A Monophenyl Phenanthroline Complex of Copper(I) That Binds to DNA but Not by Intercalation

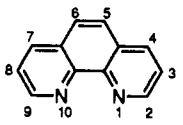
Kelley A. Meadows, Fang Liu, Brian P. Hudson, and David R. McMillin*

Department of Chemistry, 1393 Brown Building, Purdue University, West Lafayette, Indiana 47907-1393

Received April 1, 1993

Introduction

Since Sigman and co-workers first reported that copper phenanthrolines can function as artificial nucleases,[1] there has been considerable interest in using these systems as chemical probes of DNA structure.[2,3] Strand cleavage occurs via a series of reactions beginning with hydrogen atom abstraction from a ribose unit.[2] The abstracting agent is probably a metal–oxo or metal–hydroxyl intermediate which forms when hydrogen peroxide combines with a $Cu(phen)_2^+$ molecule that is noncovalently bound to DNA[2,4] (phen denotes the 1,10-phenanthroline ligand).



phen

On the bases of competitive binding studies,[5] the regiochemistry of hydrogen atom abstraction,[6] the offset in the cutting pattern from one side of the minor groove to the other,[7] and substituent effects on the phenanthroline ligand,[4] Sigman and co-workers have proposed that $Cu(phen)_2^+$ binds to the surface of DNA within the minor groove. Independent information about the binding interactions of these systems has come from spectral studies of the related $Cu(dmp)_2^+$ system, where dmp denotes 2,9-dimethyl-1,10-phenanthroline. In this system the presence of the methyl substituents destabilizes the copper(II) oxidation state relative to copper(I),[8] such that the complex does not catalyze the formation of hydrogen peroxide. Hence, binding interactions of the dmp complex can be investigated in the absence of cleavage chemistry. Our earlier results showed that the bound form of $Cu(dmp)_2^+$ is fully accessible to solvent since the charge-transfer (CT) emission from the complex remains quenched.[9,10] This result is consistent with groove binding, but it would be hard to reconcile with a more intimate mode of binding such as intercalation that, for steric reasons, would be unlikely to permit facile changes in the coordination number of the copper center.

However, Williams et al. have carried out systematic cleavage studies of oligonucleotides containing mismatched strands, and their data suggest that the phen complex preferentially causes cleavage near bulges where intercalative binding is assumed to be more favorable.[11] In addition, Rill and co-workers have interpreted cleavage results[12] and viscometry data[13] in terms of a binding model which involves intercalation of a portion of one of the ligands of $Cu(phen)_2^+$.

In the case of the more hydrophobic complex $Cu(bap)_2^+$, where bap denotes 4,7-diphenyl-1,10-phenanthroline, Sigman and co-workers have also entertained the notion of partial intercalation.[4] They invoked this mode of binding because the cutting occurs in a less sequence-dependent fashion and because Barton and co-workers had suggested that phenyl substituents in the 4,7-positions of the phenanthroline ligand(s) promote partial intercalation in systems derived from $Ru(phen)_3^{2+}$.[14] In the case of $Cu(bcp)_2^+$, where bcp denotes 2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline, we have confirmed that the phenyl substituents dictate an altered mode of binding.[9,10] The interaction with DNA somehow protects the copper center in $Cu(bcp)_2^+$ from solvent attack because the complex is emissive in aqueous media in the presence of DNA. Furthermore, polarization measurements reveal that the chromophore hardly changes its orientation during the lifetime of the excited state. This implies that the complex is bound rigidly enough to lessen dramatically the tumbling rate in solution. The emission data are consistent with intercalative binding, but recent viscometry measurements cast doubt on this model.[15] Intercalation is an important mode of binding for fused ring aromatics, but steric clashes between hydrogen atoms force the phenyl substituents to be out of the plane of the phenanthroline ring in $Cu(bcp)_2^+$ [16] and $Ru(bap)_3^{2+}$.[17] Barton and co-workers suggested that the problem presented by the twisted phenyl groups could be minimized if asymmetric docking occurred such that only one of the phenyl substituents passes between the base pairs of DNA. To test this hypothesis, we have studied the complex of a phenanthroline ligand with only one phenyl substituent, and the results are described below.

Experimental Section

Materials. All solvents were from Burdick and Jackson (spectral grade) or Fisher Chemical (ACS grade). Deuterated chloroform was from Cambridge Isotope Laboratories. Methyl lithium (MeLi) and 1,4-diazabicyclo[2.2.2]octane (DABCO) were purchased from Aldrich. The $MgSO_4$ was from Fisher, and the MnO_2 was from Fluka. Analytical and preparative alumina thin-layer chromatography (tlc) plates were from Merck. The $NaNO_3$ was from Fisher Chemicals. Salmon testes (ST) DNA was purchased from Sigma Chemical Co. Poly(dA-dT)·poly(dA-dT), poly(dG-dC)·poly(dG-dC), poly(A)·poly(U), and poly(C)·poly(I) were all purchased from Pharmacia. Trisma base and Trisma·HCl were from Sigma Chemical Co. G. F. Smith Chemical Co. supplied an impure sample of 4-phenyl-1,10-phenanthroline as a custom synthesis.

Methods. After we extracted the crude solid containing 4-phenyl-1,10-phenanthroline into a dilute solution of HCl(aq), we obtained the hydrochloride salt by reducing the volume and adding acetone. (Anal. Calcd for $C_{18}H_{12}N_2 \cdot HCl \cdot 2.5H_2O$: C, 63.99; H, 5.38; N, 8.29. Found: C, 63.65; H, 5.33; N, 8.14.) We isolated the free base as white needles (mp 126–128 °C) by dissolving the hydrochloride salt in water and inducing precipitation with NaOH(aq).

ST DNA was purified by precipitation with ethanol, while the other biopolymers were used without further purification. In order to reduce viscosity and average chain length, the ST DNA was sonicated for 5 min until the average chain length was 600 base pairs, as determined by viscometry.

* Author to whom correspondence should be addressed.

(1) Sigman, D. S.; Graham, D. R.; D'Aurora, V.; Stern, A. M. *J. Biol. Chem.* **1979**, *254*, 12269–12272.
(2) Sigman, D. S. *Acc. Chem. Res.* **1986**, *19*, 180–186.
(3) Sigman, D. S.; Chen, C.-H. B. *Annu. Rev. Biochem.* **1990**, *59*, 207–236.
(4) Thederahn, T. B.; Kuwabara, M. D.; Larsen, T. A.; Sigman, D. S. *J. Am. Chem. Soc.* **1989**, *111*, 4941–4946.
(5) Kuwabara, M. D.; Yoon, C.; Goyne, T.; Thederahn, T. B.; Sigman, D. S. *Biochemistry* **1986**, *25*, 7401–7408.
(6) Goyne, T. E.; Sigman, D. S. *J. Am. Chem. Soc.* **1987**, *109*, 2846–2848.
(7) Yoon, C.; Kuwabara, M. D.; Law, R.; Wall, R.; Sigman, D. S. *J. Biol. Chem.* **1988**, *263*, 8458–8463.
(8) Tamilarasan, R.; McMillin, D. R.; Liu, F. In *Metal-DNA Chemistry*; Tullius, T. D., Ed.; ACS Symposium Series 402; American Chemical Society: Washington, DC, 1989; pp 48–58.
(9) Tamilarasan, R.; Ropartz, S.; McMillin, D. R. *Inorg. Chem.* **1988**, *27*, 4082–4084.
(10) Tamilarasan, R.; McMillin, D. R. *Inorg. Chem.* **1990**, *29*, 2798–2802.
(11) Williams, L. D.; Thivierge, J.; Goldberg, I. H. *Nucleic Acids Res.* **1988**, *16*, 11607–11615.
(12) Veal, J. M.; Rill, R. L. *Biochemistry* **1989**, *28*, 3243–3250.
(13) Veal, J. M.; Rill, R. L. *Biochemistry* **1991**, *30*, 1132–1140.
(14) Barton, J. K.; Raphael, A. L. *J. Am. Chem. Soc.* **1984**, *106*, 2466–2468.
(15) Liu, F.; Meadows, K. A.; McMillin, D. R. *J. Am. Chem. Soc.* **1993**, *115*, 6699–6704.
(16) Klemens, F. K.; Fanwick, P. E.; Bibler, J. K.; McMillin, D. R. *Inorg. Chem.* **1989**, *28*, 3076–3079.
(17) Goldstein, B. M.; Barton, J. K.; Berman, H. M. *Inorg. Chem.* **1986**, *25*, 842–847.

0020-1669/93/1332-4663$04.00/0

All glassware was soaked for 24 h in a 50% sulfuric acid solution to remove any adventitious metal ions. Spectral measurements were made at a copper concentration of 30.4 μM in 0.025 M, pH 7.8 Tris buffer. For solubility reasons, a mixed solvent system containing methanol and buffer was used. Samples were typically prepared by adding aliquots of a buffered DNA or RNA solution to a copper stock solution in methanol followed by an appropriate amount of buffer to a final composition of 20% methanol. Concentrations of the nucleic acid solutions were determined from the reported molar absorptivity values at 260 nm: 6600 M^{-1} cm^{-1} for ST DNA[18] and poly(dA-dT)·poly(dA-dT),[19] 8400 M^{-1} cm^{-1} for poly(dG-dC)·poly(dG-dC),[20] 7140 M^{-1} cm^{-1} for poly(A)·poly(U),[21] and 5000 M^{-1} cm^{-1} for poly(C)·poly(I).[22] Viscometry studies with ST DNA were performed in 20% and 10% methanol in 0.025 M Tris pH 7.8 buffer solutions. For the viscometric measurements, the ST DNA concentration was always 0.1 mg/mL while the concentration of copper complex was variable.

Synthesis of dmpp. The 2,9-dimethyl-4-phenyl-1,10-phenanthroline ligand (dmpp) was prepared by an adaptation of a literature procedure.[23] We added the 4-phenyl-1,10-phenanthroline starting material (200 mg) to benzene along with 6 equiv of DABCO to prevent aggregation of MeLi.[24] Before the addition of the lithium reagent, however, we distilled off several milliliters of solvent in order to remove adventitious water. Then we immersed the reaction flask in an ice bath for 10 min and added 6 equiv of MeLi under argon over a period of 30 min through a dry canula. We allowed the resulting dark red solution to warm to room temperature and stirred for 2 h. After first cooling the reaction mixture in an ice bath for 10 min, we then quenched the reaction with about 30 mL of water. The resulting solution was a bright yellow color. After collecting the organic layer, we extracted the aqueous layer with methylene chloride three times. Treatment of the pooled organic layers with 20 g of (activated) MnO_2 gave a black solution. After stirring for 30 min, we added $MgSO_4$ (20 g) and then stirred for another 30 min. Filtration produced a light yellow solution that contained monomethylated ligand, and a small amount of dmpp ligand, as verified by tlc. We isolated the dmpp ligand on a preparative tlc plate of basic alumina (eluting solvent: 70/30 ethyl acetate/hexane) and reexposed the monomethylated ligand to MeLi to generate more dmpp.

Ultimately, we extracted the crude product into 0.1 M HCl, stirred overnight, and filtered. Treatment of the filtrate with 0.1 M NaOH yielded a fine white powder of dmpp (mp 202–203 °C). The yield was approximately 70%. Identification was by NMR and mass spectral analysis. NMR (in chloroform): δ 8.12 (1 d), 7.83 (1 d), 7.65 (1 d), 7.55 (5 m), 7.52 (1 d), 7.45 (1 s), 2.98 (3 s), 2.99 (3 s). MS parent ion: 284 amu.

$Cu(dmpp)_2NO_3$. This complex was prepared by a variation on a previously reported procedure.[25] We recrystallized the initial product from hot acetone by the dropwise addition of a saturated solution of $NaNO_3$ in water until there was a persistent cloud point. We then reheated the solution and added a few drops of acetone to redissolve the precipitate. We collected red crystals after 2 days. (Anal. Calcd for $[Cu(dmpp)_2]NO_3 \cdot {}^1/_2(CH_3C(O)CH_3) \cdot H_2O$: C, 67.23; H, 5.04; N, 9.45. Found: C, 67.46; H, 5.04; N, 9.12.) The molar absorptivity of the complex at 462 nm in methanol was determined to be 11 500 M^{-1} cm^{-1}.

Instrumentation. We recorded absorption spectra on a Perkin-Elmer Lambda 4C spectrophotometer. We measured emission and excitation spectra with an SLM/Aminco SPF-500C spectrofluorometer with a 520-nm long-wave-pass filter between the sample and the emission monochromator. The excitation wavelength for the emission was 462 nm. For emission lifetimes we used a Princeton Applied Research nitrogen-pumped dye laser with a boxcar averager and an Isaac analog/digital converter as described previously.[26] The excitation wavelength was 464 nm. To isolate the detector from scattered light, we used a combination of Corning color filters, 2–58 and 2–64, which cut-off from 600 to 660 nm, along with a 520-nm long-wave-pass filter and a 600-nm long-wave-pass filter. In each case we collected data for five lifetimes. In order to eliminate the laser pulse, we deleted 27 ns of data from the initial portion of the decay. We analyzed the decay curves with an iterative, nonlinear least-squares algorithm and weighted the squared residuals by $1/e_i^2$ where e_i is the experimental intensity at the ith sampling time. If the first few nanoseconds were ignored, plots of the weighted residuals were consistent with simple exponential decays. We obtained circular dichroism (CD) spectra at 25 °C with a Jasco J-600 spectropolarimeter and a 2-cm-path cell for the visible region readings or a 1-mm-path cell for the UV measurements. For the viscosity measurements we used a Cannon-Fenske No. 25 viscometer thermostated at 23 °C. A Varian VXR 500-MHz spectrometer yielded the NMR spectral data, and a Finnigan 4000 mass spectrometer provided the mass spectrum.

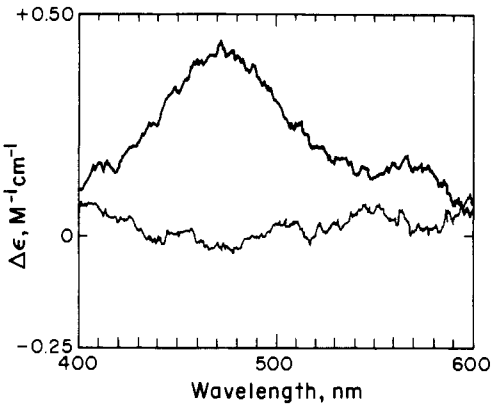


Figure 1. Induced CD spectrum of $Cu(dmpp)_2^+$ in 20% MeOH/Tris buffer at 25 °C. The DNA-P/Cu ratio was 50 for the top trace and 0 for the bottom trace. The DNA was from salmon testes.

Results

Electronic Absorption. Most of our previous work dealing with the binding of $Cu(bcp)_2^+$ to DNA has been carried out in 33% MeOH.[8,10] One of the signatures of the DNA-binding interaction is a red shift in the CT absorption maximum; however, this did not occur with $Cu(dmpp)_2^+$ until the percentage of MeOH was reduced to about 25%. Below 15% MeOH there was a distinct offset in the base line of the absorption spectrum which was indicative of light scattering due to heterogeneity. Below 10% MeOH the solutions were also visibly turbid. As a compromise, we elected to carry out subsequent experiments in 20% MeOH solution. Under these conditions the CT absorption maximum of $Cu(dmpp)_2^+$ red-shifted by 2 nm regardless of the source of polynucleotide. In addition there was hypochromism. One unexpected result was the bleaching of the sample when we combined poly(dG-dC)·poly(dG-dC) with $Cu(dmpp)_2^+$ in solution. After 24 h, solutions containing poly(dG-dC)·poly(dG-dC) with DNA–phosphate-to-copper (DNA-P/Cu) ratios of 10 and greater were almost totally colorless.

Circular Dichroism. The addition of $Cu(dmpp)_2^+$ produced a small, but reproducible attenuation of the UV circular dichroism (CD) spectrum of double-stranded RNA and DNA. Since the copper complex also absorbs in the UV region, some of the change in signal could be due to induced CD intensity. The important point is that there was no evidence of a significant conformational change of the biopolymer. At low DNA-P/Cu or RNA-P/Cu ratios the CD signals were very noisy, probably due to light scattering artifacts.[27] In the presence of all types of DNA studied and both types of RNA, a nonconservative CD signal with positive ellipticity appeared between 470 and 480 nm, in the CT absorption region of $Cu(dmpp)_2^+$. Figure 1 shows a representative CD spectrum when excess ST DNA was present.

Luminescence. In the absence of DNA or RNA the emission from $Cu(dmpp)_2^+$ is extremely weak in tris/MeOH solution. As can be appreciated from Figure 2, however, sizable enhancements

(18) Felsenfeld, G.; Hirschman, S. Z. *J. Mol. Biol.* **1965**, *13*, 407–427.
(19) Inman, R. B.; Baldwin, R. L. *J. Mol. Biol.* **1962**, *5*, 172–184.
(20) Wells, R. D.; Larson, J. E.; Grant, R. C.; Shortle, B. E.; Cantor, C. R. *J. Mol. Biol.* **1970**, *54*, 465–497.
(21) Schmechel, D. E. V.; Crothers, D. M. *Biopolymers* **1971**, *10*, 465–480.
(22) Chamberlin, M. J.; Patterson, D. L. *J. Mol. Biol.* **1965**, *12*, 410–428.
(23) Dietrich-Buchecker, C. O.; Marnot, P. A.; Sauvage, J. P. *Tetrahedron Lett.* **1982**, 23, 5291–5294.
(24) Screttas, C. G.; Eastham, J. F. *J. Am. Chem. Soc.* **1965**, *87*, 3276–3277.
(25) McMillin, D. R.; Buckner, M. T.; Ahn, B. T. *Inorg. Chem.* **1977**, *16*, 943–945.
(26) Gamache, R. E., Jr.; Rader, R. A.; McMillin, D. R. *J. Am. Chem. Soc.* **1985**, *107*, 1141–1146.
(27) Urry, D. W.; Krivacic, J. *Proc. Natl. Acad. Sci. U.S.A.* **1970**, *65*, 845-852.

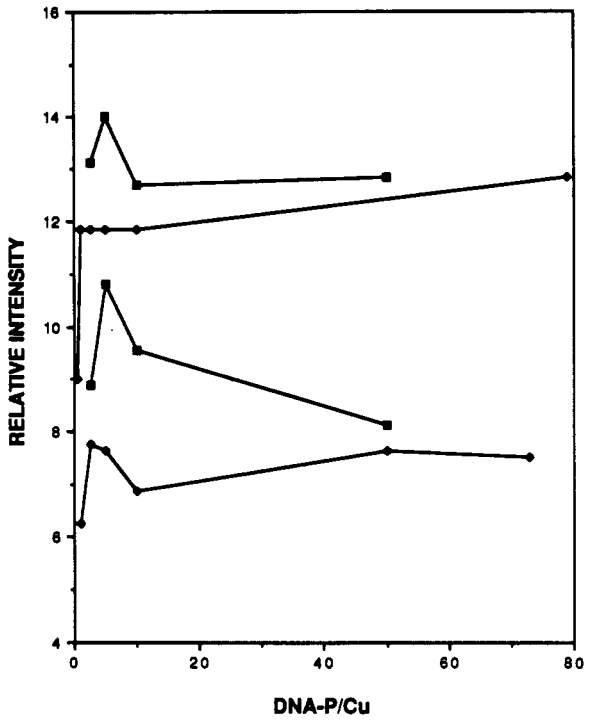


Figure 2. Emission intensity increase as a function of the RNA-P/Cu or DNA-P/Cu ratio. For clarity, the initial data are not shown, but each curve originates at unit intensity ratio in the absence of any polynucleotide. From top to bottom the polynucleotides are poly(A)·poly(U), poly(C)·poly(I), poly(dA-dT)·poly(dA-dT), and ST DNA. The copper concentration was 30.4 μM in Tris/MeOH at 25 °C.

Table I. Relative Viscosity Due to ST DNA in a Tris Buffer Containing 20% MeOH at 23 °C

Cu/DNA-P	η/η_0	Cu/DNA-P	η/η_0
0.0	1.0	0.02	1.0
0.013	1.0	0.10	0.6

in emission intensity occur in the presence of the biopolymers. In all cases the (uncorrected) emission spectrum is broad band with a maximum at around 680 nm and a full width at half-height of about 125 nm. Figure 2 describes the way in which the emission intensity depended on the DNA-P/Cu or RNA-P/Cu ratio. Scattering artifacts were apparent in the emission spectrum at DNA-P/Cu or RNA-P/Cu ratios below 10. The emission lifetime was 80 ± 5 ns in the presence of excess DNA or double-stranded RNA.

Viscometry Studies. At low copper-to-DNA ratios we obtained a specific viscosity for ST DNA that was identical to the value we obtained from solutions that did not contain $Cu(dmpp)_2^+$ (Table I). However, at higher copper concentrations we observed a decrease in the relative viscosity. A similar decrease in solution viscosity occurs when $Cu(bcp)_2^+$ binds to RNA,[28] but viscosity increases can be observed for low loadings of $Cu(bcp)_2^+$ on ST DNA or poly(dA-dT)·poly(dA-dT).[15]

Discussion

Peripheral substituents on the ligands clearly influence the way in which copper phenanthrolines bind to DNA. Previous work has shown that $Cu(bcp)_2^+$ binds more intimately than $Cu(dmp)_2^+$ in 33% MeOH,[8-10] but exposure to DNA or RNA has no effect on the absorption spectrum of $Cu(dmpp)_2^+$ under the same conditions. The CT absorption does, however, exhibit a red shift at lower levels of MeOH. The binding interaction also induces a nonconservative CD signal in the region of the CT absorption. However, despite the unsymmetrical substitution pattern within the dmpp ligand, the $\Delta\epsilon$ value is quite small and not much different from the $\Delta\epsilon$ value observed with $Cu(bcp)_2^+$ under comparable conditions.[15] No CD signal could be detected from the bound form of $Cu(dmp)_2^+$.

In most instances, the emission data reveal at least two phases in the binding of $Cu(dmpp)_2^+$. Below RNA-P/Cu or DNA-P/Cu values of ca. 10 the emission intensity generally reaches a maximum value and then drops off to a lower intensity plateau with the addition of more polynucleotide. We have observed similar behavior from $Cu(bcp)_2^+$ and assigned the emission seen at low DNA-P/Cu ratios to some type of aggregated species.[10,15] This type of sequestration protects the copper center from the solvent and allows the CT emission process to occur. Related aggregates have been observed when polyanions are exposed to high loadings of aromatic dyes.[29-31]

The CT emission persists at higher polynucleotide-to-copper ratios where the aggregates are dispersed. Early on, in the case of $Cu(bcp)_2^+$ we suggested that in this regime the emission might be explained by an intercalative mode of binding which would inhibit solvent attack and expansion of the coordination number at copper.[8,10] However, recent viscometry studies do not support this model,[15] and the same is true here, since the addition of $Cu(dmpp)_2^+$ does not enhance the specific viscosity of the ST DNA in solution. Classic work has shown that intercalative binders cause elongation of the double helix and an attendant increase in viscosity.[32] In $Cu(bcp)_2^+$ and by inference in $Cu(dmpp)_2^+$, unfavorable steric interactions involving the ortho hydrogens of the phenyl substituents and the C5 and C6 hydrogens of the parent phenanthroline cause the phenyl groups to be out of plane. This probably destabilizes the intercalated form. In previous studies Gabbay et al. have argued that 1-methyl-4,7-diphenyl-1,10-phenanthroline does not intercalate into DNA like other phenanthrolinium ions due to the nonplanarity.[33]

If there is no intercalation, an alternative structure must be considered. Sigman and co-workers have suggested that $Cu(phen)_2^+$ may bind along the minor groove with the edge of the ligands in the groove.[4] Call this the C3 edge because it contains carbons 2–4 of the ligand. This type of interaction may occur with $Cu(dmp)_2^+$, but $Cu(bcp)_2^+$ and $Cu(dmpp)_2^+$ must be bound in a much less exposed fashion in view of the fact that the CT emission persists. The presence of the phenyl substituents may induce local melting of the DNA structure and thus permit deeper penetration into the macromolecular structure. At higher loadings the copper complex may bridge between different segments of double-stranded polynucleotide.[15] This type of encapsulation would also be consistent with the emission data. At the same time, the condensation of the polynucleotide in solution may explain the viscosity decrease if there are fewer independently diffusing species in solution.

Although the structural details remain to be worked out, there can be little doubt but that the hydrophobic effect is central to the binding interactions involving copper(I) or ruthenium(II) phenanthrolines and polynucleotides in aqueous media. Thus, the extra fused ring in the phenanthroline ligand always produces more intimate binding by comparison with a bipyridine analogue.[2,34] Even more striking is the influence that phenyl

(28) Meadows, K. A.; Liu, F.; Sou, J.; Hudson, B. P.; McMillin, D. R. *Inorg. Chem.* **1993**, *32*, 2919–2923.

(29) Michaelis, L. *Cold Spring Harbor Symp. Quant. Biol.* **1947**, *12*, 131–142.

(30) Bradley, D. F.; Wolf, M. K. *Proc. Natl. Acad. Sci. U.S.A.* **1959**, *45*, 944-952.

(31) Chu, D. Y.; Thomas, J. K. In *Photophysics of Polymers*; Hoyle, C. E., Torkelson, J. M., Eds.; ACS Symposium Series 358; American Chemical Society: Washington, DC, 1987; pp 434–451.

(32) Wilson, W. D. In *Nucleic Acids in Chemistry and Biology*; Blackburn, G. M., Gait, M. J., Eds.; Oxford University Press: Oxford, England, 1990; Chapter 8.

(33) Gabbay, E. J.; Scofield, R. E.; Baxler, C. S. *J. Am. Chem. Soc.* **1973** *95*, 7850–7857.

substituents in the 4- and 7-positions of the phenanthroline have on the binding. The effect probably derives from the release of solvent molecules from the solvation shells of both partners in the adduct, i.e., the DNA and the metal phenanthroline. The importance of hydrophobic interactions in DNA binding is also apparent from recent studies of copper-assisted nucleases derived from 5-alkylresorcinols where greater activities have been observed with longer alkyl substituents.[35,36]

Acknowledgment. We are grateful to Dr. Robert P. Meadows for running and interpreting the NMR spectrum of the dmpp ligand and to Dr. Daniel Lee for many helpful discussions on the synthesis of the dmpp ligand. We also acknowledge support of this work by the National Science Foundation through Grant No. CH-9024275.

(34) Turro, N. J.; Barton, J. K.; Tomalia, D. A. *Acc. Chem. Res.* **1991**, *24*, 332-340.
(35) Scannell, R. T.; Barr, J. R.; Murty, V. S.; Reddy, K. S.; Hecht, S. M. *J. Am. Chem. Soc.* **1988**, *110*, 3650–3651.
(36) Barr, J. R.; Murty, V. S.; Yamaguchi, K.; Singh, S., Smith, D. H.; Hecht, S.M. *Chem. Res. Toxicol.* **1988**, *1*, 204–207.