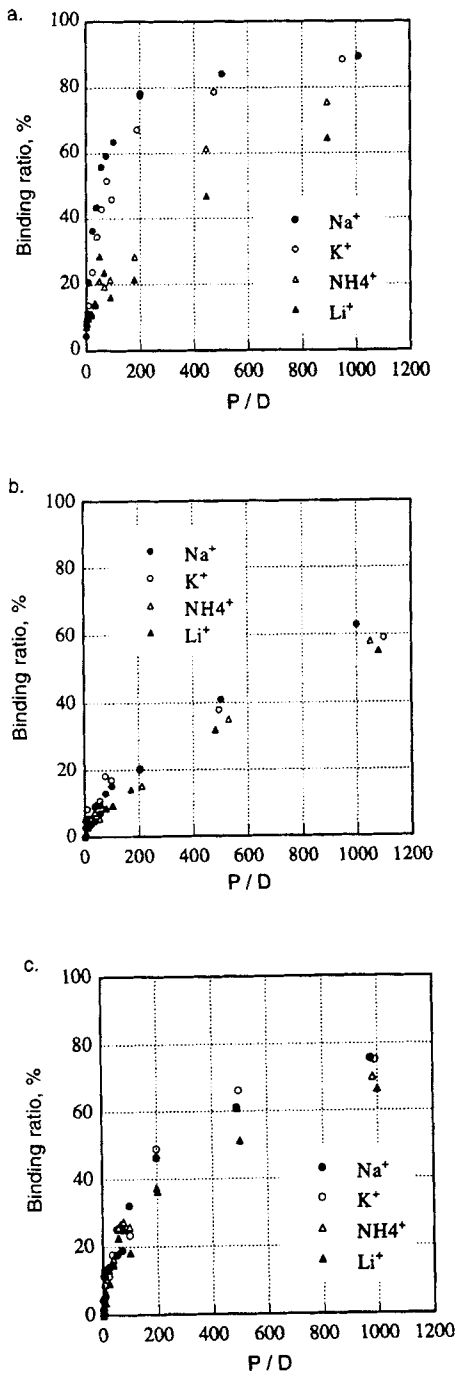


FIGURE 4 Binding profiles of ether-anthraquinone conjugates with calf thymus DNA in the presence of various monovalent cations (25°C). Filter-binding assay were carried out in the presence of 10 μM of (a) **1**, (b) **2**, and (c) **3** and increasing amount of DNA in borate buffer (pH 7.5) containing 0.1 M of alkali metal or ammonium ion. P/D indicates the ratio of DNA phosphate to ligands.

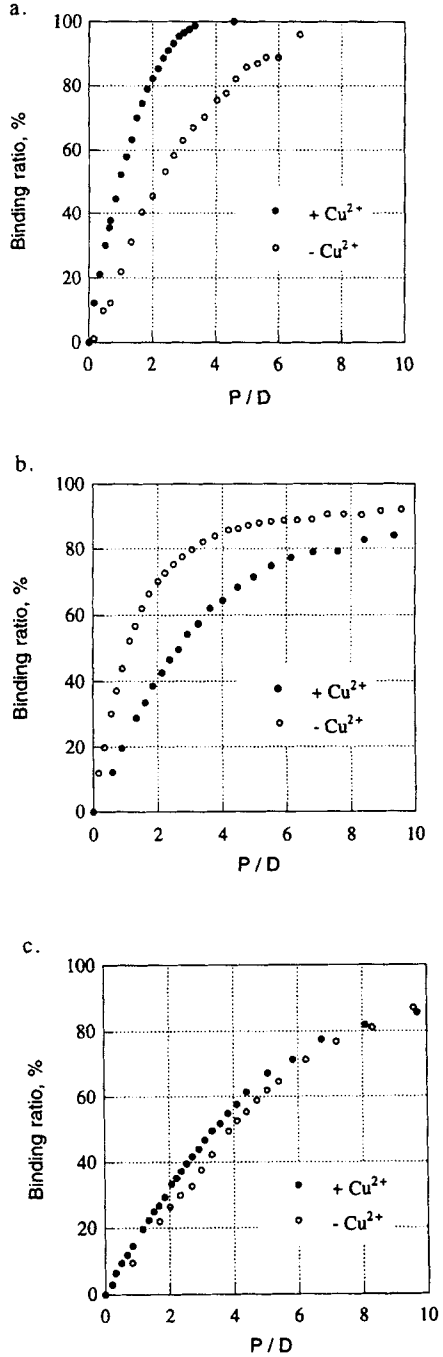


FIGURE 5 Binding profiles of polyamine-anthraquinone conjugates with calf thymus DNA in the presence and absence of Cu^{2+} (25°C). Calf thymus DNA were titrated into 30 μM of (a) **4**, (b) **5**, and (c) **6** aqueous solutions, which contain 0.2 mM HEPES (pH 7.0) and 0.1 M NaCl. In the experiments for Cu^{2+} effect, 30 μM Cu^{2+} were added on the above experimental condition. P/D indicates the ratio of DNA phosphate to ligands.

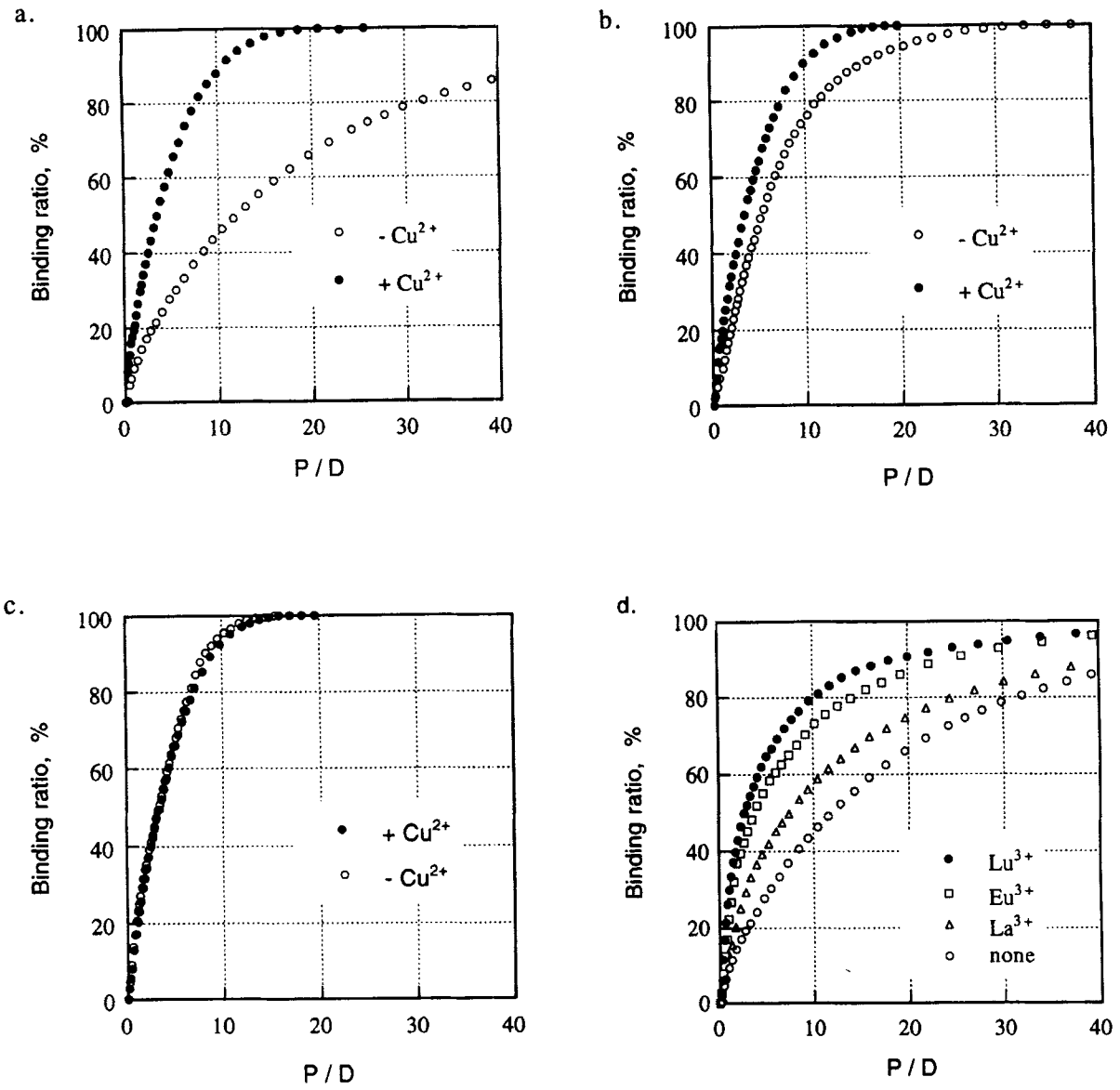


FIGURE 6 Binding profiles of complexane-anthraquinone conjugates with calf thymus DNA (a–c) in the presence and absence of Cu^{2+} and (d) in the presence of lanthanoid metals (25°C). Calf thymus DNA were titrated into 30 μM of (a, d) **7**, (b) **8**, and (c) **9** aqueous solutions, which contain 0.2 mM HEPES (pH 7.0) and 0.1 M NaCl. In the experiments for metal ion effect, 30 μM (a, b, c) Cu^{2+} and lanthanoid ions (d) were added on the above experimental condition. P/D indicates the ratio of DNA phosphate to ligands.

into the structure of metal complexes involved, the solutions of polyamine-anthraquinone conjugates were titrated with a Cu^{2+} standard solution in the presence of excess amount of DNA. Figure 7 shows the changes in the absorption spectra of the ligands. The existence of isosbestic points in each set of spectra indicates that the complexation is based on a single equilibrium reaction. Job's plots show that the stoichiometry of the complexations are clearly one molecule of Cu^{2+} ion to one molecule of polyamine-anthraquinone conjugates (data not shown). Although it is known that Cu^{2+} can bind to the phosphate groups and the bases of DNA, Cu^{2+},

TABLE I Binding constants[a] of polyamine type anthraquinone derivatives with calf thymus DNA at 298 K in the presence and absence of Cu(II).

ligand	binding constant, $K \times 10^{-4}/dm^3mol^{-1}$	
	without Cu(II)	with Cu(II)[b]
4	14	29
5	5.3	2.8
6	4.6	6.4

[a]The constants were obtained for 30 μM of ligands in a 0.3 mmol dm^{-3} HEPES (2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid) buffer with 0.1 mol dm^{-3} NaCl (pH 7.0). Experimental error is within ±4%.
[b]Equimolar Cu(II) to the ligands were used.

in the present system, binds strongly to the polyamine moieties of the DNA ligands even in the presence of a large excess of DNA phosphates and bases. This is a good indication of an intimate ternary interaction among DNA strand, heterocycle, and Cu^{2+} ion.

Unusually large spectral change was observed in the Cu^{2+} titration of **5**-DNA system. In fact, completely the same spectral behavior was observed in the absence of DNA. Therefore, the Cu^{2+} complexation reactions were also studied by pH titration photometry (data not shown). The data suggested the following points: (i) the structures in Figure 8 dominate at pH 7.0, (ii) the Cu^{2+}-**5** is formed at pH 6.0 and stays unchanged at higher pH, and (iii) Cu^{2+}-**4** and Cu^{2+}-**6** undergo further deprotonation at pH above 7.0. It

TABLE II Binding constants[a] of polyamine type anthraquinone derivatives with calf thymus DNA at 298 K in the presence and absence of Cu(II).

ligand	binding constant, $K \times 10^{-4}/dm^3mol^{-1}$	
	without Cu(II)	with Cu(II)[b]
7	1.0	6.9
		6.3
8	4.8	8.2
9	12	11

[a]The constants were obtained for 20 μM of ligands in a 0.2 mmol dm^{-3} HEPES (2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid) buffer with 0.1 mol dm^{-3} NaCl (pH 7.0). Experimental error is within ±4%.
[b]Equimolar Cu(II) to the ligands were used except for the data indicated by italic, which was obtained in the presence of one-half molar Cu(II) to **7**.

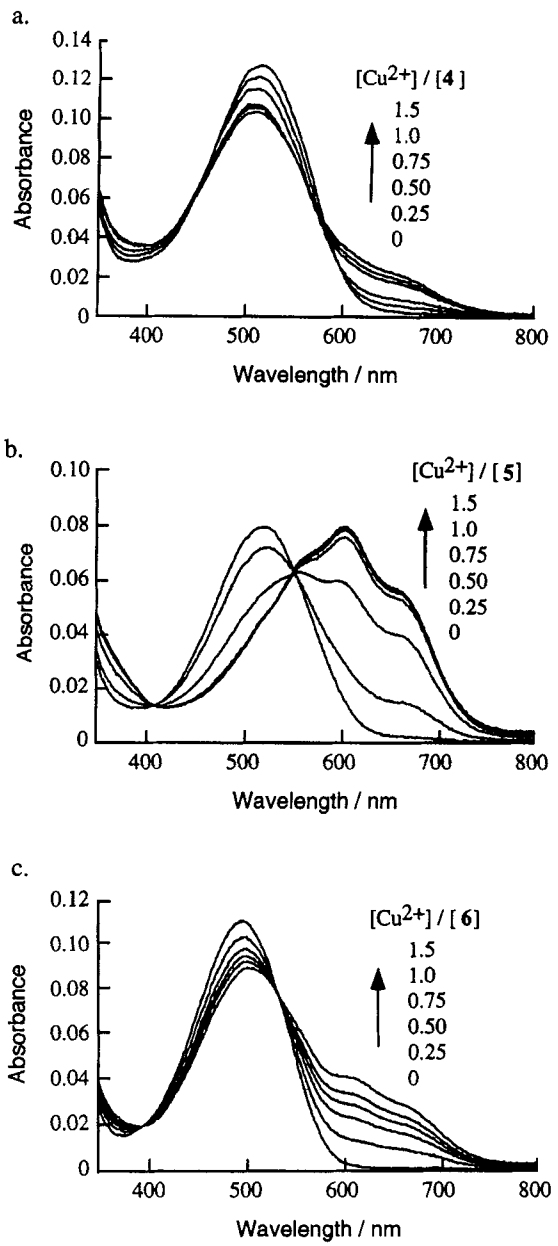
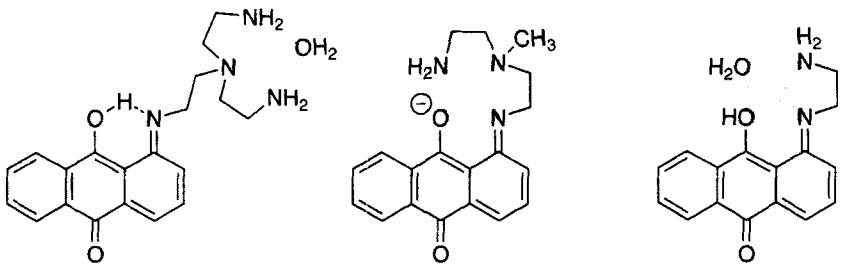


FIGURE 7 Spectral change of polyamine-anthraquinone conjugates on titration with Cu^{2+} in the presence of calf thymus DNA. The Cu^{2+} ratio to the ligands is (bottom to top curves at 650 nm) 0, 0.25, 0.50, 0.75, 1.0, and 1.5. The titration was conducted for 30 μM of (a) **4**, (b) **5**, and (c) **6** in the presence of 300 μM DNA phosphate in 1-cm cuvette in 0.2 mM HEPES buffer (pH 7.0) with 0.1 M NaCl at 25°C by using Hitachi U-3210 spectrophotometer.

is emphasized that the deprotonation of aromatic hydroxyl group takes place only for



Cu^{2+} - **4** Cu^{2+} - **5** Cu^{2+} - **6**

FIGURE 8 Possible structures of the complexes of polyamine-anthraquinone conjugates with Cu^{2+} at pH 7.0. Square-planer coordination around Cu^{2+} is indicated by shade.

Cu^{2+}-**5** at moderately low pH (pH7.0). This is in agreement with the observed decrease of DNA binding constant of **5** at pH 7.0 in the presence of Cu^{2+} (in the absence of Cu^{2+}, **5** can behave as dipositive cation at pH7.0). The slight increase in binding constant of **6** in the presence of Cu^{2+} may be attributed to the increase of its net charge, from +1 to +2, with the Cu^{2+} complexation. It is notable that **4** forms the most stable associates with DNA among polyamine-anthraquinone conjugates both in the absence and in the presence of Cu^{2+} ion. It is undoubtedly due to its stable dipositive charge (both in the absence and in the presence of Cu^{2+}) in the neutral pH region, but it could also be due to an increased hydrogen bonding interaction between the amino groups in **4** and the nucleic bases. It is known that an intercalation is stabilized by an additional hydrogen bonding with guanine base in minor groove of double stranded DNA if the intercalator has an aliphatic side chain that carries a functional group capable of hydrogen bonding.[12] Therefore, it is possible that the DNA-**4** complex involves additional hydrogen bonds between amino group in tren moiety and DNA bases, which is likely strengthened by the conformational change of tren moiety induced by the complexation with Cu^{2+}.

The possible structures and the net associated charge of Cu^{2+}-**7**,-**8**, and-**9** complex species predominant at pH 7.0 are indicated in Figure 9. Basically, the Cu^{2+} effect on the binding constant of complexane-anthraquinone conjugate with DNA is also explicable by the charge effect of the complexes (Table II). The binding constants of **7** and **8** are increased by complexation with Cu^{2+}, while that of **9** (control compound for **7** and **8**) is scarcely affected by the presence of Cu^{2+}. In fact, Cu^{2+} titration revealed that **9** essentially did not complex with Cu^{2+} (data not shown); this complexation requires **9** to form unstable aliphatic 6-membered ring including the coordination of tertiary amine nitrogen, which compares with a Table V-membered ring formed by **5**. On the other hand, **7** was characterized by seven-fold increase in the binding constant reflecting the induction of cationic charge on the ligand.

It is notable that, only for **7**, significant enhancement of binding with DNA was observed even in the presence of a half molar amount of Cu^{2+}, which was almost the same as that enhancement in the presence of equimolar Cu^{2+} (Table II). This can not be account for by simple charge effect. Figure 10 shows the Job's plots based on the spectral change of **7** and **8** on Cu^{2+} addition. The behaviors of the two complexane type DNA ligands are totally different from each other. Contrary to the existence of only one complex species for **8**, two kinds of complexes are obviously formed according to the molar ratio of Cu^{2+} to **7**. Exactly the same behaviors were observed even in the presence of excess amount of DNA (data not shown). It is apparent that the complex of **7** formed at Cu^{2+}/ligand molar ratios below 0.5 is 1:2 complex and that formed

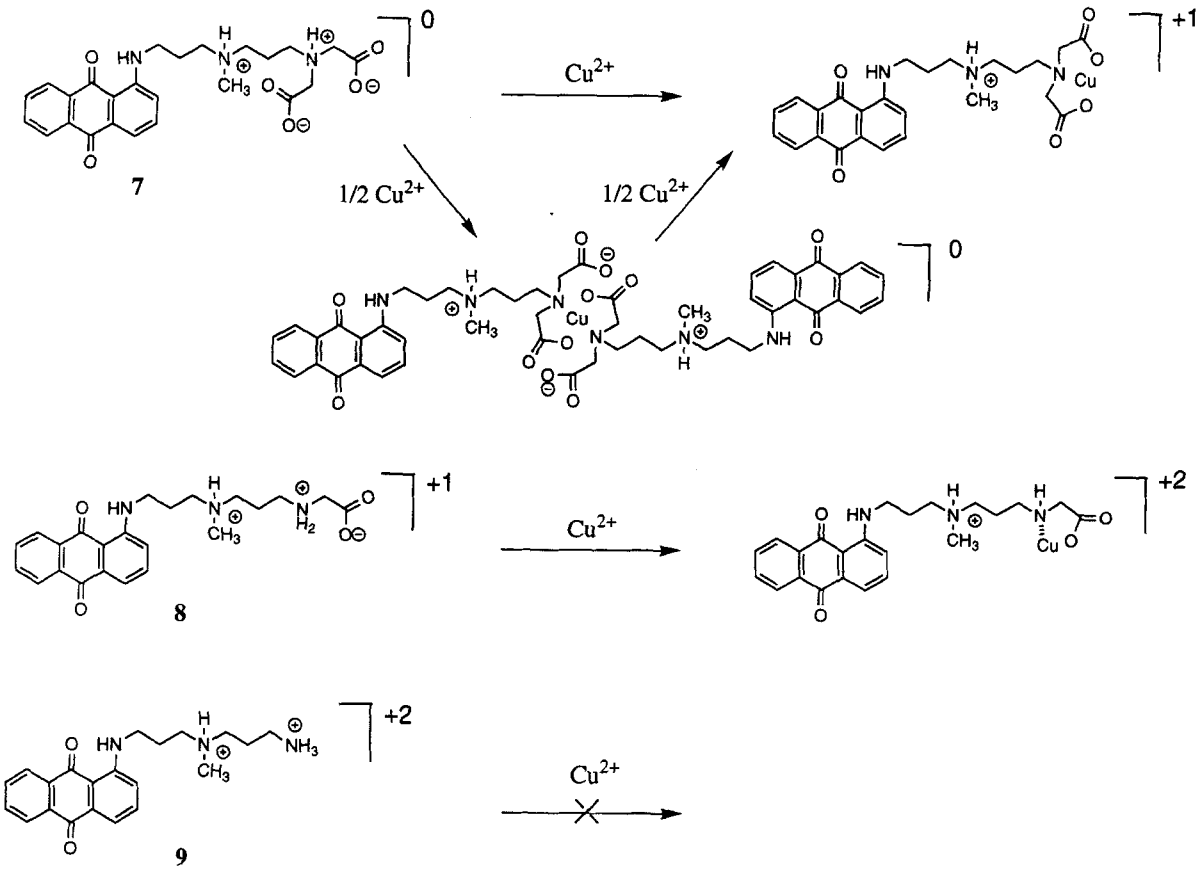


FIGURE 9 Possible structures and net charge of complexane-anthraquinone conjugate and their complex with Cu^{2+} at pH 7.0.

above 0.5 is 1:1 complex. Both the complexes show almost the same binding affinity to DNA which is 6–7 times greater than that of the free ligand 7 (Table II). It is interesting to point out that 1:2 Cu^{+2} complex and the free ligand 7 are both uncharged under the conditions of DNA binding measurements (Figure 9) but the former shows much greater affinity to DNA than the latter. It is presumably related to the molecular conformation of the 1:2 Cu^{2+} complex that allows the two anthraquinone nuclei to intercalate simultaneously when bound to the DNA double strand, i.e., the complex presumably is functioning as a bisintercalator. Spectral measurements are in support of this; the hypochromic and bathochromic shift in the absorption spectrum of 7 on binding to DNA in Cu^{2+}/7 ratio 0.5 is as remarkable as when the Cu^{2+}/7 ratio is 1–2. That the enhancement in affinity in changing from mono- to bis-intercalating reaction is limited to less than one order level, rather than the factor of 10^4 which one may expect, may be attributed to ensuing unfavorable distortions in the Cu^{2+}-7 complex and/or DNA on complex formation.[13] Optimization and fine tuning of linker structure between the two anthraquinone units should lead to realizing the more effective control of DNA binding property. Anyway, this indicates the possibility of regulating the binding affinity of DNA ligand through the dimerization of functional (intercalating) units by using a complexation with metal ion.

It is known that iminodiacetic acid captures lanthanoid metal moderately; the former is a "hard" base and the latter, a "hard" acid, respectively, according to the Lewis theory. DNA bind-

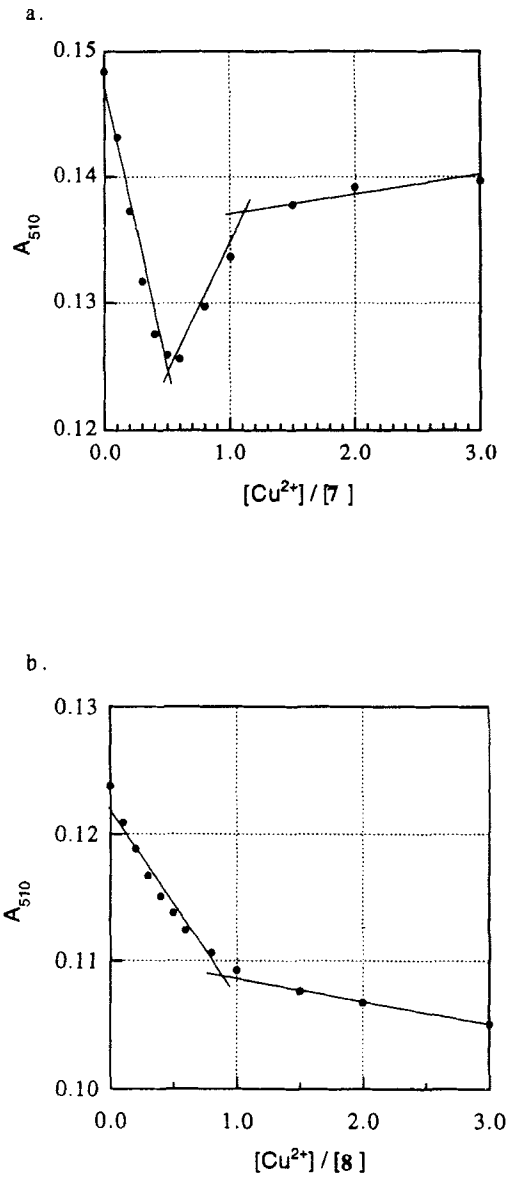


FIGURE 10 Absorbance changes of complexane-anthraquinone conjugates on titration with Cu^{2+}. Copper solution were titrated into 20 μM (a) **7** and (b) **8** solution containing 0.2 mM HEPES (pH 7.0) and 0.1 M NaCl at 25°C. The processes were monitored by the change of optical absorbance at 500 nm by using Hitachi U-3210 spectrophotometer.

ing behaviors of **7** in the presence of some lanthanoid ions are indicated in Figure 6 (d). These data show that lanthanoid ions also stabilize the DNA-ligand complex. The order of this effect is $Lu^{3+} > Eu^{3+} > La^{3+}$, which coincides with the order of the magnitude of the binding affinity of iminodiacetic acid to these lanthanoid ions.[14] The extent of the stabilization effect of lanthanoid ions is, however, smaller than that of Cu^{2+}. Treating the complexation behavior of ternary system is very complicated. It can not be discussed on a simplified picture based on the separate binary systems. However, some comments though oversimplified, may deserve mention concerning the difference in behavior among the metal ions which affect or regulate the binding of **7** to DNA. Both DNA and iminodiacetic acid can complex metal ions in binary system, including of course Cu^{2+}. However, the complexing affinity of Cu^{2+} to iminodiacetic acid function is so strong under neutral pH that the complexation between Cu^{2+} and **7** is unaffected at all even in the presence of a large excess of DNA, i.e., an isolated Cu^{2+}-DNA binary interaction is not important in the Cu^{2+}-DNA-**7** ternary interaction. On the other hand, for Ln^{3+}-DNA-**7** ternary system, Ln^{3+}-DNA binary interaction seems to be already quite strong as is suggested by the observation that even under micromolar concentrations of DNA and Ln^{3+} a charge-neutralized DNA-Ln^{3+} complex or their salt precipitates out from the solution.[15,16] The complex stability constants of iminodiacetic acid with Ln^{3+} are not so high as that with Cu^{2+}. Therefore, a possibility arises that in Ln^{3+}-DNA-**7** ternary system a considerable portion of Ln^{3+} is consumed in Ln^{3+}-DNA binary interaction and a relatively small remainder of Ln^{3+} is left for interaction with **7**. This may explain the observed behavior of Ln^{3+}-DNA-**7** ternary interaction mentioned above. A ternary system Hg^{2+}-DNA-**7** was briefly studied in this connection. The addition of Hg^{2+} to DNA-**7** binary system caused a significant reduction of DNA-**7** binding (data not shown). This may be attributed to the induction of large conformational change in double stranded DNA by Hg^{2+} which makes intrinsically strong interaction with DNA.[17] These data show that we have to consider some inherent affinity of metal ions to DNA as well as the affinity to metal chelator when we choose an ap-

propriate combination of chelator and targeted metal ion for designing good metalloregulation system.

Metal Assisted DNA Cleavage

The effect of these conjugated DNA ligands on DNA cleaving activity of metal ions such as Cu^{2+} and lanthanoid ions was studied to shed light on the chemistry of the ternary complex formation and to obtain a clue for controlling the DNA cleaving activity of metal ions. Copper (II) has been widely used as a catalyst for the hydrolysis of organic phosphate esters and for radical degradation of nucleic acid under the combined use of oxidant and reductant such as hydroperoxide and ascorbic acid. Recently, lanthanoid metals are drawing much attention as an effective catalyst for hydrolysis of nucleic acid[18].

Figure 11 and 12 shows the results of supercoil DNA relaxation assay in the presence of ether-anthraquinone conjugates and Cu^{2+} by using pBR322 plasmid DNA. The relaxation reactivity under the combined use of Cu^{2+} and **1** is much greater than the sum of that in the presence of Cu^{2+} alone and that in the presence of **1** alone (Figure 11). Thus, a cooperation with Cu^{2+} is observed for **1**, but not in the case for **2** and **3**. Although the complex formation of Cu^{2+} with 15C-5 is negligible in water or even in methanol, it is very interesting that Cu^{2+} and **1** function in a synergistic way for DNA cleavage.

As mentioned earlier the DNA binding equilibria were not accurately assayed for intercalating conjugates **1**, **2**, and **3** because of their low binding affinity. However, spectrophotometric measurements definitely indicated that the extent of DNA binding under standard conditions is much lower for **1** as compared with **2** and **3** (data not shown). In spite of this, **1** showed the greatest DNA cleaving effect among the three ether-derivatized intercalators in the presence of Cu^{+2} as mentioned above. These behaviors of **1** should certainly be associated with the steric

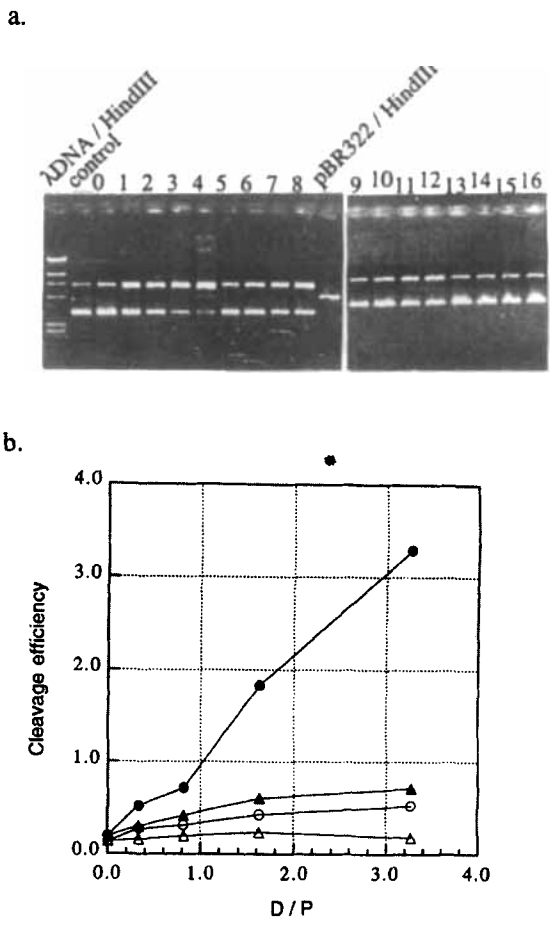


FIGURE 11 Concentration effect of ether-anthraquinone conjugates on the DNA cleavage by Cu^{2+}. (a) lane 0, 2 μM Cu^{2+} alone; lane 1–4, 2 μM Cu^{2+} and 20, 50, 100, and 200 μM **1**, respectively; lane 5–8, 2 μM Cu^{2+} and 20, 50, 100, and 200 μM **3**, respectively; lane 9–12, 20, 50, 100, and 200 μM **1**, respectively; lane 13–16, 20, 50, 100, and 200 μM **3**, respectively; reaction condition, the 10 μl of aqueous solutions of 60 μM-p pBR322 DNA containing 5 mM Tris-HCl (pH 6.7), ligands and Cu^{2+} were incubated for 12 h at 37°C. (b) The cleavage efficiency plotted in the ordinate represents the normalized ratio of the nicked open circular DNA over the supercoiled DNA. D/P indicates the ratio of the ligands to DNA phosphate. closed circle, Cu^{2+} and increase amount of **1** (lane 1–4); closed triangle, Cu^{2+} and increase amount of **3** (lane 5–8); open circle, **1** alone (lane 9–12); open triangle, **3** alone (lane 13–16).

bulkiness of crown ether group in **1** when bound to DNA; the binding of **1** to DNA is supposed to cause much greater strain to the phosphate ester bond connecting the neighboring base-pairs at the intercalated anthraquinone site. The strained phosphate ester bond should be more susceptible to cleavage. Two more points may be further

made on this suggested importance of intercalated **1**. 1) linear (form III) DNA began to appear in the condition where closed circular (form I) DNA still existed (Figure 11). Presumably, it is due to some localized strain on the both strands, which was induced by the intercalation effect as well as by some concentration effect of Cu^{2+} around a peculiar microenvironment in the vicinity of DNA-ligand complex. 2) The cleavage activity dependence on the concentration of

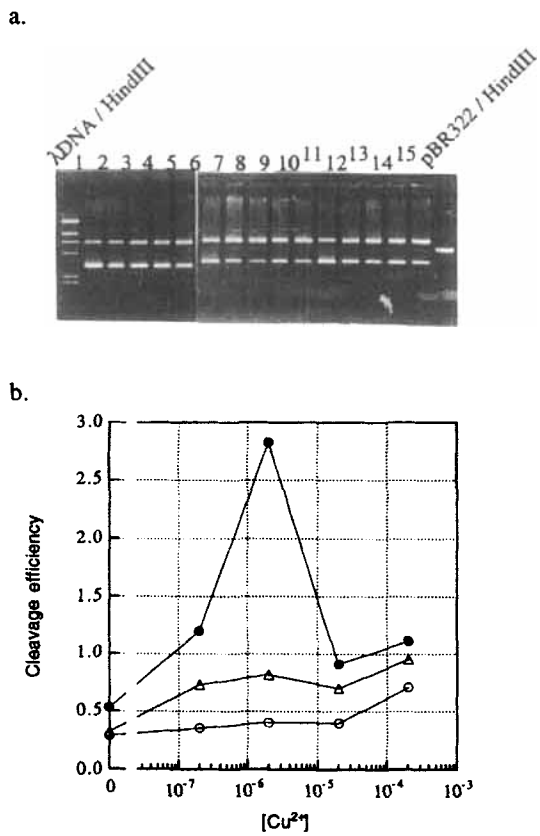


FIGURE 12 Concentration effect of Cu^{2+} on the DNA cleavage in the presence of ether-anthraquinone conjugates. (a) lane 1–5, 0, 0.2, 2.0, 20, and 200 μM Cu^{2+}, respectively; lane 6–10, 200 μM **1**, 0, 0.2, 2.0, 20, and 200 μM Cu^{2+}, respectively; lane 11–15, 200 μM **3**, 0, 0.2, 2.0, 20, and 200 μM Cu^{2+}, respectively; reaction condition, the 10 μl of aqueous solutions of 60 μM-p pBR322 DNA containing 5 mM Tris-HCl (pH 6.7), ligands and Cu^{2+} were incubated for 12 h at 37°C. (b) The cleavage efficiency plotted in the ordinate represents the normalized ratio of the nicked open circular DNA over the supercoiled DNA. D/P indicates the ratio of the ligands to DNA phosphate. open circle, Cu^{2+} alone (lane 1–5); closed circle, Cu^{2+} and **1** (lane 6–10); open triangle, Cu^{2+} and **3** (lane 11–15).

Cu^{2+} in this system exhibited a definite maximum at Cu^{2+}/DNA = 0.03, which was followed by a rapid drop at 0.33 (Figure 12). It is known that the interaction of Cu^{2+} with DNA depends on the molar ratio fed in the system.[19] Under the conditions of the ratio below 0.25, Cu^{2+} interacts with the phosphate anion in the DNA backbone and stabilizes the double stranded structure. On the other hand, under the conditions above this ratio (Cu^{2+}/DNA > 0.25), the double helix structure is destabilized because of the Cu^{2+} binding to the nucleic bases of DNA. The maximum point of cleaving activity is coincide with this Cu^{2+}/DNA critical ratio. It seems reasonable to suppose that intercalator **1** lose its binding pocket above the critical ratio and can not induce an effective strain for cleaving DNA strands.

Synergistic effect was also observed in the case of La^{3+} (Figure 13). This is the first example of cooperative DNA hydrolysis that is attained by using La^{3+} and DNA intercalator bearing metal binding capability. Lanthanoid ions as typical "hard" acids strongly bind to DNA phosphate groups as mentioned above while they are reasonably assumed to have very low affinity to "soft" nitrogen bases of nucleic bases; lanthanoid ions are already concentrated on the periphery of DNA even in the absence of **1**. It is likely that the strain on the DNA double strand backbone induced by the intercalation plays important role in the hydrolysis of phosphodiester bond. There are many enzymes which accelerate chemical reactions by inducing a bond strain on substrate (stabilization of transition state). This notion has been widely accepted as one of the most common strategy in facilitating the chemical process especially in biomimetic chemistry.

Figure 14 shows the DNA cleaving efficiencies in the presence of Cu^{2+} and polyamine-anthraquinone conjugates by relaxation assay. It should be emphasized that a cooperation of **4** with Cu^{2+} was observed, but not in the case of **5** or **6**. In a reference assay, en (ethylenediamine), dien (*N*, *N*-bis(2-aminoethyl)methylamine) or

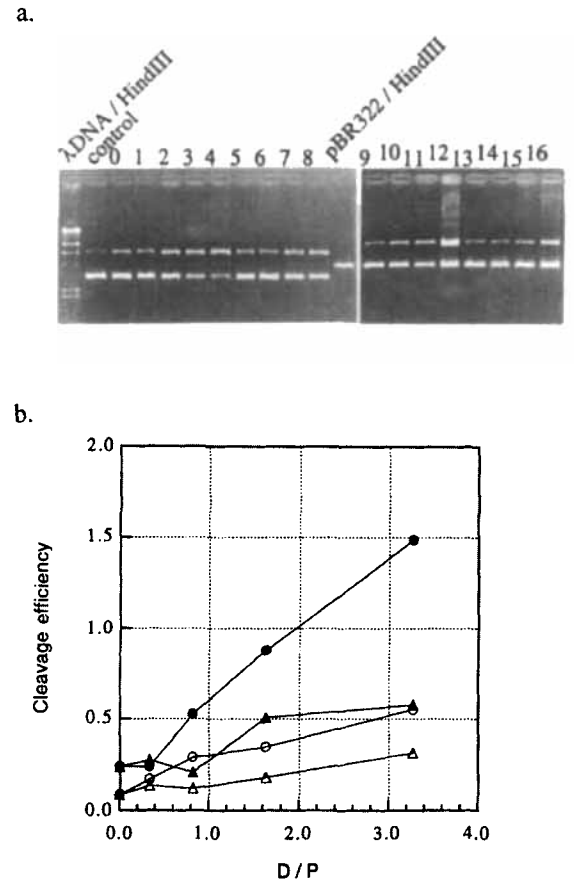


FIGURE 13 Concentration effect of ether-anthraquinone conjugates on the DNA cleavage by La^{3+}. (a) lane 0, 2 μM La^{3+} alone; lane 1–4, 2 μM La^{3+} and 20, 50, 100, and 200 μM **1**, respectively; lane 5–8, 2 μM La^{3+} and 20, 50, 100, and 200 μM **3**, respectively; lane 9–12, 20, 50, 100, and 200 μM **1**, respectively; lane 13–16, 20, 50, 100, and 200 μM **3**, respectively; reaction condition, *the 10 μl of aqueous solutions of 60 μM-p* pBR322 DNA containing 5 mM Tris-HCl (pH 8.0), ligands and La^{3+} were incubated for 24 h at 37°C. (b) The cleavage efficiency plotted in the ordinate represents the normalized ratio of the nicked open circular DNA over the supercoiled DNA. D/P indicates the ratio of the ligands to DNA phosphate. closed circle, La^{3+} and **1** (lane 1–4); closed triangle, La^{3+} and **3** (lane 5–8); open circle, **1** alone (lane 9–12); open triangle, **3** alone (13–16).

tren (tris(2-aminoethyl)amine) did not cause any cleavage enhancement (Only the data for tren was indicated in Figure 14 as a representative.). What is notable is that the apparent difference in DNA cleaving capability between **4**, **5** and **6** in the DNA relaxation study in spite of their comparable DNA binding ratio; under this condition, the binding sites on DNA are likely to be saturated with these conjugates. Therefore, the reason of the large activity difference observed here should be attributed to some special conformational or structural effect of Cu^{2+}-DNA ligand complexes. In this connection, the proposed structure of Cu^{2+}-ligand complexes may be consults (Figure 8). The Cu^{2+}-**4** complex can afford the anthraquinone nucleus for full intercalation with double stranded DNA, while its Cu^{2+} site can be placed at the same time at a favorable position for a direct interaction with the phospho-diester backbone of DNA. Thus, the phospho-diester chain at the complexation site is endowed with both bond strain caused by intercalation and Lewis acid activation through coordination to Cu^{2+}. A general acid- and general base-catalysis effect by amine moieties of **4** activated by Cu^{2+} is also conceivable as a part of the mechanism for this acceleration effect.[20] For **5** and **6**, meanwhile, anthraquinone and Cu^{2+} moieties are held together into an intimate and compact entity, which obviously can not extend an intercalation strain and Lewis acid activation

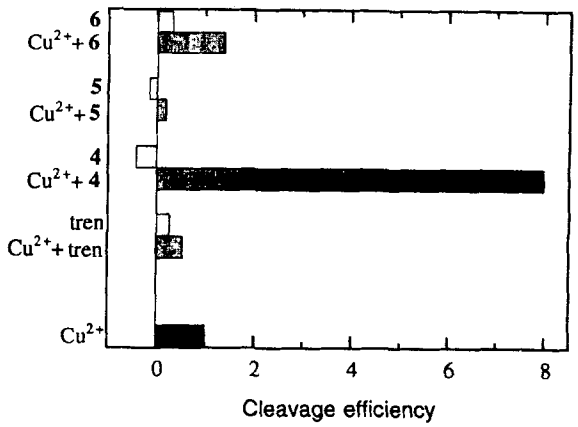


FIGURE 14 Synergistic effect between polyamine-anthraquinone conjugates and Cu^{2+} on the DNA cleavage. Cleaving efficiencies were obtained by supercoiled *DNA* relaxation assay. After incubation of 50 μM DNA-p (pBR322), 50 μM Cu^{2+} and 50 μM ligands in 0.5 mM phosphate buffer (pH 7.0) at 37°C for 12 h, DNA fragments were separated by agarose gel (1%) electrophoresis. Supercoiled and relaxed DNAs are determined by conventional densitometry. Cleavage efficiency refers to the relaxed DNA produced, Cu(II) reaction (bottom row) being taken as reference (cleavage efficiency 1).

at the same time in a cooperative manner. Neutral chelators such as crown ether (**1**) and polyamine (**4**) appear to cooperate with metal ions to accelerate the DNA cleavage through the following two factors: 1) the strain in the DNA backbone induced by the intercalation and 2) the presence of neighboring metal ions when their Lewis acidity is not seriously suppressed through the complexation.[21]

The relaxation assay in the presence of Cu^{2+} and Eu^{3+} with complexane-anthraquinone conjugates **7** are shown in Figure 15 (a) and (b), respectively; almost the same results were obtained for **8**. In contrast to the previously mentioned two series of conjugates, both the complexane-anthraquinone conjugates, **7** and **8**, significantly suppressed the cleaving activities of metal ions (That is why Eu^{3+} is adopted instead of La^{3+} here; DNA hydrolysis activity of Eu^{3+} is stronger than that of La^{3+} because of its higher Lewis acidity.).

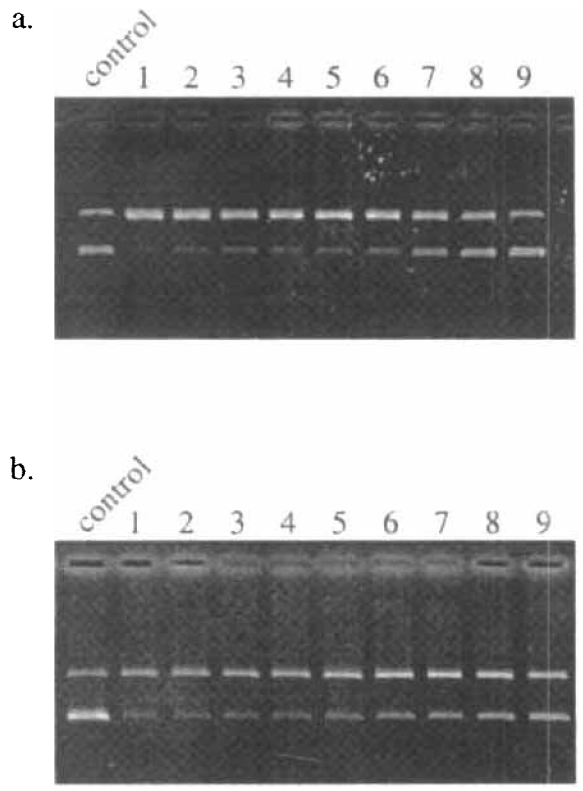


FIGURE 15 Concentration effect of complexane-anthraquinone conjugates on the DNA cleavage by (a) Cu^{2+} and (b) Eu^{3+}. (a) 10 μl of reaction mixture containing 50 μM-p pBR322 DNA, 10 μM $CuSO_4$, 0.5 mM phosphate buffer (pH 7.0), and 0, 1.0, 2.0, 3.0, 5.0, 7.0, 10, 15, and 20 μM **7** for lane 1–9, respectively, were incubated for 12 h at 37°C. (b) 10 μl of reaction mixture containing 50 μM-p pBR322 DNA, 50 μM $Eu(NO_3)_3$, 0.5 mM HEPES buffer (pH 7.0), and 0, 5.0, 10, 20, 30, 40, 50, 70, and 100 μM **7** for lane 1–9, respectively, were incubated for 12 h at 37°C.

In the complex conjugates DNA-Cu^{2+}-**7** and DNA-Cu^{2+}-**8**, it is difficult to figure out the detailed complex structure of Cu^{2+}-**7** or Cu^{2+}-**8** interaction. The iminodiacetic moiety (**7**) and iminoacetic moiety (**8**) are most certainly involved in Cu^{2+} complexation considering the high complex stability of these basic structures. However, the coordination of the tertiary nitrogen three carbons apart from the imino-nitrogen is uncertain. Whichever is the case, the complexed Cu^{2+} ion can be located reasonably close to the phosphodiester chain of DNA if one take the molecular model of **7** or **8** and allow it to intercalate from the minor groove side of DNA; the situation is not much different from those of **4**. Therefore, the main cause for the DNA cleavage suppression by **7** and **8** is to be ascribed to the lowering of the catalytic activity of the complexed metal. That is, the retardation of the DNA cleaving reaction observed here is due to the metal masking effect generally known in organic ester hydrolysis, which is carried out under the action of metallic Lewis acids. The coordination by negatively charged carboxylate strongly diminishes the residual Lewis acidity of the central metal. The carboxylato-metal complexes carry decreased net positive charge as compared with a free metal ion, while the charge of the metal complexes is invariant from the metal ion on complexation with amines. This makes the difference between the two families of metal chelator-intercalator conjugates.

Conclusions

The regulation of DNA binding property of newly designed metal chelator-intercalator conjugates was attained by using the targeted metal

ions (metalloregulation of DNA binding). The main principle governing their affinity seems to be the change of their net charge through complexation with metal ions. It is interesting to note that a conformational effect of the conjugate induced by metal ion is also important for the affinity control for **7**. We were able to demonstrate that the DNA ligand dimerization by using metal ion should be a hopeful strategy for the regulation of ligand binding to DNA. Living system widely adopts this type of dimerization strategy, i.e., the targeted DNA sequence in nature has a dyad symmetry. Therefore, it should be possible to develop the model of natural protein that is capable of recognizing C_2 symmetric sequence and to construct the artificial gene regulatory system. Construction of more effective regulation system by using complexation of DNA ligands through metal ion is in progress in our laboratory.

1 and **4**, which carry 15C-5 and tren, respectively, as a metal binding moiety enhanced the DNA cleaving activity of Cu^{2+} and lanthanoid ions in a synergistic way. On the other hand, DNA cleaving activities of these metal ions were appreciably diminished in the presence of **7** which carries iminodiacetic acid chelator. It was obvious that the activity of metal ion for DNA cleavage was highly dependent on the nature of metal chelating moiety of DNA ligand. These results were explicable by Lewis acidity or residual coordination activity of central metal in the complex with DNA ligands. Although these tendency is very common in traditional study in the organic ester hydrolysis by using metal ion as a catalysis, the results obtained here is the first example of systematic study of the chelator effect in conjugated DNA ligands on the metal catalyzed DNA hydrolysis.

MATERIALS AND METHODS

Materials

Calf thymus DNA was purchased from Sigma Chemical Co. and sonicated as previously described[22] and lyophilized for storage. The concentration of it was determined using molar absorptivity per unit base of 6,600 M^{-1} cm^{-1} at 260 nm and was expressed in terms of nucleotide equivalents per L. Plasmid pBR 322 DNA was obtained from Takara Co., Ltd. and used after ethanol precipitation for removal of EDTA contained in stock solution. Topoisomerase I was also obtained from Takara Co., Ltd. Ultracent-30 (MWCO: 30,000) was used as a ultrafiltration filter, which was a gift from Tosoh Co.

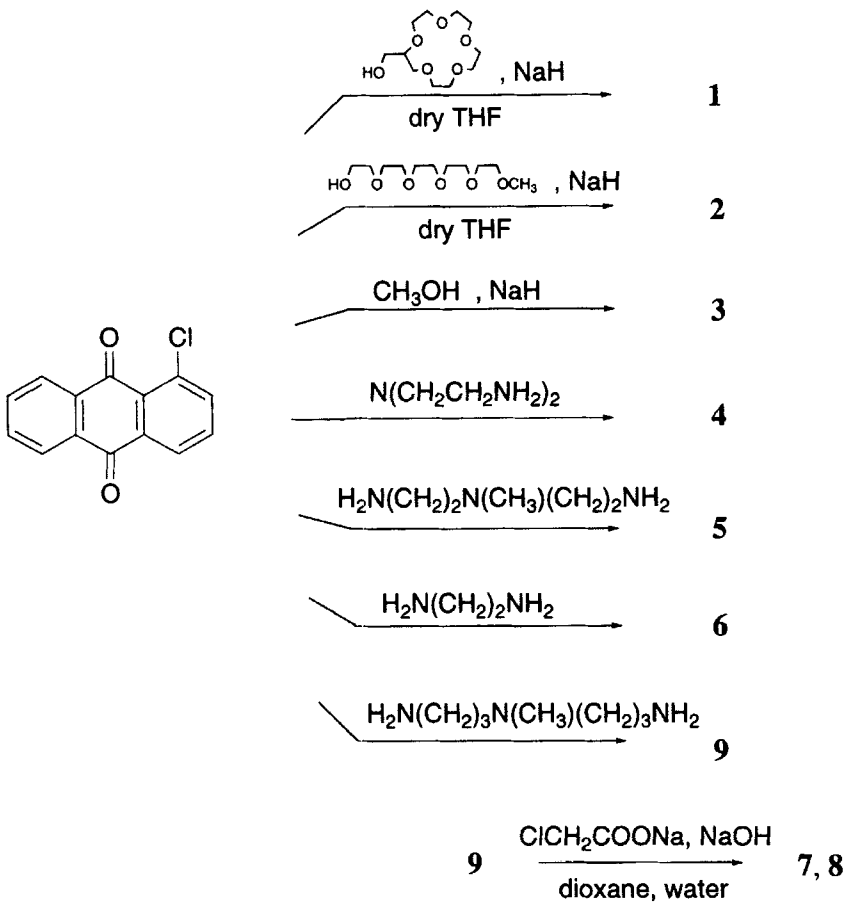
Synthesis of DNA ligands were carried out according to Scheme 1.

Preparation of Ether-Anthraquinone Conjugates

Ether-anthraquinone conjugates, **1–3**, were prepared as described previously.[23] For example, **1**: yellow powder; mp 80–81°C; ^{1}H-NMR (60 MHz, $CDCl_3$) δ 3.5–4.4 (25.0H, m), 7.2–8.5 (7.0H, m); Anal. Found: C, 65.48; H, 6.16%. Calcd for $C_{25}H_{28}O_8$: C, 65.79; H, 6.14%. **2**, **3**: NMR data and elemental analysis of **2** and **3** also supported their structures (data not shown).

Preparation of Polyamine-Anthraquinone Conjugates

1-chloroanthraquinone (1.88 g, 7.8 mmol) was dissolved in ethylenediamine (25 ml) and stirred at 70°C for 2 h. The reaction mixture was cooled and added hydrochloric acid up to pH 1. After three times extraction by ether, the aqueous phase was corrected and added sodium hydroxide up to pH 9. Three times extraction was carried out with dichloromethane, and the organic phase was dried in vacuo. Column chromatography (silica gel 60 (Merck), chloroform: methanol: diethylamine = 10:1:1) gave **6** as a red powder (hygroscopic) (2.1 g, 79%). The same treatment of 1-chloroanthraquinone by *N,N*-bis(2-aminoethyl)methylamine, *N,N*-bis(2-aminopropyl)methylamine, and tris(2-aminoethyl)amine instead of diethylamine were afforded **5**, **9**, and **4**, respectively. **4**: red powder (19%), ^{1}H-NMR (400 MHz, CD_3OD) δ 2.63 (4.2H, t, J = 5.8



SCHEME I

Hz), 2.78–2.81 (6.4H, m), 3.38 (2.1H, m), 7.06 (1.0H, d, J = 7.0), 7.43–7.49 (2.0H, m), 7.72–7.76 (2.2H, m), 8.12 (1.1H, d, J = 7.0), 8.18 (1.0H, d, J = 6.1); Anal. Found: C, 66.72; H, 6.67; N, 15.37 %. Calcd for $C_{20}H_{24}N_4O_2$ + 0.5 H_2O; C, 66.47; H, 6.96; N, 15.50%; **5**: red powder (hygroscopic) (80%), ^{1}H-NMR (400 MHz, $CD_3OD/CDCl_3$) δ 2.36 (3.0H, s), 2.64 (2.0H, t, J = 5.5 Hz), 2.82 (1.9H, t, J = 6.0), 2.89 (1.9H, t, J = 5.6), 3.45 (2.0H, t, J = 6.0), 7.11 (1.0H, m), 7.58 (2.0H, m), 7.74 (1.0H, t, J= 7.5), 7.79 (1.1H, t, J = 7.5), 8.21 (1.0H, d, J = 7.6), 8.27 (1.0H, d, J = 7.6); Anal. Found: C, 67.33; H, 6.38; N, 12.46%. Calcd for $C_{19}H_{21}N_3O_2$ + 0.8 H_2O; C, 67.57; H, 6.74; N, 12.44%; **6**: red powder (hygroscopic) (79%), ^{1}H-NMR (400 MHz, $CDCl_3$) δ 3.08 (2.1H, t, J = 6.1Hz), 3.44 (2.1H, t, J = 6.1Hz), 7.10 (2.0H, dd, J = 1.2, 8.2), 7.55 (2.0H, t, J = 7.3), 7.61 (2.0H, dd, J = 1.2, 7.3), 7.71 (2.0H, dt, J = 1.5, 7.3), 7.77 (2.0H, dt, J = 1.2, 7.3), 8.24 (2.0H, dd, J = 1.5, 7.6), 8.30 (2.0H, dd, J = 1.2, 7.6); Anal. Found: C, 67.33; H, 5.97; N, 9.36%. Calcd for $C_{16}H_{14}N_2O_2$ + 1H_2O; C, 67.60; H, 5.67; N, 9.39%; **9**: red powder (hygroscopic) (54%), ^{1}H-NMR (400 MHz, $CDCl_3$) δ 1.66 (1.9H, quin, J = 7.0 Hz), 1.80 (2.9H, s), 1.92 (2.1H, quin, J = 7.0), 2.26 (3.0H, s), 2.44 (2.0H, t, J = 7.1), 2.51 (2.0H, t, J = 7.0), 2.78 (1.9H, t, J = 6.9), 3.39 (2.1H, q, J = 6.7), 7.08 (1.0H, d, J = 8.4), 7.53 (1.0H, dd, J = 7.3, 8.3), 7.59 (1.0H, d, J = 7.3), 7.69 (1.0H, t, J = 7.5), 7.75 (1.0H, t, J = 7.5), 8.23 (1.0H, d, J = 7.8), 8.26 (1.0H, d, J = 7.8), 9.77 (0.9H, t, J = 6.7); Anal. Found: C, 69.63; H, 7.16;

N, 11.28%. Calcd for $C_{21}H_{25}N_3O_2$ + $0.5H_2O$; C, 69.96; H, 7.28; N, 11.66%.

Preparation of Complexane-Anthraquinone Conjugates

9 (0.9 g, 2.6 mmol), monochloroacetic acid sodium salt (0.3 g, 2.6 mmol), and sodium hydroxide (0.2 g, 5.2 mmol) were dissolved in 15 ml dioxane-water (2:1) and stirred at 50°C for 48 h. After neutralizing by hydrochloric acid, the reaction mixture was concentrated in vacuo. Column chromatography (silica gel 60 (Merck), chloroform:methanol:diethylamine = 5:1:1, R_f = 0.3) and recrystallization from chloroform gave **8** as a red powder (hygroscopic) (2.6%). ^{1}H-NMR (400 MHz, D_2O) δ 1.93 (2.0H, m), 2.27 (2.0H, m), 2.89 (2.1H, t, J = 6.72 Hz), 2.98 (3.0H, s), 3.24 (3.9H, t, J = 8.40), 3.36 (1.9H, t, J = 8.40), 3.71 (2.0H, s), 6.36 (1.0H, d, J = 10.42), 6.67 (1.0H, d, J = 7.29), 6.92 (1.0H, t, J = 7.29), 7.30 (1.0H, m), 7.50 (3.0H, m); Anal. Found: C, 59.74; H, 6.34; N, 9.10%. Calcd for $C_{23}H_{26}N_3O_4Na$ + $1.7H_2O$; C, 59.78; H, 6.42; N, 9.10%.

9 (1.1 g, 3.1 mmol), monochloroacetic acid sodium salt (1.8 g, 15.7 mmol), and sodium hydroxide (0.4 g, 9.3 mmol) were dissolved in 20 ml dioxane-water (1:1) and stirred at 100°C for 3 h. After neutralizing by hydrochloric acid, the reaction mixture was concentrated in vacuo. Red powder obtained was dissolved in 30 ml water and hydrochloric acid was added into this aqueous solution up to pH 1. The solution was allowed to stand overnight at room temperature. Red precipitate was corrected and dried in vacuo. Reversed-phase HPLC (Asahipak ODP-50, 4.6 i. d. × 150 (mm); temperature, 25°C; flow rate, 1.0 ml min^{-1}; buffer A, 0.1 mol dm^{-3} triethylammonium acetate (TEAA, pH 7.0); buffer B, acetonitril; linear gradient, 25–35% B in 20 min) gave **7** as a red powder (hygroscopic) (25%). ^{1}H-NMR (250 MHz, D_2O) δ 1.26 (6.8H, t, j = 6.85 Hz), 1.77 (2.0H, m), 1.90 (1.7H, s), 2.15 (2.1H, m), 2.66 (1.9H, m), 2.87 (3.1H, s), 3.10–3.30 (10.8H, m), 3.72 (4.0H, s), 6.12 (0.9H, d, J = 8.57), 6.40 (0.9H, d, J = 7.14), 6.70 (0.9H, t, J = 7.14), 7.03 (0.9H, m), 7.30 (2.9H, m); Anal. Found: C, 62.09; H, 7.40; N, 8.84%. Calcd for $C_{25}H_{29}N_3O_6$ + 0.57 CH_3COOH + 0.76 $N(CH_2CH_3)_3$ + 0.8 H_2O (the ratio of counter ions were based on the result of ^{1}H-NMR); C, 62.16; H, 7.54; N, 8.88%.

Gel Electrophoresis

Electrophoresis of DNA in agarose gel (agarose LE, Nakarai Tesque. Inc.) were performed as described previously.[24] Quantification of the relaxation reaction was performed by densitometric scanning (Shimadzu CS-9000) of the photographs of the ethidium-stained gels. The staining of relaxed DNA was founds to be 1.5 times that of supercoiled plasmid.[25] The results were corrected based on this difference in staining intensity.

Topoisomerase I Assay

[9] In typical experiment, treatment of pBR322 DNA with topoisomerase I were carried out in reaction mixtures of 10 μl containing 5 mM Tris-HCl (pH 7.5); 50mM KCl; 1 mM $MgCl_2$; 0.5 mM 2-mercaptoethanol; 5% v/v glycerol; 60 μM (nucleotide) pBR 322 DNA; 4 unit topoisomerase I; and DNA ligand ranging from 0 to 0.2 mM. After incubation at 37°C for 2 h, the mixture were extracted with phenol. The aqueous phases were recovered and subjected to ethanol precipitation. After drying, DNA were solved with 10 μl of 5 mM Tris-HCl (pH 7.5) solution.

Binding Isotherms

Filter binding assay[26] was performed at 25°C by using Ultracent-30. 10 μM of ligands and various amount of calf thymus DNA were dissolved in borate buffer (pH 7.5) containing 0.1 M alkali

metal or ammonium ion and incubate for 1 h at 25°C. After centrifuge (3,000 g), the concentration of DNA ligands in filtrate were determined by the analysis of chromatogram obtained by RP-HPLC (Tosoh, TSKgel, Octadecyl-4PW).

Spectroscopic binding analysis was performed at 25°C on a Hitachi U-3210 spectrophotometer equipped interfaced with a Hitachi SPR-10 temperature controller. The concentration of bound ligand was determined by the analysis of the spectrum in each condition, and the analysis were carried out according to the following equation proposed by McGhee and von Hippel:[10]

$$\frac{\nu}{C} = K(1 - n\nu)\left\{\frac{1 - n\nu}{1 - (n-1)\nu}\right\}^{n-1}$$

where ν, number of bound ligand per DNA nucleotide unit; C, concentration of free ligand; K, binding constant; n, binding site size. Levenberg-Marquardt nonlinear least-squares method was used to fit the corrected data with above theoretical equation.

DNA Relaxation Assay

In typical experiment, the reaction mixtures (10 µl) containing 50 µM pBR322 and varying concentration of ligands and metal ions were incubated in an aqueous buffer solution (0.5 mM phosphate buffer (pH 7.0)) at 37°C for 12 h. After extraction with phenol, DNA was purified by precipitation from aqueous ethanol.[27] The precipitate was dissolved in 10 µl of TE buffer solution (10 ml Tris-HCl (pH 7.0), 1 mM EDTA). 3 µl of loading buffer (30% glycerol) was added into this solution, and the solution applied to 1% agarose gel for electrophoresis.

Acknowledgements

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