 Nucleic Acids Research, 1999, Vol. 27, No. 12 **2487–2493**

A 2,2'-bipyridine ligand for incorporation into oligodeoxynucleotides: synthesis, stability and fluorescence properties of ruthenium–DNA complexes

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Received March 4, 1999; Revised and Accepted April 27, 1999

ABSTRACT

A non-nucleoside linker based upon the ligand 2,2'-bipyridine and ethylene glycol is prepared and placed into the backbone of a number of oligonucleotides. The bipyridine ligand is reacted with *cis*-dichloro bis(2,2'-bipyridyl) Ru(II) to generate the relatively substitutionally inert complex based upon the well-characterized *tris*-2,2'-bipyridyl Ru(II). The ruthenium-containing DNA complexes exhibited UV and fluorescence characteristics that are consistent with those previously observed for simple *tris*-2,2'-bipyridyl Ru(II) complexes. Oligonucleotides containing the ruthenium complex will form both DNA duplexes and triplexes with stabilities that are slightly better than those formed from simple tethered oligonucleotide probes in which the two hybridizing sequences are tethered by simple tri(ethylene glycol) or hexa(ethylene glycol) linkers.

INTRODUCTION

The incorporation of transition metal complexes into oligonucleotides has been a topic of recent interest. Such conjugates have been employed for the study of electron transfer processes, most notably those in which electrons are transferred through the DNA double helix (1–14). Cleavage processes for both DNA and RNA can occur as the result of the action of transition metals. In one approach various metal–ligand complexes are tethered to an oligonucleotide probe sequence (15–18) and the probe targets a complementary sequence. In the complex, the metal facilitates cleavage by hydrolytic or free radical chemistry.

Oligonucleotides containing ligands for the formation of metal–DNA complexes have typically been prepared by covalent attachment of a ligand to either terminus of the probe sequence (19–21) or by tethering the ligand to a base residue. In the former case, the ligand, usually containing a small linker, can be converted to a phosphoramidite for the 5'-terminal coupling. It is also possible to introduce a suitable linker into the DNA sequence during assembly, and then subsequently post-synthetic conjugation can be employed to introduce the metal center. In the latter case, a base residue, usually T, is functionalized with a linker that tethers the metal-binding ligand (16,18,22,23). Base modifications of this type permit the incorporation of the metal–ligand complex at internal sites within the probe sequence.

Regardless of the site of ligand attachment in the oligonucleotide, the metal ion can be introduced by one of two general techniques. The metal can be complexed to the tethered ligand by a post-synthetic procedure (15,16,18,19,23). In this procedure it is necessary to monitor non-specific binding by the metal, and when present, to employ a scavenging ligand to remove excess metal from the complex. Alternatively, the metal can be complexed to the desired ligand at the monomer stage and then the nucleotide/ligand/metal building block incorporated into the DNA sequence during the assembly process (22,24–27). In the latter case, the metal–ligand complex must survive the conditions of DNA assembly, deprotection and isolation.

Here we describe the preparation of a bipyridine linker that can be incorporated into the backbone of oligonucleotides to provide a site for the formation of metal complexes. We report on the synthesis of the linker and the preparation of a metal complex, as well as the stability of DNA duplexes and triplexes containing this complex, and their fluorescent properties.

MATERIALS AND METHODS

Materials

Thin layer chromatography (TLC) was performed using either aluminum-backed precoated silica gel 60 F254 plates purchased from EM Science (Gibbstown, NJ) or Baker-flex aluminum oxide IB-F plates from J. T. Baker (Phillipsburg, NJ). Preparative chromatography was performed using aluminum oxide, activated, neutral, Barokmann I, standard grade from the Aldrich Chemical Co. (Milwaukee, WI) and Alumina G-F preparative plates from Analtech (Newark, DE). ^{1}H-NMR were performed at 300 or 400 MHz on varian multiprobe NMR spectrometers. The small molecule mass spectrometry was performed by the Mass Spectrometry Laboratory at the University of Illinois, Champaign-Urbana, IL and mass spectrometry of the DNA was performed by Dr Guobing Xiang, Sequenome Inc., San Diego, CA. Ruthenium (III) chloride, *cis*-dichloro bis(2,2'-bipyridine) ruthenium (II) dihydrate and ammonium hexachloro ruthenate (IV) were purchased from Strem Chemicals (Newburyport, MA).

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Methods

*4,4'-dimethyl-2,2'-bipyridine (**1**).* This compound was prepared essentially as described elsewhere (28). To 50 ml of 4-picoline freshly distilled from potassium hydroxide and calcium hydride was added 2.23 g of 5% Pd/C and the suspension was refluxed. After 4 days, 25 ml of benzene was added and the mixture was refluxed for an additional 30 min. While still hot, the solution was filtered through celite using a glass frit and washed with benzene. The filtrate was cooled and reduced in volume by ~50% and the white solid, 1.9 g (10.5 mmol, 11% yield) was collected by filtration, washed with petroleum ether and dried. R_f (alumina in ethyl acetate: dichloromethane 5: 95) = 0.65. ^{1}H-NMR ($CDCl_3$): δ = 8.45 (d, 2H), 8.20 (s, 2H), 7.10 (d, 2H), 2.41 (s, 6H). ^{13}C-NMR ($CDCl_3$): δ = 156.0, 148.9, 148.2, 124.7, 122.0, 21.2. HRMS: calculated for $C_{12}H_{12}N_2$ (M^+): 184.1000, found: 184.0995.

*2,2'-bipyridine-4,4'-dicarboxylic acid (**2**).* This compound was prepared by a procedure similar to that described elsewhere (29). To a cooled solution (<5°C) of 5 g (27.1 mmol) of 4,4'-dimethyl-2,2'-bipyridine in 250 ml of 25% sulfuric acid was added 10 g of potassium permanganate. After stirring for 30 min the solution was allowed to warm to ambient temperature. A second 10 g portion of potassium permanganate was added after the solution was chilled on ice. The reaction was refluxed overnight during which time the color changed from a dark purple to a milky white. The reaction was cooled and filtered through a glass frit and the light brown product was collected. Yield: 3.4 g (14.0 mmol, 52% yield). This compound exhibited poor solubility characteristics, but TLC analysis indicated that it was of sufficient purity to be used in the following step and mass spectral analysis agreed with the expected product. R_f (alumina in ethyl acetate:dichloromethane 3:97) = 0.45; HRMS: calculated for $C_{12}H_8N_2O_4$ (M^+): 244.0487, found: 244.0491.

*4,4'-dimethoxycarbonyl-2,2'-bipyridine (**3**).* To 3.42 g (14.0 mmol) of 4,4'-dicarboxy-2,2'-bipyridine in 50 ml of methanol was added 7 ml of concentrated sulfuric acid. After refluxing overnight the solution was poured onto 150 ml of water forming a white slurry. The pH of the slurry was adjusted to 8 with 25% (w/v) sodium hydroxide. The product was then extracted with dichloromethane, dried over magnesium sulfate and evaporated to yield 3.24 g (11.9 mmol, 85%) of product. The white solid was recrystallized from toluene to give a white crystalline product. mp = 210–211°C. R_f (alumina in ethyl acetate:dichloromethane 3:97) = 0.7. ^{1}H-NMR ($CDCl_3$): δ = 8.90 (s, 2H), 8.80 (d, 2H), 7.90 (d, 2H), 4.90 (s, 6H). ^{13}C-NMR ($CDCl_3$): δ = 162.5, 156.5, 150.1, 138.6, 129.2, 120.6, 52.8. HRMS: calculated for $C_{14}H_{12}N_2O_4$ (M^+): 272.0797, found: 272.0799.

*4,4'-Bis(hydroxymethyl)-2,2'-bipyridine (**4**).* Prepared by a procedure similar to that described elsewhere (30). To 3.24 g (13.3 mmol) of 4,4'-dimethoxycarbonyl-2,2'-bipyridine dissolved in 240 ml of anhydrous methanol was added 9.72 g of sodium borohydride. The reaction refluxed for 3 h and then was quenched by adding a small amount of saturated ammonium chloride solution. The reaction mixture was evaporated to dryness and enough water was added to dissolve the residue. The product was extracted with ethyl acetate, dried over magnesium sulfate, and evaporated to dryness. Yield: 2.16 g (7.96 mmol, 60%). R_f (alumina in methanol:dichloromethane 3:97) = 0.43. ^{1}H-NMR (DMSO-d_6) δ = 8.60 (d, 2H), 8.40 (s, 2H), 7.40 (d, 2H), 5.20 (t, 2H), 4.60 (d, 4H). ^{13}C-NMR (DMSO-d_6) δ = 156.7, 154.3, 150.5, 122.9, 119.4, 63.2. HRMS: calculated for $C_{12}H_{12}N_2O_4$ (M^+): 215.0821, found: 215.0821.

2[-2-(2-Triphenylmethoxyethoxy)ethoxy]ethanol. To a solution of 7.9 g (52.6 mmol) tri(ethyleneglycol) in anhydrous pyridine was added 4.36 g (15.6 mmol) of triphenyl methyl chloride with a catalytic amount of dimethylamino pyridine. The reaction was allowed to warm to room temperature and stir overnight. The pyridine was evaporated and the mixture was suspended in dichloromethane, extracted three times with saturated sodium bicarbonate, and dried with magnesium sulfate. The product was purified on silica gel and eluted with 1:1 ethyl acetate: petroleum ether. Yield: 18.56 g (47.34 mmol, 90%). R_f (silica gel in ethyl acetate: petroleum ether 1:1) = 0.27. ^{1}H-NMR ($CDCl_3$) 7.50–7.15 (m, 15H), 3.60–3.70 (m, 8H), 3.59 (t, 2H), 3.24 (t, 2H). HRMS: calculated for $C_{25}H_{28}O_4$ (M^+): 392.1988, found: 392.1989.

*2-(2-Triphenylmethoxyethoxy)ethoxy-1-bromo-ethane (**5**).* Carbon tetrabromide (0.829 g, 2.5 mmol) and 1 g (2.5 mmol) of 2-[2-(2-Triphenylmethoxyethoxy)ethoxy]ethanol were stirred together in dichloromethane with ice cooling. A solution of triphenylphosphine (0.667 g, 2.5 mmol) in dichloromethane was added dropwise over 30 min. After stirring for 2 h the solution was evaporated to dryness and resuspended in diethyl ether. The triphenylphosphine oxide (white solid) was removed by filtration and the product was purified further on silica gel using dichloromethane. Yield: 0.70 g (1.55 mmol, 62%). R_f (silica gel in dichloromethane) = 0.49. ^{1}H-NMR ($CDCl_3$) 7.50 –7.15 (m, 15H), 3.85 (t, 2H), 3.65 (m, 6H), 3.42 (t, 2H), 3.22 (t, 2H). HRMS: calculated for $C_{25}H_{27}BrO_3$ (M^+): 454.1144, found: 454.1147.

*4,4'-Bis{2-[2-(2-triphenylmethoxyethoxy)ethoxy]ethoxymethyl}-2,2'-bipyridine (**6**).* To 0.3 g (1.39 mmol) of 4,4'-bis(hydroxymethyl)-2,2'-bipyridine dissolved in anhydrous tetrahydrofuran (~100 ml) was added 0.105 g (4.4 mmol) of sodium hydride. After refluxing the reaction mixture for 3 h under nitrogen 1.99 g (4.37 mmol) of **5** was added. The reaction was refluxed overnight and TLC analysis indicated the presence of some starting material and some mono substituted product. Another 0.105 g (4.4 mmol) of sodium hydride was added and the reaction was refluxed overnight after which TLC analysis showed the absence of starting material. The reaction was quenched with methanol and evaporated to dryness. The residue was resuspended in dichloromethane washed three times with saturated ammonium bicarbonate, and dried over magnesium sulfate. The reaction was purified on alumina in dichloromethane with a gradient of ethyl acetate. Yield: 0.69 g (0.714 mmol, 50%). R_f (alumina in ethyl acetate:dichloromethane 9:1) = 0.31. ^{1}H-NMR ($CDCl_3$) δ = 8.60 (d, 2H), 8.30 (s, 2H), 7.20–7.50 (m, 32H), 4.70 (s, 4H), 3.7–3.8 (m, 20H), 3.20 (m, 4H). ^{13}C-NMR ($CDCl_3$) δ = 156.0, 149.3, 148.5, 144.1, 128.7, 127.9, 127.7, 126.9, 121.9, 119.3, 71.7, 70.8, 70.7, 70.4, 70.1, 63.3. HRMS: calculated for $C_{62}H_{64}N_2O_8$ (M^+): 965.4741, found: 965.4740.

*4,4'-Bis{2-[2-(2-hydroxyethoxy)ethoxy]ethoxymethyl}-2,2'-bipyridine (**7**).* To 4.5 g (4.66 mmol) of **6** in 50 ml of dichloromethane was added 3.8 ml (5.3 g, 46.6 mmol) of trifluoroacetic

acid. The solution was stirred at room temperature for 1 h and then washed with saturated ammonium bicarbonate. The mixture was purified on alumina using a gradient of methanol in dichloromethane. Yield: 1.35 g (2.81 mmol, 60%). R_f (alumina in methanol:dichloromethane 2:98) = 0.28. ^{1}H-NMR (400 MHz CDCl3) δ = 8.6 (d, 2H), 8.3 (s, 2H), 7.4 (D,2H), 4.65 (s,4H), 3.7–3.8 (m, 12H). ^{13}C-NMR (400 MHz $CDCl_3$) δ = 155.9, 149.3, 148.7, 122.0, 119.5, 72.6, 71.7, 70.7, 70.6, 70.3, 70.1, 61.6. LRMS: 481.3 (M^+).

*4-‹2-{2-[2-(4,4'-dimethoxytriphenylmethoxy)ethoxy]ethoxy}-ethoxymethyl›-4'-‹2-[2-(2-hydroxyethoxy)ethoxy]ethoxymethyl›-2,2'-bipyridine (**8**).* To 0.57 g (1.19 mmol) of **7** in anhydrous pyridine dimethoxytrityl chloride (0.45 g, 1.10 mmol) was added with a catalytic amount of dimethylamino pyridine. The reaction was allowed to stir overnight at room temperature and then quenched with methanol. The mixture was washed with saturated sodium bicarbonate and purified on alumina with a methanol gradient in dichloromethane. Yield: 0.214g (0.271 mmol, 23%). R_f (alumina in methanol:dichloromethane 2:98) = 0.59. ^{1}H-NMR (400 MHz $CDCl_3$) δ = 8.6 (d, 2H), 8.25 (s, 2H), 7.45–6.9 (m, 15H), 4.65 (d, 4H), 3.2–3.6 (m, 16H), 3.2(t, 2H). HRMS: calculated for $C_{45}H_{54}N_2O_{10}$ (M^+): 783.3857, found: 783.3856.

*4-‹2-{2-[2-(4,4'-dimethoxytriphenylmethoxy)ethoxy]ethoxy}-ethoxymethyl›-4'-(2-‹2-{2-O-[(2-cyanoethoxy)-N,N-diisopropylaminophosphino]ethoxy}ethoxy›-ethoxymethyl)–2,2'-bipyridine (**9**).* To 0.210 g (0.268 mmol) of **8** in 2 ml of anhydrous dichloromethane 102 µl (0.097 g, 0.321 mmol) of 2-cyanoethyl tetraisopropylphosphoramidite (1.2 equivalents) was added with a catalytic amount of tetrazole. The reaction was stirred for 30 min after which time the TLC showed complete disappearance of starting material. The reaction was stopped with methanol and the product was purified on an alumina prep plate using dichloromethane and a trace of methanol and resulted in a colorless oil. Yield: 0.223 g (0.227 mmol, 85%). R_f (alumina in methanol:dichloromethane 1:99) = 0.83. ^{31}P-NMR ($CDCl_3$) δ = 149.0.

Oligonucleotide synthesis. DNA sequences containing bipyridine ligand were synthesized using standard phosphoramidite protocols. The bipyridine linker was dissolved in anhydrous acetonitrile. Coupling efficiencies for the bipyridine linker were equivalent to those of the common nucleoside phosphoramidites. Intermediate I_2/H_2O/THF oxidation conditions were employed. Fully protected oligonucleotides containing a terminal DMT group were deprotected overnight in concentrated ammonium hydroxide at 55°C. The sequences were isolated and purified using a 9.4 × 250 mm column of C-8 Hypersil with a flow rate of 3 ml/min in 50 mM triethyl ammonium acetate (pH 7.0) and an acetonitrile gradient (0–60% B 45 min). The terminal DMT was removed by reaction in 80% acetic acid for 30 min at 0°C. After removal of the acid by rotary evaporation, the oligonucleotides were desalted using a column of Sephadex G-10 and lyophilized to dryness. MALDI-TOF analyses were performed in selected cases: 5'-CGCACCCAT-bipy-CTCTCC, calculated: 4952.5, found: 4951.5; 5'-TTTTTTTT-bipy-TTTTTTTT, calculated: 5348.9, found: 5347.7.

DNA metal conjugates. DNA ruthenium conjugates were prepared by refluxing 1 A_{260} unit of oligonucleotide (or in some cases as much as 5 A_{260} units) containing a bipyridine linker in 600 µl of 100 mM NaH_2PO_4 (pH = 7.0), and 400 µl of degassed ethanol for 3–4 h with 1.1 equivalents of *cis*-dichlorobis(2,2'-bipyridine) ruthenium (II) dihydrate. After this period of time, ~1 ml of pyridine was added and the reaction was refluxed for another 3–4 h to remove any non-specifically bound ruthenium (i.e., from the N^7 of guanine). The solutions containing the DNA metal conjugates were then evaporated to dryness, resuspended in 1 ml of water and purified by using a disposable desalting column containing Sephadex G-25 to remove any unincorporated metal. Quantitative incorporation of the metal was confirmed by gel-shift assays and by UV-Vis spectroscopy (λ_{max} = 260, 450 nm) and fluorescence spectroscopy (λ_{ex} = 456 nm, λ_{em} = 590 nm). Complexes were analyzed by denaturing PAGE and FPLC using a mono-Q column. Buffer A = 10 mM KH_2PO_4/30% CH_3CN and buffer B = 10 mM KH_2PO_4/30% CH_3CN 1 M KCl. Gradient = 0–100% B over 60 min.

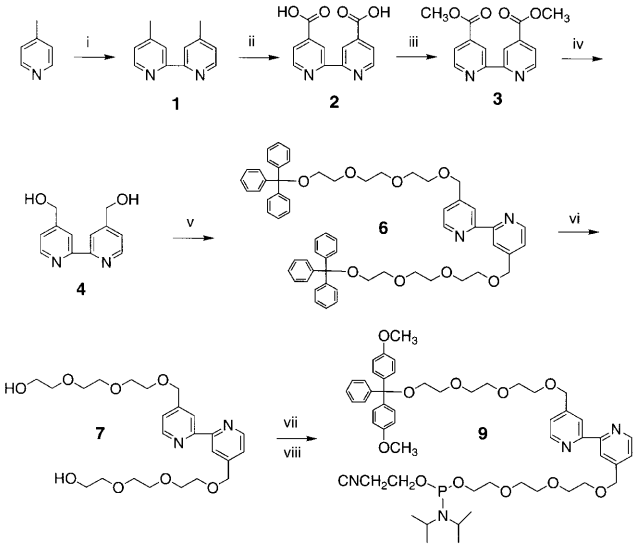
T_m values. T_m values were obtained by forming the DNA duplex by heating and cooling a 1:1 mixture of complementary strands typically at 1 µM (concentrations were determined by absorbance values at 260 nm—the biypyridine had a minimal contribution to the extinction coefficient at this wavelength). For the DNA triplexes the duplex was formed in the same manner and then 1 µM of the third strand was added and the complex was allowed to equilibrate at ambient temperature overnight. The buffer conditions used were either 50 mM HEPES (pH = 7.5) or 50 mM PIPES (pH = 6.4) containing 10 mM $MgCl_2$, and 100 mM NaCl. Measurements were made by heating the samples from 0 to 95°C in 1° increments using an AVIV 14DS spectrophotometer (AVIV Associates, Lakewood, NJ). After temperature stabilization absorbance readings were taken. Absorbance versus temperature plots were made using Igor Pro software (AVIV Associates) and the T_m was determined by first derivative analyses.

RESULTS AND DISCUSSION

We chose to base a metal-complexing linker on the 2,2'-bipyridyl heterocycle since its coordination chemistry is well characterized (31). Bipyridine seemed to offer a good solution for a general ligand since it is known to coordinate a variety of metals including copper, zinc, platinum, ruthenium, osmium, etc. However, to provide some flexibility to the metal-binding site, it was necessary to introduce appropriate linkers such that the ligand could be incorporated into the backbone of the oligonucleotide between two phosphodiester residues. We chose glycol linkers for this purpose, they are hydrophillic in nature and have been used in a variety of studies (31–34) to link two sequences of DNA or RNA, as exemplified in tethered oligonucleotide probes (35,36), in which two hybridizing probes are tethered by a glycol linker.

Synthesis

Reductive coupling of 4-picoline (28) generated the 4,4'-dimethyl-2,2'-bipyridine (**1**) which could subsequently be oxidized (29) to the corresponding diacid (**2**) and converted to the diester (**3**) (Scheme 1). Finally, reduction (30) of the diester (**3**) yielded the dialcohol (**4**). Preparation of the hydrophillic linker involved protection of tri(ethylene glycol) as the mono triphenylmethyl ether and its conversion to the corresponding



Scheme 1. i, 5% Pd/C, reflux 5 days; ii, $KMnO_4$/25% H_2SO_4, reflux 12 h; iii, H_2SO_4/methanol, reflux 24 h; iv, $NaBH_4$/ethanol, reflux; v, NaH/THF/$Ph_3C(OCH_2CH_2)_3Br$ (**5**); vi, trifluoroacetic acid/dichlromethane; vii, DMT-Cl/pyridine; viii, tetraisopropyldiamino-β-cyanoethylphosphine/0.5 eq. tetrazole.

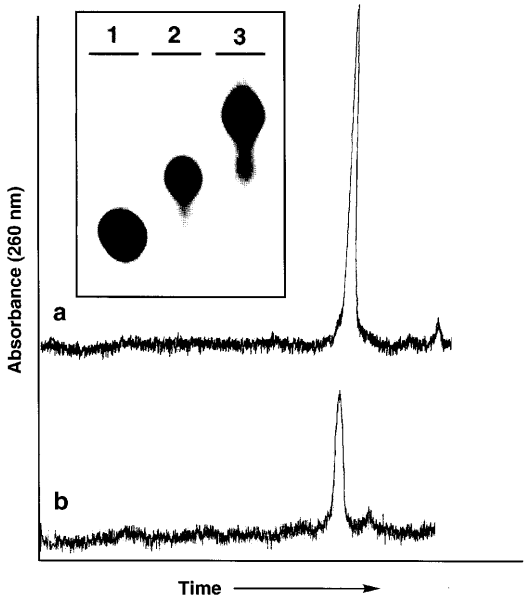


Figure 1. FPLC analysis of (**a**) TTTTT-bipy-TTTTT or (**b**) TTTTT-bipy[$bipy_2$Ru(II)]-TTTTT using a mono-Q column. Buffer A = 10 mM KH_2PO_4/30% CH_3CN and buffer B = 10 mM KH_2PO_4/30% CH_3CN 1 M KCl. Gradient + 0–100% B over 60 min. Inset: PAGE analysis (20% acrylamide) of T_{10} (lane 1), TTTTT-bipy-TTTTT (lane 2) and TTTTT-bipy[$bipy_2$Ru(II)]-TTTTT after reaction of TTTTT-bipy-TTTTT with *cis*-dichloro bis(2,2'-bipyridyl) Ru(II) (lane 3).

bromide derivative (**5**) (the corresponding DMT protecting group proved to be unstable). In the presence of sodium hydride, ether formation resulted and a bipyridine chelator was obtained with two triethylene glycol linkers (**6**). The triphenyl methyl groups were then removed and the linker was converted to the dimethoxytrityl protected phosphoramidite, suitable for DNA synthesis.

DNA synthesis using the bipyridine linker proceeded without difficulty. HPLC on a reversed-phase column was used for the isolation of the 2,2'-bipyridine containing sequences. After removal of the DMT group and desalting of the oligonucleotides, they eluted as single peaks using an FPLC assay (mono-Q column) (Fig. 1a). The linker-containing sequences, such as 5'-TTTTT-bipy-TTTTT were retarded (e.g., relative to T_{10}, Fig. 1, inset) when analyzed by PAGE and compared with the native DNA sequence lacking the bipyridine linker.

Although bipyridine ligands can be used to bind a variety of metals, we have initially examined complexes formed from the DNA bound linker and *cis*-dichloro bis(2,2'-bipyridyl) Ru(II) to generate the relatively substitutionally inert *tris*-2,2'-bipyridyl Ru(II) complex. These complexes could be formed by simply refluxing the oligonucleotide containing the bipyridine linker with *cis*-dichloro bis(2,2'-bipyridyl) Ru(III) in aqueous ethanol. A subsequent treatment with pyridine was employed to scavenge non-specifically bound ruthenium (e.g., to the N^7 of guanine). It was difficult to monitor the reaction with *cis*-dichloro bis(2,2'-bipyridine) Ru (II), or to purify the product by either reversed-phase or ion-exchange (mono-Q) chromatography (compare Fig. 1a with b). In both cases the oligonucleotide starting material, that containing the bipyridine ligand, and the product *tris*-2,2'-bipyridyl Ru(II) complex, essentially co-eluted from both types of columns, as illustrated for the mono-Q column and the sequences 5'-TTTTT-bipy-TTTTT and 5'-TTTTT-Ru$(bipy)_3$-TTTTT (Fig. 1a and b). However, PAGE analysis could resolve both sequences (Fig. 1, inset). Analysis of the crude reaction (Fig. 1, inset) indicated that metal complex formation proceeded with near quantitative yields. It should in principle also be possible to perform the conjugation reaction with the linker **7** and then convert this derivative to the corresponding phosphoramidite for use in DNA synthesis similar to other described procedures (22). However, the post-synthetic conjugation approach described here permits the use of a variety of conjugating metal complexes for each sequence prepared.

UV and fluorescence characteristics

In all cases, the purified conjugates had UV spectra that were characteristic of DNA (λ_{max} = 260 nm) and had additionally absorbance characteristics of a *tris*-2,2'-bipyridyl Ru(II) complex (λ_{max} = 450 and 286 nm). The linker by itself is not significantly fluorescent, but when reacted with *cis*-dichloro bis(2,2'-bipyridyl) Ru(II) a brightly fluorescent complex results with an emission maximum of 590 nm (Fig. 2a). Similar properties are conferred upon oligonucleotides containing the bipyridine linker and complexed with ruthenium (e.g., Fig. 2b). The emission maximum for all the complexes studied remained at 590 nm regardless of whether the complex was the linker alone, a homo- or hetero-oligomer, or the corresponding double-stranded or triple-stranded complex. No significant change in quantum yield was detected as the single-stranded Ru-containing sequence was complex with either a complementary single-stranded or double-stranded sequence (generating the corresponding duplex and triplex, respectively), suggesting that the *tris*-2,2'-bipyridyl Ru(II) complex does not interact with single-stranded, double-stranded or triple-stranded DNA, but rather functions as an effective reporter group with a bright spectroscopic signal.

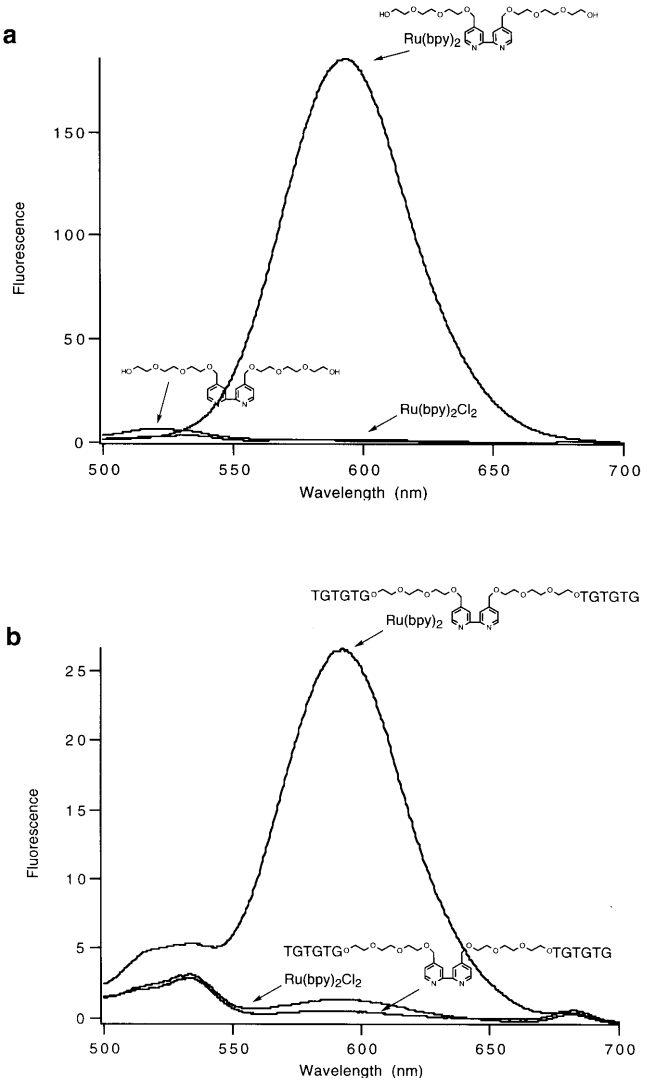


Figure 2. Fluorescence spectra of (**a**) **7** and its complex with ruthenium and (**b**) a corresponding oligonucleotide tethering **7** and its complex with ruthenium.

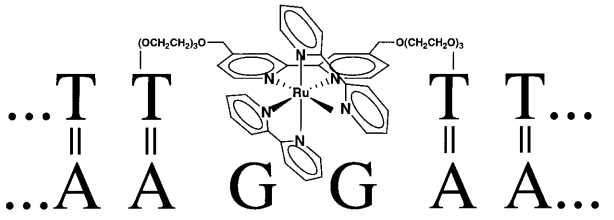


Figure 3. Illustration of a DNA duplex containing a tethered *tris*-(2,2'-bipyridine) Ru(II) complex formed from a tethered 2,2'-bipyridine ligand and *cis*-dichloro bis(2,2'-bipyridine) Ru(II). The internal attachment of the conjugate is to the 3' and 5' hydroxyls of the sugar residues of the relevant thymidine residues.

Table 1. DNA-2,2'-bipyridyl oligonucleotide conjugates*

Sequence	T_m (°C)
5'-CGCACCCATCTCTCTCC-3' 3'-GCGTGGGTAGAGAGAGG-5'	68
5'-CGCACCCAT-bipy-CTCTCC-3' 3'-GCGTGGGTA -GA- GAGAGG-5'	55
5'-CGCACCCAT-bipy-CTCTCC-3' 3'-GCGTGGGTA -CC- GAGAGG-5'	55
5'-CGCACCCAT-bipy-CTCTCC-3' 3'-GCGTGGGTA -CCC- GAGAGG-5'	48

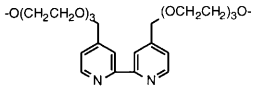
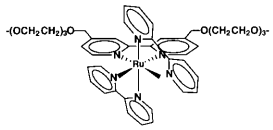
*T_m values were obtained in 50 mM buffer, 10 mM $MgCl_2$, 100 Mm NaCl at strand concentrations of 1 μM. Each value is the average of at least two determinations.

DNA duplexes and triplexes

We have examined the thermal denaturation and fluorescence characteristics of the ruthenium conjugates when present in either DNA duplexes or triplexes. Model building studies indicated that the bipyridyl linker would span the distance of at least two nucleoside residues, so in the prepared duplex and triplex complexes the conjugate strand was initially designed to hybridize to adjacent sequences with a two residue gap in the complex (illustrated for the Δ isomer in Fig. 3). A series of T_m values for simple duplex complexes of the DNA–bipyridine conjugate is illustrated in Table 1. The native 17mer duplex exhibited a T_m value of 68°C, and when two residues were removed from middle of one strand and replaced by the bipyridine linker the T_m was reduced to 55°C. This reduced T_m value would be expected due to the loss of two hydrogen-bonding base residues, but still results in duplexes of reasonable stability. Changing to two residue across from the bipyridine from two purines (A and G) to two pyrimidines (C residues) did not alter the T_m value. To test the model building studies, we increased the number of unpaired residues in the target strand from two to three (all C residues) and the T_m value dropped an additional 7°C. These experiments suggest that the most effective duplexes formed with the bipyridine conjugate are those that place the conjugate across from two unpaired residues in the target strand. Although in the present case and under the present conditions the ruthenium complex is largely inert, with other metal complexes or under proteolysis conditions this approach to sequence targeting permits the metal complex to access a short single-stranded region of the target sequence and facilitate chelation, redox or cleavage chemistries.

The T_m values measured for the simple 18 residue duplex, composed largely of dT-dA base pairs with two central dC-dG base pairs was 52°C (Table 2). Removal of the central two dC, and their replacement with tri(ethylene glycol) or hexa(ethylene glycol) resulted in a 20 and 17°C decrease in T_m values, respectively. While the loss of the two central base pairs significantly alters helix stability, the T_m values of 32 and 35°C are still within a range that permits stable duplex formation. The use of two independently hybridizing oligonucleotides bridged

Table 2. T_m values (°C) for duplexes and triplexes containing Ru(bipy)$_3$ or related linkers

L	5' TTTTTTTT L TTTTTTTT 3' 3' AAAAAAAA-GG-AAAAAAAA 5'	5' TTTTTTTT L TTTTTTTT 3' 5' AAAAAAAA-GG-AAAAAAAA 3' 3' TTTTTTTT-CC-TTTTTTTT 5'	5' TTTTTTTT L TTTTTTTT 3' 5' GCGCGAAAAAAAA-GG-AAAAAAAACCCGG 3' 3' CGCGCTTTTTTTT-CC-TTTTTTTTGGGCC 5'
-CC-	**52**	**25, 52**	**25, 71**
-O(CH$_2$CH$_2$O)$_3$-	**32**	**30, 53**	**27, 71**
-O(CH$_2$CH$_2$O)$_6$-	**35**	**29, 52**	**27, 72**
	**36**	**33, 53**	**34, 71**
	**35**	**32, 53**	**31, 70**

*T_m values were obtained in 50 nM buffer, 10 Mm $MgCl_2$, 100 Mm NaCl at strand concentrations of 1 μM. Each value is the average of at least two determinations and are estimated to be ±1°C.

by a glycol linker is the basis for a series of tethered oligonucleotide probes (35,36). Introduction of the bipyridine linker (in place of the glycol based linkers) resulted in no additional disruption of helix stability as determined from T_m values, and the presence of the *tris*-2,2'-bipyridyl Ru(II) complex as part of the bridging linker still resulted in a T_m value of 35°C. While the difference in T_m values for the conjugated duplexes and a fully native duplex is ~15°C, these differences appear largely related to the loss of 2 bp that occurs with the introduction of the linker (Table 2). A comparison of the T_m value for either the bipyridine-containing duplex or that complexed with ruthenium, with the T_m value of the corresponding duplex containing a bridging hexa(ethylene glycol) linker indicates no significant change in thermal stability (Table 2).

Two types of triplexes were examined; both employed an 18 residue $dA_8G_2A_8$ target site with an oligo-dT third strand. In one case the target strand was the minimal 18mer sequence necessary to form the three stranded complex, while in the second case the 18mer target was embedded within a 28mer duplex. In both cases, the hybridization target was also designed with a 2 bp gap that was bridged by either a simple glycol-based linker, or the bipyridine-based linker, in the probe sequence. A third strand composed solely of nucleoside residues (such that two C^+–GC base triplets were present in the center of the complexes) resulted in two thermally induced transitions, one at 25°C and a second at 52 or 71°C (depending upon the length of the duplex). The first transition is interpreted as the triplex to duplex transformation, while the second represents the duplex to random coil transformation. For the DNA triplexes formed with glycol-based linkers, the temperatures of the first transitions are raised slightly (Table 2) while those of the second representing duplex denaturations remain essentially constant. This apparent increase in T_m is likely to be partially related to the presence of two adjacent charged C^+–G-C base triplets in the native complex. Charge–charge interactions may destabilize the native triplex relative to those containing linkers as suggested by the differences in T_m values for the native triplex sequences and those containing either the tri(ethylene glycol) or hexa(ethylene glycol) linkers (Table 2). Relative to the two glycol-containing conjugates, introduction of the bipyridine linker further increased the temperatures for the first transitions (triplex) without affecting the second (duplex) suggesting the presence of some slight stabilizing interactions with the bipyridine. With a third strand containing the *tris*-2,2'-bipyridyl Ru(II) complex across from two dG-dC base pairs (Table 2), the first transitions were largely unchanged relative to the uncomplexed linker (20mer target) or reduced slightly (28mer target). The results from these last two complexes suggest that introduction of the bisbipyridyl ruthenium complex into the third strand containing the bipyridyl linker does not result in significant disruption of the complex. These results suggest that linkers of this type can be used effectively to incorporate metals into higher order DNA structures.

Double-stranded complexes containing the *tris*-2,2'-bipyridyl Ru(II) complex can likely be formed also with RNA targets and these conjugates may be useful in antisense technologies.

ACKNOWLEDGEMENTS

We thank Dr Guobing Xiang, Sequenome Inc., San Diego, CA for the MALDI-TOF mass spectrometry, Professor Michael Clarke for scientific discussions and Dr Javier Rojo for critical reading of the manuscript. (NIH GM53201.)

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