time would reduce ionization of the rare tautomer, a 2-oxo-4hydroxo tautomer of thymine (or uracil) might be sufficiently long lived to accomplish base mispairing (Scheme V).

In any case, the here-proposed model for a metal-assisted tautomerization of 1-methyluracil or 1-methylthymine<sup>12</sup> could provide a rationale for findings on the increase of GC content in bacterial DNA at the expense of AT under the influence of Cu-(II), $^{62}$  although we note that there is an alternative possibility (cf. Figure 8).

Isolation of the two Pt complexes 1 and 3 containing rare nucleobase tautomers has been possible because kinetics of the complex decomposition are sufficiently slow. With Pd(II) or first-row transition elements the preparation of analogues is difficult, if not impossible. The results of the X-ray structure

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analysis of 1 have been used to estimate the geometry of the rare 2-oxo-4-hydroxo tautomer.

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Registry No. 1, 121809-96-9; 2, 121809-97-0; 3, 121844-93-7; 1-MeUH, 615-77-0; cis-(NH<sub>3</sub>)<sub>2</sub>Pt(1-MeU)<sub>2</sub>, 83350-97-4; Na<sub>2</sub>PtCl<sub>6</sub>, 16923-58-3; H2PtCl6, 16941-12-1.

Supplementary Material Available: Table A listing positional and anisotropic thermal parameters of 1, Table B listing positional parameters and temperature factors for 3, Table C listing possible hydrogen-bonding interactions in 1 and 3, and Figure 1 showing <sup>1</sup>H NMR spectra of 3 in MeSO- $d_6$  (4 pages); tables of observed and calculated structure factors (37 pages). Ordering information is given on any current masthead page.

# DNA Oligomers and Duplexes Containing a Covalently Attached Derivative of Tris(2,2'-bipyridine)ruthenium(II): Synthesis and Characterization by Thermodynamic and Optical Spectroscopic Measurements<sup>†</sup>

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Abstract: Oligonucleotides having the base sequence 5'-GCA(C\*)TCAG-3' and 5'-GCAC(T\*)CAG-3' were synthesized where C\* and T\* equal, respectively, a chemically modified cytidine or thymidine base containing a linker arm terminating in a primary amine. The primary amine of these modified oligomers reacted specifically with the N-hydroxysuccinimide ester of 4-carboxy-4'-methyl-2,2'-bipyridine to form bipyridine-labeled oligomers, and these oligomers reacted with  $Ru(bpy)_2(H_2O)_2^{2+}$ to give oligonucleotides with covalently attached derivatives of  $Ru(bpy)_3^{2+}$ . Oligonucleotides with nonspecifically bound  $Ru(bpy)_2(H_2O)_x^{2+}$ , where x = 0 or 1, were also formed, but were chromatographically separated from the former product. Duplexes of the Ru(bpy)<sub>3</sub><sup>2+</sup>-labeled oligonucleotides were formed upon addition of their unmodified complementary strands and were studied by melting temperature behavior as a function of concentration and by absorption and emission optical spectroscopies. Both hybridization behavior and the spectroscopic properties of the ruthenium label itself were retained in these labeled duplexes. This work shows that it is possible to use DNA duplexes as molecular scaffolds to organize covalently attached polypyridyl-substituted transition-metal complexes and constitutes an initial step in the construction of macromolecules with specifically located, redox-active subunits.

The ability of DNA oligomers to hybridize allows a complementary probe strand to bind to a specific, target base sequence. Additionally, such probe/target hybridization can be detected by spectroscopic methods if the probe sequence is appropriately labeled. There are a number of reports of synthetic oligonucleotides that contain a covalently attached label,<sup>1-9</sup> and label attachment can be either to a base<sup>1,2,9</sup> or to a phosphate.<sup>4-8</sup> We have previously studied a series of oligonucleotides and duplexes with a variety of labels attached at either thymidine or cytidine.<sup>9</sup> However, in that study as well as in most others,<sup>4-8</sup> the labels were organic molecules, usually with good fluorescence properties. Examples include derivatives of pyrene,<sup>8,9</sup> acridine,<sup>3-6</sup> phenanthridine,<sup>7</sup> and fluorescein.<sup>9</sup> Very recently, Helene and co-workers attached a number of metalloporphyrins to oligonucleotides.<sup>10</sup> However in

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that study, as in many others, the labels were attached was at 3' or 5' terminal phosphates. Importantly, Dreyer and Dervan attached an inorganic coordination complex, Fe-EDTA, to a

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modified internal base to produce a DNA-binding probe, which could chemically cleave DNA in the presence of dioxygen.<sup>2</sup> This study extends their labeling work and attaches a fluorescent as well as redox-active inorganic label to an internal base; the label used here is a derivative of tris(2,2'-bipyridine)ruthenium(II)  $(Ru(bpy)_3^{2+})$ . Polypyridyl complexes of iron, ruthenium, and osmium have been widely studied due to their interesting photochemical and photophysical properties.<sup>11</sup> The metal-to-ligand charge-transfer (MLCT) excited states of these complexes are long-lived (500-1000 ns) and possess sufficient free energy to participate in a variety of redox reactions. In addition, the interaction of polypyridyl-substituted complexes of ruthenium and other transition metals with DNA has been extensively studied, particularly by Barton and co-workers.<sup>12-26</sup> These studies demonstrated that exogenous ruthenium polypyridyl complexes can interact strongly with DNA duplexes. In particular, ruthenium(II) complexes with two or more phenanthroline<sup>12-15</sup> or phenanthroline derivative ligands<sup>18,19</sup> intercalate into the DNA base-pair stack. The DNA binding and photoredox properties of ruthenium(II) [and cobalt(III)] have also been successfully used to effect light-induced DNA strand scission.<sup>20-24</sup> Therefore, DNA oligomers with covalently attached ruthenium polypyridine complexes could potentially be used as photochemically activated DNA cleavage agents. This work, therefore, also complements the above intermolecular studies involving DNA and ruthenium polypyridyl complexes by examining an intramolecular system comprised of a DNA octamer covalently attached to a ruthenium polypyridyl complex. Although the procedure for internally labeling DNA with ruthenium polypyridyl complexes can be optimized further, we have found that the desired labeled oligonucleotides can be prepared in isolable quantities. Importantly, the absorption and emission properties of the attached ruthenium complex are maintained in the labeled oligomer, and duplex formation between the labeled oligomer and its complementary strand is not seriously perturbed. Given the large number of transition-metal ions that can bind polypyridyl ligands and the large variety of such ligands themselves, an extensive family of labeled oligomers of the type described here can be produced and used as the basis for exploring label/label interactions in DNA-based, ordered macromolecules.

#### **Experimental Section**

Synthesis of Succinimidyl-4-carboxy-4'-methyl-2,2'-bipyridine. 4,4'-Dimethyl-2,2'-bipyridine (10.0 g, 54 mmol),  $KMnO_4$  (17.5 g, 110 mmol), and  $MnSO_4$  (0.10 g, 0.7 mmol) were dissolved in 1:1 pyridine/water (300 mL) and allowed to stir at room temperature for 3-5 days. Excess  $KMnO_4$  was then destroyed by addition of 2-propanol (50 mL) and

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refluxing. After 1 h, the solution was filtered while hot and the solvent removed by rotary evaporation. The solid was Soxhlet extracted with chloroform for 12 h to remove unreacted 4,4'-dimethyl-2,2'-dipyridine. The remaining solid was dissolved in water and 6 M HCl was added to pH 2. The acid product precipitated and was filtered, washed with water, and dried in vacuum. The desired monoacid product was separated from the diacid by Soxhlet extraction with acetone for 2-3 days. Evaporation of solvent and drying yielded the monoacid (0.5-0.8 g, 2-4 mmol, 4-7%). The succinimidyl ester of the monoacid is prepared by standard methods. 4-Carboxy-4'-methyl-2,2'-bipyridine (0.214 g, 1 mmol) and N-hydroxysuccinimide (NHS; 0.115 g, 1 mmol) were dissolved in dry N,N-dimethylformamide (DMF, 40 mL). Dicyclohexylcarbodiimide (DCC; 0.206 g, 1 mmol) dissolved in DMF (10 mL) was added dropwise. The solution was allowed to stir for 12 h and then filtered to remove the dicyclohexylurea byproduct. Solvent was removed by vacuum, and the resulting solid was recrystallized with dichloromethane to yield the succinimidyl ester (0.23 g, 0.74 mmol, 74%). The identity of the product was confirmed by <sup>1</sup>H NMR through comparison with authentic NHS and both 4,4'-dimethyl- and 4-carboxy-4'-methyl-2,2'-bipyridine. The same procedure was used to prepare the disuccinimidyl ester of 4,4'-dicarboxy-2,2'-bipyridine, except with 2 equiv of NHS and DCC.

Synthesis of Modified Oligonucleotides. Cytidine and thymidine nucleosides (C\* and T\*, respectively), both containing a linker arm terminating in a primary amine, were synthesized as described previously.<sup>9</sup> These were converted to phosphoramidite reagents and used for automated oligonucleotide synthesis by standard procedures.<sup>9,27</sup> Purification of the modified and corresponding unmodified oligonucleotides was accomplished by using a previously developed chromatographic protocol.<sup>9</sup> Concentrations of the modified and unmodified oligonucleotides were determined by standard methods for calculating extinction coefficients.<sup>28</sup>

Bipyridine Labeling of Modified Oligonucleotides. The oligonucleotides containing the modified C\* or T\* bases were labeled with succinimidyl-4-carboxy-4'-methyl-2,2'-bipyridine by the same procedure reported previously for labeling reactions with succinimidyl-1-pyrenebutanoic acid.9 This involved reaction between the C\*- or T\*-modified octanucleotide and a large excess (100-200 fold, added as a DMF solution) of the bipyridine derivative in a borate buffer solution (0.05 M, pH = 9.3) for 12 h at room temperature. Purification of the bipyridine-labeled oligomer was accomplished with a Pharmacia FPLC system equipped with a ProPRC HR 10/10 column. A linear gradient of 1:1 acetonitrile/10 mM triethylammonium acetate was used ranging from 0 to 35% over 45 min at 2 mL/min. The chromatogram (not shown) of the reaction of the C\*- or T\*-modified oligomer with the bipyridine derivative gave three fractions: at 5 min (NHS), 20-22 min (bipyridine and unreacted DNA), and 28 min (bipyridine-labeled DNA). The last fraction was absent in a chromatogram of the reaction of the corresponding unmodified oligomer with the bipyridine derivative, indicating no bipyridine labeling. Yields ranged from 40 to 60% based on modified oligomer starting material. Importantly, a second FPLC run on the fraction at 20-22 min using a gradient ranging from 0 to 70% afforded separation of unreacted DNA from bipyridine, allowing recovery of the modified oligomer. The bipyridine-labeled oligomer could be identified by its UV-visible spectrum in which the DNA absorption band at 260 nm exhibited a shoulder not present in normal DNA. This feature extended from 290 to 340 nm and is due to  $(\pi,\pi^*)$  absorption by bipyridine. Note that the absorption maximum of authentic bipyridine shifts from 290 to 300 nm upon acidification. This behavior was paralleled in the bipyridine-labeled oligomer, as addition of acid made the shoulder more pronounced. Because absorption at 260 nm from a single bipyridine molecule is small relative to that of the octanucleotide, no correction was made for label absorption in calculations of the concentration of bipyridine-labeled DNA for reaction with ruthenium. The same procedure using disuccinimidyl-4,4'-dicarboxy-2,2'-bipyridine afforded C\*- or T\*-modified oligomers labeled with this bipyridine derivative. Since bipyridine was in large excess, it was not likely that a single, bifunctional label would react with two oligomers. Additionally, unreacted NHS ester groups were hydrolyzed during the lengthy reaction, so that the final product had the 4-carboxy converted to an amide bound to DNA, and the 4'-carboxy was left as a carboxylate. A control reaction of this bipyridine with the unmodified octamer showed no product formation.

Reaction of Ruthenium with Bipyridine-Labeled Oligomers. Bis(2,2'- bipyridine)ruthenium(II) dichloride dihydrate (Aldrich, 6 mg, 12  $\mu$ mol) was dissolved in 1:1 ethanol/water under nitrogen (20 mL). The solution

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### Oligonucleotides with Covalently Attached $Ru(bpy)_3^{2+}$

was refluxed for 3 h, after which the UV-visible spectrum indicated conversion to  $Ru(bpy)_2(H_2O)_2^{2+}$  had occurred. An aliquot of this solution (0.1 mL, 0.06 µmol) was added to a solution of the bipyridine-labeled DNA (0.9 mL,  $\sim$ 0.06  $\mu$ mol) in phosphate buffer (0.1 M, pH 7) contained in a 2-mL disposable sample vial. This solution was stirred for 2-3 days at room temperature in the dark. A control reaction under identical conditions was run using the unmodified oligomer. The solution was then run through a Pharmacia Sephadex NAP-10 column for desalting. The resulting solution was then purified by reversed-phase liquid chromatography under the same conditions used for the purification of the bipyridine-labeled product. In all cases, whether the DNA oligomer was modified or not, several fractions resulted. The reversed-phase chromatograms of the reaction products of  $Ru(bpy)_2(H_2O)_2^{2+}$  with the two types of base-modified oligomers, as well as the unmodified oligomer, had qualitatively the same appearance (not shown). Several reactions were run and each type of reaction produced identical chromatograms for the different runs. All the reversed-phase chromatograms consisted of an early, colorless fraction (i.e., DNA only) followed by several colored fractions (i.e., ruthenated DNA). These latter fractions were then further purified by ion-exchange chromatography using the Pharmacia FPLC system equipped with a MonoQ HR 5/5 column (50  $\times$  5 mm diameter, 10-µm particle size anion-exchange resin). A linear gradient of 7:3 (1 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>3</sub>, 0.05 M KH<sub>2</sub>PO<sub>4</sub>)/methanol against 7:3 0.05 M  $KH_2PO_4$ /methanol was used ranging from 0 to 35% over 35 min at 1 mL/min.

The various fractions were identified by UV-visible absorption and emission spectroscopies. Figure 2 presents the absorption spectra of representative fractions from the chromatographic separations for the ruthenium-labeling step. Each of the fractions in Figure 2 corresponded to a single, sharp peak after ion-exchange chromatography. Four types of spectra are shown: the unmodified octamer (A); the unmodified octamer with bound  $Ru(bpy)_2(H_2O)_x^{2+}$  where x = 0 or 1 (B); the bi-pyridine-labeled T\*-modified octamer (C); and the  $Ru(bpy)_3^{2+}$ -labeled T\*-modified octamer (D). The basis for the assignments is as follows. Identification of ruthenium-free DNA fractions (A and C) was straightforward, since these exhibited no absorption beyond 310 nm for the unmodified oligomer and none beyond 340 nm for the bipyridinelabeled oligomers. As expected, no emission was observed for these samples with excitation at 450 nm. Several ruthenium-containing fractions were obtained upon reaction of  $Ru(bpy)_2(H_2O)_2^{2+}$  with the unmodified oligomer (B). These product fractions exhibited absorption bands at 285 (shoulder on the DNA band), 340, and 480 nm. The positions and relative intensities of these bands (after accounting for absorption due to DNA) are virtually identical with those for Ru- $(bpy)_2(H_2O)_2^{2+}$  (not shown). Importantly, each of these rutheniumcontaining fractions lacked emission over the visible range despite significant absorbance at 450 nm. Similar nonfluorescent ruthenated fractions were also isolated from the reaction of  $Ru(bpy)_2(H_2O)_2^{2+}$  with the bipyridine-labeled oligomers. The desired product, DNA with bound  $Ru(bpy)_{3}^{2+}$ , was also isolated from the above reaction (D). This fraction has absorption bands at 290 nm (shoulder on the DNA band) and 460 nm, with none at 340 nm. Authentic  $Ru(bpy)_3^{2+}$  exhibits bands at 290 and 454 nm. Both authentic  $Ru(bpy)_3^{2+}$  and this fraction emit strongly from 600 to 800 nm when excited at 450 nm. This combination of reversed-phase and ion-exchange chromatographies gave clean separations of the various reaction products allowing isolation of the octamer with the covalently attached tris(bipyridine)ruthenium derivative in 5-10% yield, based on the starting amount of bipyridine-labeled oligomer.

The same reaction using the oligomers labeled with 4,4'-dicarboxy-2,2'-bipyridine displayed more complex chromatographic behavior. Fractions were recovered that exhibited the characteristic MLCT emission of tris(polypyridyl)ruthenium(II) complexes; however, the quantities were too small for thermodynamic or spectroscopic characterization.

Thermodynamic and Spectroscopic Measurements. Absorption and emission spectra were measured as described previously.<sup>9</sup> Emission quantum yields were calculated with fluorescein as a standard (0.1 M NaOH, QY = 0.92<sup>29</sup>). DNA melting curves were obtained on the automated multicell system described previously.<sup>9</sup> In the calculation of concentrations of the ruthenium-labeled oligomers, it was necessary to account for the contribution to the absorbance at 260 nm from the ruthenium complex itself. This was done by taking the ratio of the absorption of free Ru(bpy)<sub>3</sub><sup>2+</sup> at 260 nm to that at 454 nm ( $\lambda_{max}$ ). The absorption of the ruthenium-labeled oligomer at 260 nm was then corrected for label absorption by using the above ratio of Ru(bpy)<sub>3</sub><sup>2+</sup> absorbances and the absorbance in the labeled oligomer at 460 nm ( $\lambda_{max}$ ). The melting temperature ( $T_m$ ) buffer was 0.01 M Na<sub>2</sub>PO<sub>4</sub>, 0.1 mM Na<sub>2</sub>EDTA, and 1 M NaCl at pH 7. On several occasions, samples were run through Sephadex NAP-10 columns after a  $T_m$  run. These columns



**Figure 1.** Structures of the modified cytidine  $(C^*)$  and thymidine  $(T^*)$  bases as units in an oligonucleotide. The site of label attachment is indicated by X (X = H or  $H_2^+$  for a modified, unlabeled oligomer and X = L for an attached label).



Figure 2. UV-visible absorption spectra of the products of the Ru-(bpy)<sub>2</sub>(H<sub>2</sub>O)<sub>2</sub><sup>2+</sup> labeling reaction: (A) unmodified single-strand DNA; (B) unmodified single-strand DNA with nonspecifically bound Ru-(bpy)<sub>2</sub>(H<sub>2</sub>O)<sub>x</sub><sup>2+</sup>, where x = 0 or 1; (C) bipyridine-labeled, T\*-modified DNA; (D) T\*-Ru(bpy)<sub>3</sub><sup>2+</sup> single-strand DNA. Spectra were taken in water and have been normalized to allow presentation on the same vertical scale.

hold up free Ru(bpy)<sub>3</sub><sup>2+</sup> and other salts. Despite the presence of EDTA in the  $T_{\rm m}$  buffer, no leaching of ruthenium was observed since the absorbance spectra of the recovered ruthenium-labeled DNA remained unchanged. Computer fitting of the melting curves to the "all-or-none" model<sup>30</sup> was performed as described previously.<sup>9</sup>

### **Results and Discussion**

The same octanucleotide base sequence used in an earlier study with organic labels was used here for ruthenium labeling; 5'-GCACTCAG-3'. It contained all four heterocyclic bases, testing the generality of the labeling procedures, and internal cytidine and thymidine bases, which could be replaced by their modified analogues. The structures of these modified cytidine (C\*) and thymidine (T\*) bases as units in an oligonucleotide are shown in Figure 1. Duplexes formed with this unmodified oligomer, and its C\*- and T\*-modified oligomers, gave melting temperature ( $T_m$ ) values in a convenient range and the melting curves were fitted by the all-or-none model<sup>30</sup> to give thermodynamic parameters for duplex formation.

**Bipyridine Labeling Chemistry.** The reaction conditions used earlier to prepare fluorescein- and pyrene-labeled oligonucleotides<sup>9</sup> were used here to prepare both 5'-GCA(C\*-bpy)TCAG-3' and 5'-GCAC(T\*-bpy)CAG-3' oligomers. The activated bipyridine derivative used was succinimidyl-4-carboxy-4'-methyl-2,2'-bipyridine. This molecule was easily prepared from 4,4'-dimethyl-2,2'-bipyridine. It reacted preferentially with primary amine of the linker arm on the modified oligonucleotides to yield an amide. The desired bipyridine-labeled oligonucleotides were

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easily separated from the reaction mixture by the reversed-phase liquid chromatographic procedures (see above). The attachment of bipyridine to a modified octanucleotide was indicated by a long-wavelength shoulder on the first DNA band in a UV-visible absorption spectrum. No such evidence was seen for reaction of the bipyridine derivative with the corresponding unmodified octanucleotide.

Ruthenium Labeling Chemistry. The bipyridine-labeled C\* and T\* oligonucleotides were reacted with  $Ru(bpy)_2(H_2O)_2^{2+}$  in neutral phosphate buffer. Unfortunately, the ruthenium complex did not react exclusively with the bipyridine label. Instead, a variety of products were obtained both for the bipyridine-labeled octamers and for the corresponding unmodified ones. There are two likely reasons for this. First, in contrast to a neutral, hydrophobic organic species such as pyrene (fluorescein is water soluble, but anionic),  $Ru(bpy)_2(H_2O)_2^{2+}$  is a water-soluble cationic complex. Since DNA strands are anionic, there can be nonspecific electrostatic attractions between these strands and cationic labels. More importantly though, the heterocyclic bases on DNA have a variety of nitrogen donor atoms, which are much better ligands for ruthenium than is water. Fortunately, the bases are inferior ligands relative to bipyridine.<sup>31</sup> However, covalent binding of  $Ru(bpy)_2(H_2O)_2^{2+}$  to base sites in DNA oligomers is possible. Such covalent attachment to DNA bases is apparently far more important than electrostatic attraction under the reaction conditions used here, since  $Ru(bpy)_3^{2+}$  showed no association with the unmodified octamer. This complex has also been shown to have a negligible interaction with biological DNA duplexes.<sup>14,25</sup> To limit the nonspecific binding of  $Ru(bpy)_2(H_2O)_2^{2+}$  to the octamer, only 1 equiv of ruthenium complex was used in the labeling reaction. Fortunately, most bis(bipyridine) complexes of ruthenium(II) have no easily detectable steady-state emission.<sup>11</sup> Additionally, their absorption spectra are different from those of tris(bipyridine)ruthenium(II) complexes (see Experimental Section). Thus, chromatographic separation combined with optical absorption and emission spectroscopies allowed isolation and characterization of the modified oligonucleotide with the desired ruthenium tris(bipyridyl) complex bound to it.

The ruthenated nonfluorescent fractions most likely contain complexes with the formula  $Ru(bpy)_2(H_2O)_x(L)_{2-x}^{2+}$ , where x = 0 or 1, and L = nitrogen donor group on a heterocyclic base. The nature of this binding is of interest, but is beyond the scope of this work. A number of related studies have been conducted, chiefly by Clarke and co-workers, on the binding of aquopentaamine complexes of ruthenium(II) and -(III) to DNA and to the individual heterocyclic bases.<sup>32-38</sup> These studies established that the primary coordination site on DNA is N-7 of guanine.<sup>33,35-38</sup> This has also been proposed as a binding site for Ru(phen)<sub>2</sub>Cl<sub>2</sub>.<sup>12</sup> Additionally, coordination to N-1 and N-6 of adenine and, to a lesser extent, N-3 of cytosine can occur.33,36-38 These latter binding modes occur more readily in single-strand DNA than in duplexes.<sup>36</sup> The ruthenium-labeling reaction used here involved a single-strand octanucleotide with terminal guanosines and internal cytidines and adenosines. Thus, by analogy with the studies on Ru- $(\rm NH_3)_5(\rm H_2O)^{2+/3+}$  complexes, even an unmodified oligomer has a number of potential binding sites for  $Ru(bpy)_2(H_2O)_2^{2+}$ , which could lead to several products over the lengthy reaction period.

Thermodynamic Characterization. Given that the desired ruthenium label could be attached to C\*- and T\*-modified strands of DNA, it was then possible to examine this label's effect on



Figure 3. Melting curves of octanucleotide duplexes: (A)  $C^*-Ru(bpy)_3^{2+}$ duplex; (B)  $T^*-Ru(bpy)_3^{2+}$  duplex. The high temperature to low temperature experimental curve is shown (plus signs) together with a best nonlinear least-squares fit (solid line) using the all-or-none model. The absorbance was monitored at 260 nm in a 1 cm path length cell, and the temperature was ramped at 10 °C/h.

Table I. Duplex Thermodynamic Parameters<sup>4</sup>

sequence	$\Delta H^{\circ} (\pm 5\%),$ kcal/mol	$\Delta S^{\circ} (\pm 10\%),$ cal/mol K	$\Delta G^{\circ} (\pm 5\%),$ kcal/mol
GCACTGCG <sup>b</sup>	-56	-150	-10.9
$GCA(C^*)TCAG^b$	-56	-160	-9.8
$C^*-Ru(bpy)_3^{2+}$	-41	-110	-8.3
GCATCAG <sup>b</sup>	-42	-110	-8.5
$GCAC(T^*)CAG^b$	-54	-150	-9.9
$T^*-Ru(bpy)_3^{2+}$	-41	-100	-9.3
GCACCAG <sup>b</sup>	-50	-140	-9.9
T*-biotin <sup>b</sup>	-52	-140	-9.2

<sup>a</sup> Parameters at 25 °C. All duplexes were in the  $T_{\rm m}$  buffer: 0.01 M Na<sub>2</sub>PO<sub>4</sub>, 0.1 mM Na<sub>2</sub>EDTA, and 1 M NaCl, at pH 7. <sup>b</sup>Reference 9.

duplex formation in each case. Duplex solutions were prepared of the  $Ru(bpy)_3^{2+}$ -labeled C\*- and T\*-modified oligomers with their common complementary strand, 5'-CTGAGTGC-3', and melting curves were recorded as a function of concentration. Figure 3 shows representative melting curves for the two types of ruthenium-labeled duplexes along with the best nonlinear least-squares fit to the data by the all-or-none model.<sup>30</sup> Both of these melting curves have the same qualitative appearance as that of the corresponding unmodified octameric duplex, with only a small difference between the curves for the two types of label attachment. This result contrasts with an earlier study on the internal labeling of octameric duplexes with organic labels.9 In that work, the duplexes labeled at a T\* site gave melting curves similar in appearance to that of an unmodified duplex, while those labeled at C\* gave multiphasic melting curves that could not be fit to the all-or-none model. Since the C\* linkage is to an amino group involved in Watson-Crick base pairing, label/duplex association could easily perturb duplex formation. The T\* linkage, however, has no connection with base pairing, so duplex formation may not be as easily perturbed as with a C\* linkage. In support of this proposal, a duplex with C\* but lacking an attached label gave "normal" melting curve behavior. The ruthenium complex used here is hydrophilic and spherical. Thus it is not likely to intercalate into the base-pair stack. Indeed the studies of Barton and co-workers<sup>12-19</sup> on the interaction of a number of ruthenium

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polypyridyl complexes with DNA show that while phenanthroline (phen) complexes do intercalate, those with only bipyridine ligands do not. The result is that C\*-modified duplexes labeled with this complex show melting curve behavior that is reasonably close to that predicted by the all-or-none model (see Figure 3A). Not surprisingly Figure 3B shows that the T\*-modified duplex labeled with  $Ru(bpy)_3^{2+}$  has nearly ideal all-or-none melting behavior.

Even though a slightly better fit to the data obtains for the T\* oligomer (Figure 3B) than for C\* (3A), both fits are sufficiently good that the all-or-none model can be used as a means of measuring  $T_{\rm m}$  values. Table I summarizes the thermodynamic parameters for duplex formation that were deterimined from plots of  $1/T_m$  versus in [DNA].<sup>30b</sup> For comparison purposes, the previously determined parameters for a number of other duplexes are also included to allow a quantitative estimate of the effect of ruthenium labeling on duplex stability. The bipyridine-labeled duplexes were not studied, since this label alone was of no intrinsic interest, and several duplexes with attached organic labels have been previously studied.<sup>9</sup> The C\* and T\* base modifications both destabilize the duplex by 1 kcal/mol (as measured by  $\Delta G^{\circ}$ ). However, attachment of  $Ru(bpy)_3^{2+}$  leads to a divergence in relative duplex stability between the two types of label attachments. The T\*-Ru(bpy)<sub>3</sub><sup>2+</sup> duplex is destabilized by 1.6 kcal/mol compared to the unmodified duplex (or 0.6 kcal/mol compared to one with a T\* modification alone). In contrast, the C\*-Ru(bpy) $_3^{2+}$ duplex is destabilized by 2.6 kcal/mol compared to the unmodified duplex (or 1.5 kcal/mol relative to the one with a C\* modification alone). Thus, label attachment at C\* reduced duplex stability much more than did label attachment at T\*.

The  $\Delta G^{\circ}$  value obtained for the T\*-Ru(bpy)<sub>3</sub><sup>2+</sup> duplex is the same as that found earlier for a T\*-biotin one (the analogous C\*-biotin duplex could not be quantitatively studied).<sup>9</sup> Biotin is not expected to interact with DNA and thus provides an estimate of the effect of attaching a relatively "inert" label to a T\*-modified duplex. While the  $\Delta G^{\circ}$  values for biotinylated and ruthenated T\*-modified duplexes are the same, indicating similar degrees of label "inertness", there are large differences in their  $\Delta H^{\circ}$  and  $\Delta S^{\circ}$ values. In particular, the  $\Delta H^{\circ}$  and  $\Delta S^{\circ}$  values for the biotinlabeled duplex are very close to those for the duplex with a T\* modification alone. However, the  $\Delta H^{\circ}$  and  $\Delta S^{\circ}$  values for the ruthenated duplex are substantially less negative (see Table I for values). These results likely reflect the hydrophilic nature of the  $Ru(bpy)_{3}^{+2}$  label. Since this label is well solvated, the initial state (single strands) would be more ordered than the one for the duplex with a T\* modification alone. Similarly, label solvation would also stabilize the single-strand configuration and thus reduce the decrease in enthalpy found for duplex formation relative to the T\*-modified duplex. The  $\Delta H^{\circ}$  and  $\Delta S^{\circ}$  values for the C\*-Ru- $(bpy)_3^{2+}$  duplex are similar to those of the T\* one. Taken together, the above thermodynamic comparisons indicate that the ruthenium label attached to either a C\*- or T\*-modified duplex does not strongly associate with the duplex and is in fact likely to be well solvated in aqueous media. Spectroscopy data given below also support this model.

The destabilizing effect of attaching  $\operatorname{Ru}(\operatorname{bpy})_3^{2+}$  to a duplex can also be compared to the effect of removing the base pair involved in the labeling and thus producing a heptameric duplex. Removing the T\*/A pair increases  $\Delta G^\circ$  by only 1 kcal/mol relative to the unmodified duplex. In this case the nearest-neighbor interactions in the resulting heptameric duplex are more favorable (CC/GG neighbors are created) than in the original octamer,<sup>39</sup> so that some of the stability loss due to removing the T\*/A pair is masked. Removing the C\*/G pair increases  $\Delta G^\circ$  by much more, 2.4 kcal/mol. In this case the nearest-neighbor effects are roughly constant. Thus, attaching Ru(bpy)<sub>3</sub><sup>2+</sup> to a C\*-modified duplex produces nearly the same  $\Delta G^\circ$  increase as removing the C\*/G pair, 2.6 versus 2.4 kcal/mol, both relative to the unmodified duplex. Attaching this same label to a T\*-modified duplex increases  $\Delta G^\circ$  by 1.6 kcal/mol relative to the unmodified duplex.

Table II. Spectroscopic Data<sup>a</sup>

sample	absorptn λ <sub>max</sub> , nm	emissn λ <sub>max</sub> , <sup>b</sup> nm	excitatn spectrum λ <sub>max</sub> , <sup>c</sup> nm	quantum yield <sup>d</sup> (±10%)
C*-Ru(bpy) <sub>3</sub> <sup>2+</sup> duplex	460	660	470	0.013
T*-Ru(bpy) <sub>3</sub> <sup>2+</sup> duplex	460	660	470	0.012
$Ru(bpy)_3^{2+}$	454	620	455	0.012

<sup>a</sup> All samples in air-saturated  $T_{\rm m}$  buffer at 20 °C. <sup>b</sup> Excitation at 450 nm. <sup>c</sup> Emission monitored at 620 nm. <sup>d</sup> Using fluorescein as a standard (0.1 M NaOH, QY = 0.92<sup>29</sup>); excitation = 450 nm, emission = 500-800 nm.

Thus, if the extra CC/GG stabilization in the corresponding heptamer is accounted for in the case of T\* removal, the amount of destabilization for Ru(bpy)<sub>3</sub> labeling at either a C\* or T\* site is comparable to that produced by the loss of the base pair involved in the labeling. However, the destabilizing effect of Ru(bpy)<sub>3</sub><sup>2+</sup> labeling is far less than that of a base-pair mismatch: duplex destabilization of roughly 4 kcal/mol was reported for conversion of an A/T base pair to A/A or T/T pairs.<sup>40</sup>

**Spectroscopic Characterization.** Spectroscopic data for the two ruthenium-labeled duplexes are summarized in Table II. A representative absorption spectrum is shown in Figure 2D. Both the absorption and emission bands are red-shifted in the Ru- $(bpy)_3^{2+}$ -labeled oligomer relative to the symmetrically substituted Ru $(bpy)_3^{2+}$  complex. The shift is from 454 to 460 nm in absorption and from 620 to 650 nm in emission. The unsymmetrical ruthenium complex bound to DNA contains two 2,2'-bipyridine ligands and a third 2,2'-bipyridine with 4'-methyl and 4-carbamide groups. Similar spectroscopic shifts have been observed for related ruthenium(II) complexes containing other substituted polypyridyl ligands. For example, a red shift of 7 nm in absorption and of 14 nm in emission is seen in dichloromethane for tris(4,4'-dimethyl-2,2'-bipyridine)ruthenium(II) relative to Ru(bpy)\_3^{2+.41}

Emission quantum yields were calculated for the rutheniumlabeled duplexes as well as for  $\text{Ru}(\text{bpy})_3^{2+}$ . The values reported here are lower than those commonly reported,<sup>11</sup> because these samples were aerated and held in the 1 M NaCl  $T_m$  buffer. All of the quantum yield values were similar, indicating that ruthenium label emission was neither quenched nor enhanced as a result of either C\* or T\* modes of duplex attachment. The previously reported lack of association between free  $\text{Ru}(\text{bpy})_3^{2+}$  and natural, duplex DNA<sup>14</sup> is consistent with our results; in contrast, enhanced emission for phenanthroline-based ruthenium(II) complexes was observed due to their association with added DNA.<sup>14,15</sup> Finally, no differences were seen in absorption and emission spectra or in quantum yields between the two types of label attachment sites nor between single-strand and duplex forms of ruthenium-labeled DNA.

These spectroscopic results for duplexes with a covalently bound  $\operatorname{Ru}(\operatorname{bpy})_3^{2+}$  label differ from those for pyrene labels. Emission from a pyrenebutyrate label, for example, was extensively quenched ( $\geq 500$  times) when it was attached to a DNA duplex.<sup>9</sup> There are two important differences between the pyrenebutyrate and  $\operatorname{Ru}(\operatorname{bpy})_3^{2+}$  labels. First, the pyrene label showed 1.5 kcal/mol increased duplex stabilization ( $\Delta G^\circ$  decrease), while the Ru-(bpy)\_3^{2+} label decreased duplex stabilization by 0.6 kcal/mol ( $\Delta G^\circ$  increase), both for T\* base attachment. Thus the pyrenebutyrate label was strongly associated with (perhaps intercalated into) the DNA duplex, while the Ru(bpy)\_3^{2+} label for oxidative quenching of the pyrene label's excited singlet state is in the range of -1.8 to -2.2 eV, <sup>9,42</sup> while the free energy available for oxidative quenching of the ruthenium label's excited state is about -0.8 eV.<sup>43</sup> A ruthenium polypyridyl

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label, therefore, has significantly less driving force to photoreduce a DNA base than does a pyrene label. Consequently, the combination of lowered excited state reducing power and lessened label/duplex association for Ru(bpy)3<sup>+2</sup> complexes makes them much more suitable as "innocent" labels for DNA duplex attachment than are planar aromatic labels such as pyrene.

Concluding Remarks. The above data demonstrate that for short strands of ruthenium tris(bipyridyl) labeled DNA neither the label's spectroscopic properties nor the duplex's hybridization stability are appreciably impaired. Thus, DNA-based supramolecules related to the ones described here could specifically locate molecular subunits to accomplish particular photochemical and photophysical tasks. To facilitate such research the synthesis

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of this type of labeled oligomer should be improved. This could be accomplished in several ways. A derivative of tris(2,2'-bipyridine)ruthenium(II) could be prepared that reacts specifically with a functional group of a modified base in an oligonucleotide. This approach contrasts with the semispecific reaction of diaquobis(2,2'-bipyridine)ruthenium(II) used here. Another possibility would be to prepare a ruthenium-labeled nucleoside and convert it to the appropriate reagent for automated oligonucleotide synthesis. Finally, ruthenium and other transition-metal complexes could also be attached to DNA oligomers at an internucleotide phosphate rather than at a modified base.

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# DNA Duplexes Covalently Labeled at Two Sites: Synthesis and Characterization by Steady-State and Time-Resolved **Optical Spectroscopies**

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Abstract: A series of oligonucleotides having the base sequence 5'-GCAC(T\*-L)CAG-3' and its complement, 5'-CTGAG-(T\*-L)GC-3', were synthesized where T\* is a chemically modified thymidine base with a covalently attached linker arm terminating in a primary amine and L is a molecular label: pyrenebutyrate, pyrenesulfonate, or anthraquinone. Melting temperature studies on duplexes of these labeled oligonucleotides established their thermodynamic parameters for duplex formation and suggested strong label/duplex association (perhaps intercalation) for anthraquinone and pyrenebutyrate. Thus, label/label interactions were absent in the duplex with two pyrenebutyrate labels as well as in the one with both pyrenebutyrate and anthraquinone. In contrast, the duplex doubly labeled with pyrenesulfonate showed pronounced label/label quenching. In this case, emission lifetimes were significantly shortened relative to the duplex with a single pyrenesulfonate label. The above results are discussed in terms of the design requirements for constructing duplexes in which label/label interactions dominate label/duplex association.

Duplex formation between oligonucleotides with complementary base sequences is one of the salient features of DNA chemistry. The sequence-specific nature of this interaction for short oligomers allows confidence in the chemical identity and structure of the duplex formed. It is possible to take advantage of this hybridization specificity by using DNA as a template or scaffold to bring together molecular labels covalently attached to individual, complementary strands of DNA. DNA does not naturally contain functional groups to which molecules of interest (or labels) can be covalently attached. However, a number of workers have developed methods for synthesizing chemically modified nucleosides and nucleotides that allow subsequent labeling reactions to proceed. These modifications can be to the heterocyclic bases<sup>1,2</sup> or to phosphorus.<sup>3,4</sup> Additionally, the properties of single labels covalently attached to oligonucleotide single strands and duplexes have been studied by a number of research groups using optical<sup>5-11</sup> and magnetic resonance spectroscopic techniques,<sup>7</sup> as well as thermodynamic analysis based on melting curves.<sup>8,9</sup> However, less attention has been paid to DNA duplexes in which there is more than one label, and in these studies the labels were attached to the 3' or 5' oligonucleotide termini. Several types of such systems have been studied. The first was comprised of a single

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