Efficient Cleavage of DNA by Iron(III) Triazacyclononane Derivatives

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Abstract: Compounds based on (1,4,7-trimethyl-1,4,7-triazacyclononane)iron(III) chloride have been synthesized. Exceedingly low concentrations (approximately 0.5 μM) of these reagents are required to effect single-stranded oxidative cleavage of plasmid DNA at physiological pH and temperature. Approximately 3 breaks per plasmid per micromolar of reagent occur in 1 h at 37 °C. The addition of dithiothreitol dramatically increased the effectiveness of these compounds; only 0.05 μM of reagent was required for DNA cleavage. When psoralen (a DNA photo-cross-linking agent) was attached to the iron complex, irradiation further increased the cleavage efficiency. The DNA cleaving abilities rival those of the cytotoxic antitumor drug bleomycin. Unlike bleomycin, the synthetic agents cut DNA with little sequence specificity. The lability of the chloride ligands, the hard acid character of iron(III), and the absence of base specificity in the DNA cleaving reaction suggest that a cationic iron species binds to the phosphate backbone of DNA. The reaction's dependence on reductants and dissolved oxygen suggests that it proceeds by a redox mechanism. Crystals of (1,4,7-trimethyl-1,4,7-triazacyclononane)$FeCl_3$ ($L'FeCl_3$) belong to the monoclinic space group $P2_1/c$, with a = 12.321(2) Å, b = 7.3220(10) Å, c = 15.903(3) Å, V = 1434.7(5) Å^3, and Z = 4 at 293 K. Refinement of 145 least squares parameters for 2613 independent reflections with $F > 4.0\sigma(F)$ converged to R = 3.43% and R_w = 6.45%. The coordination geometry around iron(III) approximates a trigonally distorted octahedron. The N–Fe–N bond angles (77.8°–78.8°) are compressed, while the Cl–Fe–Cl angles (96.7°–97.0°) are expanded from the octahedral value.

Introduction

Chemical methods for nicking DNA have several uses that include probing structural variations in nucleic acids,[1,2] identifying binding sites of DNA ligands,[1,3] designing artificial nucleases,[4–6] and serving as chemotherapeutic agents.[7] The use of coordination complexes to promote such DNA cleavage has also been the subject of research. This application generally employs metal redox chemistry to generate oxygen-based radicals that attack the deoxyribose backbone of DNA. Examples include $[Cu(phen)_2]^{2+}$,[8] EDTA–Fe(II) as a free coordination complex[9,10] or linked to DNA binding agents,[11,12] and agents based on oxoruthenium(IV).[13] Some reagents such as uranyl salts[14] and tris(phenanthroline) complexes of Co and Rh[15,16] that require photochemical activation for the cleavage reaction have also been used.

Our previous studies have focused on developing hydrolysis catalysts for the phosphate diester backbone of DNA. Both Cu(bpy)$(OH_2)_2^{2+}$ and Ni(tren)$(OH_2)_3^{3+}$ were shown to catalyze the hydrolysis of simple phosphate diesters but were unable to cleave DNA hydrolytically.[17,18] Those coordination complexes discovered that did cleave DNA did so through redox mechanisms.[17,19]

This paper describes a new oxidative DNA cleavage agent (1,4,7-trimethyl-1,4,7-triazacyclononane)Fe(III) ($L'FeCl_3$).[20] Derivatives in which the $L'FeCl_3$ is attached to the DNA intercalant, psoralen, have also been synthesized, and the ability of these derivatives to cross-link DNA was established. Experiments examining the ability of $L'FeCl_3$ and several other metal derivatives to cleave plasmid DNA were carried out, and quantitative comparisons with other DNA cleavage agents are presented. The $L'FeCl_3$-based derivatives are shown to efficiently cleave DNA in the presence of oxygen. With the addition of reducing agents the efficiency is comparable to that of bleomycin–Fe(II) (BLM–Fe(II)). The $L'FeCl_3$ complex is

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(20) Abbreviations used: 8-MOP, 8-methoxypsoralen; BLM, bleomycin; bp, base pair(s); bpy, 2,2′-bipyridine; CHES, 2-(cyclohexylamino)ethanesulfonic acid; DMF, dimethylformamide; des, desferrioxamine; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EthBr, ethidium bromide; HEPES, *N*-(2-hydroxyethyl)piperzine-*N*′-2-ethanesulfonic acid; L′, 1,4,7-trimethyl-1,4,7-triazacyclononane; phen, 1,10-phenanthroline; ps3L″, 8-((3-(4,7-dimethyl-1,4,7-triazacyclononyl-1)propyl-1)oxy)psoralen; ps4L″, 8-((4-(4,7-dimethyl-1,4,7-triazacyclononyl-1)butyl-1)oxy)psoralen; ps6L″, 8-((6-(4,7-dimethyl-1,4,7-triazacyclononyl-1)hexyl-1)oxy)psoralen; TBE, Tris–borate buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA); tosyl, *p*-toluenesulfonyl; TE, Tris–EDTA buffer (10 mM Tris, 1 mM EDTA); tren, tris(2-aminoethyl)amine.

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easy to synthesize and is stable in aqueous solution. This makes it an attractive reagent for DNA cleavage.

Experimental Section

Melting points were taken in Kimax glass capillary tubes with the use of a Thomas melting point apparatus. Nuclear magnetic resonance spectra (^{1}H and ^{13}C) were recorded with a General Electric QE-300 MHz spectrometer, and the significant chemical shifts are reported in ppm (δ units) referenced to the solvent peak. Coupling constants (J) are given in Hertz. Elemental analyses were determined by Desert Analytics, Tucson, AZ. Thin layer chromatography was performed on Merck Kieselgel 60 F_{254}, and spots were visualized with UV light. Flash column chromatography was performed on silica gel (Fisher, 100−200 mesh). Methanol, pyridine, and DMF were dried by standard procedures. Other commercial reagents were used without purification. 1,4,7-Trimethyl-1,4,7-triazacyclononane[21−26] and 1,4-dimethyl-1,4,7-triazacyclononane[27] were prepared by known methods. Desferrioxamine (desferal mesylate, CIBA No. S-778) was a gift of Dr. Paul Saltman. The sodium salt of calf thymus DNA was purchased from Sigma. Milli-Q water was used to prepare all solutions. An Orion Model 501 research digital ion analyzer was used for pH measurements. Absorption measurements were made with an IBM 9420 UV−visible spectrophotometer. Fluorescence measurements employed an SLM Aminico SPF-500C spectrofluorometer. UV irradiations were performed at room temperature using a Kratos LH150 Hg−Xe arc lamp equipped with a 250 W Kratos Universal Arc Lamp Supply, LPS251HR, a 10 cm water filter, and a 320−400 nm filter (Corning CS 7-39).

I. Syntheses. 8-Hydroxypsoralen.[28] To a 100 mL round-bottom flask containing a stir bar and pyridinium chloride (13.4 g, 116 mmol, 115.56 g/mol) was added 8-methoxypsoralen (2.5 g, 11.6 mmol, 216.18 g/mol). The mixture was refluxed (220−230 °C) with stirring for 25 min. While still hot, the solution was poured into 350 mL of 1 M hydrochloric acid and the flask was rinsed with 1 M HCl. The precipitate was filtered, rinsed with water, and placed in an oven (80 °C) for 4 h to yield 2.21 g of crude product. The crude product was recrystallized from acetone and water to give 1.76 g of yellow crystals (77%). Mp = 246−248 °C. ^{1}H NMR (acetone-d_6): δ 6.32 (d, 1H, J = 9.6 Hz), 6.97 (d, 1H, J = 2.2 Hz), 7.43 (s, 1H), 7.92 (d, 2H, 2.1 Hz), 8.02 (d, 2H, J = 9.6 Hz). ^{13}C NMR (acetone-d_6, ppm): 107.5, 110.9, 114.7, 117.0, 126.4, 130.8, 140.4, 145.6, 146.0, 147.8, 160.3.

8-((3-Bromopropyl-1)oxy)psoralen. 8-Hydroxypsoralen (1.76 g, 10 mmol, 202.16 g/mol), 1,3-dibromopropane (8.8 mL, 1.989 g/mL, 87 mmol, 201.90 g/mol), and potassium carbonate (12.04 g, 87 mmol, 138.21 g/mol) were refluxed in 150 mL of acetone for 10 h. After the solution cooled to room temperature, it was filtered and the filtrate was reduced to an oil on a rotary evaporator. The product was purified by flash chromatography (pentane, followed by chloroform). A pale yellow solid was obtained (2.46 g, 87%). Mp = 102−105 °C. ^{1}H NMR ($CDCl_3$): δ 2.40 (p, 2H, J = 6.1 Hz), 3.77 (t, 2H, J = 6.5 Hz), 4.63 (t, 2H, J = 5.8 Hz), 6.38 (d, 1H, J = 9.6 Hz), 6.83 (d, 1H, J = 2.2 Hz), 7.38 (s, 1H), 7.70 (d, 1H, J = 2.1 Hz), 7.77 (d, 1H, J = 9.6). ^{13}C NMR ($CDCl_3$, ppm): 29.8, 33.0, 71.3, 76.4, 106.6, 113.2, 114.6, 116.3, 125.8, 131.4, 144.1, 146.5, 147.8, 160.2.

8-((4-Bromobutyl-1)oxy)psoralen. A protocol similar to that used for the preparation of 8-((3-bromopropyl-1)oxy)psoralen was employed. Yield = 91%. Mp = 73−75 °C. ^{1}H NMR ($CDCl_3$): δ 2.02 (p, 2H, J = 8.3 Hz), 2.22 (p, 2H, J = 7.9 Hz), 3.57 (t, 2H, J = 6.5 Hz), 4.52 (t, 2H, J = 6.0 Hz), 6.37 (d, 1H, J = 9.6 Hz), 6.82 (d, 1H, J = 2.2 Hz), 7.37 (s, 1H), 7.69 (d, 1H, J = 2.2 Hz), 7.77 (d, 1H, J = 9.6).

8-((6-Bromohexyl-1)oxy)psoralen. A protocol similar to that used for the preparation of 8-((3-bromopropyl-1)oxy)psoralen was employed. Yield = 89%. ^{1}H NMR ($CDCl_3$): δ 2.02 (p, 2H, J = 8.3 Hz), 2.22 (p, 2H, J = 7.9 Hz), 3.57 (t, 2H, J = 6.5 Hz), 4.52 (t, 2H, J = 6.0 Hz), 6.37 (d, 1H, J = 9.6 Hz), 6.82 (d, 1H, J = 2.2 Hz), 7.37 (s, 1H), 7.69 (d, 1H, J = 2.2 Hz), 7.77 (d, 1H, J = 9.6). ^{13}C NMR ($CDCl_3$, ppm): 24.8, 27.7, 29.8, 32.6, 33.9, 73.7, 74.6, 106.7, 113.0, 114.5, 116.4, 125.9, 131.8, 143.3, 144.3, 146.5, 148.0, 160.4.

8-((3-(4,7-Dimethyl-1,4,7-triazacyclononyl-1)propyl-1)oxy)psoralen. 8-((3-Bromopropyl-1)oxy)psoralen (2.4 g, 7.4 mmol, 323.15 g/mol), 1,4-dimethyl-1,4,7-triazacyclononane (2.14 g, 13.6 mmol, 157.27 g/mol), triethylamine (1.03 mL, 0.726 g/mol, 19.7 mmol, 101.19 g/mol), and chloroform (80 mL) were placed together in a 100 mL round-bottom flask. The solution was refluxed for 5 h. After cooling, the solvent was removed. The triethylamine was removed under vacuum, and 8 mL of DMF was added. DMF and excess 1,4-dimethyl-1,4,7-triazacyclononane were removed by Kugelrohr distillation at 50−60 °C. The residue was dissolved in 0.1 M hydrochloric acid with gentle warming. The aqueous layer was extracted twice with ether and was then made basic with sodium hydroxide. The product was extracted into ether and dried over magnesium sulfate, and the ether was removed. The product is a yellow oil (1.9 g, 64% (based on 8-((3-bromopropyl-1)oxy)psoralen)) and was stored under nitrogen at −20 °C. ^{1}H NMR ($CDCl_3$): δ 2.02 (m, 2H, J = 6.9 Hz), 2.35 (s, 6H), 2.71 (m, 12 H), 2.79 (t, 2H, J = 7.2 Hz), 4.59 (t, 2H, J = 6.4 Hz), 6.38 (d, 1H, 9.6 Hz), 6.83 (d, 1H, J = 2.1 Hz), 7.37 (s, 1H), 7.70 (d, 1H, J = 2.1 Hz), 7.78 (d, 1H, J = 9.6). ^{13}C NMR ($CDCl_3$, ppm): 28.3, 46.3, 55.1, 56.0, 56.4, 57.0, 72.1, 106.5, 112.8, 114.2, 116.1, 125.6, 131.6, 143.0, 144.1, 146.2, 147.7, 160.1. Anal. Calcd for ps3L″ $C_{22}H_{29}O_4N_3$: C, 66.14; H, 7.32; N, 10.52. Found: C, 65.10; H, 6.89; N, 10.16.

8-((4-(4,7-Dimethyl-1,4,7-triazacyclononyl-1)butyl-1)oxy)psoralen. The same method as that used for the preparation of 8-((3-(4,7-dimethyl-1,4,7-triazacyclononyl-1)propyl-1)oxy)psoralen was employed. Yield = 79%. ^{1}H NMR ($CDCl_3$): δ 1.70 (p, 2H, J = 6.5 Hz), 1.88 (p, 2H, J = 6.9 Hz), 2.35 (s, 6H), 2.54 (m, 2H), 2.67 (s, 8H), 2.72 (s, 4H), 4.51 (t, 2H, J = 6.5 Hz), 6.36 (d, 1H, 9.6 Hz), 6.82 (d, 1H, J = 2.1 Hz), 7.35 (s, 1H), 7.69 (d, 1H, J = 2.2 Hz), 7.77 (d, 1H, J = 9.6). ^{13}C NMR ($CDCl_3$, ppm): 24.0, 27.9, 46.2, 55.9, 56.3, 56.9, 58.7, 73.6, 106.5, 112.9, 114.3, 116.2, 125.7, 131.6, 143.1, 144.2, 146.4, 147.9, 160.3.

8-((6-(4,7-Dimethyl-1,4,7-triazacyclononyl-1)hexyl-1)oxy)psoralen. The same method as that used for the preparation of 8-((3-(4,7-dimethyl-1,4,7-triazacyclononyl-1)propyl-1)oxy)psoralen was employed. Yield = 79%. ^{1}H NMR ($CDCl_3$): δ 1.33−1.62 (m, 6H), 1.89 (p, 2H, J = 7.5 Hz), 2.36 (s, 6H), 2.49 (m, 2H), 2.68 (s, 8H), 2.74 (s, 4H), 4.51 (t, 2H, J = 9 Hz), 6.38 (d, 1H, 9.6 Hz), 6.83 (d, 1H, J = 2.1 Hz), 7.36 (s, 1H), 7.70 (d, 1H, J = 2.1 Hz), 7.78 (d, 1H, J = 9.6). ^{13}C NMR ($CDCl_3$, ppm): 25.7, 27.3, 27.9, 30.1, 46.7, 56.2, 56.9, 57.5, 59.2, 74.0, 106.7, 112.9, 114.7, 116.5, 125.9, 132.0, 143.4, 144.3, 146.5, 148.1, 160.5.

(8-((3-(4,7-Dimethyl-1,4,7-triazacyclononyl-1)propyl-1)oxy)psoralen)$CuCl_2$. Copper(II) chloride (34 mg, 0.25 mmol, 134.45 g/mol) dissolved in methanol (2 mL) was added dropwise to a solution of 8-((3-(4,7-dimethyl-1,4,7-triazacyclononyl-1)propyl-1)oxy)psoralen (100 mg, 0.25 mmol, 399.51 g/mol) in methanol (2 mL). The solution was heated under reflux for 20 min and then stirred at room temperature overnight. Solvent was removed under reduced pressure, and the residue was rinsed with ether and some ethanol. A yellow-green powder was obtained in 62% yield (83 mg). Anal. Calcd for ps3L″$CuCl_2$ $C_{22}H_{29}O_4N_3CuCl_2$: C, 49.49; H, 5.47; N, 7.87. Found: C, 48.07; H, 5.01; N, 7.53.

(8-((3-(4,7-Dimethyl-1,4,7-triazacyclononyl-1)propyl-1-)oxy)psoralen)$Pb(NO_3)_2$. An 80 mM ethanolic solution of 8-((3-(4,7-dimethyl-1,4,7-triazacyclononyl-1)propyl-1)oxy)psoralen (500 mg in 16 mL ethanol, 1.3 mmol, 399.51 g/mol) was added to a 80 mM ethanolic solution of lead(II) nitrate (415 mg in 16 mL ethanol, 1.3 mmol, 331.23 g/mol) in a 50 mL round-bottom flask. The solution was stirred at

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room temperature overnight. The small amount of precipitate was filtered, and the filtrate was dried on a rotary evaporator. The residue was washed twice with ether to yield 729 mg of a pale yellow solid (80%). ^{1}H NMR (MeOD-d_4): δ 2.18 (m, 2H), 2.69 (s, 6H), 3.22–3.43 (m, 14H), 4.47 (t, 2H, J = 5.5 Hz), 6.33 (d, 1H, J = 9.6 Hz), 6.91 (d, 1H, J = 2.2 Hz), 7.54 (s, 1H), 7.84 (d, 1H, J = 2.2 Hz), 7.98 (d, 1H, J = 9.6 Hz). Anal. Calcd for ps3L″Pb$(NO_3)_2$ $C_{22}H_{29}O_{10}N_5Pb$: C, 36.16; H, 4.00; N, 9.58. Found: C, 36.05; H, 3.85; N, 9.42.

[(8-((3-(4,7-Dimethyl-1,4,7-triazacyclononyl-1)propyl-1)oxy)-psoralen)Zn$(H_2O)_3$]$(NO_3)_2$. A method similar to that used for (8-((3-(4,7-dimethyl-1,4,7-triazacyclononyl-1)propyl-1)oxy)psoralen)Pb$(NO_3)_2$ was employed using zinc(II) nitrate hexahydrate. The residue was dissolved in ethanol, and ether was added. After refrigeration for 2 h, a pale yellow precipitate was collected in 54% yield. ^{1}H NMR (MeOD-d_4): δ 2.13 (m, 2H), 2.57 (s, 6H), 2.75–3.03 (m, 14H), 4.45 (t, 2H, J = 5.5 Hz), 6.32 (d, 1H, J = 9.6 Hz), 6.90 (d, 1H, J = 2.1 Hz), 7.53 (s, 1H), 7.82 (d, 1H, J = 2.2 Hz), 7.97 (d, 1H, J = 9.7 Hz). Anal. Calcd for [ps3L″Zn(H_2O)]$(NO_3)_2$ $C_{22}H_{31}O_{11}N_5Zn$: C, 43.54; H, 5.15; N, 11.54. Found: C, 43.58; H, 4.93; N, 10.98.

(8-((3-(4,7-Dimethyl-1,4,7-triazacyclononyl-1)propyl-1)oxy)-psoralen)$FeCl_3$. Iron(III) trichloride hexahydrate (135 mg, 0.50 mmol, 270.32 g/mol) dissolved in methanol (1 mL) was added slowly to a solution of 8-((3-(4,7-dimethyl-1,4,7-triazacyclononyl-1)propyl-1)oxy)-psoralen (128 mg, 0.32 mmol, 399.51 g/mol) in methanol (2 mL) in a 5 mL round-bottom flask. The solution was refluxed for 1 h. After cooling, a bright yellow precipitate was collected by filtration and rinsed twice with methanol and once with ether. The product (140 mg) was obtained in 78% yield. It can be recrystallized from methylene chloride layered with pentane. Mp = 184–187 °C (dec). Anal. Calcd for (ps3L″)$FeCl_3$·$^1/_2$$CH_2Cl_2$ $C_{22.5}H_{30}O_4N_3FeCl_4$: C, 44.73; H, 5.01; N, 6.96. Found: C, 45.27; H, 4.95; N, 5.81.

(8-((4-(4,7-Dimethyl-1,4,7-triazacyclononyl-1)butyl-1)oxy)psoralen)-$FeCl_3$. The same methodology as that used for the preparation of (8-((3-(4,7-dimethyl-1,4,7-triazacyclononyl-1)propyl-1)oxy)psoralen)-$FeCl_3$ was adopted. Yield = 74%. Mp = 171–178 °C (dec). Anal. Calcd for ps4L″$FeCl_3$ $C_{23}H_{31}O_4N_3FeCl_3$: C, 47.98; H, 5.43; N, 7.30. Found: C, 48.80; H, 5.43; N, 7.15.

(8-((6-(4,7-Dimethyl-1,4,7-triazacyclononyl-1)hexyl-1)oxy)psoralen)-$FeCl_3$. The same methodology as that used for the preparation of (8-((3-(4,7-dimethyl-1,4,7-triazacyclononyl-1)propyl-1)oxy)psoralen)-$FeCl_3$ was employed. Yield = 22%. The compound could be recrystallized from methylene chloride layered with pentane. Mp = 172–175 °C (dec). Anal. Calcd for (ps6L″)$FeCl_3$ $C_{25}H_{35}O_4N_3FeCl_3$: C, 49.73; H, 5.84; N, 6.96. Found: C, 49.45; H, 5.81; N, 6.98.

(1,4,7-Trimethyl-1,4,7-triazacyclononane)$FeCl_3$. This compound was prepared as described previously[22] in 88% yield. Mp = 220 °C (dec). X-ray quality crystals were obtained by slow recrystallization from acetone.

II. DNA Cleavage. The plasmid DNA, pDD436, was a generous gift from April Meyer and Daniel Donoghue of UCSD. It is a construct whose parent plasmid is pSP64(polyA)[29] with a length of 4451 bp. It contains several inserts including cyclinB1. It was further purified on a Sephacryl S-1000 column equilibrated with 10 mM TE and 1 M NaCl buffer. The plasmid was stored frozen in 5 mM HEPES (pH 7.3).

Cleavage Experiments with Metal–Ligand Complexes Generated *In Situ*. Reagent grade inorganic salts, namely, $Mn(SO_4)\cdot H_2O$, $FeCl_3\cdot 6H_2O$, $Co(NO_3)_2\cdot 6H_2O$, $Ni(NO_3)_2\cdot 6H_2O$, $Cu(NO_3)_2\cdot {}^5/_2H_2O$, $Zn(NO_3)_2\cdot 6H_2O$, $Cd(NO_3)_2\cdot 4H_2O$, $Pb(NO_3)_2$, $LaCl_3\cdot 7H_2O$, $NdCl_3$, $SmCl_3$, $Gd(NO_3)_3\cdot 6H_2O$, $Tb(NO_3)_3\cdot 5H_2O$, and $Yb(NO_3)_3\cdot 5H_2O$ were purchased from commerical sources and used without further purification. The metal–ligand complex was generated by mixing each of these metal salts with a solution of the ligand in water to make a final concentration of 75 mM. The ligands used for this experiment were ps3L″ and ps6L″. The reactions were done at three different pH's. HEPES was used to buffer for pH 7 and pH 8, while CHES was used for pH 9. Each experiment was done in a 0.5 mL Eppendorf tube using a 20 μL solution consisting of 25 μg/mL supercoiled pDD436 (~40 μM bp), 12 mM buffer, and 40 μM metal–ligand complex. After making the solution, it was then split in two. One half was kept in the dark, and the other half was irradiated for 5 min at room temperature. All the samples were incubated for 17 h at 37 °C. The extent of DNA cleavage was assessed by analysis on 1% agarose gels containing 0.3 μg/mL EthBr.[30] The bands were viewed using a transilluminator (Haake-Büchler) and photographed (Photodyne camera with red filter) with Polaroid 667 film.

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Cleavage Experiments with Previously Isolated Metal–Ligand Complexes. Experiments similar to the ones described above were done using metal–ligand complexes that had been isolated and characterized. Compounds tested included ps3L″$FeCl_3$, ps4L″$FeCl_3$, ps6L″$FeCl_3$, ps3L″$CuCl_2$, [ps3L″Zn$(H_2O)_3$]$^{2+}$, ps3L″Pb$(NO_3)_2$, and L′$FeCl_3$. Only the iron complexes cleaved DNA efficiently. These compounds were further tested at varying concentrations and incubation and irradiation times, with different reductants and oxidants, at different temperatures, and with several quenchers. When necessary, 10 mM NaCN was used to quench the iron triazacyclononane reactions. For most experiments with ps3L″$FeCl_3$ and L′$FeCl_3$, 50 μM aqueous stock solutions containing 2% DMSO (282 mM) were used. In the stock solution, the DMSO is present in 5600 times the concentration of the cutting reagent (282 mM). When added to the DNA solutions, final concentrations of DMSO ranged from 50 μM to 6 mM.

III. Ability of the Psoralen Derivatives to Cross-Link DNA. The alkaline fluorescence assay was performed as previously described,[31,32] at pH 11.8. Reactions volumes of 500 μL containing 50 μg/mL calf thymus DNA, 10 mM HEPES (pH 7.0), and 77 μM of the appropriate psoralen derivative were irradiated (320–400 nm). Aliquots of 2 × 25 μL were removed over time. One aliquot was denatured as described,[32] and 1 mL of alkaline fluorescence buffer was then added to both. The percentage of DNA mass connected to a cross-link (%XL) was calculated as F_d/F_n, where F_d is equal to the fluorescence of the denatured sample and F_n is equal to the fluorescence of the nondenatured DNA sample.

IV. Quantitative Comparisons with Other DNA Cleavage Reagents. Comparisons of cleavage efficiency were made with BLM, BLM–Fe(II), Fe^{2+}, Fe^{3+}, and EDTA–Fe(II). Blenoxane (Mead Johnson), a clinical mixture of BLM A_2 (60%) and BLM B_2 (30%) was used. BLM–Fe(II) was made by mixing equimolar amounts of Blenoxane and $FeSO_4\cdot 7H_2O$ solutions together. EDTA–Fe(II) was made just before the experiment by mixing equal volumes of 5 mM $(NH_4)FeSO_4\cdot 6H_2O$ (freshly prepared solution) with 10 mM EDTA. The 50 μM aqueous stock solutions containing 2% DMSO (282 mM) were used for ps3L″$FeCl_3$ and L′$FeCl_3$. Each 10 μL final volume reaction mixture contained 22 μg/mL pDD436 (34 μM bp) and 10 mM HEPES (pH 7.4). The metal reagent concentration was varied. In the cases where the samples were irradiated, a Hg–Xe Lamp was used (320–400 nm filter) to irradiate for 5 min at room temperature. Then the samples were incubated for 55 min at 37 °C. When DTT was added to the samples, the addition occurred after the irradiations or after 5 min if no irradiation was used. Samples were then incubated for 55 min. The final concentration of DTT was 1 mM. The ps3L″$FeCl_3$ and L′$FeCl_3$ reactions were quenched with 1 mM NaCN when necessary. Analysis of the cleavage was done on 1% agarose gels containing 0.3 μg/mL EthBr.[30] A CCD Video Image Analysis System was used to quantitate the amount of DNA cleavage. The amount of supercoiled plasmid was corrected by a factor of 1.4.[33]

A Poisson distribution was used to describe the cleavage reaction. The average number of strand scissions per DNA plasmid, μ, is equal to −ln(fraction of form I). When only DNA forms I and II are formed (as is the case with all the complexes tested except for BLM), this number equals the average number of single-stranded cuts per molecule of plasmid DNA. In the case of BLM, most of the linear DNA is formed from specific double-stranded cuts under the reaction conditions, as opposed to random single-stranded breaks that happen to occur

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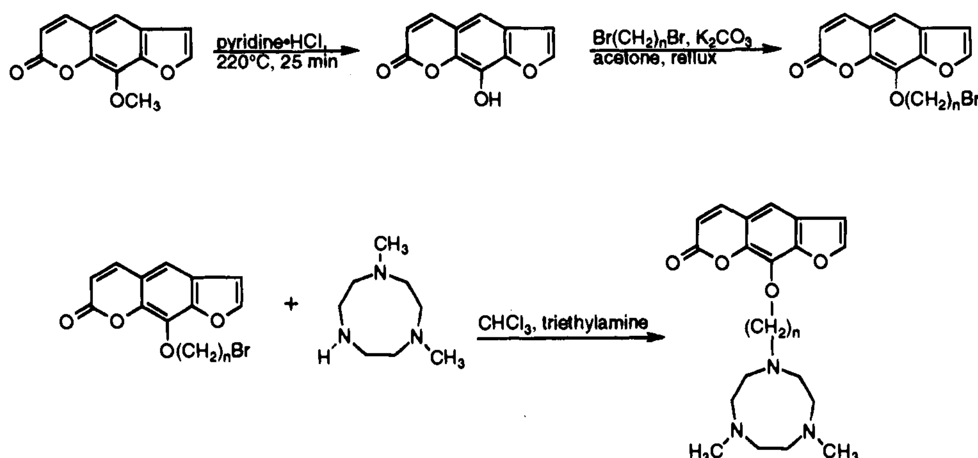
Scheme 1. Synthesis of ps3L″, ps4L″, and ps6L″



nearby on complementary strands.[33,34] The number of double-stranded cuts per plasmid therefore equals the fraction of form III. Subtracting the number of double-stranded breaks from the total average number of breaks gives the number of single-stranded breaks when using BLM−Fe(II).

The average number of strand breaks made in each plasmid per micromole of metal complex per hour at 37 °C was calculated for all systems. It was determined by plotting the μ versus the concentration of the metal complex and computing the slope of the line. An average of 3−6 runs was used, and the errors are reported as the standard deviation. The average number of strand breaks for BLM−Fe(II) includes both single-stranded and double-stranded breaks.

V. Specificity of DNA Cleavage. The restriction endonucleases *Xba*I and *Eco*RI were obtained from New England Biolabs. The Klenow fragment of DNA polymerase I came from Promega Corp. Deoxynucleoside triphosphates were obtained from Boehringer Mannheim, and the [α-^{32}P]dCTP was obtained from ICN Pharmaceuticals. pT5110 plasmid was generously donated by Mark van Doren and James Posakony of UCSD. The plasmid is pBluescriptI KS+ with a 110 bp insert. The 144 bp restriction fragment was prepared by digesting pT5110 with *Xba*I followed by *Eco*RI. The 3′ ends were labeled with [α-^{32}P]dCTP using the Klenow fragment to do a fill-in reaction. After the remaining deoxynucleosides were removed using a G-25 spin column, the labeled restriction fragment was isolated on a 5% nondenaturing polyacrylamide 1:29 cross-linked gel, run in 1× TBE.[30,35]

Maxam−Gilbert G and A + G lanes were prepared by standard protocols.[35,36] Both the EDTA−Fe(II) and the iron triazacyclononane derivative samples were prepared as follows: end-labeled DNA was added to calf thymus carrier DNA. Sodium ascorbate and the cutting reagent were placed on the inner wall of the 1.5 mL Eppendorf tube. In the case of EDTA−Fe(II), hydrogen peroxide was also placed on the side of the tube. The reaction was initiated by spinning the samples down and incubating at room temperature for 5 min. In the cases where the sample was irradiated, all the reagents except for the sodium ascorbate were added, the sample was irradiated for 5 min, and the reaction was then initiated by the addition of the sodium ascorbate. Final sample concentrations were 100 μM bp calf thymus DNA, 20 000 cpm/lane of labeled restriction fragment, 1 mM sodium ascorbate (freshly prepared), and 7.5 μM (iron triazacyclononane derivatives) or 10 μM (EDTA−Fe(II)) cutting reagent. The EDTA−Fe(II) sample also contained 0.03% H_2O_2. The EDTA−Fe(II) complex was made immediately before use by mixing equal volumes of 5 mM (NH_4)-$FeSO_4 \cdot 6H_2O$ and 10 mM EDTA. Reactions were quenched by precipitating the DNA with ethanol.

Samples were electrophoresed on 10% polyacrylamide, 0.4 mm thick, 1:19 cross-linked denaturing gels at 1575 V (44 V/cm) for ~2 h in ½× TBE. After the gel was dried, autoradiography was conducted at −70 °C with the use of an intensifying screen. The autoradiograms were scanned. Densitometric analysis was performed using Adobe Photoshop 2.5 and Image 1.47 on a MacIntosh Quadra, with the density of the film as a baseline.

VI. Crystal Structure Determination. X-ray data for a 0.18 × 0.4 × 0.7 mm^3 green crystal of $C_9H_{21}Cl_3FeN_3$ were collected with a Siemens R3m/V automated diffractometer equipped with a Mo X-ray tube and a graphite crystal monochromator. Orientation matrix and unit cell parameters were determined by the least squares fitting of 25 machine-centered reflections (15° < 2θ < 30°) and confirmed by examination of axial photographs. Intensities of three check reflections were monitored every 197 reflections, throughout the data collection. Structure solutions and data workup were performed on a DEC Microvax II computer with SHELXTL PLUS software. All non-hydrogen atoms were refined anisotropically, and the hydrogen atoms were generated in idealized positions for the structure factor calculations but were not refined.

Results

I. Syntheses. The metal complexes tested in this study were synthesized according to Scheme 1. The trimethyltriazacyclononane ligand, whose coordination chemistry has been extensively developed,[37] was chosen because of its ability to bind transition metal ions tightly. In addition, the three methyl groups sterically prevent two such ligands from binding to a single metal ion. This latter feature was important for maintaining one or more coordination sites open for binding to DNA. The metal complex was attached to a drug that could covalently bind DNA as a further measure to enhance binding and DNA cleavage. Psoralen is an intercalator (K_d DNA = 1.3 mmol/L for 8-methoxypsoralen)[38] which is also used as a phototherapy drug that cross-links DNA. Irradiation (360 nm) causes an intercalated psoralen to undergo two [2 + 2] cycloaddition reactions with adjacent thymine bases on complementary strands. The 360 nm UV light does not photodamage DNA directly.[39]

II. DNA Cleavage. The DNA cleaving ability of metal complexes can be monitored by reacting them with supercoiled plasmid DNA. One nick in the supercoiled plasmid (form I) causes the DNA to unwind into the open circular form (form II). When nicking occurs on both DNA strands and the sites are nearby, this results in the linear form (form III). The amount of conversion of supercoiled DNA to open circular and linear DNA was monitored by agarose gel electrophoresis with

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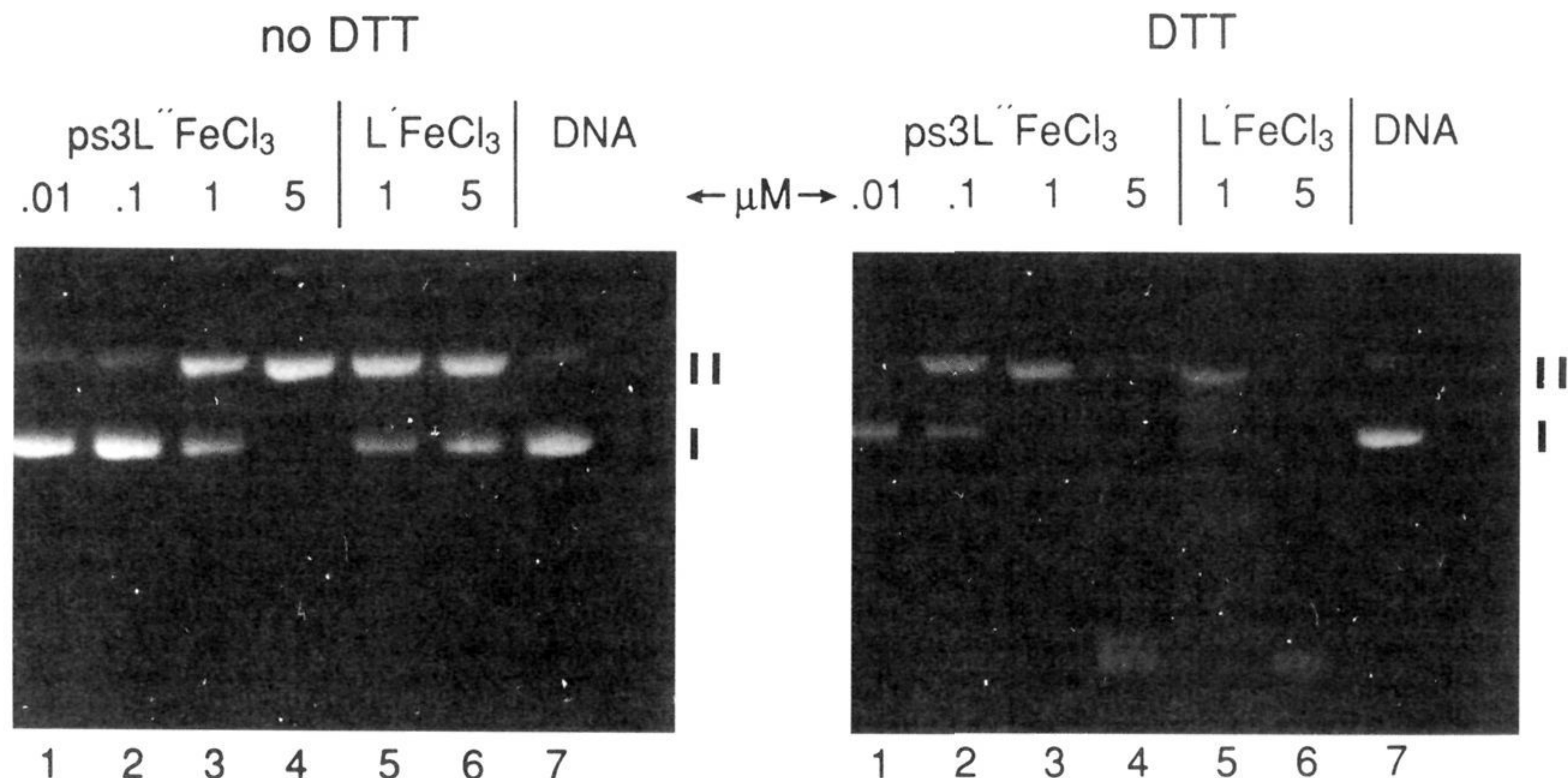

Figure 1. Cleavage of pDD436 by ps3L″$FeCl_3$ and L′$FeCl_3$ with and without dithiothreitol. Each lane contains 10 mM HEPES (pH 7.4), 22 μg/mL pDD436, and concentrations as given of ps3L″Fe^{3+} and L′Fe^{3+}. The gel on the left contains no reducing agent. The lanes in the gel on the right contain 1 mM DTT: lane 1, 0.01 μM ps3L″Fe^{3+} (0.06 mM DMSO); lane 2, 0.1 μM ps3L″Fe^{3+} (0.6 mM DMSO); lane 3, 1 μM ps3L″Fe^{3+} (5.6 mM DMSO); lane 4, 5 μM ps3L″Fe^{3+} (28 mM DMSO); lane 5, 1 μM L′Fe^{3+} (5.6 mM DMSO); lane 6, 5 μM L′Fe^{3+} (28 mM DMSO); lane 7, DNA control. The samples were incubated at 37 °C for 1 h and run on a 1% agarose gel.

ethidium bromide staining. The compact supercoiled form I migrates through the gel pores most rapidly, followed by form III and then form II.

Equimolar solutions of the ligands and the following metal ions were tested: Mn^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Pb^{2+}, La^{3+}, Nd^{3+}, Sm^{3+}, Gd^{3+}, Tb^{3+}, and Yb^{3+}. Some minor cleavage was seen with the iron complexes and sporadic cleavage was seen with lead and copper. Though the formation constants are generally quite high for metal triazacyclononane complexes,[37] the fact that little or no cleavage was seen with these solutions suggests that the kinetics is slow. As well, there are problems with precipitation of metal hydroxides with longer times.

Because little cleavage was seen when metal−ligand complexes were generated *in situ*, experiments were repeated with isolated metal−ligand complexes. Compounds tested included ps3L″$CuCl_2$, [ps3L″$Zn(H_2O)_3$]$^{2+}$, ps3L″$Pb(NO_3)_2$, ps3L″$FeCl_3$, and L′$FeCl_3$. Negligible amounts of cleavage were seen with the non-iron complexes. Irradiation at 360 nm to photo-cross-link the psoralen had no enhancing effect on the inactive complexes. The copper complex showed a slight cleaving ability when the reducing agent DTT was added, but the iron complexes cleaved DNA remarkably well under all conditions. The comparative DNA cleaving abilities of some of the L′$FeCl_3$-based complexes are illustrated by Figure 1. The lowermost band in each lane represents intact supercoiled plasmid (form I), the upper band represents a singly-nicked open-circular plasmid (form II). The linear plasmid (form III) runs slightly below form II, but it is not formed with the L′$FeCl_3$-based complexes. Disappearance of sharp bands and the appearance of streaking in the gels accompanies extensive DNA damage. (Band streaking can also result from slow binding equilibria and other changes that affect DNA mobility.) Bands which migrate near the top of the gel are likely due to plasmid dimers, though they may sometimes be due to metal compound−DNA complex formation.

With ps3L″$FeCl_3$, ps4L″$FeCl_3$, and ps6L″$FeCl_3$, the effect of the length of the hydrocarbon chain between the psoralen and the triazacyclononane moiety was investigated. Presumably, with different length linkers, the iron triazacyclononane segment would have variable accessibility to positions on the DNA backbone such as the bases, the phosphate diester bonds, and the sugar moieties. Initial experiments showed a difference in the cleavage abilities with various length linker arms; however, this arose from a difference in solubility. When the reactions were performed by first solubilizing the complexes in a small amount of DMSO, no differences in cleavage efficiencies were seen. Apparently an $-O(CH_2)_3-$ linking group imparts enough flexibility to permit intercalation of the tethered psoralen without interfering with the metal−DNA interaction. The ps3L″$FeCl_3$ complex, which is conveniently isolated, was chosen for the remaining experiments.

Typically the reactions were incubated at 37 °C for 1 h. Comparisons were made for incubation times of 0−3 h. The cleavage reaction began immediately and continued with time. At the various times, 10 mM NaCN was added to quench the reaction. Irradiation times at 360 nm, which causes psoralen cross-linking, were varied from 0 to 20 min. A gradual increase in cleavage efficiency was seen with ps3L″$FeCl_3$, whereas the DNA control sample showed no effect on irradiation.

Experiments with ps3L″$FeCl_3$ (1 μM) were repeated with and without the addition of reductants, namely, sodium ascorbate (1 μM) and dithiothreitol (1 μM−1 mM), and also with and without oxidants such as O_2 and $(NH_4)_2Ce^{IV}(NO_3)_6$ (1 μM). Both the reductants significantly increased the amount of cleavage seen. The oxidant, $(NH_4)_2Ce^{IV}(NO_3)_6$, had no effect. In solutions where most of the oxygen was removed, cleavage decreased. Similar results were obtained with L′$FeCl_3$. This suggests that the active species derives from the reaction between a reduced iron(II) complex and dissolved oxygen.

Several reagents with the potential to quench the iron triazacyclononane reaction with DNA were investigated. The reagents tested included desferrioxamine (an extremely efficient chelator of Fe^{3+}),[40,41] sodium formate (a hydroxyl radical

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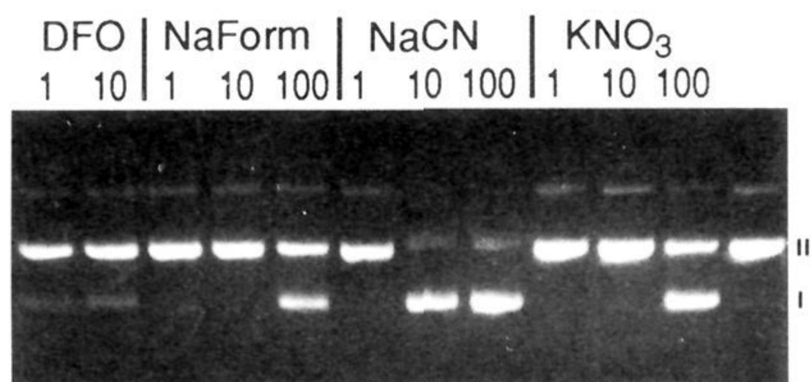

Figure 2. Effect of various inhibitors on the cleavage of pDD436 by ps3L″$FeCl_3$. Each lane contains 10 mM HEPES (pH 7.4), 22 μg/mL pDD436, and 1 μM ps3L″$FeCl_3$ (DMSO was added as a cosolvent): lane 1, 1 mM desferrioxamine; lane 2, 10 mM desferrioxamine; lane 3, 1 mM sodium formate; lane 4, 10 mM sodium formate; lane 5, 100 mM sodium formate; lane 6, 1 mM NaCN; lane 7, 10 mM NaCN; lane 8, 100 mM NaCN; lane 9, 1 mM potassium nitrate; lane 10, 10 mM potassium nitrate; lane 11, 100 mM potassium nitrate; lane 12, DNA control. The quenching agent was added with the ps3L″$FeCl_3$. The samples were incubated for 1 h at 37 °C.

scavenger[42] and also a potential ligand for Fe^{3+}), sodium cyanide (a strong ligand for Fe^{2+}), and potassium nitrate (to vary ionic strength). Each was used at concentrations of 1, 10, and 100 mM (except for desferrioxamine) with and without DTT. The results without DTT are shown in Figure 2. All the reagents partially inhibited the cleavage reaction at 100 mM. Desferrioxamine partially inhibits the reaction at 1 and 10 mM. Sodium cyanide was the most effective inhibitor. It partially quenched the cleavage reaction at 1 mM and quenched the reaction completely at concentrations of 10 mM. It was effective with or without DTT present, and it was selected as a general purpose quenching reagent.

As noted, the less polar psoralen derivatives dissolved readily, if first solubilized in a small amount of DMSO. Because DMSO is a known radical scavenger,[43] it is not usually used with DNA cleaving agents that work by a redox mechanism. However, added DMSO shows no deleterious effect on the amount of cleavage seen for any of the iron triazacyclononane complexes. Concentrations of DMSO ranged from 50 μM to 6 mM. Even much higher concentrations of DMSO (75 mM) did not reduce the DNA cutting efficiency. When similar concentrations of DMSO were added to the EDTA−Fe(II) cleaving system, the reaction was quenched. This verifies DMSO's ability to intercept the free OH radicals in the bulk solution generated by EDTA−Fe(II). Apparently, the oxidative cleavage of DNA by the iron triazacyclononane complexes does not involve the generation of diffusible OH radicals in bulk solution.

One of the more remarkable things about the iron triazacyclononane complexes is the relatively low concentrations needed to effect DNA cleavage. Concentrations as low as 0.1 μM ps3L″$FeCl_3$ and 0.5 μM L′$FeCl_3$ cause cleavage without added reducing agent. Further results on the effect of concentration with these compounds are given below in the discussion of comparative cleavage abilities.

III. Ability of the Psoralen Derivatives to Cross-Link DNA. An alkaline ethidium bromide fluorescence assay is commonly used to quantify cross-linking of DNA[32,44,45] and has been used previously with psoralens.[31,46] The technique is based on the observation that native double-helical DNA binds ethidium bromide efficiently, whereas denatured DNA binds ethidium bromide poorly. The ethidium intercalated into duplex structures shows a 25-fold enhancement in fluorescence.[47] In the concentration range of this assay, the correlation between EthBr binding to duplex DNA and the resulting fluorescence is linear.[48,49] When cross-linked DNA is denatured by heating and then rapidly renatured by cooling, it reverts to the double-helical form. (This occurs because cross-linking keeps the bases in register.) At the same time, there is a recovery in the ability of the DNA to bind ethidium bromide. When non-cross-linked DNA is heated and cooled rapidly, the strands do not reanneal effectively and EthBr does not intercalate effectively. The use of a pH of 11.8 further inhibits the re-annealing of non-cross-linked DNA.[45,50a] The ratio of the fluorescence before and after denaturation yields the amount of DNA cross-linking, as described in the Experimental Section.

Because of the cleavage reaction effected by ps3L″$FeCl_3$, unreactive [ps3L″$Zn(H_2O)_3]^{2+}$ was used to test whether an appended metal complex hinders cross-linking of DNA by the psoralen group. Comparisons were made to 8-MOP and to a control reaction with no added psoralen derivative. The results shown in Figure 3 and Table 1 show that photo-cross-linking is complete within minutes, even with an appended metal complex. This agrees with the insensitivity of the DNA cleaving reaction to the length of the linker arm. The $-O(CH_2)_3-$ linking group provides enough conformational flexibility so that the tethered psoralen can intercalate and photo-cross-link.

It is important to recognize that this method measures *relative* cross-linking efficiencies. The percent of cross-linking (%XL) calculated in Table 1 does not correspond to the absolute amount of cross-linking. With a DNA substrate that contains duplex strands of heterogeneous molecular size, such as calf thymus DNA, the mass of DNA that becomes cross-linked does not linearly follow the number of cross-links. Initially, the long strands will be cross-linked more efficiently than small duplexes. Therefore, the %XL value depends on the average length of the DNA strands in the sample and the concentrations of DNA, ethidium bromide, and the psoralen derivative.

IV. Species Present in Solution. Iron chlorides often hydrolyze in aqueous solutions to yield aquo or hydroxo complexes. When the iron complexes were dissolved in organic solvents, such as acetonitrile and DMSO, the solutions were a bright yellow. In water, or in organic solutions containing water, the solutions were clear. The spectral changes that occur are shown in Figure 4. Dissolved ps3L″$FeCl_3$ in acetonitrile gave electronic absorption peaks at 249, 297, and 394 nm. The peaks at 249 nm and 297 nm are $\pi \rightarrow \pi^*$ electronic transitions typical of psoralens.[38] The intense (ϵ, 4000 M^{-1} cm^{-1}) absorption at 394 nm is attributable to a $Cl^- \rightarrow Fe^{3+}$ charge transfer transition. On the basis of the optical electronegativities[50b] of Fe^{3+} and Cl^-, this transition should occur near 370 nm, which is close to the absorption in question. The disappearance of the 394 nm absorption on addition of water to the acetonitrile solution suggests loss of the chloride ligands to produce a triaquo or mixed aquo−hydroxo complex.

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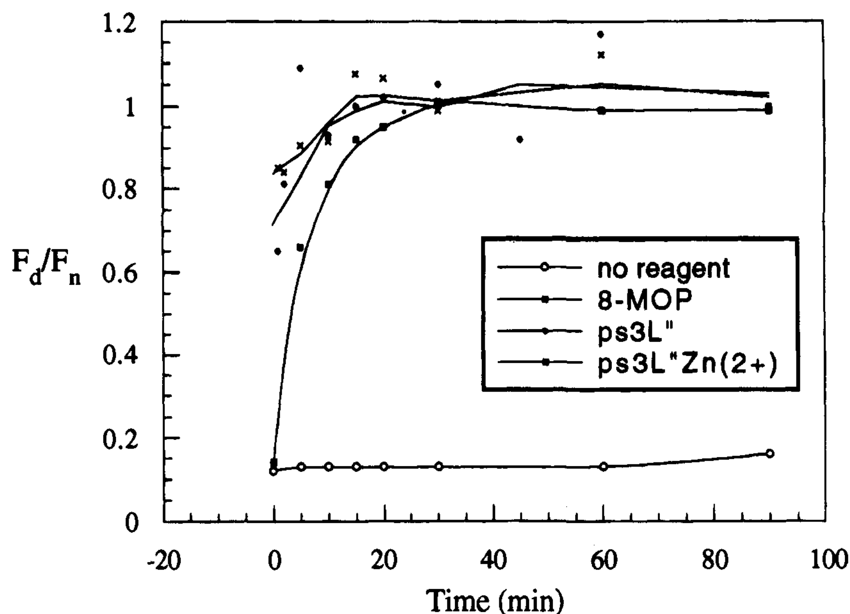

Figure 3. Percent of cross-linking as a function of time for 8-MOP, ps3L″, and $[ps3L''Zn(H_2O)_3]^{2+}$. The curves represent the lines of best fit.

Table 1. Relative Photo-Cross-Linking Efficiencies with Calf Thymus DNA

compd	$\%XL_{50}$[a] (min)
8-methoxypsoralen	3
ps3L″	1
$[ps3L''Zn(H_2O)_3]^{2+}$	1

[a] Irradiation (320–400 nm) time at which 50% of DNA by mass is connected to a cross-link.

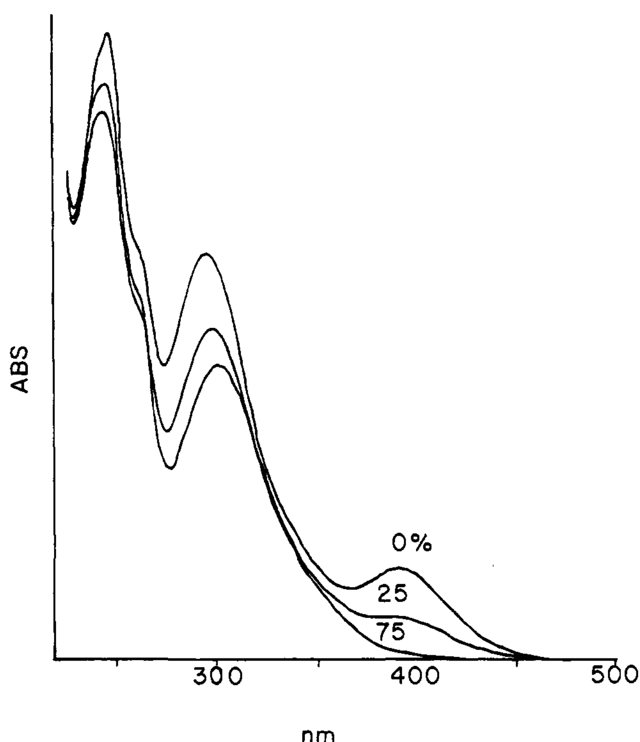

Figure 4. Absorption spectra of $ps3L''FeCl_3$ (28 μM) as a function of the percent of added water. For the parent complex in acetonitrile, $\epsilon_{249} = 2.6 \times 10^4$, $\epsilon_{297} = 1.7 \times 10^4$, and $\epsilon_{394} = 4.0 \times 10^3$.

V. Quantitative Comparisons with Other DNA Cleavage Reagents. Iron triazacyclononane complexes are highly effective reagents for DNA cleavage. Concentrations as low as 0.1 μM $ps3L''FeCl_3$ and 0.5 μM $L'FeCl_3$ cause strand scission without added reducing agent. The effectiveness of the iron triazacyclononane complexes was quantitatively compared with Fe^{3+}, Fe^{2+}, BLM–Fe(II), and EDTA–Fe(II). Bleomycin is effective in combination chemotherapy against several cancers. It requires a metal ion, such as Fe^{2+}, and molecular oxygen for its drug action.[51,52] This DNA cleavage reaction has been the source of extensive study.[53–56] While the actual structure of the activated BLM species is not known with certainty, the DNA cleavage reaction is thought to result from oxygen-based free radicals, such as hypervalent metal–oxo species, formed near the DNA helix. This induces both single-strand and double-strand breaks in the DNA backbone.[53] By contrast, EDTA–Fe(II) is a metal complex that generates free hydroxyl radicals through the Fenton reaction.[3,9,10] It cleaves DNA in a random fashion with single-strand breaks.

Reactions of both these cleaving agents with plasmid DNA were compared with those of the iron triazacyclononane complexes. Comparisons were also made with aqueous Fe^{3+}(aq) and Fe^{2+}(aq) salts ($FeCl_3{\cdot}6H_2O$ and $FeSO_4{\cdot}7H_2O$). The latter compounds showed no detectable cleavage at the concentration ranges used in this study. Without added reducing agent, slight cleavage was seen only above 50 μM for the simple salts, and with added reducing agent, cleavage could be detected at concentrations of 5 μM. Table 2 and Figure 5 summarize the results of the comparative studies.

The DNA cleaving abilities of $ps3L''FeCl_3$, $L'FeCl_3$, and BLM–Fe(II) complexes are comparable. All are highly active and yield approximately 3 breaks per plasmid per micromolar of reagent in 1 h. This corresponds to cleaving plasmid DNA with reagent concentrations of 10^{-7} M. The number of double-stranded breaks for BLM–Fe(II) was found to be 11–17% of the total amount of strand breaks. With added dithiothreitol (DTT), which reduces Fe^{III} to Fe^{II}, a large enhancement is seen in the cleavage efficiency for all three compounds. The $ps3L''FeCl_3$ and $L'FeCl_3$ complexes show no significant dif-

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Table 2. Comparisons of Quantitative DNA Cleaving Efficiencies[a]

reagent	$h\nu$−DTT−[b]		$h\nu$− DTT+		$h\nu$+ DTT−		$h\nu$+ DTT+	
	SB[c]	μM[d]	SB	μM	SB	μM	SB	μM
ps3L″$FeCl_3$	1.3 ± 0.4	0.6	17 ± 2	0.04	2.4 ± 0.7	0.3	26 ± 4	0.03
L′$FeCl_3$	4.5 ± 1.2	0.3	16 ± 6	0.05	3.7 ± 0.2	0.2	13 ± 3	0.06
BLM−Fe(II)	2.6 ± 0.6	0.3	29 ± 6	0.01				
EDTA−Fe(II)			0.3 ± 0	2.1				

[a] Experiments were performed as described in section IV of the Experimental Section. [b] $h\nu$ refers to with or without irradiation, and DTT refers to with or without 1 mM DTT. [c] Average number of strand breaks in each plasmid per micromolar of reagent in 1 h. [d] Concentration at which 50% of form I has been cleaved.

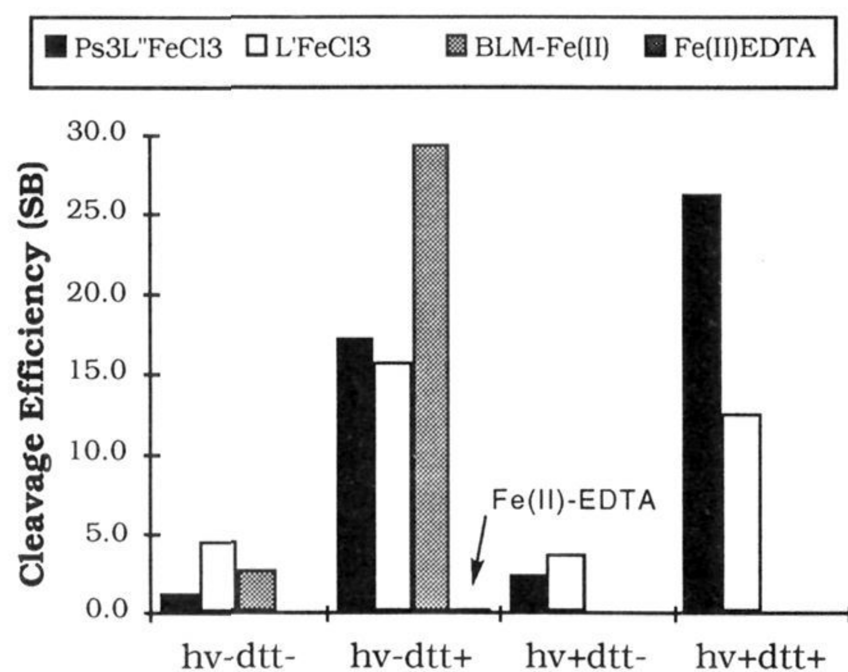

Figure 5. Bar graph comparing quantitative cleavage efficiencies. SB = average number of strand breaks in each plasmid per μM of reagent in 1 h. Experiments were performed as described in section IV of the Experimental Section.

ference in cleavage ability. Both cause about 17 single-stranded breaks per plasmid per micromolar of reagent in 1 h. In comparison to EDTA−Fe(II), the ps3L″$FeCl_3$, L′$FeCl_3$, and BLM−Fe(II) complexes are more active and cause detectable cleavage of plasmid DNA with as little as 10^{-8} M of reagent (added DTT).

The effect of UV irradiation was also investigated. Psoralen photo-cross-links to thymine bases in DNA with near UV irradiation (320−400 nm). Irradiation increased the cleavage ability of the ps3L″$FeCl_3$ complex by about 50%, but it had no significant effect on the L′$FeCl_3$ complex. This trend is seen with and without the addition of DTT.

VI. Specificity of DNA Cleavage. To further characterize DNA cleavage by the iron triazacyclononane complexes, the compounds were reacted with a 144 bp DNA 3′-labeled with ^{32}P. The specificity of the cleavage reaction was examined by high-resolution gel electophoresis. It was thought that L′$FeCl_3$ might exhibit little cleavage specificity but that the psL″$FeCl_3$ could on irradiation, as psoralen prefers photo-cross-linking with 5′-TA-3′ residues[57,58] (intercalation occurs at all sites[59]). As is shown in Figure 6, the iron triazacyclononane complexes tested showed no sequence specificity. They all cleave with same non-specific pattern as obtained with EDTA−Fe(II). Use of UV irradiation with the ps3L″$FeCl_3$ derivative also had no detectable effect. These results were verified by densitometric analysis, and no significant differences were seen even in the AT-rich regions.

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VII. Crystal Structure Determination. The product for the reaction between 1,4,7-trimethyl-1,4,7-triazacyclononane and $FeCl_3 \cdot 6H_2O$ is a hexacoordinate Fe(III) complex, chelated to the three nitrogens of the triazacyclononane and to three chloride ligands. The structure was defined by X-ray crystallography. Results are summarized in Figure 7 and in Tables 3−5. The most noteworthy feature of the structure is the trigonal distortion of the octahedral geometry about iron(III). The N−Fe−N angles of 77.8°−78.8° are constrained by the ring size of the macrocycle, whereas the Cl−Fe−N angles of 96.7°−97.0° are somewhat larger than 90°. The bond lengths to Fe are all unexceptional. The Fe−N bond distances in this complex are 2.232−2.264 Å, whereas the average Fe−N distance is 2.241 ± 0.115 Å determined for 25 small molecules containing Fe(III) coordinated to a tertiary amine.[60] The Fe−Cl bond distances in L′$FeCl_3$ are 2.300−2.309 Å, whereas the average is 2.308 ± 0.036 Å determined for 22 small molecule structures with an octahedral Fe(III).[60]

Discussion

The complex (1,4,7-trimethyl-1,4,7-triazacyclononane)$FeCl_3$ efficiently cleaves DNA with single-stranded nicks at physiological pH and temperature. The quantitative comparisons indicate that it is nearly as efficient as BLM−Fe(II). It cuts plasmid DNA at reagent concentrations of 0.3 μM. Adding a reductant reduces the concentration required to induce cleavage by 10-fold.

Attaching psoralen to the iron complex with a hydrocarbon chain offers a strategy for placing the metal ion near DNA. Psoralen covalently binds DNA on UV irradiation by first undergoing a [2 + 2] cycloaddition reaction to a thymine base (other bases are less likely targets) on one strand (photobinding). Then it undergoes another [2 + 2] cycloaddition reaction with a thymine base on the other strand (photo-cross-linking). The attached psoralen did little to further enhance cleavage by the metal complex. Without an added reducing agent, the psoralen complexes performed worse than L′$FeCl_3$. Only in the case where both irradiation and dithiothreitol were used was there a significant enhancement in cleavage seen through the addition of the psoralen moiety. The psoralen derivative did show a 50% enhancement in its cleavage ability on irradiation with DTT. This enhancement is not seen in the case of the L′$FeCl_3$. Presumably, this results from cross-linking to the DNA and covalent anchoring of the complex.

To test whether an appended metal complex interfered with photo-cross-linking of psoralen, an ethidium fluorescence assay was used. Addition of the triazacyclononane moiety to 8-methoxypsoralen does not inhibit the photo-cross-linking process markedly. Neither does the addition of the Zn(II) triazacyclononane complex inhibit the ability of the appended psoralen to photo-cross-link DNA. On the contrary, these changes slightly enhanced the amount of photo-cross-linking. This

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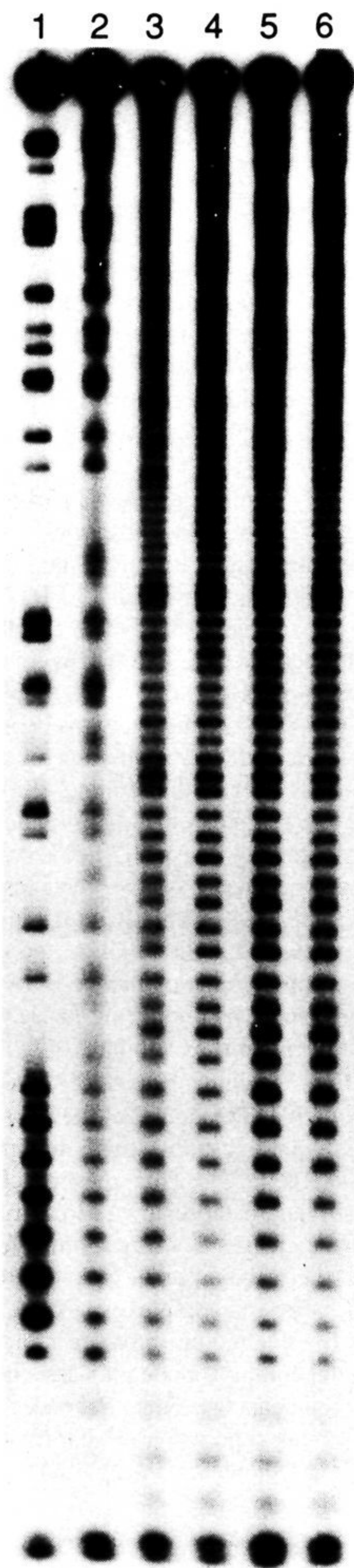

Figure 6. Autoradiogram of high-resolution denaturing gel: lane 1, Maxam–Gilbert G reaction; lane 2, Maxam–Gilbert G + A reaction; lane 3, EDTA–Fe(II); lane 4, $L'FeCl_3$; lane 5, $ps3L''FeCl_3$; lane 6, $ps3L''FeCl_3$ plus 5 min of UV irradiation.

enhancement of cross-linking efficiency may stem from the cationic charge of the appended group. Polyamines bind well to DNA[61–63] because of their positive charge at physiological pH. The zinc complex could also bind with the phosphate backbone or base groups of DNA to enhance the intercalative binding of psoralen. It should be pointed out that these studies were performed with calf thymus DNA, while the other studies in this paper were done with plasmids or shorter duplexes. Shorter duplexes are expected to show about the same efficiency. Less cross-linking may occur with plasmid DNA, since supercoiling may hinder intercalation.

The addition of psoralen offers little increase in cleavage efficiency, which suggests that the $L'FeCl_3$ moiety itself already possesses a strong affinity for DNA. Spectral changes suggest that the chloride ions are readily displaced from $L'FeCl_3$ in aqueous solution. We postulate that these are replaced by either aquo or a mixture of aquo and hydroxo ligands. This produces a cationic complex with two or three labile coordination sites that could bind to the DNA backbone. This probably occurs through the phosphate diester oxygens given the hard acid

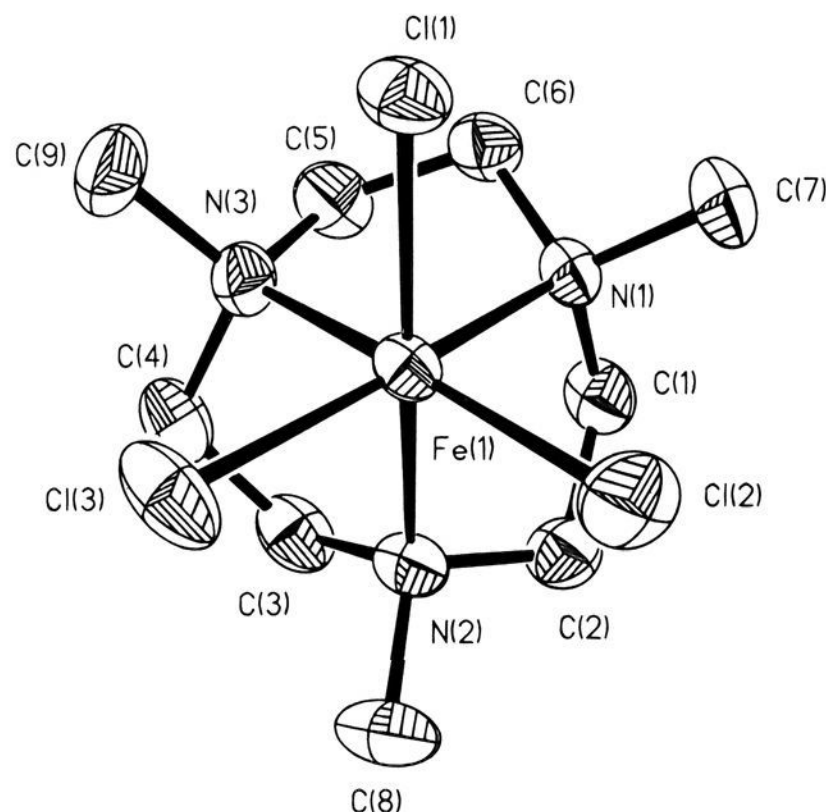

Figure 7. Thermal ellipsoid plot of $L'FeCl_3$ with 50% probability ellipsoids.

Table 3. Crystallographic Data for $L'FeCl_3$

emp form	$C_9H_{21}Cl_3FeN_3$	form wt	33.5
a, Å	12.321 (2)	T, °C	20
b, Å	7.3220 (10)	λ, Å	0.717073
c, Å	15.903 (3)	ϱ_{calcd}, g cm^{-1}	1.544
V, Å^3	1434.7 (5)	μ, cm^{-1}	15.95
Z	4	transmn coeff	0.6817–0.9755
space group	$P2_1/c$	$R(F_o)$, %	3.43
		$R_w(F_o)$, %	6.45

Table 4. Atomic Coordinates ($\times 10^4$) and Equivalent Isotropic Displacement Coefficients (Å $\times$ 10^3) for $L'FeCl_3$[a]

	x	y	z	U(eq)
Fe(1)	2437(1)	289(1)	1280(1)	28(1)
Cl(1)	922(1)	−1418(1)	969(1)	43(1)
Cl(2)	2516(1)	−427(1)	2688(1)	58(1)
Cl(3)	3699(1)	−1825(1)	830(1)	56(1)
N(1)	1432(2)	2784(3)	1486(1)	32(1)
N(2)	3727(2)	2429(3)	1431(2)	35(1)
N(3)	2456(2)	1613(3)	−6(1)	38(1)
C(1)	2135(3)	4368(4)	1736(2)	42(1)
C(2)	3240(2)	3773(4)	2016(2)	45(1)
C(3)	3998(2)	3297(4)	601(2)	46(1)
C(4)	3594(2)	2192(5)	−133(2)	47(1)
C(5)	1704(2)	3212(5)	−43(2)	48(1)
C(6)	890(2)	3151(4)	667(2)	40(1)
C(7)	567(3)	2504(5)	2126(2)	49(1)
C(8)	4746(2)	1704(5)	1816(3)	58(1)
C(9)	2162(3)	291(6)	−685(2)	59(1)

[a] Equivalent isotropic U defined as one-third of the trace of the orthogonalized U_{ij} tensor.

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Table 5. Selected Bond Lengths (Å) and Angles (deg) in L′FeCl$_3$

	Bond Lengths		
Fe(1)−Cl(1)	2.300(1)	Fe(1)−Cl(2)	2.301(1)
Fe(1)−Cl(3)	2.309(1)	Fe(1)−N(1)	2.232(2)
Fe(1)−N(2)	2.244(2)	Fe(1)−N(3)	2.264(2)
N(1)−C(1)	1.501(4)	N(1)−C(6)	1.486(3)
N(1)−C(7)	1.490(4)	N(2)−C(2)	1.482(4)
N(2)−C(3)	1.504(4)	N(2)−C(8)	1.493(4)
N(3)−C(4)	1.479(4)	N(3)−C(5)	1.493(4)
N(3)−C(9)	1.494(4)	C(1)−C(2)	1.496(4)
C(3)−C(4)	1.504(4)	C(5)−C(6)	1.512(4)
	Bond Angles		
Cl(1)−Fe(1)−Cl(2)	96.7(1)	Cl(1)−Fe(1)−Cl(3)	96.7(1)
Cl(2)−Fe(1)−Cl(3)	97.0(1)	Cl(1)−Fe(1)−N(1)	91.5(1)
Cl(2)−Fe(1)−N(1)	93.8(1)	Cl(3)−Fe(1)−N(1)	165.6(1)
Cl(1)−Fe(1)−N(2)	167.6(1)	Cl(2)−Fe(1)−N(2)	91.6(1)
Cl(3)−Fe(1)−N(2)	91.4(1)	N(1)−Fe(1)−N(2)	78.8(1)
Cl(1)−Fe(1)−N(3)	92.8(1)	Cl(2)−Fe(1)−N(3)	167.5(1)
Cl(3)−Fe(1)−N(3)	89.9(1)	N(1)−Fe(1)−N(3)	77.8(1)
N(2)−Fe(1)−N(3)	77.8(1)	Fe(1)−N(1)−C(1)	110.5(2)
Fe(1)−N(1)−C(6)	105.5(2)	C(1)−N(1)−C(6)	110.5(2)
Fe(1)−N(1)−C(7)	112.7(2)	C(1)−N(1)−C(7)	109.8(2)
C(6)−N(1)−C(7)	107.7(2)	Fe(1)−N(2)−C(2)	104.1(2)
Fe(1)−N(2)−C(3)	111.1(2)	C(2)−N(2)−C(3)	111.2(2)
Fe(1)−N(2)−C(8)	113.0(2)	C(2)−N(2)−C(8)	108.6(2)
C(3)−N(2)−C(8)	108.8(2)	Fe(1)−N(3)−C(4)	104.9(2)
Fe(1)−N(3)−C(5)	111.3(2)	C(4)−N(3)−C(5)	111.0(2)
Fe(1)−N(3)−C(9)	111.9(2)	C(4)−N(3)−C(9)	108.4(2)
C(5)−N(3)−C(9)	109.3(2)	N(1)−C(1)−C(2)	112.2(2)
N(2)−C(2)−C(1)	112.1(2)	N(2)−C(3)−C(4)	112.3(2)
N(3)−C(4)−C(3)	111.2(2)	N(3)−C(5)−C(6)	111.1(3)
N(1)−C(6)−C(5)	111.2(2)		

character of Fe(III) and the absence of base specificity observed in the DNA cleavage reaction. Strong metal−DNA association may explain why a tethered DNA binding agent (psoralen) offers a minimal enhancement in DNA cleavage efficiency.

The addition of reducing agents greatly increased the amount of cleavage seen with the iron(III) triazacyclononane complexes; however, removing O_2 reduces the efficiency of cleavage. This suggests a redox mechanism that involves initial formation of a Fe(III)−superoxide complex. Several inhibition studies were performed. Desferrioxamine (Fe(III) chelator), potassium nitrate (control for ionic strength), sodium cyanide (Fe chelator), sodium formate (hydroxyl radical scavenger and Fe(III) chelator), and DMSO (hydroxyl radical scavenger) were all examined. With the exception of DMSO, all were effective to some degree at very high concentrations. Sodium cyanide was effective at relatively low concentrations. It partially quenched the reaction at 1 mM and totally quenched the reaction at 10 mM both with and without DTT. This may result from several factors. Cyanide binding will favor Fe(II) over Fe(III), so it becomes a poorer reducing agent for molecular oxygen. For example, the redox couple is $Fe(CN)_6^{4-/3-} = -0.69$ V (1 M H_2SO_4) or −0.46 V (0.1 M NaOH). Cyanide, a strong ligand, may also displace the $Fe^{2+/3+}$ from coordination sites on the DNA and block the coordination sphere. Furthermore, a $L'Fe(CN)_3^{0/-}$ species would lose any electrostatic component to DNA−metal complex binding.

Though DMSO was not effective at quenching the reactions of the iron triazacyclononanes with DNA, it did effectively quench cleavage by EDTA−Fe(II). The anionic EDTA−Fe(II) complex operates by generating diffusable hydroxyl radicals in bulk solution. These contrasting results suggest that any oxygen radical species formed from the iron triazacyclononane complexes is being generated in close contact with DNA. If a hydroxyl radical is being formed, it would have to react immediately with DNA and not diffuse into solution. Alternatively, an iron oxo species may be involved. The observation that EDTA−Fe(II) must be present in much higher concentrations than L′FeCl$_3$ for effective cleavage further supports the notion that the reactive species is generated very close to the DNA with the L′FeCl$_3$ system.

Though reducing agents enhanced the DNA cleaving ability of ps3L″FeCl$_3$ and L′FeCl$_3$, they were not essential co-reagents. Without added reducing agent, the iron(III) complexes still cleaved plasmid DNA, even though iron(II) is required to generate oxygen radical species. It seems likely that the iron(III) complexes are being reduced to iron(II) complexes by adventitious reducing agents present in biological preparations. Given the low concentrations (10^{-7}) necessary for cleavage, this seems a reasonable explanation; however, we cannot exclude the possibility that a second (less efficient) non-redox pathway for DNA cleavage exists.

While the L′FeCl$_3$ complex exhibits a cleaving ability which rivals that of a specific DNA binding species, such as bleomycin, it exhibits non-sequence-specific cleavage characteristic of a bulk solution component, such as seen for EDTA−Fe(II) (Figure 6). The cleavage patterns of ps3L″FeCl$_3$, L′FeCl$_3$, and EDTA−Fe(II) show little sequence specificity. In contrast, BLM−Fe(II) shows specificity for GC = GT > GA ≫ GG and efficiently cleaves regions of $(AT)_n$, cutting exclusively at ApT, but not at TpA.[55] It was expected that irradiation of the ps3L″FeCl$_3$ complex might show preferential cutting of DNA in the AT-rich regions because psoralen cross-links to these regions. The lack of specificity seen with the ps3L″FeCl$_3$ complex may arise from overwhelming cleavage by the noncross-linked species. Although BLM−Fe(II) is capable of making double-stranded as well as single-stranded nicks in DNA, the iron triazacyclononane complexes predominantly cleave DNA through single-stranded nicks. It would be interesting to see whether L′FeCl$_3$ possesses antitumor properties similar to BLM−Fe(II).

Many other DNA cleaving agents have been synthesized that react through oxidative degradation of the DNA. For example, Dervan and his co-workers studied methidiumpropyl−EDTA−Fe(II) (MPE−Fe(II)).[11,12,64] This reagent cleaves DNA at concentrations similar to those of the iron triazacyclononane complexes. Since it cleaves DNA by forming hydroxyl radicals in bulk solution, the mechanism of cleavage differs from that of the iron triazacyclonone complexes. Whereas in the MPE−Fe(II) system it is the ethidium bromide moiety that binds to the DNA, the metal complex alone may associate with DNA in the iron triazacyclononane systems. Second, the iron triazacyclonone systems do not appear to involve formation of diffusible hydroxyl radicals, as shown by the DMSO quenching experiments.

The L′FeCl$_3$ complex offers an alternative to MPE−Fe(II) as a DNA cleaving system. The reaction of MPE−Fe(II) is inhibited by iron chelators, such as EDTA and desferrioxamine, as well as other metals such as Ni^{II} and Zn^{II}. In contrast, L′FeCl$_3$ is inhibited weakly by these chelators, which suggests that the L′FeCl$_3$ complex is more kinetically stable in solution. The second difference is the ease with which L′FeCl$_3$ can be synthesized. Both 1,4,7-trimethyl-1,4,7-triazacyclononane and FeCl$_3$ are readily obtained from commercial sources.

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Supplementary Material Available: Tables of crystal data collection parameters, complete atomic positional parameters, anisotropic temperature factors, and bond distances and angles for L′$FeCl_3$ (6 pages); listing of observed and calculated structure factors for L′$FeCl_3$ (12 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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