Sequence-Specific Cleavage of DNA by Oligonucleotide-Bound Metal Complexes

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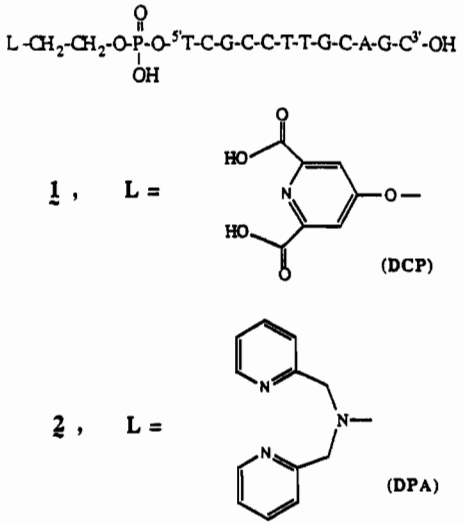
2,6-Dicarboxypyridine (DCP) and *N,N*-bis(2-picolyl)amine (DPA) ligands were synthesized and attached via ethylene groups to the 5′-ends of 12-base oligonucleotides. The base-sequence of the oligonucleotide probes were chosen to be 5′-T-C-G-C-C-T-T-G-C-A-G-C-3′, which is complementary to a 12-base sequence in pUC9 plasmid DNA. When hybridized to a denatured BamHI/PvuI restriction fragment of pUC9 in the presence of Fe^{2+}, oxygen, and a reducing agent, these probes afforded specific cleavage at their complementary sequences in the 135-base-pair template. Analysis of the cleavage fragments by high-resolution polyacrylamide gel electrophoresis indicated that both probes cleaved DNA at a single stretch of bases near the position of the tethered ligand. The cleaving activity of DPA-12-mer was unusually high and extended over eight contiguous nucleotides. DCP-12-mer showed an unprecedented high cleavage specificity extending over two nucleotides only.

Introduction

Molecular recognition and sequence-specific cleavage of DNA by synthetic probes have been the subject of intensive studies for more than a decade.[1,2,3,4] Such restriction enzyme analogues, if proven to be both efficient and specific, could have many applications, such as DNA structure and sequence determination, recombinant DNA manipulations, and gene isolation and analysis.[5,6,7] Until now, such applications have been confined to a number of restriction endonucleases that are limited in their specificities and natural availability. For cleavage of single-stranded DNA, specificity could be provided by oligonucleotides equipped with DNA-cleaving functionality.[8,9,10] Several examples of oxidative cleavage of DNA by metal chelating groups bound to short oligonucleotides have been reported.[11,12,13,14] Although such DNA probes have shown high binding-specificity toward the target DNA, they cleave it at several nucleotides around the tethered ligand. In all cases, achieving complete cleavage-specificity has proven to be difficult, possibly for two reasons. First, the metal-binding group may not be tethered tightly to a single site; second, free radical intermediates such as OH• believed to be formed under such oxidative reaction conditions can diffuse in all directions and cleave DNA at a range of several nucleotides.

We report here the synthesis of two 12-base oligonucleotide probes (**1** and **2**) that we modified at the 5′-end by covalently attaching metal-chelating ligands, 2,6-dicarboxylpyridine (DCP) and 2,2′-dipicolylamine (DPA). A short ethylene bridge was used to tether the ligands to the 12-mers. The oligonucleotide part of these probes was chosen to be complementary to a 12-nucleotide sequence in the BamHI/PvuI restriction fragment of pUC9 plasmid DNA. In the presence of Fe^{2+} and a reducing agent, both probes were able to cleave the complementary DNA strand with high sequence-specificities. The DCP–iron complex (**1**) was found to produce cleavage largely at a single base site while DPA–iron complex showed unusually high cleaving activity over a range of several contigeous bases.



Experimental Section

General Methods. ^1H NMR spectra were recorded at 500 MHz on JOEL GSX500 or at 300 MHz on General Electric QE300 spectrometers. ^{31}P NMR spectra were recorded on a Bruker WM250 instrument, using aqueous phosphoric acid as an external reference. Precoated GF-254 silica gel plates (1000 micron, Analtech) were used for thin-layer chromatography. Column chromatography was performed with EM Reagents silica gel grade 62 (60–200 mesh). High-performance liquid chromatography (HPLC) purification of oligonucleotides and their derivatives was carried out on a C-18 reverse-phase column, using a 0.1 M triethylammonium acetate/acetonitrile gradient. High-resolution polyacrylamide gel electrophoresis was used for purification of oligonucleotides and analysis of DNA cleavage fragments. Electrophoresis was conducted in 89 mM Tris borate containing 2.5 mM EDTA at pH 8.3. Autoradiography of the gels was carried out at −70 °C on Kodak X-Omat AR film, using Cronex Lightning Plus intensifying screens.

Reagent grade chemicals were used without further purification unless otherwise stated. 2,6-Dicarboxy-4-hydroxypyridine (chelidamic acid)

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was purchased from ICN Biomedicals. Ferrous ammonium sulfate and dithiothreitol were purchased from Sigma. 5′-[α^{32}P]dGTP, 5′-[α^{32}P]-dCTP, 5′-[γ^{32}P]dATP (each of specific activity of 3000 Ci/mmol), and 5′-[α^{32}P]cordycepin triphosphate (5000 Ci/mmol) were from New England Nuclear (Boston, MA). Plasmid pUC9 DNA (27 Kb) was purchased from International Biotechnologies, and enzymes were from Boehringer Manheim. All other reagents were from Aldrich.

Synthesis of 2,6-Bis(methoxycarbonyl)-4-(2-hydroxyethoxy)pyridine, 3. 2,6-Dicarboxy-4-hydroxypyridine was converted into the dimethyl ester by refluxing in anhydrous methanol containing 1% (v/v) sulfuric acid. Dimethyl 4-hydroxy-2,6-pyridinedicarboxylate (2.11 g, 10 mmol) was then dissolved in dimethyl sulfoxide (10 mL) containing potassium *tert*-butoxide (2.24 g, 20 mmol). 2-(2-Bromoethoxy)tetrahydropyran (20 mmol, prepared fresh by reacting 2-bromoethanol with 1 equiv of 3,4-dihydropyran and purified by vacuum distillation) was added and stirred at room temperature for 4 h. Water (25 mL) was then added, and the solution was extracted with diethyl ether (3 × 25 mL). The organic layer was washed with water (2 × 40 mL), dried over anhydrous sodium sulfate, and evaporated to give an oil. The tetrahydropyran protecting group was then hydrolyzed by stirring the crude product for 3 h, at room temperature, in 50 mL of 90% aqueous methanol containing five drops of concentrated hydrochloric acid. Evaporation of solvents and purification of the oily residue by preparative TLC (R_f = 0.5, silica gel, 10% methanol in methylene chloride) afforded 3 in 80% yield. Recrystallization from ethyl acetate gave white plates, mp 98–102 °C. ^{1}H NMR ($CDCl_3$): δ 3.99 (6H, s), 4.05 (2H, t), 4.25 (2H, t), 7.82 (2H, s). ^{13}C NMR ($CDCl_3$): 53.50 (OCH_3), 61.80 (CH_2OH), 70.60 (OCH_2), 114.40 (C_3 and C_5, pyridine), 149.70 (C_4, pyridine), 165.00 (C_2 and C_6, pyridine), 167.50 ppm (–COO). MS (EI) [*m*/*z* (relative intensity)]: 255 (M^+, 2), 223 (11), 196 (100), 164 (43), 152 (35), 120 (38). IR (Nujol): 3175–3500 (broad), 3000, 2850, 1750, 1700, 1600, 1460, 1350, 1200, 1100, 1050 cm^{-1}. Anal. Calcd for $C_{11}H_{13}NO_6$: C, 51.77; H, 5.13; N, 5.49. Found: C, 51.64; H, 5.28; N, 5.24.

***N,N*-Bis(2-picolyl)-2-aminoethanol, 4.** To a solution of 2-pyridinecarboxaldehyde (2.14 g, 20 mmol) in anhydrous methanol (20 mL) containing sodium cyanoborohydride (0.63 g, 10 mmol) was added dropwise a solution of 2-aminoethanol (0.61 g, 10 mmol) in anhydrous methanol (20 mL) containing glacial acetic acid (0.07 g, 20 mmol). After complete addition, the solution was stirred at room temperature for 48 h, solvent was evaporated under vacuum, and water (20 mL) was added. The solution was acidified (pH 2) by concentrated HCl and washed with chloroform (2 × 25 mL). The aqueous layer was treated with concentrated KOH to pH 9. Extraction with methylene chloride (3 × 30 mL) gave yellow oil, which was purified by column chromatography (silica gel, 5% methanol/methylene chloride) to afford 4 as a light yellow oil (1.10 g; 45% yield). ^{1}H NMR ($CDCl_3$): δ 2.37 (2H, t), 3.58 (2H, t), 3.79 (4H, s), 5.10 (1H, broad), 7.05 (2H, m), 7.25 (2H, m), 8.45 (2H, m). ^{13}C NMR ($CDCl_3$): 56.80 (CH_2OH), 59.70 (CH_2N), 60.20 (CH_2N), 122.00 (C_5, pyridine), 123.00 (C_3, pyridine), 136.50 (C_4, pyridine), 148.90 (C_6, pyridine), 159.40 ppm (C_2, pyridine). MS (EI) [*m*/*z* (relative intensity)]: 243 (M^+, 2), 212 (17), 378 (100), 119 (27), 93 (98). IR (film): 3100–3500 (broad), 2950, 2800, 1750, 1675, 1600, 1550, 1450, 1420, 1375, 1150, 1075, 1000 cm^{-1}.

[(2,6-Bis(methoxycarbonyl)pyridin-4-yl)oxy]ethyl Methyl *N,N*-Diisopropylphosphoramidite, 5. 2,6-Bis(methoxycarbonyl)-4-(2-hydroxyethoxy)pyridine, 3 (2.55 g, 10 mmol), was dissolved in methylene chloride (20 mL) containing 2 equiv of *N,N*-diisopropylethylamine (2.80 g, 20 mmol), in a round-bottomed flask sealed with a septum. Methyl *N,N*-diisopropylchlorophosphoramidite (1.97 g, 10 mmol) was added dropwise under argon. The reaction was monitored by TLC and stopped after 30 min when all the alcohol starting material reacted. Methanol (0.5 mL) was then added, and the solution was stirred for 5 min. Methylene chloride (30 mL) was added, and the solution was washed once with a saturated solution of sodium carbonate and once with a saturated NaCl solution (10 mL each). The organic layer, after drying over anhydrous sodium sulfate, was evaporated to give an oil. The crude product was purified by running it through a short column of silica gel, eluting with CH_2Cl_2/EtOAc/Et_3N, 4.5/4.5/1, to give pure 5 (3.54 g; 85% yield). ^{1}H NMR (d_6-acetone): δ 1.13 (12H, d), 3.36 (3H, d), 3.54 (2H, m), 3.93 (6H, s), 4.15 (2H, m), 4.35 (2H, m), 7.82 (2H, s). ^{31}P NMR (d_6-acetone): 151.62 ppm. ^{13}C NMR (d_6-acetone): 24.08 (d, *i*-pr CH_3), 42.70 (d, NCH), 49.96 (d, $POCH_3$), 52.10 (ester CH_3), 61.64 (d, $POCH_2$), 69.31 (d, CH_2), 114.40 (C_3 and C_5, pyridine), 150.02 (C_4, pyridine), 164.89 (C_2 and C_6, pyridine), 166.87 ppm (COO). MS (EI) [*m*/*z* (relative intensity)]: 416 (M^+, 13), 315 (53), 205 (33), 161 (100). IR (film): 3000, 2900, 1750, 1730, 1600, 1430, 1400, 1350, 1250,1175, 1100, 1030, 975 cm^{-1}.

***N,N*-Bis(2-picolyl)-2-aminoethyl Methyl *N,N*-Diisopropylphosphoramidite, 6.** A procedure similar to that of 5 was followed using *N,N*-bis(2-picolyl)-2-aminoethanol, to give the desired phosphoramidite in 62% yield. ^{1}H NMR (d_6-acetone): δ 1.15 (12 H, d), 2.80 (2H, m), 3.35 (3H, d), 3.58 (2H, m), 3.76 (2H, m), 3.90 (4H, s), 7.20 (2H, m), 7.60 (2H, m), 7.70 (2H, m), 8.48 (2H, m). ^{31}P NMR (d_6-acetone): 149.36 ppm. ^{13}C NMR (d_6-acetone): 24.16 (d, *i*-pr CH_3), 42.60 (d, *i*-pr CH), 50.30 (d, $POCH_3$), 54.95 (d, NCH_2), 60.78 (s, CH_2), 61.75 (d, $POCH_2$), 121.85 (s, C_5, pyridine), 121.90 (s, C_3, pyridine), 122.65 (s, C_4, pyridine), 136.11 (s, C_6, pyridine), 148.83 ppm (s, C_2, pyridine). IR (film): 2950, 2800, 1750, 1600, 1550, 1490, 1450, 1400, 1250, 1175, 1050, 975 cm^{-1}.

Synthesis of Ligand-12-mer Probes 1 and 2. The solid-phase phosphoramidite method was used to synthesize the fully protected 12-mer.[15] The synthesis was carried out on Applied Biosystems DNA Synthesizer Model 380A, using a 10-μmol scale. After detritylation of the oligonucleotide, while it is still attached to the solid support, a solution of the phosphoramidite (100 μmol) in anhydrous THF (0.5 mL) containing tetrazole (17.5 mg, 0.5 M) was introduced to the column in the 13th addition cycle. After oxidation, deprotection and cleavage of the oligonucleotide from the solid support were accomplished upon treatment with 0.1 M NaOH (0.5 mL) for 12 h, with occasional shaking. The solution was then neutralized by glacial acetic acid and lyophilized, and the pellet was desalted on a C-18 Sep-Pak cartridge (Waters Associates). Salt was removed by washing the column with 10 mL of water, and the 12-mer was eluted with 3 mL of methanol/water (6:4, v/v). Oligonucleotides were purified by reverse-phase HPLC, on a C-18 column (using 0–70% acetonitrile/0.1 M triethylammonium acetate gradient at pH 7.0). Further purification was carried out by polyacrylamide gel electrophoresis (20%, 1.5 mm; at 200 V for 6 h). The ligand-12-mers were eluted from the excised gel with water, passed through a G-25 column, and lyophilized to give 18 mg of pure 1. The base-sequence of the oligonucleotide was confirmed by the Maxam–Gilbert sequencing method,[16] after labeling the 3′-end with 5′-[α^{32}P]cordycepin triphosphate in the presence of terminal polynucleotidyl transferase.[17]

Isolation and Labeling of Template DNA Fragment. A 135-bp double-stranded restriction fragment, containing the 12-nucleotide complement of the ligand-12-mer probes, was obtained by enzymatic digestion of pUC9 plasmid DNA. The plasmid DNA was first digested with BamHI, labeled at the 3′-end with [α^{32}P]dGTP in the presence of dATP and Klenow fragment of DNA polymerase I, and then digested with PvuI. This restriction fragment was isolated from other fragments by column chromatography, using Sephacryl-S500 (Pharmacia LKB), and purified by polyacryamide gel electrophoresis. Digestion of pUC9 DNA by BamHI, followed by successive treatment with bacterial alkaline phosphatase, [γ^{32}P]dATP in the presence of T_4-polynucleotide kinase, and then PvuI, gave the 5′-end labeled fragment containing a 12-nucleotide sequence identical to that of the probes.

DNA Cleavage Reactions. In a typical DNA cleavage experiment, 6 μL of a solution of the end-labeled template DNA (3000 cpm, approximately 3.3 nM) in 50 mM Tris·HCl (pH 7.5), in a small Eppendorf tube, was denatured by heating at 90–95 °C for 4 min and then rapidly chilling in ice. Template/probe hybridization was accomplished by adding 1 μL of 10 μM solution of the probe and 1 μL of 0.5 M NaCl to the template solution and then incubating at 25 °C for 30 min. Cleavage reactions were started by adding 1 μL of 100 μM Fe^{2+} solution, followed by 1 μL of 50 mM dithiothreitol (DTT). The final reaction solution (10 μL) contained ~2 nM of template DNA, 1 μM of ligand-12-mer probe (template:probe ratio of ~1:500; 1:45 in bp's), 50 mM NaCl/50 mM Tris·HCl, 10 μM Fe^{2+}, and 5 mM DTT. After incubation at 25 °C for 1.5 h, the reaction was terminated by adding EDTA to a final concentration of 10 mM. Carrier t-RNA (2.5 μL of 1 mg/μL solution) was added, and the solution was diluted to 25 μL with 0.5 M sodium acetate. DNA was then precipitated by adding 75 μL of ethanol and dried in vacuum. To analyze cleavage fragments, the samples were redissolved in 8 μL of 80% formamide, heat denatured, loaded on 8% polyacrylamide gel (0.4 mm thick, 1:19 cross-linked/7 M urea), and electrophoresed at 1500 V for 2.0 h. After drying, the gel was autoradiographed at –70 °C on a Kodak X-Omat AR film.

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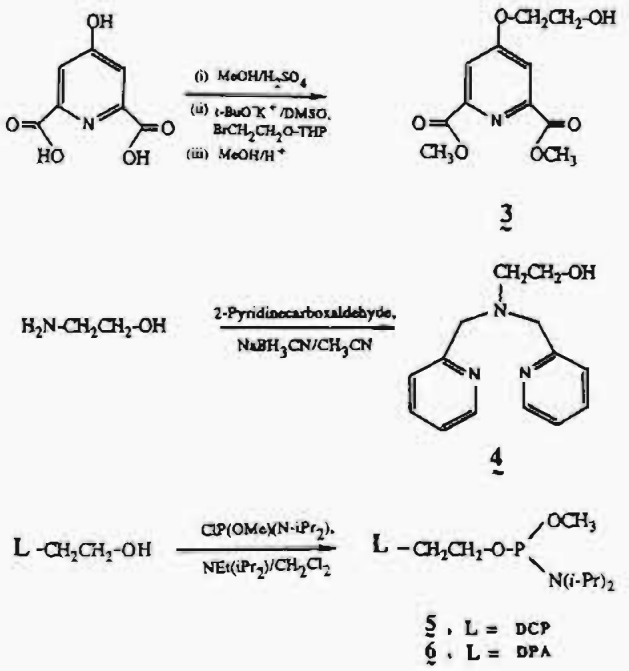
Scheme I



Results and Discussion

Syntheses of Ligands. Ligand **3** was synthesized from the commercially available 2,6-dicarboxy-4-hydroxypyridine (chelidamic acid). In order to circumvent any possible interference of the carboxylic groups in the following phosphoramidite reaction, the starting material was converted into its dimethyl ester derivative. Nucleophilic substitution reaction between dimethyl 4-hydroxy-2,6-pyridinedicarboxylate and 2-bromoethanol was successful only after protecting the hydroxyl group in the latter in the form of tetrahydropyran derivative (Scheme I). Synthesis of ligand **4** was achieved by reductive amination of 2-pyridinecarboxaldehyde with 2-aminoethanol, in the presence of sodium cyanoborohydride. Ligands **3** and **4** were converted into their phosphoramidite derivatives upon reaction with methyl *N,N*-diisopropylchlorophosphoramidite. The syntheses of compounds **1** and **2** were achieved by coupling the phosphoramidite derivatives of **3** and **4**, respectively, to the 5′-hydroxyl groups of the oligonucleotides.

Oligonucleotides Syntheses and Modifications. The 12-mer oligonucleotides were synthesized by standard automated solid-phase phosphoramidite procedures.[15] High coupling efficiencies were achieved upon reacting the ligand–phosphoramidite derivatives with the 5′-hydroxyl group of the support-attached oligonucleotide. Anhydrous tetrahydrofuran was substituted for acetonitrile in the coupling reaction due to the low solubility of the ligand–phosphoramidites in the latter. Hydrolysis and deprotection steps were carried out in aqueous NaOH instead of the standard ammonium hydroxide, to avoid possible ammonolysis of the ester groups of the DCP ligand. Base-sequence of the oligonucleotides was verified by chemical sequencing methods.[16] The ligand-bound 12-mers displayed reduced electrophoretic mobilities relative to unmodified oligonucleotides of similar base-sequences.

DNA Cleavage and Controls. A 135-bp BamHI/PvuI restriction fragment of pUC9 DNA, labeled at 3′-end, was used to examine the specificity of DNA cleavage by the modified oligonucleotides. Figure 1 shows a polyacrylamide gel analysis of cleavage fragments of the template when hybridized to a 500-fold molar excess of the probes and reacted with 10 μM Fe^{2+}/5 mM dithiothreitol, at 25 °C and pH 7.5. Excess amounts of the probes were needed to ensure effective hybridization and selectivity. Significant reduction in the cleavage efficiency was observed at 1 order of magnitude lower concentration of the probe. However, increasing the probe/template ratio by more than 500-fold resulted in an insignificant enhancement in cleavage efficiency.

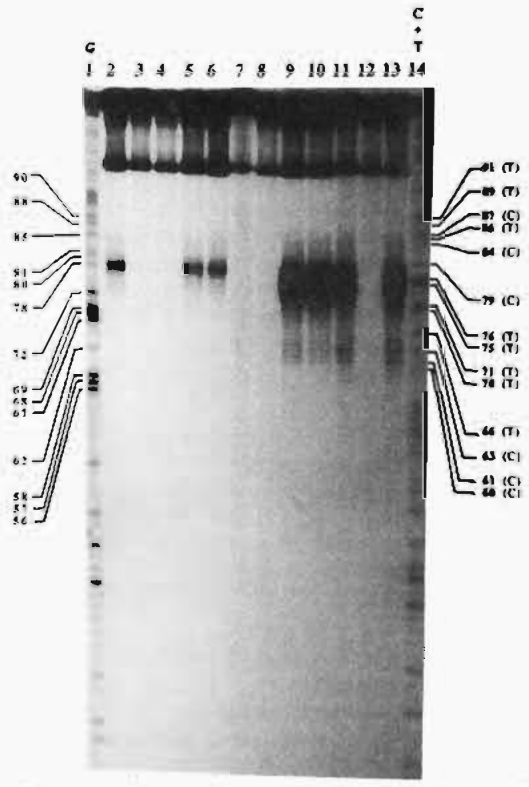


Figure 1. Autoradiogram of denaturing gel showing cleavage of 3′-end labeled 135-bp BamHI/PvuI restriction fragment of pUC9 plasmid DNA by DCP-12-mer (**1**) and DPA-12-mer (**2**). Lanes 1 and 14 are Maxam–Gilbert G and C + T ladders of 3′-labeled 135-bp DNA fragment, respectively. Lanes 2 and 13 represent the cleavage products of the 3′-end labeled fragment by **1** and **2**, respectively [1 μM probe, 10 μM Fe^{2+}, 5 mM DTT]. Lane 3 is a control showing the 3′-end labeled fragment after treatment with 1 μM of unbound ligand (4-hydroxy-2,6-dicarboxypyridine), 10 μM Fe^{2+}, and 5 mM DTT. Lane 4 description is as in lane 2 and in the presence of 1 mM ethidium bromide. Lane 5 description is as in lane 2 and in the presence of catalase [60 μg/mL]. Lane 6 description is as in lane 2 and in the presence of 100 μM H_2O_2. Lane 7 shows the 5′-end labeled DNA fragment treated as in lane 2. Lane 8 is a control showing the 3′-end labeled fragment under the cleavage reaction conditions, including 1 μM of an unmodified probe, 10 μM Fe^{2+}, and 5 mM DTT. Lane 9 description is as in lane 13 and in the presence of 100 μM H_2O_2. Lane 10 description is as in lane 13 but using 2 μM of **2**. Lane 11 description is as in lane 13 and in the presence of 1 mM ethidium bromide. Lane 12 description is as in lane 13 and in the presence of catalase [60 μg/mL]. (Refer to the text for details.)

Both probes (**1** and **2**) affected the cleavage of template at a single site (i.e., a stretch of nucleotides) near the end of the probe bearing the ligand. The cleavage site, in the case of DCP-12-mer (**1**), covered two consecutive nucleotides only (Figure 1, lane 2, and Figure 3A). On the other hand, DPA-12-mer (**2**) introduced a wider range of cleavage which extended over eight contiguous nucleotides (Figure 1, lane 13, and Figure 3B). No DNA cleavage was observed when any of the ligand-bound probes, Fe^{2+}, or the reducing agent was omitted from the reaction. Cleavage did not occur when the template was not denatured prior to reaction. No cleavage was observed when an unbound ligand (Figure 1, lane 3) or an unmodified probe of identical sequence but lacking the ligand (Figure 1, lane 8) was used instead of the ligand-bound probes. Under similar conditions, no cleavage was observed when the 5′-end labeled strand, containing a sequence identical to that of the probes, was used instead of the complementary 3′-end labeled strand (Figure 1, lane 7). Both probes failed to cleave

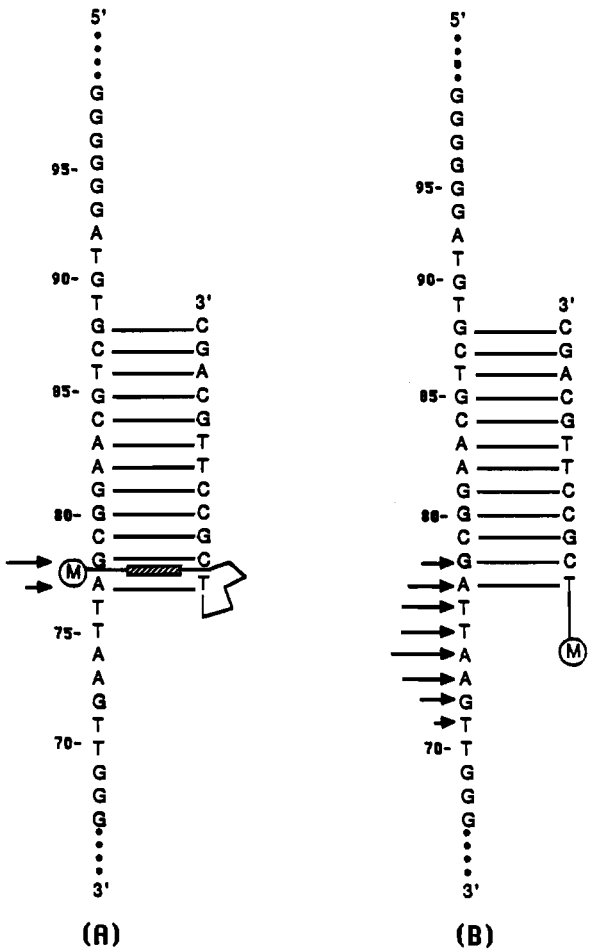


Figure 2. A schematic presentation showing a histogram of the cleavage pattern of 135-bp DNA template: (A) Cleavage by **1**, lane 2 (the rectangle represents the aromatic portion of DCP and circle represents the metal center); (B) cleavage by **2**, lane 13. The length of the arrow represents the relative intensity of cleavage at the indicated base.

DNA when Cu^{2+} was substituted for Fe^{2+}. The presence of catalase in the reaction mixture showed no significant effect on DNA cleavage by **1** while resulting in total inhibition of the cleavage by **2** (Figure 1, lanes 5 and 12, respectively). The presence of H_2O_2 (100 × 6dM) resulted in 25% enhancement of DNA cleavage by **2** (Figure 1, lane 9) but showed no effect on the activity of **1** (Figure 1, lane 6). When excess ethidium bromide (1000 equiv relative to oligonucleotide) was added to the DNA/probe hybrid, prior to initiation of the cleavage reaction, total inhibition of DNA cleavage by **1** was observed (Figure 1, lane 4). On the other hand, ethidium bromide had no effect on the activity of **2** (Figure 1, lane 11).

Autoradiography showed that the DNA cleavage occurred at only one stretch of nucleotides that is complementary to the ligand-12-mer probe. This suggests that, after forming a stable duplex with the template, the ligand-bound probes chelate Fe^{2+} and, in the presence of O_2 and dithiothreitol, bring about the localized DNA cleavage. Control experiments showed that cleavage reactions depended on the presence of the ligand-probe, Fe^{2+}, and dithiothreitol. The order of the following steps was maintained in order to minimize the probes' autocleavage: hybridization of the probe to substrate, addition of metal, followed by activation by dithiothreitol. Lack of DNA cleavage in the absence of the reducing agent indicates that oxidative pathways are involved in the reactions of both **1** and **2** with the template. However, the active intermediate generated by **1** during the cleavage reaction seems to be significantly different from the one generated by **2**. The fact that activity of **2** was enhanced by the presence of H_2O_2 and inhibited by catalase indicates that H_2O_2 and/or OH radical

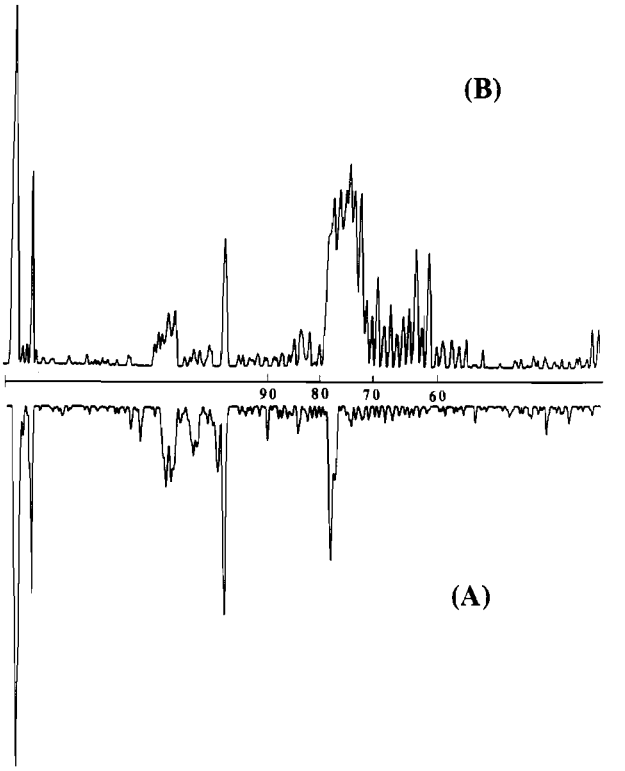


Figure 3. Comparison of densitometer plots of (A) lane 2 and (B) lane 13 of Figure 1. Conditions are as stated in Figure 1.

could be intermediates in the reaction. The cleavage pattern of the template by **2** is consistent with the intermediacy of diffusible species.[11] By contrast, neither H_2O_2 nor catalase had any effect on the activity of **1** which cleaved the template DNA at two consecutive nucleotides only. Such a very short range of cleavage pattern indicates that reaction of **1** involves either nondiffusible metal-associated oxidizing species[18] capable of initiating DNA cleavage or free radicals that do not diffuse far from the site of generation.

To compare specificity versus reactivity of the probes, cleavage reactions were conducted at low concentration of **2** to tune down its activity to a level comparable to that of **1**. Under such conditions, **2** cleaved the template at a stretch of bases covering eight contiguous nucleotides while **1** cleaved DNA at two bases. In a separate experiment, neither probe cleaved DNA when Cu^{2+}, Co^{2+}, Ni^{2+}, or Cd^{2+} was used instead of Fe^{2+}.

DNA-Fragment Analysis. Probe **2**, in the presence of Fe^{2+} and dithiothreitol, cleaves the complementary template DNA at a single site which covers eight contiguous nucleotides. The cleavage site is centered at residues A-74 and T-75 of the template, three nucleotides down from the 5'-end of the probe bearing the ligand (Figures 2B and 3B). This suggests that diffusible species may be responsible for this pattern of cleavage. However, the ability of this probe to cleave at one stretch of bases rather than two could be attributed to the short ethylene bridge between the ligand and the 5'-end of the probe. This is different from what was previously observed by Dervan[11] and others[12] in the case of EDTA–oligonucleotide probes, which, under similar reaction conditions, cleaved the template DNA at two stretches of bases, each extending over several nucleotides on both sides of the base carrying the tethered ligand.

A unique pattern of DNA cleavage was observed in the case of probe **1**. The cleavage extended over two nucleotides only, at residues A-77 and G-78 of the template (Figures 2A and 3A). One interpretation for these results may be that the planar

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aromatic portion of the ligand intercalates between the last two base-pairs of the probe/template hybrid, which, in the presence of Fe^{2+} and dithiothreitol, forms a metal-bound active intermediate that brings about this high specificity of DNA cleavage. Such ligand intercalation does not hinder the probe/template hybridization but rather stabilizes the double-stranded hybrid.[19] Inhibition of the activity of **1** by strong intercalating agents such as ethidium bromide supports this hypothesis and indicates that intercalation of ligand may be required for DNA cleavage.

In conclusion, two significantly different patterns of DNA cleavage were obtained by probes **1** and **2**. One probe (**1**) showed a high DNA cleaving specificity, possibly through a mechanism that does not involve free radical intermediates. Probe **2**, on the other hand, was less specific than **1** and cleaved DNA through a mechanism consistent with Fenton chemistry involving diffusible free radicals. Currently we are investigating the mechanism of DNA cleavage by **1** and the nature of the active intermediates involved.

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