Synthesis of a New Copper Complex of a Macrocyclic Compound Consisting of 1,10-Phenanthroline, and Its DNA Binding and Cleaving Activity

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A copper complex of a macrocylcic compound consisting of two 1,10-phenanthroline molecules bridged by two sulfur atoms, [Cu(smc)], was synthesized. The interaction of [Cu(smc)] with DNA was examined by UV-vis absorption spectroscopy, circular dichroism spectroscopy, and viscometry. The binding constant of [Cu^{I}(smc)] with DNA is 4.2×10^{4} M^{-1}, which is almost identical with that of bis(1,10-phenanthroline)copper, [$Cu^{I}(phen)_2$]. A viscosity measurement indicates that [Cu^{I}(smc)] binds to DNA by an intercalative mode. [Cu(smc)] cleaves DNA in the presence of a reducing agent under an aerobic condition. In this process ·OH plays an important role.

The metal complexes of 1,10-phenanthroline have been reported to demonstrate a unique interaction with double-stranded DNA and to induce the cleavage of DNA.[1,2] Sigman and co-workers have shown that bis(1,10-phenanthroline)copper, [$Cu(phen)_2$], noncovalently binds to DNA by intercalation, and exhibits nuclease-like activity in the presence of a reducing agent under an aerobic condition.[3—9] They observed that [$Cu(phen)_2$] interacts best with B-form DNA, while it scarcely does so with Z-form DNA,[10] and that the type and position of substitutes in the ligand molecule alter the peculiarity of the reaction with DNA.[3,11—13] For example, substitution at the 2- and 9-positions of 1,10-phenanthroline blocks nuclease activity by hindering the formation of a square-planar complex. In contrast, copper complexes of 1,10-phenanthroline substituted at the 5-position with Br, NO_2, methyl, phenyl, and aminoacetyl, exhibit the same digestion pattern of DNA as dose the parent complex, while phenyl substitution at the 4- and 7-position gives a different result. The cleavage of DNA is considered to be brought about by the action of hydrogen peroxide generated by a redox reaction of the [$Cu(phen)_2$] complex.[14—16] Meanwhile, Barton and co-workers have reported that the tris(4,7-diphenyl-1,10-phenanthroline)ruthenium complex has the ability to recognize different forms of DNA.[17—20] They proposed that the structure of a major groove in DNA is concerned with recognition. It appears that both the structure of each metal complex and the kind of the metal contained determine the interaction of the metal complex with DNA of a particular conformation and specific sequence. To gain further information concerning the interaction and cleavage of DNA with metal complexes, and to create a new DNA binding and cleaving compound, we have explored novel metal complexes with different structures.

We previously synthesized several macrocyclic compounds consisting of two 1,10-phenanthroline molecules (Fig. 1), and investigated the properties of their metal

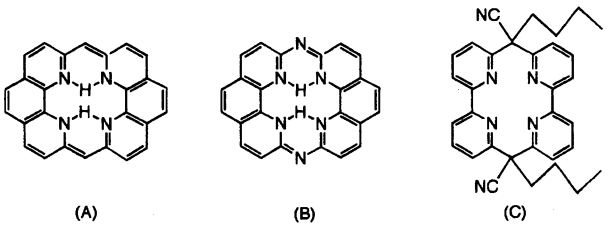


Fig. 1. Derivative of macrocylcic compounds.

complexes. Among them, the two transition-metal complexes of (A) and (B) were found to be useful as an oxygen-reducing catalyst.[21,22] The dicyano dibutyl macrocycle (C) showed high selectivity toward the lithium ion, and will be useful as a selective ionophore for lithium.[23]

Since the compounds (A) and (B) are bridged by carbon and nitrogen, respectively, the metal complexes of these compounds have a planar or nearly planar structure, which appears to endow them with a unique chemical character. However, since the solubility of these complexes in water was shown to be very poor, studies of the reaction of these complexes with DNA were impossible. We thus explored a similar type of complex with high water solubility, and prepared a copper complex of a novel macrocyclic compound consisting of two 1,10-phenanthroline molecules bridged by sulfur, bis(1,10-phenenthroline)[2,1,10,9-*bcdef*:2′,1′,10′,9′-*ijklm*][1,8]dithia [3,6,10,13] tetraazacyclotetradecine (SMC). Having a planar structure of the ligand, the copper complex is expected to possess different properties as well as a different binding ability with DNA from those of [$Cu(phen)_2$], which has a tetrahedral structure. In our preliminary study we found that the Cu complex, [Cu(smc)], is soluble in water. We examined the interaction of the metal complexes, [Cu^{II}(smc)] and [Cu^{I}(smc)], with DNA by UV-vis absorption and a circular dichroism spectroscopic method.[24] In the current study the binding mode of the Cu complex with DNA was further investigated by a viscosity measurement,

which provided a critical test for the detection of classical intercalation. The DNA-cleaving activity of the complex was also investigated under several conditions. Here, we describe the synthesis, properties and DNA-binding and -cleaving activity of [Cu(smc)] in detail.

Experimental

Materials. Copper acetate ($Cu(CH_3COO)_2$), sodium hydroxide (NaOH), 3-mercaptopropionic acid (MP), dimethylsulfoxide (DMSO), and sodium perchlorate ($NaClO_4$) were purchased from Kanto Chemical. 1,10-Phenanthroline, *N,N*-dimethylacetamide (DMA), 1-propanol, L(+)-ascorbic acid sodium salt (Asc), agarose 1600, superoxide dismutase from bovine erythrocyte (SOD), and 1,4-diazabicylco[2.2.2]octane (DABCO) were from Wako Pure Chemical, and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), catalase, and sodium 4,5-dihydroxy-1,3-benzenedisulfonate (Tiron) were from Tokyo Kasei Kogyo. Calf thymus DNA, *N*-(*t*-butyl)-α-phenylnitrone (PBN) and ethidium bromide were purchased from Sigma. DMA was purified by distillation under reduced pressure, and 1-propanol was purified by distillation. All other reagents were used without purification.

Method. UV-vis spectra were measured with a Hitachi U-3200 spectrometer. The circular dichroism spectra were measured with a JASCO J20-A spectropolarimeter using a 1 cm cell. ESR were taken with a JEOL RE-2X. The mass spectra were measured with a JEOL JMS-DX 302 apparatus, the ^{1}H NMR spectra with a Varian Gemini 200 FT-NMR spectrometer, and the IR spectra with a Perkin–Elmer FT-IR 1600. The melting points were taken with a Yanaco MP-500.

Synthesis of Cu Complexes. A copper(II) complex of SMC, [Cu^{II}(smc)], was prepared by three different procedures, as shown in Scheme 1. The starting material, 9-chloro-1,10-phenanthroline-2(1*H*)-thione, was synthesized from 2,9-dichloro-1,10-phenanthroline.[21]

Method 1: A mixture of 9-chloro-1,10-phenanthroline-2(1*H*)-thione (**1**) (0.6 g) and NaOH (0.1 g) DMA (30 ml) was refluxed for 2.5 h. After cooling, the resulting yellow solid was collected by filtration and washed with water, methanol, and ether. Chloride of sodium complex **2** was obtained as a yellow solid in 71% yield (0.38 g). [Na(smc)]Cl: mp 372 °C (dp); MS (FAB) m/z=443 [Na(smc)]; IR (KBr) 1584, 1440, and 1119 cm^{-1}; ^{1}H NMR (CF_3COOD–$(CD_3)_2SO$) δ=8.49 (4H, s), 9.27 (4H, s), 9.32 (4H, s). Found: C, 59.62; H, 3.10; N, 12.04%. Calcd for $C_{24}H_{12}N_4S_2NaCl$: C, 59.99; H, 2.82; N, 11.70%.

[Na(smc)] (**2**) (0.1 g) and $Cu(CH_3COO)_2\cdot 3H_2O$ (0.07 g) were suspended in 1-propanol (20 ml); the mixture was heated at 97 °C for 2.5 h. After cooling, the solution was concentrated by a rotary evaporator, and the resulting brown solid was collected by filtration and washed with water and methanol. [Cu^{II}(smc)] (**3**) was obtained as an acetate salt in 65% yield (0.071 g). [Cu^{II}(smc)]$(CH_3COO)_2$: mp 385 °C (dp); MS (FAB) m/z=483 ([Cu(smc)]; IR (KBr) 1569, 1473, 1130, and 1028 cm^{-1}. Anal. Found: C, 56.17; H, 3.14; N, 9.96%. Calcd for $C_{24}H_{12}N_4S_2Cu(C_2H_3O_2)_2$: C, 55.86; H, 2.99; N, 9.81%,

Method 2: [Cu^{II}(smc)] (**3**) was prepared from metal-free SMC (**4**) instead of [Na(smc)] (**2**). SMC (**4**) was obtained by the following method. A mixture of **1** (0.1 g) and 1,8-diazabicyclo[5.4.0]undec-7-ene (200 ml) in DMS (10 ml) was stirred for 1 h at 80 °C. After cooling, a precipitated yellow solid was collected by filtration, and then washed with water, methanol, and ether. A metal-free macrocyclic compound (**4**) was obtained as a yellow solid in 71% yield (0.06 g). SMC: mp 398 °C (dp); Mp (FAB) m/z=421 (SMC); IR (KBr) 1558, 1472, and 1060 cm^{-1}; ^{1}H NMR (CF_3COOD–$(CD_3)_2SO$) δ=8.19 (4H, d, J=8.8 Hz), 8.40 (4H, s), 8.94 (4H, d, J=8.8 Hz). Anal. Found: C, 63.16; H, 3.07; N, 12.28%. Calcd for $C_{24}H_{12}N_4S_2$: C, 62.95; H, 3.43; N, 12.10%.

Metal-free SMC (**4**) (0.1 g) and $Cu(CH_3COO)_2\cdot 3H_2O$ (0.07 g) were suspended in 1-propanol (20 ml); the mixture was then heated at 97 °C for 2.5 h. After cooling, the solution was concentrated by a rotary evaporator, and the resulting brown solid was collected by filtration and washed with water, methanol, and ether. [Cu^{II}(smc)] (**3**) was obtained as a tetrahydrate in 40% yield (0.046 g). [Cu^{II}(smc)]-$(CH_3COO)_2$: mp 388 °C (dp); MS (FAB) m/z=483 ([Cu(smc)]); IR (KBr): 1622, 1495, 1137 cm^{-1}. Anal. Found: C, 49.60; H, 3.89; N, 8.23%. Calcd for $C_{24}H_{12}N_4S_2Cu$-$(C_2H_3O_2)_2\cdot 4H_2O$: C, 49.89; H, 3.86; N, 8.31%.

Method 3: [Cu^{II}(smc)] was prepared by direct cyclization of **1** in the presence of $CuCl_2$ and DBU. A mixture of **1** (0.1 g), $CuCl_2$ (0.04 g) and DBU (200 ml) in DMA (5 ml) was stirred for 8 h at 80 °C. After cooling, the resulting red solid was collected by filtration and washed with ether. Dichloride of **3** was obtained as a red solid in 64% yield (0.122 g) [Cu^{II}(smc)]Cl_2: mp 402 °C (dp); MS (FAB) m/z=483 ([Cu(smc)]); IR (KBr): 1555, 1502, 1441, and 1181 cm^{-1}. Anal. Found: C, 46.94; H, 2.80; N, 9.18%. Calcd for $C_{24}H_{12}N_4S_2CuCl_2\cdot 3H_2O$: C, 47.33; H, 2.95; N, 9.20%.

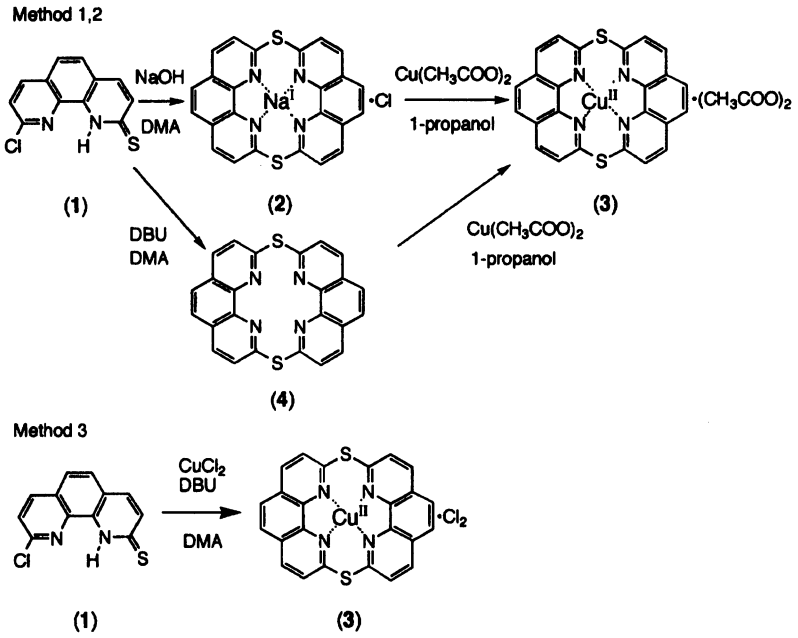
As [Cu^{II}(smc)]Cl_2 was very poorly soluble in water, we used [Cu^{II}(smc)]$(CH_3COO)_2$ for the following studies.

A copper(I) complex of SMC, [Cu^{I}(smc)] (**5**) was obtained by direct reduction of [Cu^{II}(smc)] (**3**) in a deoxygenated 0.1 M Tris-acetate buffer (pH 7.2) with an excess amount of 3-mercaptpropionic acid or L(+)-ascorbic acid, as in Scheme 2. The conversion of the Cu(II) complex to the Cu(I) complex was confirmed based on the UV absorption.

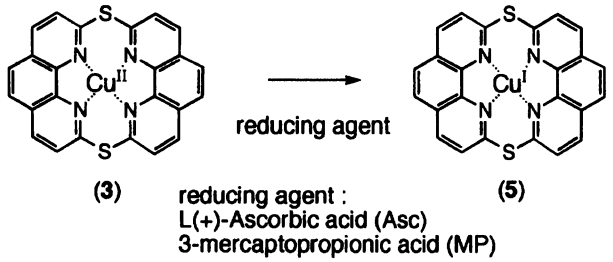
Studies of [Cu^{I}(smc)] were carried out in the buffer solution in the presence of the reducing agent, since [Cu^{I}(smc)] is very susceptible to oxidation.

Absorption Spectral Titration. The interaction of [Cu^{II}(smc)] or [Cu^{I}(smc)] with calf thymus DNA was investigated by UV-vis spectrophotometric titration in a 0.1 M Tris-acetate buffer (pH 7.2) (1 M=1 mol dm^{-3}). The binding constant of the complex with DNA was estimated by the Scatchard plot method[25—28] based on the result of titration.

To avoid DNA degradation by the Cu complex, dissolved oxygen in the 0.1 M Tris-acetate buffer (pH 7.2) was effectively removed by bubbling N_2 gas. Fixed amounts of [Cu(smc)] were titrated with DNA over the range of DNA concentration form 0 to 5 mM. The calf thymus DNA concentration was determined spectrophotometrically using a molar absorption coefficient of $\varepsilon_{260}=6.55\times10^3$ $M^{-1}cm^{-1}$.[26] The final concentration of [Cu(smc)] was 0.2 mM. The UV spectra were measured after equilibration. The differences in the absorbance at 340 nm in [Cu^{I}(smc)] and 330 nm in [Cu^{II}(smc)] were applied to the following Scatchard



Scheme 1. Synthesis of $[Cu^{II}(smc)]$.



Scheme 2. Reduction of $[Cu^{II}(smc)]$.

equation,[26] and the binding constant was calculated:

$$r/m = K(n - r). \quad (1)$$

Here, K is the association constant of the complex with DNA, r is the number of complex molecules bound per DNA nucleotide residue, where $r = Cb/Cp$, m is the molar concentration of free complex, and n is the number of binding sites per DNA. Cb is the concentration of the bound complex and Cp is the input concentration of the DNA. Cb was evaluated based on the relation $Cb = \Delta A/\Delta\varepsilon$. ΔA denotes the decrease in the absorbance of the complex due to the addition of DNA and $\Delta\varepsilon = \varepsilon_f - \varepsilon_b$, where ε_f is the extinction coefficient of the free complex, and ε_b is the extinction coefficient of the bound ligand, as measured from the absorbance of the complex in the presence of a 25-fold excess of DNA.

The binding constants of $[Cu^{I}(smc)]$ and $[Cu^{I}(phen)_2]$ with DNA were determined by the same method. The Cu^{I} complex was produced form a solution of the Cu^{II} complex in a 0.1 M Tris-acetate buffer (pH 7.2) by the addition of 5 mM Asc, and the spectra were recorded in the presence of Asc.

Circular Dichrosim Spectra. A solution of $[Cu^{II}(Smc)]$ or $[Cu^{I}(smc)]$ for a CD measurement was prepared in an oxygen-free 0.1 M Tris-acetate buffer (pH 7.2). After the CD base line was recorded, a DNA stock solution was added to each solution to the final DNA concentration of the nucleotide residue in the range from 0 to 5 mM; the final concentration of $[Cu^{I}(smc)]$ was made to 0.2 mM, and spectra were recorded. The binding constant was calculated based on the difference in the CD spectra of $[Cu^{I}(smc)]$ at 328 nm by, the Scatchared equation in a similar way as in UV titration. The $[Cu^{I}(smc)]$ solution was prepared from a solution of $[Cu^{II}(smc)]$ in a 0.1 M Tris-acetate buffer (pH 7.2) by the addition of 5 mM Asc.

Viscosity Measurements. The viscosity measurements were made with an Ubbelohde viscometer secured in a constant-temperature water bath at 26±0.5°C.[28—31] Tris-acetate buffer (pH 7.2, 0.1 M) was added into an empty viscometer vigorously purged with N_2 gas. Then, N_2 gas was bubbled through the solution in order to remove any oxygen. Flow measurements were made at least five times, and were accepted if they agreed within 0.2 s, although repeated measurements were typically within 0.1 s. At first, the flow time for the buffer was measured; a 1 mM stock solution of calf thymus DNA was then added to the viscometer and the solution was mixed by gentle bubbling with N_2 gas. The final DNA concentration was 0.2 mM. After a 0.3 mM $[Cu^{II}(smc)]$ stock solution was added into the DNA solution at the molar ratio of the Cu complex to DNA from 0 to 0.5, the solution was mixed by N_2 gas bubbling and left for 30 min until equilibrium was reached. Then, the flow time (t)

was determined.

The viscosity of a mixture of [Cu^{I}(smc)] and DNA was measured by the same method. The flow time (t) of the buffer in the presence of 5 mM Asc was measured. Then, stock solutions of 1 mM DNA and 0.3 mM [Cu^{I}(smc)] that were produced from a 0.3 mM [Cu^{II}(smc)] solution by the addition of 5 mM Asc were added into the solution in the viscometer. After bubbling with N_2 was, the flow time (t) was determined. All of the data were converted to the reduced specific viscosity, $\eta_{rel}=t/t_0$.

A viscosity measurement was also made for a DNA solution titrated with either $CuSO_4$ or 1,10-phenanthroline for a control experiment.

ESR Spectra. An aqueous solution of a 4 mM Cu complex was prepared in a 0.1 M Tris-acetate buffer (pH 7.0), which was bubbled with N_2 gas for 30 min; a drop of 1,2-ethanediol was then added into each solution. The ESR spectrum of the Cu complex was recorded at 77 K. ESR scans were made with a 100 kHz field modulation of 0.03 mT amplitude; the microwave power level was maintained at 5 mW.

The formation of radical species during a redox reaction of [Cu(smc)] was observed in the presence of a reducing agent and oxygen by an ESR spin-trapping method using *N*-(*t*-butyl)-α-phenylnitrone (PBN) as a radical trapping agent. A solution of 4 mM Cu complex (0.1 ml) and 50 mM Asc in a 0.1 M Tris-acetate buffer (pH 7.0) and, a 0.1 ml ethanol solution of PBN 200 mM were mixed, and were then incubated at 37 °C for 10 min; the ESR spectra were measured at room temperature.

DNA Cleaving Activity. The DNA cleaving activity of the [Cu(smc)] complex was assessed by conversion of a closed circular form of pBR322 DNA to nicked and the linear form of DNA, which were analyzed by agarose gel electrophoresis. Four μl of [Cu(smc)] (0.3—0.075 mM), 1 μl of 3-mercaptopropionic acid (MP) (30 mM) and 1 μl of pBR322 DNA (0.3 μg) were mixed in a 0.1 M Tris-borate buffer (pH 7.2). The reaction mixtures were incubated for 1 h at 37 °C. The reaction was quenched by adding 1 μl of EDTA (1 mM).

Agarose gel (0.9%) was prepared in a pH 7.2 buffer comprising Tris-borate (0.1 M) and EDTA (2 mM). Electrophoresis was carried out at 100 V for 1 h with 1 μl of a loading buffer (0.25% Bromphenol Blue, 0.25% Xylene Cyanol FF, 30% glycerol in H_2O) as a marker. The gel patterns were developed by soaking the gels in ethidium bromide solution (1 μg/1 ml). After dying, the gel was irradiated with UV (short wave) and pictures were taken.

Moreover, we carried out DNA cleaving with the [Cu(smc)] complex in the presence of several types of scavengers in order to investigate what kind of active oxygen species was responsible for the DNA cleavage. The scavangers were SOD, which converts O_2^- to H_2O_2 and O_2; catalase, which decomposes H_2O_2; 4,5-dihydroxy-1,3-benzenedisulfonic acid (Tiron), which scavenges O_2^-; 1,4-diazabicyclo[2.2.2]octane (DABCO), which scavenges $^1O_2$; and dimethyl sulfoxide (DMSO), which scavenges ·OH. Four μl of [Cu(smc)] (0.3 mM), 0.5 μl of MP (120 mM), 1 μl of pBR322 DNA (0.3 μg), and 0.5 μl of the quencher (SOD 300 mg ml^{-1}, catalase 300 mg ml^{-1}, DABCO 120 mM, Tiron 120 mM, DMSO 6 M) were mixed in the Tris-buffer. The reaction mixtures were incubated for 1 h at 37 °C. Then, the DNA cleaving activity was assayed as described above.

Results

Synthesis of [Cu^{II}(smc)]. [Cu^{II}(smc)] (**3**) was prepared from [Na(smc)] (**2**) or SMC (**4**), as described in Scheme 1. [Na(smc)] (**2**) was prepared by cyclization of 9-chloro-1,10-phenanthroline-2(1*H*)-thione (**1**) with NaOH. The ^{1}H NMR, mass spectrum, and elementary analysis of compound (**2**) showed that this is a sodium complex, [Na(smc)], in which the macrocyclic ligand is coordinated to Na^+ and the chloride ion is a counter anion. A similar base-mediated macrocyclization of **1** with DBU, instead of NaOH, gave a metal-free macrocyclic compound, SMC (**4**), which was confirmed by ^{1}H NMR, the mass spectrum, an elementary analysis, and the IR spectrum.

Transmetallation of [Na(smc)] with copper acetate yielded [Cu^{II}(smc)] (**3**) as an acetate. Complexation of metal-free SMC (**4**) with copper acetate also formed [Cu^{II}(smc)] (**3**) as an acetate tetrahydrate. The structure of [Cu^{II}(smc)] (**3**) was confirmed by the mass spectrum, an elemental analysis, and the IR spectrum.

Alternatively, [Cu^{II}(smc)] was prepared as a chloride by DBU-mediated cyclization in the presence of copper(II) chloride as a template. The chloride of [Cu^{II}(smc)] synthesized by method 3 was poorly soluble in water. We therefore used [Cu^{II}(smc)] acetate synthesized by method 1 or 2 in our studies with DNA.

[Cu^{I}(smc)] (**5**) was obtained by the reduction of [Cu^{II}(smc)] (**3**) with a reducing agent, such as L(+)-ascorbic acid (Asc) or 3-mercaptopropionic acid (MP), in a 0.1 M Tris-acetate buffer (pH 7.2), as shown in Scheme 2. By the addition of a reducing agent, the spectrum of [Cu^{II}(smc)] (λ_{max} 289 nm, $\varepsilon_{289}=2962$ $mol^{-1}\,dm^3\,cm^{-1}$) was converted to that of [Cu^{I}(smc)] (λ_{max} 349 nm, $\varepsilon_{349}=3020$ $mol^{-1}\,dm^3\,cm^{-1}$ and $\lambda_{max}=$ 432 nm, $\varepsilon_{432}=2481$ $mol^{-1}\,dm^3\,cm^{-1}$). [Cu^{II}(smc)] was easily regenerated by passing oxygen to a solution of [Cu^{I}(smc)]. An attempt to isolate the [Cu^{I}(smc)] complex was unsuccessful, since the Cu(I) complex was too unstable to isolate.

Interaction of [Cu(smc)] and DNA Studied by Absorption and Circular Dichroism Spectroscopy. The interaction of [Cu^{I}(smc)] and [Cu^{II}(smc)] with calf thymus DNA was investigated by a spectrophotometrical titration method. The spectra of [Cu^{I}(smc)] and [Cu^{II}(smc)] in the presence of DNA are shown in Fig. 2. In both cases, the absorption of DNA at 260 nm increased; meanwhile, the absorption of the d→d* transition of the copper complex at 300—400 nm decreased as the amount of DNA was increased. The observed hypochromicity indicates that the interaction between [Cu(smc)] and DNA proceeded in each case. The decrease in the absorption of [Cu^{I}(smc)] at 340 nm, where DNA has no absorption, was converted to Scatchard plots (Fig. 3), and the binding constant (K) was estimated by using the Scatchard equation.

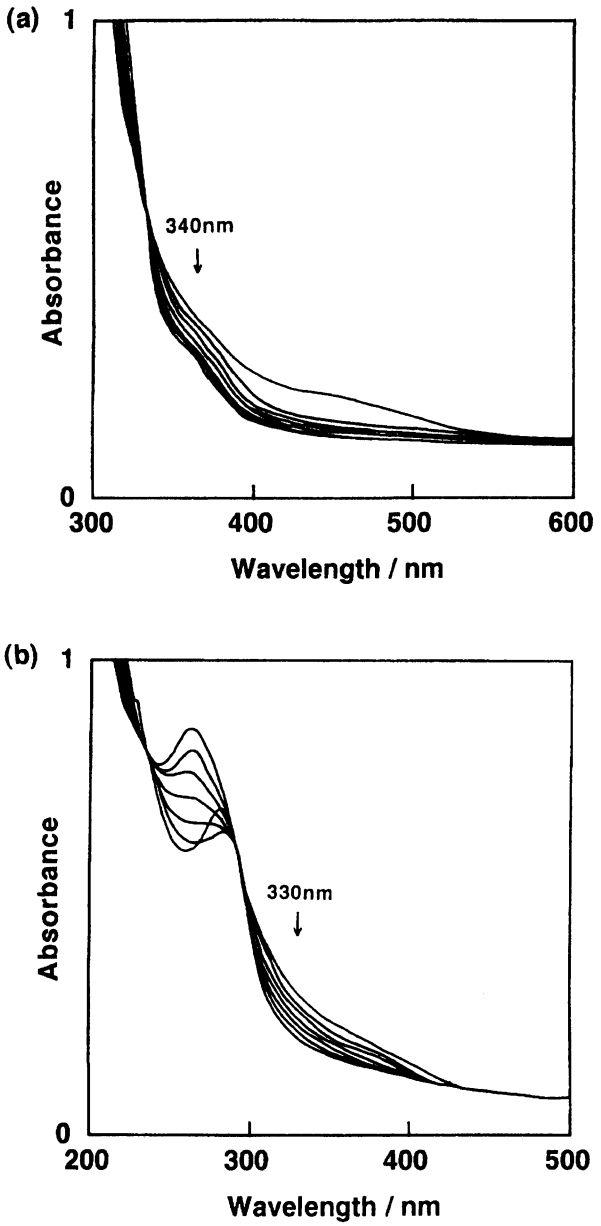


Fig. 2. Spectrophotometric titration of (a) [Cu^{I}(smc)] and (b) [Cu^{II}(smc)] with DNA. (a) [Cu^{I}(smc)] concentration is 0.2 mM. (b) [Cu^{II}(smc)] concentration is 0.2 mM. The DNA concentration ranges from 0 at the top curve to 5 mM at the bottom one at (a) 340 nm and (b) 330 nm.

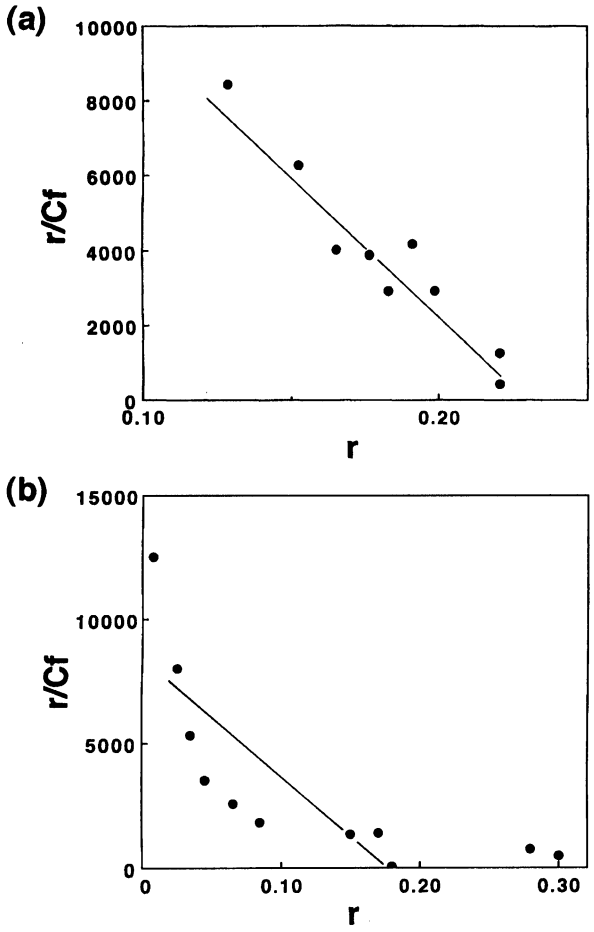


Fig. 3. Scatchard plots of (a) [Cu^{I}(smc)] and (b) [Cu^{II}(smc)] by UV-vis spectrophotometric titration.

Table 1. Binding Constant (K) of Cu Complex with DNA Esitmated from Scatchard Plot

Complex	$K(M^{-1})$	n
[Cu^{I}(smc)]	4.2×10^{4} a)	0.17
	4.0×10^{4} b)	0.20
[Cu^{II}(smc)]	1.5×10^{4} a)	0.18
	1.3×10^{4} b)	0.24
[Cu^{I}(phen)$_2$]	4.7×10^{4} 31)	0.18

a) Determined by UV titration. b) Determined by CD titration.

In a similar way, the interaction of [Cu^{II}(smc)] with DNA was investigated based on the decrease in the absorption of [Cu^{II}(smc)] at 330 nm. Deviations in the Scatchard plots were large, presumably because the spectral changes of the complexes were too small to fit the plot well, especially in case of [Cu^{II}(smc)]. We estimated the K values to be 1.5×10^{4} M^{-1} for [Cu^{II}(smc)] and 4.2×10^{4} M^{-1} for [Cu^{I}(smc)], respectively, as listed in Table 1.

The interaction of [Cu^{II}(smc)] and [Cu^{I}(smc)] to DNA was further investigated based on the circular dichroism spectrum. A CD measurement was performed at different stoichiometric ratios of the Cu complex to calf thymus DNA from 1 : 0 to 1 : 25. Since [Cu(smc)] is not an asymmetric compound, [Cu(smc)] alone did not show any CD band. When calf thymus DNA was added into a solution of the [Cu^{I}(smc)] complex, the CD bands at 328 and 382 nm were induced, as shown in Fig. 4. The CD spectrum of DNA was not observed in this region. The results indicate that the [Cu^{I}(smc)] complex was fixed in an asymmetric environment formed by right-handed B-form DNA. In the case of [Cu^{II}(smc)], CD was slightly induced by the ad-

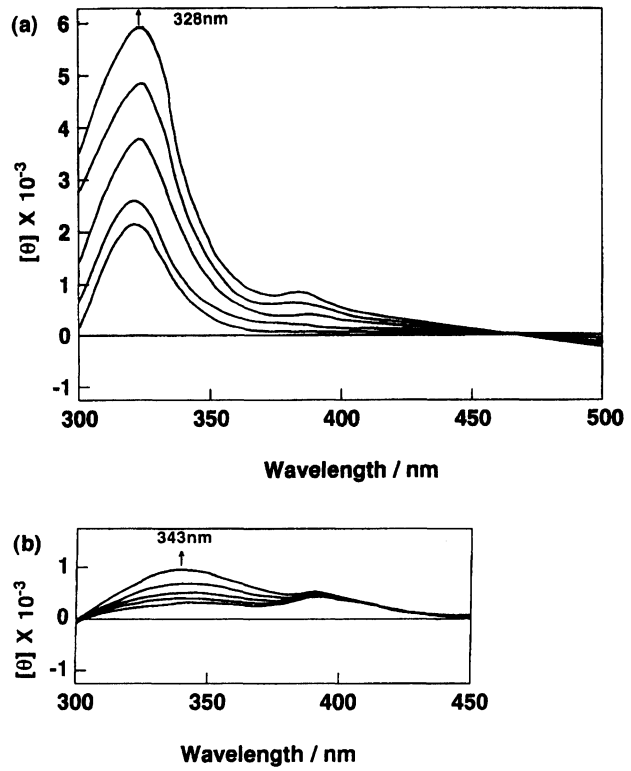


Fig. 4. Differential CD spectra of (a) [Cu^{I}(smc)] and (b) [Cu^{II}(smc)] with DNA. (a) [Cu^{I}(smc)] concentration is 0.2 mM. (b) [Cu^{II}(smc)] concentration is 0.2 mM. The DNA concentration range is from 0 to 5 mM (increasing with increasing spectral magnitude). The molecular ellipticity is expressed based on per mole of [Cu(smc)].

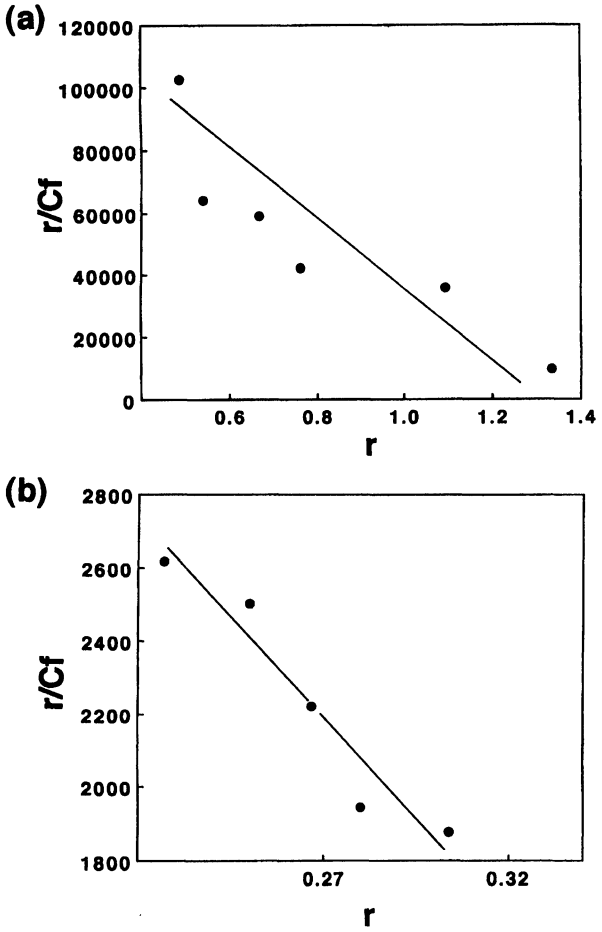


Fig. 5. Scatchard plots of (a) [Cu^{I}(smc)] and (b) [Cu^{II}(smc)] by CD spectrophotometric titration.

dition of DNA in the region of 300—400 nm. From the variation of the CD band induced by DNA, the binding constants of [Cu^{I}(smc)] and [Cu^{II}(smc)] with DNA were calculated according to the Scatchard method in a similar way as that by UV absorption. The Scatchard plots converted from the variation of CD band are shown in Fig. 5. The binding constants of [Cu^{I}(smc)] and [Cu^{II}(smc)] to DNA obtained from the CD study were 4.0×10^{4} $M^{-1}$and 1.3×10^{4} M^{-1}, respectively (Table 1). Although the deviations of the Scatchard plots were large, the K values obtained with the CD spectral titration corresponded well with the K value estimated from the absorption spectral titration.

Viscosity Study of the Interaction of [Cu(smc)] and DNA. Intercalation of a ligand to DNA causes a significant increase in the viscosity of a DNA solution due to an increase in the separation of the base pairs at the intercalation site and, hence, an increase in the overall DNA molecular length.[28—31] In contrast, a ligand that binds exclusively in the DNA grooves causes a less-pronounced change (positive or negative) or change in the viscosity of a DNA solution. The effect of [Cu^{I}(smc)] on the viscosity of a DNA solution was studied in order to assess the binding mode of [Cu^{I}(smc)] to DNA. The viscosity of the DNA solution increased as the molar ratio of [Cu^{I}(smc)] to DNA increased, as shown in Fig. 6. This suggests that [Cu^{I}(smc)] interacalates into DNA. Similarly, [Cu^{II}(smc)] caused an increase in the viscosity of the DNA solution, but to a smaller extent than [Cu^{I}(smc)]. Thus, [Cu^{I}(smc)] is likely more capable of intercalating to DNA than [Cu^{II}(smc)]. On the other hand, 1,10-phenanthroline, Cu^{I} ion or Cu^{II} ion alone caused no change in the viscosity of the DNA solution (data not shown).

ESR Spectra of Cu Complex. The ESR spectra of the Cu^{II} complexes are shown in Fig. 7. At 77 K, the ESR of [Cu^{II}(smc)] exhibited $g^{\perp}=2.05$, $g^{//}=2.26$ and $A^{//}=140$ G. The ESR parameters indicate that the complex has a pseudo-square planar structure.

The formation of a free-radical species generated from a [Cu^{II}(smc)] solution with a reducing agent under aerobic condition was monitored by a spin-trap experiment using *N*-(*t*-butyl)-α-phenylnitrone(PBN) as a spin-trap agent. The typical ESR spectrum of the PBN spin adduct was observed, as shown in Fig. 8. In the case of (a) [Cu^{II}(smc)], the PBN spin-adduct parameters, $a_N=15.8$ G and $a_H=3.3$ G, suggest that ·OH was trapped by PBN.[32,33] Spectrum (b) was obtained from

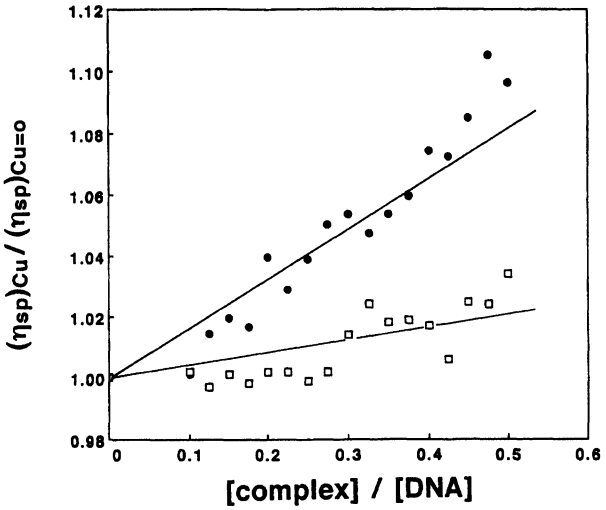


Fig. 6. Effect of [Cu^{I}(smc)] and [Cu^{II}(smc)] on the viscosity of a DNA solution. The complex is titrated into 200 μM DNA solution at 26 °C. (●) [Cu^{I}(smc)] and (□) [Cu^{II}(smc)]. The ordinate represents the ratio of the specific viscosity of the DNA solution in the presence and absence of the complex. The abscissa represents the total concentration of the complex.

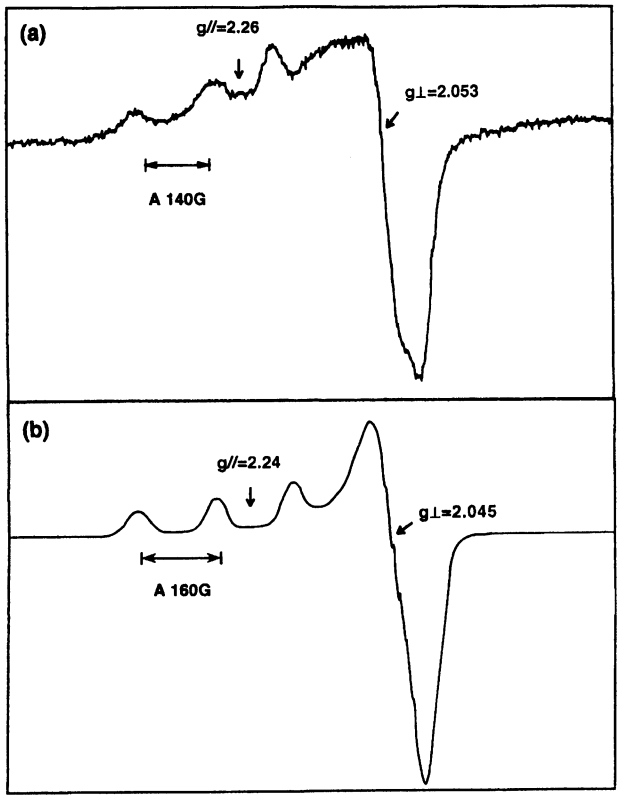


Fig. 7. ESR spectra of (a) [Cu^{II}(smc)] and (b) [$Cu^{II}(phen)_2$] at 77 K.

[$Cu^{II}(phen)_2$] under the same condition as given above. These results indicate that [Cu^{II}(smc)] is reduced to [Cu^{I}(smc)] and the ·OH is generated during the redox reaction of [Cu^{I}(smc)] and oxygen. The radical generated from [Cu^{II}(smc)] was seven-tenths less than that from [$Cu^{II}(phen)_2$].

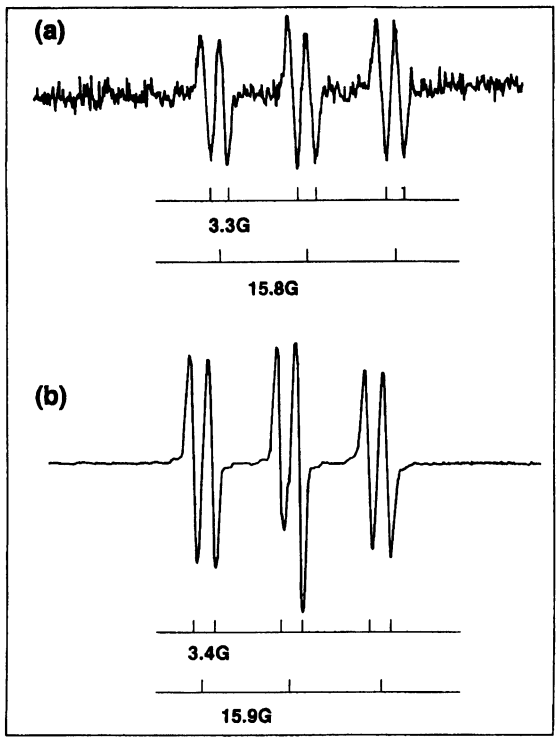


Fig. 8. ESR specta of a spin-trapped radical obtained from an aqueous solution containing (a) 2 mM [Cu^{II}(smc)] and (b) 2 mM [$Cu^{II}(phen)_2$] with 5 mM Asc at room temperature in the presence of 200 mM PBN.

DNA Cleaving Activity of [Cu(smc)]. The DNA cleaving activity of [Cu(smc)] was investigated in the presence of a reducing agent under aerobic condition. 3-Mercaptopropionic acid was used as a reducing agent (Table 2). The cleaving pattern of pBR322 DNA by [Cu(smc)] is shown in Fig. 9. In lane 4, DNA was degraded to small pieces at 0.2 mM of [Cu(smc)] and, therefore, is not visible. Closed circular DNA (form I) was cut to nicked (form II), and then to linear (form III) DNA at 0.2 to 0.05 mM concentration of [Cu(smc)] (lane 4—7). In the absence of MP (lane 3), no significant cleavage was observed.

In order to examine what kind of active species generated in the redox reaction of [Cu(smc)] is responsible for the cleavage of DNA, the cleavage reaction of DNA was carried out in the presence of several types of "in-

Table 2. Cleavage(%) of pBR322 DNA by Cu Complex

	Concentration of [Cu(smc)]			[$Cu(phen)_2$]	
	0.2 mM	0.15 mM	0.1 mM	0.05 mM	0.05 mM
Nicked	—	65	74	78	96
Linear	—	35	26	18	4
c.c.c.	—	0	0	4	0

The values were determined by photodensitometer.

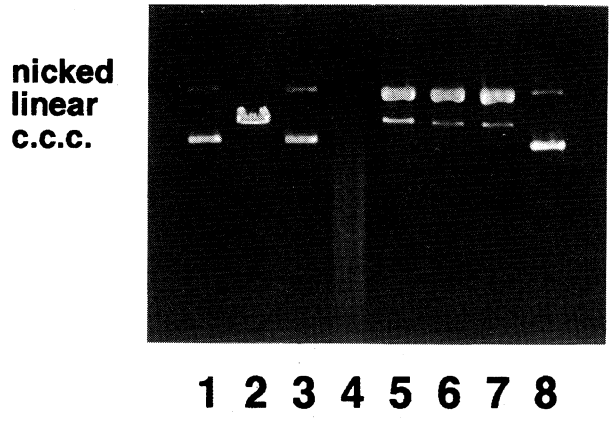


Fig. 9. Cleaving of pBR 322 c.c.c. DNA by [Cu(smc)] with MP for 30 min at 37 °C. The DNA was electrophoreased on agarose gel and treated as described under the Experimental procedure. The amount of DNA is 0.3 μg. MP concentration is 5 mM. Lane assignment: (1) c.c.c. DNA control; (2) linear DNA control; (3) 0.2 mM [Cu(smc)] control; cleaving reaction in the presence of various concentrations of [Cu(smc)] with 5 mM MP; (4) 0.2 mM; (5) 0.15 mM; (6) 0.1 mM; (7) 0.05 mM; (8) 5 mM MP control. The various forms of plasmid DNA (c.c.c. DNA, nicked DNA, linear DNA) are indicated in the figure.

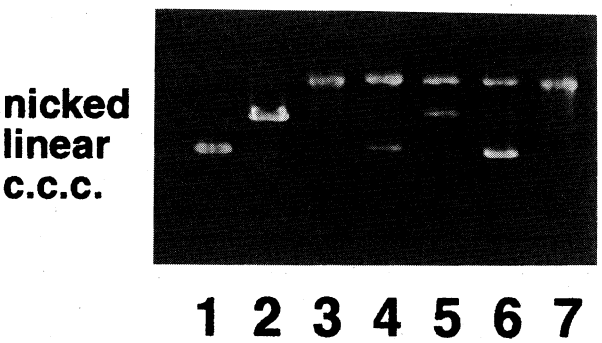


Fig. 10. Cleaving of pBR322 c.c.c. DNA by [Cu(smc)] with MP in the presence of several types of inhibitors for 30 min at 37 °C. Lane assignment: (1) c.c.c. DNA control; (2) linear DNA control; (3) 0.2 mM [Cu(smc)] with 5 mM MP; cleavage reaction of 0.2 mM [Cu(smc)] with 5 mM MP in the presence of inhibirtors, (4) 1 M DMSO; (5) 20 mM DABSO; (6) 20 mM Tiron; (7) 50 μg ml^{-1} SOD.

hibitors". Fig. 10 shows the effect of the inhibitors on the DNA cleavage. DMSO, which scavenges ·OH, and Tiron,which scavenges O_2^-, both inhibited DNA cleavage by [Cu(smc)] (lane 4 and lane 6). The inhibition by Tiron was more prominent. On the other hand, DABCO, which scavenges $^1O_2$, and SOD, which decomposed O_2^- to H_2O_2 and O_2, both did not inhibit DNA cleavage (lane 5 and lane 7). These results indicate that ·OH and O_2^- should play an important role in DNA strand scission.

Discussion

In a previous communication we reported on the synthesis of a copper complex of a novel macrocyclic ligand comprising 1,10-phenanthroline, [Cu^{II}(smc)], by transmetallation of the corresponding sodium complex, [Na(smc)].[24] In the current study we have shown alternative procedures for the synthesis of [Cu^{II}(smc)] by metallation of the metal-free SMC or by direct macrocyclization of 9-chloro-1,10-phenanthroline-2(1*H*)-thione in the presence of the Cu^{II} ion.

[Cu^{II}(smc)] undergoes reduction to [Cu^{I}(smc)] in the presence of a reducing agent. [Cu^{I}(smc)], however, is unstable and readily oxidized. The results of UV and CD studies and a viscosity measurement indicate that [Cu^{I}(smc)] and [Cu^{II}(smc)] interact with DNA. The binding constants of [Cu^{I}(smc)] and [Cu^{II}(smc)] to DNA were estimated independently based on a the measurement of the UV or CD spectra. Both methods gave almost identical values, and the results showed that [Cu^{I}(smc)] binds stronger with DNA, compared to [Cu^{II}(smc)]. The viscosity method clarified that [Cu^{I}(smc)] binds to DNA by the intercalative mode.

Based on the ESR study, [Cu^{II}(smc)] likely possesses a distorted square-planar geometry.[34] Meanwhile, the Cu^{I} complex generally possesses a tetrahedral geometry. It was reported that Cu^{I} complexes of the macrocyclic ligand have a distorted square-planar structure.[35,36] Therefore, [Cu^{I}(smc)] is assumed to be constrained to a distorted square-planar geometry by steric constraints imposed by the macrocyclic ligand, SMC, as shown in Fig. 11.

Considering the fact that [Cu^{I}(smc)] binds to DNA by the intercalative mode, the distorted square-planar [Cu^{I}(smc)] interacalates into DNA, possibly with one of the two 1,10-phenanthroline moieties, and another crooked 1,10-phenanthroline moiety may arrange in the groove of DNA. In the case of [Cu^{II}(smc)], when one moiety of 1,10-phenanthroline intercalated into DNA, another moiety causes a steric hindrance toward DNA. Thus, [Cu^{II}(smc)] binds with DNA more weakly than dose [Cu^{I}(smc)].

[Cu^{II}(smc)] cleaves DNA in the presence of oxygen and a reducing agent. In this process, radical species generated by a redox reaction of [Cu(smc)] may play an important role. The formation of ·OH during the

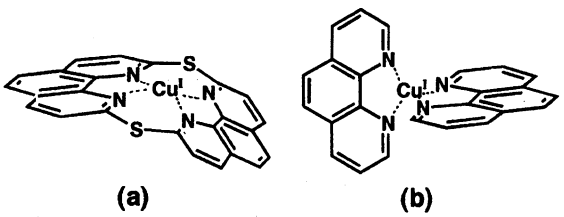


Fig. 11. Proposed structures of (a) [Cu^{I}(smc)] and (b) [Cu^{I}(phen)$_2$].

redox reaction of [Cu(smc)] was observed in a spin-trap study. Inhibition of the [Cu(smc)] induced-DNA cleavage by DMSO and Tiron suggests that DNA cleavage is concerned with ·OH and O_2^- generated from the redox reaction of [Cu(smc)].

Thus, the mechanism of DNA cleavage by [Cu(smc)] can be postulated to be as follows. The reduction of [Cu^{II}(smc)] yields [Cu^{I}(smc)], which likely reacts with O_2 to give O_2^- followed by the formation of H_2O_2. The reaction of [Cu^{I}(smc)] with H_2O_2 may form a transient complex which decomposes to give [Cu^{II}(smc)] and ·OH. [Cu^{I}(smc)] binds to DNA and the generated ·OH from the bound [Cu^{I}(smc)] possibly attacks DNA near to the site of [Cu^{I}(smc)] binding. Alternatively, the transient active complex at the binding site can react directly with DNA, and then abstract hydrogen to form a DNA radical which leads to cleavage. It is hard to conclude from the present study that either of these mechanisms is predominantly responsible for the degradation of DNA.

DNA cleaving activity of the Cu complex is influenced by the strength of DNA binding and the quantity of active species for the cleavage. In a comparison of [Cu(smc)] with bis(1,10-phenanthroline)copper, [$Cu(phen)_2$],[3] the binding constant of [Cu^{I}(smc)] with DNA is slightly smaller than that of [$Cu^{I}(phen)_2$]. The amount of radical species generated in the redox reaction of [Cu(smc)] is seven-tenths that of [$Cu(phen)_2$]. These would be reasons why the DNA cleaving activity of [Cu(smc)] is slightly lower than that of [$Cu(phen)_2$].[24]

The different DNA binding abilities may be caused by differences in the structure of the Cu complexes. [$Cu^{I}(phen)_2$] forms a tetrahedral geometry in which two coordinating molecules are orthogonal to one another. Sigman et al have reported that [$Cu^{I}(phen)_2$] displays uniqueness in binding to DNA.[1] They claimed that one phenanthroline molecule intercalated into DNA, and that another phenanthroline molecule fits to the minor groove of DNA. [Cu^{I}(smc)] can not form a tetrahedral structure, since two 1,10-phenanthroline molecules are bridged by sulfur, forming a macrocycle. In consequence, [Cu^{I}(smc)] can not fit to the minor groove of DNA as dose [$Cu^{I}(phen)_2$], and the binding constant of [Cu^{II}(smc)] is lower than that of [$Cu^{I}(phen)_2$].

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