Synthesis and Biological Activity of a Platinum(II) 6-Phenyl-2,2′-bipyridine Complex and Its Dimeric Analogue

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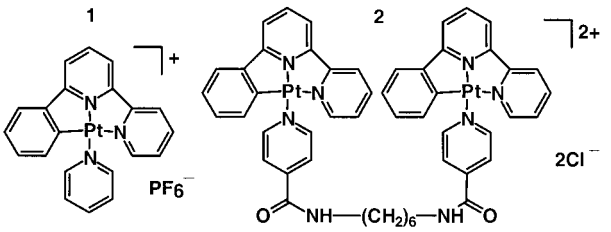
*We have synthesized (pyridyl)-(6-phenyl-2,2′-bipyridine)platinum(II) hexafluorophosphate (**1**) and its corresponding dimer, μ-N,N′-bis(isonicotinyl)-1,6-hexanediamino bis-[6-phenyl-2,2′-bipyridine-platinum(II)] dichloride (**2**). The DNA binding constants of **1** and **2** at 20 °C were determined by absorption titration to be $2.25 \times 10^4\ M^{-1}$ and $3.07 \times 10^6\ M^{-1}$, respectively. Compound **1** showed an AT preference, while **2** had no base preference. The binding site sizes of **2** for [poly(dA-dT)]$_2$, calf thymus DNA (ctDNA), and [poly(dG-dC)]$_2$, as determined by fluorescence titration, were 6.6, 4.0, and 2.8 bp, respectively. Compound **2** probably bound to [poly(dA-dT)]$_2$ through bisintercalation, and to [poly(dG-dC)]$_2$ by monointercalation. Binding of DNA by both complexes is favorable, since the binding free energies of **1** and **2** were estimated to be −5.8 and −8.7 kcal mol^{-1}, respectively. The results of viscosity measurements and gel mobility shift assay demonstrated that binding of **1** and **2** caused DNA lengthening. The cytotoxicities of the complexes in various human cancer cell lines were determined by MTT assay. Complex **2** exhibited cytotoxicity comparable to that of cisplatin, and was more toxic than **1** by an order of magnitude.*

KEYWORDS:

antitumor agents · bioinorganic chemistry · DNA · intercalation · platinum

Introduction

The development of DNA-binding metal complexes with high affinity and sequence specificity has been the principal objective of most studies in the field of anticancer chemotherapy.[1] A number of transition metal complexes have been utilized to probe nucleic acid structures and to act as molecular "light-switches" for DNA.[2] Theoretically, the DNA binding constant of a dimeric DNA-binding molecule will be the square of that of its monomeric unit.[3] In this project, a Pt^{II} complex, (pyridyl)-(6-phenyl-2,2′-bipyridine)platinum(II) hexafluorophosphate[4] (**1**),



and its corresponding dimer, μ-*N*,*N*′-bis(isonicotinyl)-1,6-hexanediamino-bis-[6-phenyl-2,2′-bipyridine-platinum(II)] dichloride (**2**) were synthesized, and their interactions with DNA and their biological activities were studied by various biophysical techniques and cell-based assays.

Square-planar platinum(II) complexes containing π-aromatic ligands are potential metallointercalators for double-stranded DNA (dsDNA) because the aromatic ring system may insert between DNA base pairs through π–π stacking interactions.[5] Studies of a variety of Pt^{II} complexes have revealed that [Pt(terpy)X]$^+$ (terpy = 2,2′:6′,2″-terpyridine, X = chloride, 2-hydroxyethanethiolate, 2-aminoethanethiolate, ethyl 2-mercaptoacetate, or cysteine), [Pt(phen)(en)]$^{2+}$ (phen = 1,10-phenanthroline, en = ethylenediamine), and [Pt(bipy)(en)]$^{2+}$ (bipy = 2,2′-bipyridine) can intercalate with DNA.[1b, 1c] Platinum complexes offer certain advantages as potential biological agents because they are usually soluble in water, kinetically stable, and do not form insoluble hydrated oxides at physiological pH values. The tendency of planar platinum(II) complexes to form one-dimensional columnar stacks in their solid lattices, the aromaticity and sizes of the terpy, phen, and bipy ligands, the positive charge on the complex, and its planarity all contribute to the ability of these Pt^{II} complexes to bind intercalatively to DNA.[6]

We had previously synthesized and characterized the square-planar Pt^{II} complex **1**, which contains 6-phenyl-2,2′-bipyridine and pyridine as the ligands.[4] This complex meets all the

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requirements of a DNA intercalator. Since the DNA-binding properties and cytological activities of **1** had not previously been studied, we have investigated these properties of the metal complex here. Initial bioassay studies of **1** showed that it has certain intercalating properties and cytotoxic effects.

DNA binding affinity should be greatly enhanced for a bisintercalator and the biological activity of intercalating agents has been correlated with their DNA binding affinity,[7] therefore it is to be expected that a bisintercalator may have greater cytotoxicity than its monomer. In addition, as the binding site size of a bisintercalator is expected to be larger than that of the monomer, the bisintercalator may potentially have an increased sequence selectivity.[8] As a result, a dimeric Pt^{II} 6-phenyl-2,2′-bipyridine complex (compound **2**) was designed and synthesized.

Here we report the detailed profiles of the DNA-binding and cytotoxic properties of the monomeric and dimeric Pt^{II} complexes.

Results and Discussion

Determination of the DNA affinities of 1 and 2

The DNA binding constants of **1** and **2** were determined by absorption and fluorescence titrations. The monomeric and dimeric platinum(II) 6-phenyl-2,2′-bipyridine complexes exhibit intense optical absorption as a result of metal-to-ligand charge transfer (MLCT) and intraligand electronic transitions.[9] Since intercalative $\pi-\pi$ stacking of the aromatic rings of the metal complexes with the DNA bases affects the transition dipoles of the molecules and usually leads to a reduction in its absorbance, absorption titrations were carried out to determine the DNA binding constants of **1** and **2**. The absorption bands of both **1** and **2** were found to change with increasing concentration of calf thymus DNA (ctDNA) in tris(hydroxymethyl)aminomethane (Tris) buffer. Hypochromism at 334 nm and isosbestic points at 300 and 355 nm were observed in the absorption titration spectra of **1**. Similarly, hypochromism at 320 nm and an isosbestic point at 297 nm were observed in the absorption titration spectra of **2** (Figure 1). The hypochromism and the appearance of isosbestic points are characteristics of intercalative binding.[10] A plot of A_0/A against [DNA]/[complex] (Figure 1 A, inset) shows that the binding of **2** to DNA reached a saturated level at a [DNA]/[complex] ratio of 1.6, where A_0 and A are the absorbances of **2** at 320 nm in the absence and in the presence, respectively, of various concentrations of ctDNA, and [DNA] and [complex] are the concentrations of ctDNA in base pairs and of **2**, respectively.

The intrinsic binding constant, K, of a metal complex to DNA can be determined from a plot of $D/\Delta\varepsilon_{ap}$ against D,[11]

$$\frac{D}{\Delta\varepsilon_{ap}} = \frac{D}{\Delta\varepsilon} + \frac{1}{(\Delta\varepsilon \times K)} \quad (1)$$

where D is the concentration of DNA, $\Delta\varepsilon_{ap} = |\varepsilon_A - \varepsilon_F|$, in which $\varepsilon_A = A_{obs}/[complex]$, and $\Delta\varepsilon = |\varepsilon_B - \varepsilon_F|$. The terms ε_B and ε_F correspond to the extinction coefficients of DNA-bound complex and free complex, respectively. From absorption measurements of **1** and **2** at 334 nm and 330 nm, respectively, in the presence of increasing amounts of ctDNA, the plots of $D/\Delta\varepsilon_{ap}$ against D (Figure 1 A, inset) were found to be linear, and the K values could be estimated as $(2.25 \pm 0.35) \times 10^4\,M^{-1}$ and $(3.07 \pm 1.27) \times 10^6\,M^{-1}$ for **1** and **2**, respectively (Table 1). The ε_B value of **1** derived from the plot ($1.5 \times 10^4\,M^{-1}cm^{-1}$) is in good agreement with that determined experimentally ($1.8 \times 10^4\,M^{-1}cm^{-1}$) at [DNA]/[complex] ratios above 9.0. Similarly, the ε_B of **2** derived from the plot ($9.0 \times 10^3\,M^{-1}cm^{-1}$) agrees well with that determined experimentally ($8.0 \times 10^3\,M^{-1}cm^{-1}$) at [DNA]/[complex] ratios above 1.7. The binding constants of **1** and **2** for ctDNA, $[poly(dA\text{-}dT)]_2$, and $[poly(dG\text{-}dC)]_2$ are listed in Table 1. The K value of **1** for $[poly(dA\text{-}dT)]_2$ is larger than that for $[poly(dG\text{-}dC)]_2$ by an order of magnitude, but there are no significant difference

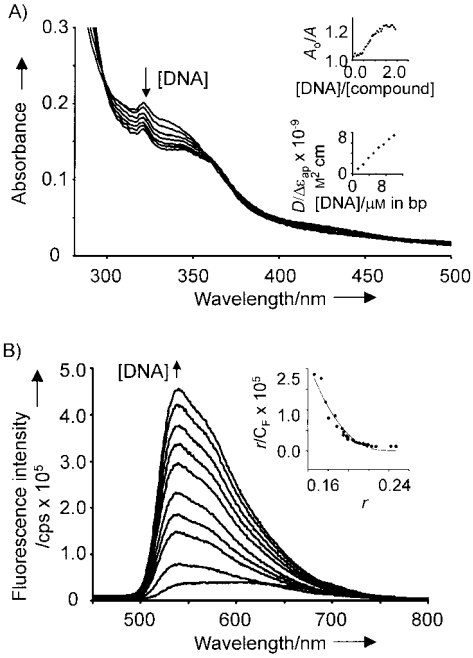

Figure 1. *A) Absorption spectra of* ***2*** *(2.13×10^{-5} M) upon addition of double-stranded calf thymus DNA (0 – 3.93×10^{-5} M in base pairs). Inset: absorption titration curves monitored at 320 nm. The* K *value is $(3.07 \pm 1.27) \times 10^6\,M^{-1}$; B) Emission spectra of* ***2*** *(1.04×10^{-5} M) in the presence of increasing amounts of calf thymus DNA (0 – 3.51×10^{-4} M in base pairs). Inset: Scatchard plot of the equilibrium binding isotherm. Initial values of* K *and* n *are $1 \times 10^5\,M^{-1}$ and 5 bp, respectively, and the fit values of* K *and* n *are $(1.88 \pm 0.20) \times 10^6\,M^{-1}$ and 4.0 bp, respectively.*

Table 1. *DNA affinity constants of* ***1*** *and* ***2*** *with different types of DNA, determined by absorption and fluorescence titrations at 20 °C.*

		$K\,[M^{-1}]$	
	ctDNA	$[poly(dA\text{-}dT)]_2$	$[poly(dG\text{-}dC)]_2$
1	$2.25(\pm 0.35) \times 10^{4}$[a] $2.24(\pm 0.11) \times 10^4$ $(n = 2.4)$[b]	$2.98(\pm 0.57) \times 10^{4}$[a]	$4.11(\pm 1.35) \times 10^{3}$[a]
2	$3.07(\pm 1.27) \times 10^{6}$[a] $1.88(\pm 0.20) \times 10^6$ $(n = 4.0)$[b]	$4.24(\pm 1.61) \times 10^{6}$[a] $1.00(\pm 0.20) \times 10^7$ $(n = 6.6)$[b]	$2.22(\pm 0.82) \times 10^{6}$[a] $5.37(\pm 0.58) \times 10^6$ $(n = 2.8)$[b]

[a] K values were determined by absorption titration; [b] K and n values were determined by fluorescence titration.

in the K values of **2** for ctDNA, [poly(dA-dT)]$_2$, and [poly(dG-dC)]$_2$. The results indicated that **1** showed a AT preference, and that there was no base preference for **2**.

Figure 1 B shows fluorescence spectra of **2** in the presence of increasing amounts of double-stranded ctDNA. The concentration of the free metal complex was determined by Eq. (2):

$$C_F = C_T \frac{(I/I_0 - P)}{(1 - P)} \quad (2)$$

where C_T is the concentration of the metal complex added, C_F is the concentration of the free metal complex, and I and I_0 are the fluorescence intensities in the presence and in the absence of DNA, respectively. P is the ratio of the observed fluorescence quantum yield of the bound metal complex to that of the free metal complex. The value of P was obtained from a plot of I/I_0 against 1/[DNA] such that it is the limiting fluorescence yield given by the y-intercept. The amount of bound metal complex (C_B) at any concentration was equal to $C_T - C_F$. A plot of r/C_F against r, where r is equal to C_B/[DNA], was constructed according to the modified Scatchard equation [Eq. (3)] reported by McGhee and von Hippel.[12]

$$\frac{r}{C_F} = K(1 - nr)\left[\frac{(1 - nr)}{[1 - (n - 1)r]}\right]^{n-1} \quad (3)$$

In Eq. (3), K is the intrinsic binding constant and n is the binding site size in base pairs. The binding data were fitted to Eq. (3) with SigmaPlot version 5.0 graph software on a personal computer to extract the binding parameters. The values of K and n were obtained from the best fit of the data to Eq. (3). The Scatchard analysis plot derived from the fluorescence intensity of **2** at 548 nm is shown in the inset of Figure 1 B. The binding constant of the metal complexes and the n value of **2** as determined by fluorescence analysis are listed in Table 1.

The K values of **1** and **2** for ctDNA, [poly(dA-dT)]$_2$, and [poly(dG-dC)]$_2$ obtained from both absorption and fluorescence titrations were in good agreement. Complex **2** has a DNA binding constant larger than that of a bis-Pt(terpy) complex with a 1,6-dithiohexane by an order of magnitude ($K = 1.9 \times 10^5\,\text{M}^{-1}$[3c]). The bis-Pt(terpy) complex has a structure similar to **2**, including the intercalative polypyridyl ligand and aliphatic hydrocarbon linker. The binding site size of **1** for ctDNA was found to be 2.4 bp, and the n values of **2** for [poly(dA-dT)]$_2$, ctDNA, and [poly(dG-dC)]$_2$ were determined to be 6.6, 4.0, and 2.8 bp, respectively. The binding site size of **2** for [poly(dG-dC)]$_2$ was close to that of the monomer **1**. The calf thymus DNA contained 43% GC.[13] From the n value of **2** for DNA with a different GC content, **2** probably bound to [poly(dA-dT)]$_2$ by bisintercalation, while binding to [poly(dG-dC)]$_2$ by monointercalation only, and by a mixture of bis- and monointercalation to ctDNA. According to ref. [3c] the n values both of the bis-Pt(terpy) complex and of its monomeric analogue (butane-1-thiolato)-2,2′:6′,2″-terpyridine)platinum(II) were 2.5 bp with ctDNA at 20 °C. It is not possible that the bisintercalating compound had the same binding site size as its monointercalating analogue. The plot of I/I_0 against [DNA] for **1** titrated with [poly(dA-dT)]$_2$ and [poly(dG-dC)]$_2$ is shown in Figure 2, and

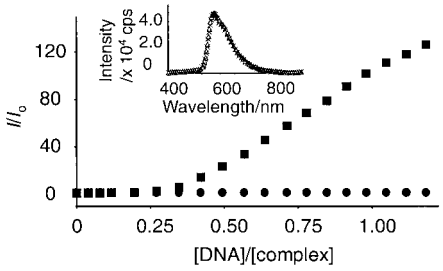

Figure 2. *Plot of I/I_0 against [DNA]/[complex] for **1**: [poly(dA-dT)]$_2$ (filled squares) and [poly(dG-dC)]$_2$ (filled circles). Inset: Emission titration for 1.64×10^{-5} M **1** with [poly(dG-dC)]$_2$ (0–8.79×10^{-5} M in base pairs). The fluorescence intensity at 537 nm remain unchanged after addition of [poly(dG-dC)]$_2$ in the absence (cross) and in the presence (open triangle) of DNA.*

the inset shows the emission spectra of **1** in the absence and in the presence of [poly(dG-dC)]$_2$. The fluorescence intensity of **1** did not increase significantly upon addition of [poly(dA-dT)]$_2$ from [DNA]/[**1**] = 0–0.3, but rose linearly when [DNA]/[**1**] was larger than 0.3. The two phases of binding indicated that **1** associated with DNA dominantly by a non-intercalative mode (for example, electrostatic interaction) at low values of [DNA]/[**1**], and shifted to intercalative mode at higher values of [DNA]/[**1**]. However, [poly(dG-dC)]$_2$ cannot enhance the fluorescence of **1** even at values of [DNA]/[**1**] larger than 1.0. Unlike **2**, **1** interacts strongly with [poly(dA-dT)]$_2$, but weakly with [poly(dG-dC)]$_2$.

The results of absorption and fluorescence titrations provide a good estimation of the binding constant for the interaction of the metal complexes with DNA. The DNA binding constant of **1** ($K = 2.25 \times 10^4\,\text{M}^{-1}$ at 20 °C) is comparable with the K values of other metallointercalators such as [Pt(dppz)(*t*N^C)]$^{2+}$ (dppz = dipyrido[3,2-*a*:2′,3′-*c*]phenazine, *t*N^CH = 4-*tert*-butyl-2-phenylpyridine) ($K = 1.3 \times 10^4\,\text{M}^{-1}$ at 20 °C) and [Pt(terpy)OH]$^+$ ($K = 7 \times 10^4\,\text{M}^{-1}$ at 25 °C).[14] The DNA binding constant of **2** was greater than that of the monomer by two orders of magnitude. Significant enhancement in the DNA binding constant of the dimeric Pt^{II}(C^N^N) complex was observed, but the K value is less than that predicted theoretically (in the order of $10^8\,\text{M}^{-1}$). Similar observations have been made with other bisintercalating agents such as bis-daunorubicin[3a] and bis-Pt(terpy) complexes.[3c]

Thermodynamic parameters of DNA binding of 1 and 2

The melting temperature (T_m) of ctDNA alone was found to be 77.0 °C, while the T_m values of ctDNA in the presence of **1** and **2** were increased to about 85.5 °C and 86.0 °C, respectively (Figure 3).

The thermodynamic profiles of the DNA binding of the two metal complexes were constructed. The DNA binding constants of **1** at 85.5 °C and of **2** at 86.0 °C were determined by McGhee's equation [Eq. (4)],[15]

$$\left(\frac{1}{T_m^0} - \frac{1}{T_m}\right) = \left(\frac{\Delta H_m}{R}\right) \ln (1 + KL)^{1/n} \quad (4)$$

where T_m^0 is the melting temperature of ctDNA alone, T_m is the melting temperature in the presence of metal complex, ΔH_m is the enthalpy of DNA melting (per bp), R is the gas constant, K is

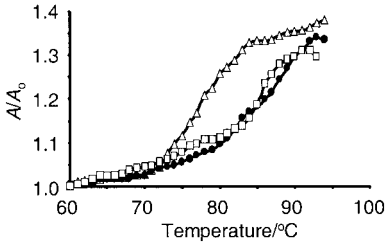

Figure 3. *Plots of A/A_0 against temperature for 1.07×10^{-5} M (in base pairs) calf thymus DNA alone (open triangles) and for calf thymus DNA in the presence of* ***1*** *(open squares) and* ***2*** *(filled circles), with a 1:1 ratio of DNA base pair to metal complex in 5 mM Tris, 50 mM NaCl, at pH 7.2.*

the DNA binding constant at T_m, L is the free ligand concentration (approximated at T_m by the total ligand concentration), and n is the binding site size. A value of $\Delta H_m = 7.0 \pm 0.3$ kcal mol^{-1} was used.[3a] On the basis of the fluorescence measurement and neighbor exclusion principle, the values of n for **1** and **2** were assumed to be 2.0 bp and 4.0 bp, respectively. By substitution of the required parameters into Eq. (4), K was determined to be 96 M^{-1} at 85.5 °C for **1** and 180 M^{-1} at 86.0 °C for **2**.

The standard enthalpy and standard entropy of the binding of **1** and **2** to ctDNA were determined by van't Hoff's equation [Eq. (5)] and the standard free energy change [Eqs. (6) and (7)],

$$\ln\left(\frac{K_1}{K_2}\right) = \left(\frac{\Delta H^0}{R}\right)\left(\frac{T_1 - T_2}{T_1 T_2}\right) \quad (5)$$

$$\Delta G^0 = -RT\ln K \quad (6)$$

$$\Delta G^0 = \Delta H^0 - T\Delta S^0 \quad (7)$$

where K_1 and K_2 are the DNA-binding constants of the metal complex at temperatures T_1 and T_2, respectively, and ΔG^0, ΔH^0, and ΔS^0 are the standard free energy change, standard enthalpy, and standard entropy of the metal complex binding to DNA, respectively. Complete thermodynamic parameters for the binding of **1** and **2** to ctDNA derived from the above equations are listed in Table 2. For the dimeric complex, the favorable binding free energy (-8.7 kcal mol^{-1}) results from the difference between the negative enthalpic contribution (-30.9 kcal mol^{-1}) and the unfavorable entropic contribution ($T\Delta S^0 = -39.6$ kcal mol^{-1}) at 20 °C. The value of ΔH for the dimer is twice that for the monomer, which indicates that both monomeric units in the dimer bind to DNA. The negative entropy values for both monomer and dimer indicate that the degree of freedom of the compounds is decreased after the binding, and that the DNA conformational freedom is also reduced upon ligand–DNA binding. In the following section, the conformational change of DNA induced by the binding of the Pt^{II}(C^N^N) complexes is discussed.

Table 2. *Thermodynamic parameters estimated by absorption titration and by the DNA melting study.*

	1	**2**
T_m[a] [°C]	85.5[b]	86.0[b]
K at T_m [M^{-1}]	0.96×10^2	1.8×10^2
K at 20 °C [M^{-1}]	2.25×10^4	3.06×10^6
ΔG^0 [kcal mol^{-1}]	-5.8	-8.7
ΔH^0 [kcal mol^{-1}]	-17.4	-30.9
ΔS^0 [cal mol^{-1} K^{-1}]	-39.6	-135.2

[a] The T_m of calf thymus DNA alone was 77.0 °C; [b] The T_m of calf thymus DNA in the presence of metal complex in a 1:1 ratio of base pair to complex.

DNA conformational changes induced by binding of the Pt^{II} complexes

The conformational changes of DNA induced by binding of **1** and **2** were investigated by viscosity measurements, gel mobility shift assay, and restriction endonuclease fragmentation assay.

A fixed volume of the Pt^{II} complex solution was added to a solution of sonicated ctDNA (approximately 200 bp). After thorough mixing, the relative viscosity of the resulting mixture was recorded. The relative viscosity ratio (η/η_0), where η and η_0 are the relative viscosities of DNA in the presence or in the absence of ligand, respectively, was plotted against r-bound, and the results are shown in Figure 4.

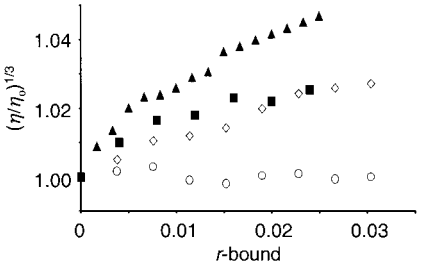

Figure 4. *The relative specific viscosity of sonicated calf thymus DNA (~200 bp) in the presence of ethidium bromide (diamonds), H33342 (circles),* ***1*** *(squares), and* ***2*** *(triangles), shown as a function of the binding ratio.*

The viscosity of DNA samples is proportional to l^3 for rodlike DNA of length l.[16] Intercalators generally increase the hydrodynamic length of DNA, while groove binders do not lengthen the DNA molecules.[17] Viscosity measurement can sensitively detect the lengthening and unwinding of the DNA helix induced by the binding of intercalators,[18] and thus provides evidence of intercalation of small DNA-binding molecules. The viscosity of a 5.0×10^{-4} M DNA solution increased by 2.5%, 2.5%, and 4.6% in the presence of 1.2×10^{-5} M ethidium bromide (EB), **1**, and **2**, respectively, while remaining unchanged in the presence of the same concentration of H33342.

The gel mobility shift assay technique is also sensitive to changes in DNA length or conformation, since the electrophoretic mobility of nucleic acid is proportional to the length of the nucleic acid molecules.[19] The results of gel electrophoresis of a 100-bp DNA ladder are shown in Figure 5 A. Lanes 1 and 8 are the DNA ladder alone. In the presence of EB (lane 2), retardation of DNA movement in the gel was observed, while in the presence of H33342 (lane 3) no shift in DNA mobility relative to lanes 1 and 8 was found. Mobility retardation, smearing, and tailing were observed in lanes 4–7. In the presence of **1** and **2**, the mobility shift of DNA increased with the concentration of metal complexes. Both the viscosity and the gel mobility shift

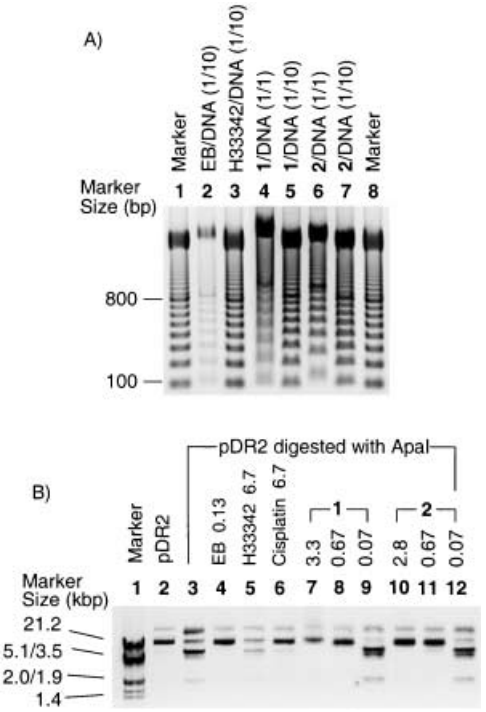

Figure 5. *A) Electrophoresis of a 100-bp DNA ladder in a 1.5% (w/v) agarose gel showing the mobility of DNA (1.52×10^{-3} M in base pairs) in the absence (lanes 1 and 8) and in the presence (lanes 2 – 7) of the indicated compounds. B) Electrophoresis of a 10.7-kbp plasmid (pDR2) in 1% (w/v)* agarose gel after restriction enzyme (ApaI) digestion in the absence (lane 3) and in the presence (lanes 4 – 12) of various compounds, labeled with the molar ratio of compound to DNA base pairs. Lane 2 is the undigested DNA.

experiments confirmed that both the monomeric and the dimeric Pt^{II} complexes bind with DNA through intercalation.

Restriction enzymatic DNA cleavage is inhibited when the local conformation of the enzyme binding site changes or the restriction site is altered upon the binding of a ligand.[20] The results of restriction enzyme digestion of a plasmid DNA (pDR2) in the absence and in the presence of various DNA binding agents are shown in Figure 5 B. Two bands corresponding to supercoiled and nicked DNA are observed in the undigested DNA (lane 2). After ApaI digestion of pDR2 (lane 3), five to six bands corresponding to DNA fragments of 8, 5, and 2 kbp are observed, together with bands for supercoiled and nicked DNA. In the presence of a classical intercalator (EB), a minor groove binder (H33342), and an intrastrand crosslinker (cisplatin), digestion of plasmid was inhibited. In the presence of high molar ratios of **1** or **2** to DNA base pair, complete inhibition of ApaI digestion was found, while partial inhibition was observed at lower molar ratios. The results indicated that the restriction enzymatic digestion of plasmid could be inhibited by DNA-binding molecules including intercalators, groove binders, and covalent crosslinkers.

From the above in vitro experiments, it is clear that **1** and **2** can intercalate with DNA and induce significant conformational change of the DNA, particularly for the dimer. Subsequently, their cytotoxicity against human carcinoma cell lines was examined and the mechanisms of cell death induced by the complexes were investigated.

Cytotoxicities of the Pt^{II} complexes

The cytotoxicities of the $Pt^{II}(\overset{\frown}{C}\ \overset{\frown}{N}\ N)$ complexes and of cisplatin in various human cell lines were determined by standard MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-tetrazolium bromide) assay (Table 3).[21] Cisplatin has a growth inhibitory concentration (IC_{50}) in the range of 4.07×10^{-6} to 1.05×10^{-5} M for the five different human cancer cell lines tested. As listed in Table 3, **2** exhibits cytotoxicity comparable with that of cisplatin, and it is more toxic than **1** by an order of magnitude in the cell lines studied.

Cell death can be divided into two types: necrosis ("accidental" cell death) and apoptosis ("programmed" cell death).[22] Necrotic cells undergo cell lysis and lose their membrane integrity, and severe inflammation is induced.[23] Apoptotic cells, however, are transformed into small membrane-bound vesicles (apoptotic bodies) which are engulfed in vivo by macrophages, and no inflammatory response is found.[24] Harmless removal of cells (cancer cells, for example) is one consideration in chemotherapy.[25] Therefore, induction of apoptosis is one of the considerations in the development of anticancer drugs. Most of the cytotoxic anticancer drugs in current use have been shown to induce apoptosis in susceptible cells.[26]

The type of cell death induced by **1** and **2** was investigated by two apoptosis assays: acridine orange/ethidium bromide (AO/EB) staining, and DNA ladder analysis. The AO/EB staining assay can detect the difference in membrane integrity between necrotic and apoptotic cells.[27] AO is a vital dye and can stain both live and dead cells. EB stains only cells that have lost their membrane integrity. Under the fluorescence microscope, live cells appear green. Necrotic cells stain red, but have a nuclear morphology resembling that of viable cells. Apoptotic cells appear green and morphological changes such as cell blebbing and formation of apoptotic bodies will be observed. In Figure 6 A, the nucleoli of living cells in the untreated sample were stained as bright green spots in the nucleus. However, cells irradiated with 60 mJ cm^{-2} Ultraviolet-C (UV-C) or treated with

Table 3. *Cytotoxicities of **1**, **2**, and cisplatin against various human carcinomas.*

	IC_{50}[a] [M]				
Cell lines	KB-3-1	KB-V1	CNE3	Hep G2	HL60
1	$3.88(\pm 1.21) \times 10^{-4}$	$6.54(\pm 2.39) \times 10^{-4}$	$1.35(\pm 0.36) \times 10^{-4}$	$4.50(\pm 1.29) \times 10^{-5}$	$1.04(\pm 0.30) \times 10^{-4}$
2	$2.44(\pm 2.13) \times 10^{-5}$	$7.11(\pm 3.42) \times 10^{-5}$	$1.60(\pm 0.12) \times 10^{-5}$	$3.66(\pm 0.13) \times 10^{-5}$	$3.16(\pm 1.21) \times 10^{-5}$
Cisplatin	$5.47(\pm 2.43) \times 10^{-6}$	$4.07(\pm 2.02) \times 10^{-6}$	$1.05(\pm 0.27) \times 10^{-5}$	$1.05(\pm 0.06) \times 10^{-5}$	$4.16(\pm 1.89) \times 10^{-6}$

[a] IC_{50} values are given as the mean ± standard error of the mean.

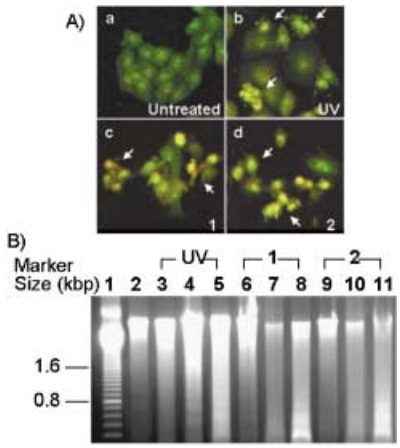

Figure 6. *A) KB-3-1 cells were stained by AO/EB and observed by laser scanning confocal microscopy: a) KB-3-1 cells without treatment; b) irradiated with 60 mJ cm^{-2} UV-C; c) in the presence of* ***1*** *(IC_{30}); d) in the presence of* ***2*** *(IC_{30}); incubated at 37 °C, 5% CO_2 for 72 h. Cells indicated by arrows are apoptotic cells. B) Electrophoretic analysis of genomic DNA extracted from KB-3-1 cells subjected to different treatments: 60 mJ cm^{-2} UV-C irradiation (lanes 3–5), incubation in the presence of* ***1*** *(IC_{30}; lanes 6–8), and incubation in the presence of* ***2*** *(IC_{30}; lanes 9–11). Genomic DNA of the treated cells was collected at different points in time during the incubation: 6 h (lanes 3, 6, and 9), 12 h (lanes 4, 7, and 10), and 24 h (lanes 5, 8, and 11). Lane 1 is a 100-bp DNA ladder (Amersham Pharmacia Biotech). Lane 2 is DNA extracted from untreated cells.*

either **1** or **2** for 72 h exhibited green apoptotic cells with apoptotic bodies. In the DNA ladder analysis,[28] the characteristic feature of apoptosis—the appearance of DNA fragments of multiples of 180 bp—was found in the UV-induced apoptotic sample as well as in the samples treated with the two compounds (Figure 6 B). Significant numbers of apoptotic cells were found in the populations treated with **1** and **2**. The results of the cell morphological study and DNA ladder analysis demonstrate that the two compounds can induce apoptosis in the cell lines studied. In order to understand how the compounds affect cellular metabolism and regulation on a genomic scale,[29] experiments involving the gene expression profiling of the KB-3-1 cell line on treatment with the metal complexes are underway in our laboratory.

Experimental Section

Materials: Reagent grade solvents and chemicals were used without purification unless otherwise noted. ctDNA was purchased from Sigma Chemical Co. and purified before use by the method reported in ref. [30]. DNA concentrations per base pair were determined by absorption spectroscopy by using the molar extinction coefficient of 12800 M^{-1} cm^{-1} bp^{-1} at 260 nm.[31] A 100-bp DNA ladder was purchased from Amersham Pharmacia Biotech Asia Pacific Ltd. (Hong Kong). Plasmid DNA, pDR2 (10.7 kb), was purchased from CLONTECH Laboratories, Inc. (Palo Alto, USA). DNA binding experiments were carried out in aerated Tris buffer solutions (5 mM Tris, 50 mM NaCl, pH 7.2), unless stated otherwise.

6-Phenyl-2,2′-bipyridine ($C\widehat{N}N$)[32] and Pt($C\widehat{N}N$)Cl[33]: These compounds were synthesized according to the methods described in refs. [32] and,[33] respectively.

(Pyridyl)-(6-phenyl-2,2′-bipyridine)platinum(II) hexafluorophosphate (1): A mixture of [Pt($C\widehat{N}N$)Cl] (0.10 g, 0.22 mmol) and pyridine (0.11 mL, 0.22 mmol) in MeCN/H_2O (1:1) was stirred for 3 h. A clear orange solution was obtained, and treatment with NH_4PF_6 gave a yellow precipitate, which was washed with diethyl ether. Recrystallization by diffusion of diethyl ether into acetonitrile solution afforded yellow crystals. Yield: 0.11 g, 80%; ^{1}H NMR (300 MHz, [D_6]dimethylsulfoxide, 25 °C, tetramethylsilane(TMS)): $\delta = 6.36$ (m, 1H), 7.12 (m, 2H), 7.53 (m, 1H), 7.70 (m, 4H), 7.87 (m, 1H), 8.01 (m, 2H), 8.20 (m, 2H), 8.94 (m, 2H)) ppm; MS-FAB: *m/z* (%): 505 (100) $[M]^+$; elemental analysis: calcd (%) for $C_{21}H_{16}N_3PF_6Pt$ (764.4): C 38.8, H 2.5, N 6.5; found: C 38.8, H 2.5, N 6.5.

***μ*-*N*,*N*′-Bis(isonicotinyl)-1,6-hexanediamino-bis-[6-phenyl-2,2′-bipyridine-platinum(II)] dichloride (2)**: Linker (0.07 g, 0.21 mmol) in H_2O was added dropwise to Pt($C\widehat{N}N$)Cl (0.2 g, 0.43 mmol) in MeCN/H_2O (1:1). The mixture was stirred at room temperature overnight and was evaporated to dryness. The product was extracted with ethanol and purified by recrystallization from ethanol. Yield: 0.09 g, 34%; ^{1}H NMR (270 MHz, CD_2Cl_2, 25 °C, TMS): $\delta = 0.91$ (quint, 2H; CH_2), 1.45 (quint, 2H; CH_2), 3.46 (q, 2H; CH_2), 6.57 (m, 1H), 7.12 (m, 1H), 7.23 (m, 1H), 7.42 (m, 1H), 7.54 (m, 5H), 7.97 (m, 3H), 8.15 (m, 1H), 8.71 (d, 2H; =CH), 9.10 ppm (m, 1H); ES-MS: *m/z* (%): 589 (40) $[M]^{2+}$, 752 (100) $[C_{34}H_{29}N_6O_2Pt]^+$; elemental analysis: calcd (%) for $C_{50}H_{44}N_8O_2Cl_2Pt_2$ (1250.0): C 48.0, H 3.5, N 9.0; found: C 47.8, H 3.4, N 8.8.

***N*,*N*′-Bis(isonicotinyl)-1,6-hexanediamine (the linker)**: A mixture of methyl isonicotinamide (5.2 mL, 38 mmol) and 1,6-hexanediamine (1.92 g, 17 mmol) in MeOH (30 mL) was heated under reflux in the presence of ammonium chloride (0.15 g) for 4 h. A pale yellow solid was formed. The product was purified by recrystallization from MeOH. Yield: 4.04 g, 75%; ^{1}H NMR (300 MHz, CD_2Cl_2, 25 °C, TMS): $\delta =$ 0.87 (quint, 2H; CH_2), 1.48 (quint, 2H; CH_2), 3.53 (q, 2H; CH_2), 6.55 (m, 1H; NH), 7.66 (d, 2H; =CH), 8.74 ppm (d, 2H; =CH); MS-FAB: *m/z* (%): 327 (100) $[M+H]^+$; elemental analysis: calcd (%) for $C_{18}H_{22}N_4O_2$ (326.4): C 66.3, H 6.7, N 17.2; found: C 66.1, H 6.6, N 17.0.

Absorption and fluorescence titrations: All absorption and fluorescence spectra were recorded on a Perkin–Elmer Lambda 19 UV/Visible spectrophotometer and a Jobin Yvon Horiba Spex FluoroMax-3 spectrofluorimeter, respectively. Titrations were performed by keeping the concentration of metal complex constant while varying the nucleic acid concentration. Either the absorbance or the fluorescence intensity was recorded after each addition of DNA.

UV melting study: UV melting studies were carried out with a Perkin–Elmer Lambda 19 UV/Visible spectrophotometer equipped with a Peltier temperature programmer (PTP-6). Solutions of ctDNA in the absence and presence of compound, with a DNA base pair to compound ratio of 1:1, were prepared in Tris buffer. The temperature of the solution was increased by 1 °C min^{-1}, and the absorbance at 260 nm was continuously monitored.

Viscosity experiments: Rodlike DNA samples of approximately 200 bp in length were prepared for viscosity measurements by sonication.[34] The method used by Suh and Chaires[17] was employed in this study. Viscosity experiments were performed on a Cannon–Manning Semi-Micro Viscometer, immersed in a thermostated water bath maintained at 27 °C. Titrations of ligand were carried out by addition of small volumes of concentrated stock solutions to the DNA sample in BPE buffer (6 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1 mM Na_2EDTA, pH 7.0) in the viscometer. Solutions in the viscometer were mixed by bubbling nitrogen through the solution. DNA concentrations of approximately 5×10^{-4} M in base pairs were used. Relative

viscosities for DNA either in the presence or in the absence of ligand were calculated from the relationship

$$\eta = \frac{(t - t_0)}{t_0} \quad (8)$$

where t is the observed flow time of the DNA-containing solution, and t_0 is the flow time of buffer alone.

Gel mobility shift assay: A 100-bp DNA ladder (Amersham Pharmacia Biotech) (1.52×10^{-3} M in base pairs) was mixed with various compounds and incubated at 37 °C for 5 min. The mixtures were analyzed by gel electrophoresis. The gel was stained by immersion in a bath of ethidium bromide (0.5 μg mL^{-1}) after electrophoresis.

Restriction endonuclease fragmentation assay: Digestion of a plasmid, pDR2 (10.7 kb), with a restriction enzyme (ApaI, Boehringer Mannheim) was performed by mixing the DNA (3.0×10^{-4} M in base pairs) in 1 × SuRE/Cut Buffer A (Boehringer Mannheim) with ApaI (1 unit μL^{-1}), followed by incubation at 37 °C for one hour.[30] A mixture of compound and plasmid pDR2 (10.7 kbp, 3.0×10^{-4} M in base pairs) in digestion buffer was first incubated at room temperature for 5 minutes before the addition of ApaI. Two controls with pDR2 in the absence and in the presence of ApaI were also prepared. After digestion, the samples were analyzed by electrophoresis.

MTT assay: Standard procedures were used.[21] Cells were plated in 96-well microassay culture plates (10^4 cells per well) and grown overnight at 37 °C in a 5% CO_2 incubator. Test compounds were then added to the wells to achieve final concentrations ranging from 10^{-6} to 10^{-4} M. Control wells were prepared by addition of culture medium (100 μL). Wells containing culture medium without cells were used as blanks. The plates were incubated at 37 °C in a 5% CO_2 incubator for 72 h. Upon completion of the incubation, stock MTT dye solution (20 μL, 5 mg mL^{-1}) was added to each well. After 4 h incubation, buffer (100 μL) containing *N*,*N*-dimethylformamide (50%) and sodium dodecyl sulfate (20%) was added to solubilize the MTT formazan. The optical density of each well was then measured on a microplate spectrophotometer at a wavelength of 570 nm. The IC_{50} value was determined from plots of % viability against dose of compound added. Five different human carcinomas were the subjects in this study: KB-3-1 (oral epidermal), KB-V1 (oral epidermal), CNE3 (nasopharyngeal), Hep G2 (hepatocellular), and HL60 (promyelocytic leukaemia).

Acridine orange/ethidium bromide (AO/EB) staining: Cell cultures of a monolayer of KB-3-1 cells were incubated in the absence and in the presence of **1** or **2** at their IC_{30} values (the concentrations that gave 30% inhibition) at 37 °C and 5% CO_2 for 72 h. After 72 h, 1 mL cell culture was stained with AO/EB solution (40 μL; 50 μg mL^{-1} AO, 50 μg mL^{-1} EB in phosphate buffer).[27] Samples were observed immediately under a laser confocal microscope (Zeiss Axiovert 100M).

DNA ladder analysis: KB-3-1 cells were cultured at a concentration of approximately 2×10^5 cells mL^{-1}. Compounds **1** and **2** were added to the cultures at their IC_{30} values. Other flasks of cells were irradiated with 60 mJ cm^{-2} UV-C to induce apoptosis. An untreated sample was used as a negative control. After treatment, all the cultures were incubated at 37 °C, 5% CO_2. Cells were collected at three different time points: 6, 12, and 24 h. Their genomic DNA was extracted[30] and analyzed by electrophoresis.

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[1] a) E. Kikuta, M. Murata, N. Katsube, T. Koike, E. Kimura, *J. Am. Chem. Soc.* **1999**, *121*, 5426 – 5436; b) M. Howe-Grant, K. C. Wu, W. R. Bauer, S. J. Lippard, *Biochemistry* **1976**, *15*, 4339 – 4346; c) S. J. Lippard, P. J. Bond, K. C. Wu, W. R. Bauer, *Science* **1976**, *194*, 726 – 728; d) E. Wong, C. M. Giandomenico, *Chem. Rev.* **1999**, *99*, 2451 – 2466.
[2] a) I. Haq, P. Lincoln, D. Suh, B. Norden, B. Z. Chowdhry, J. B. Chaires, *J. Am. Chem. Soc.* **1995**, *117*, 4788 – 4796; b) R. M. Hartshorn, J. K. Barton, *J. Am. Chem. Soc.* **1992**, *114*, 5919 – 5925; c) K. E. Erkkila, D. T. Odom, J. K. Barton, *Chem. Rev.* **1999**, *99*, 2777 – 2795.
[3] a) J. B. Chaires, F. Leng, T. Przewloka, I. Fokt, Y.-H. Ling, R. Perez-Soler, W. Priebe, *J. Med. Chem.* **1997**, *40*, 261 – 266; b) P. B. Dervan, M. M. Becker, *J. Am. Chem. Soc.* **1978**, *100*, 1968 – 1970; c) W. D. McFadyen, L. P. G. Wakelin, I. A. G. Roos, B. L. Hillcoat, *Biochem. J.* **1987**, *242*, 177 – 183.
[4] S. W. Lai, M. C. W. Chan, T. C. Cheung, S. M. Peng, C. M. Che, *Inorg. Chem.* **1999**, *38*, 4046 – 4055.
[5] a) C. W. Chan, L. K. Cheng, C. M. Che, *Coord. Chem. Rev.* **1994**, *132*, 87 – 97; b) C. M. Che, K. T. Wan, L. Y. He, C. K. Poon, V. W. W. Yam, *J. Chem. Soc. Chem. Commun.* **1989**, 943 – 944; c) H.-Q. Liu, S.-M. Peng, C. M. Che, *J. Chem. Soc. Chem. Commun.* **1995**, 509 – 510.
[6] S. J. Lippard, *Acc. Chem. Res.* **1978**, *11*, 211 – 217.
[7] L. Valentini, V. Nicolella, E. Vannini, M. Menozzi, S. Penco, F. Arcamone, *Farmaco* **1985**, *40*, 377 – 390.
[8] P. B. Dervan, *Science* **1986**, *232*, 464 – 471.
[9] a) V. M. Miskowski, V. H. Houlding, *Inorg. Chem.* **1989**, *28*, 1529 – 1533; b) V. H. Houlding, V. M. Miskowski, *Coord. Chem. Rev.* **1991**, *111*, 145 – 152.
[10] W. I. Sundquist, S. J. Lippard, *Coord. Chem. Rev.* **1990**, *100*, 293 – 322.
[11] C. V. Kumar, E. H. Asuncion, *J. Am. Chem. Soc.* **1993**, *115*, 8547 – 8553.
[12] J. D. McGhee, P. H. von Hippel, *J. Mol. Biol.* **1974**, *86*, 469 – 489.
[13] V. H. DuVernay, Jr., J. A. Pachter, S. T. Crooke, *Biochemistry* **1979**, *18*, 4024 – 4030.
[14] a) C.-M. Che, M. Yang, K.-H. Wong, H.-L. Chan, W. Lam, *Chem. Eur. J.* **1999**, *5*, 3350 – 3355; b) C. S. Peyratout, T. K. Aldridge, D. K. Crites, D. R. McMillin, *Inorg. Chem.* **1995**, *34*, 4484 – 4489.
[15] J. D. McGhee, *Biopolymers* **1976**, *15*, 1345 – 1375.
[16] G. Cohen, H. Eisenberg, *Biopolymers* **1969**, *8*, 45 – 55.
[17] D. Suh, J. B. Chaires, *Bioorg. Med. Chem.* **1995**, *3*, 723 – 728.
[18] F. Leng, W. Priebe, J. B. Chaires, *Biochemistry* **1998**, *37*, 1743 – 1753.
[19] E. C. Long, J. K. Barton, *Acc. Chem. Res.* **1990**, *23*, 271 – 273.
[20] S. Neidle, L. H. Pearl, J. V. Skelly, *Biochem. J.* **1987**, *243*, 1 – 13.
[21] T. Mosmann, *J. Immunol. Methods* **1983**, *65*, 55 – 63.
[22] a) R. A. Schwartzman, J. A. Cidlowski, *Endocr. Rev.* **1993**, *14*, 133 – 151; b) I. Vermes, C. Haanen, *Adv. Clin. Chem.* **1994**, *31*, 177 – 246.
[23] R. Van Furth, T. L. Van Zwet, *J. Immunol. Methods* **1988**, *108*, 45 – 51.
[24] J. S. Savill, P. M. Henson, J. E. Henson, C. Haslett, M. J. Walport, A. H. Wyllie, *J. Clin. Invest.* **1989**, *83*, 865 – 875.
[25] N. A. Thornberry, Y. Lazebnik, *Science* **1998**, *281*, 1312 – 1316.
[26] J. A. Hickman, *Cancer Metastasis Rev.* **1992**, *11*, 121 – 139.
[27] G. P. Amarante-Mendes, E. Bossy-Wetzel, T. Brunner, D. Finucane, D. R. Green, S. Kasibhatla in *Cells: A laboratory manual*, *Vol. 1* (Eds.: D. L. Spector, R. D. Goldman, L. A. Leinwand), Cold Spring Harbor Laboratory Press, New York, **1998**, Chapter 15.
[28] A. H. Wyllie, *Nature* **1980**, *284*, 555 – 556.
[29] M. J. Marton, J. L. DeRisi, H. A. Bennett, V. R. Iyer, M. R. Meyer, C. J. Roberts, R. Stoughton, J. Burchard, D. Slade, H. Dai, D. E. Bassett Jr., L. H. Hartwell, P. O. Brown, S. H. Friend, *Nat. Med.* **1998**, *4*, 1293 – 1301.
[30] *Molecular Cloning: A Laboratory Manual* (Eds.: J. Sambrook, E. F. Fritsch, T. Maniatis), Cold Spring Harbor Laboratory Press, New York, **1989**.
[31] J. K. Barton, J. M. Goldberg, C. V. Kumar, N. J. Turro, *J. Am. Chem. Soc.* **1986**, *108*, 2081 – 2088.
[32] C. O. Dietrich-Buchecker, P. A. Marnot, J. P. Sauvage, *Tetrahedron Lett.* **1982**, *23*, 5291 – 5294.
[33] a) E. C. Constable, R. P. G. Henney, T. A. Leese, D. A. Tocher, *J. Chem. Soc. Dalton Trans.* **1990**, 443 – 449; b) E. C. Constable, R. P. G. Henney, T. A. Leese, D. A. Tocher, *J. Chem. Soc. Chem. Commun.* **1990**, 513 – 515.
[34] J. B. Chaires, N. Dattagupta, D. M. Crothers, *Biochemistry* **1982**, *21*, 3933 – 3940.

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