2′-Ribose-Ferrocene Oligonucleotides for Electronic Detection of Nucleic Acids

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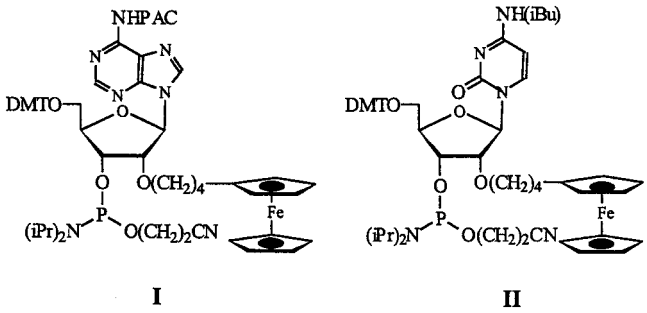
We have synthesized two novel phosphoramidites with a ferrocenyl moiety at the 2′-ribose position linked through a butoxy linker. Using automated DNA/RNA synthesis techniques, oligonucleotides containing ferrocene at various positions were prepared and characterized by HPLC, MALDI-TOF mass spectrometry, and electrochemistry. Thermal stability studies of the ferrocene-modified DNA duplexes revealed that introduction of one or two ferrocenyl complexes does not result in an observed change of the T_m values of the corresponding DNA duplexes when compared to the nonmodified hybrids. These data indicate that the introduction of a ferrocenyl group at the 2′-position of the ribose ring containing either a purine or pyrimidine base has no effect on the stability of the modified DNA. The electrochemical behavior of the ferrocene-containing DNA was examined by cyclic voltammetry. The modified 2′-ferrocene-oligonucleotides are electrochemically active and can be used as signaling probes for the electronic detection of nucleic acids on bioelectronic sensors.

Introduction

The study of energy- and electron-transfer processes through the DNA duplex and the development of DNA hybridization probes and electrochemical sensors have resulted in the synthesis of numerous transition-metal modified olionucleotides. These include ruthenium,[1−5] osmium,[4,6] iron,[7−9] rhodium,[10,11] and copper complexes.[12]

In our ongoing research efforts to develop electronic microsensors for detecting nucleic acids in unpurified samples,[13−15] we have reported that the ferrocene-conjugated deoxyuridines through unsaturated bonds at the nucleobase have been synthesized and have been successfully incorporated into DNA oligonucleotides in a site-specific manner using phosphoramidite chemistry.[16]



I **II**

Figure 1. Structures of compounds **I** and **II**.

Here, we report a new type of ferrocene-containing phosphoramidite where a ferrocenyl group is linked to the 2′-position of ribose ring (Figure 1). We have chosen ferrocene (Fc) and its derivatives because these complexes are attractive electrochemical probes as a result of their stability and convenient synthetic chemistry. By employing phosphoramidites **I** and **II** in automated DNA/RNA synthesis techniques, ferrocene derivatives can be incorporated into any position of the oligonucleotide sequence. The thermal stability and electrochemical properties of the ferrocene-containing DNA oligonucleotides are discussed.

Results and Discussion

(i) Preparation of Alkylferrocene. Preparation of alkylferrocenes relies on the reductive deoxygenation of ferrocenyl aldehydes and ketones that are easily prepared by the Friedel−Crafts acylation of ferrocene. Two methods have been developed for the reductive deoxygenation reactions: (a) utilizing Zn/Hg in toluene/aqueous HCl, a

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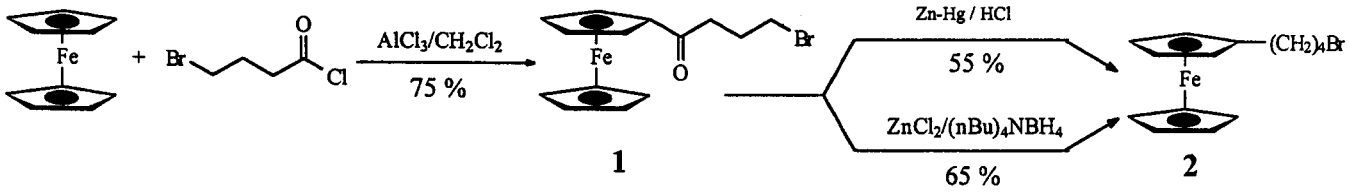
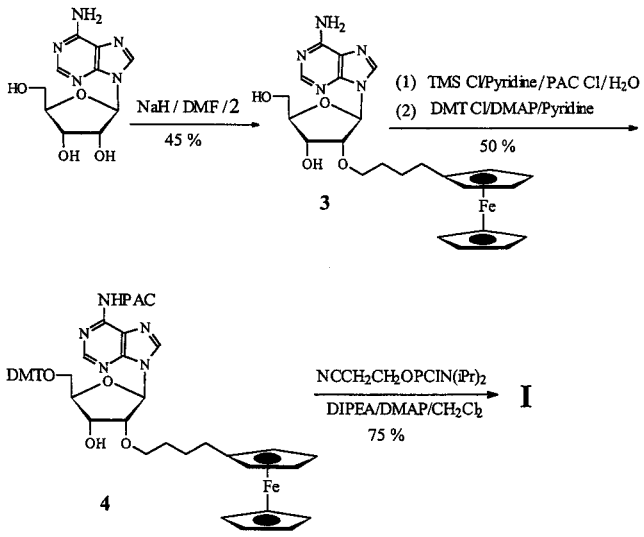
Scheme 1



Scheme 2



method that has numerous waste products and is relatively slow as a result of its heterogeneous nature, and (b) a rapid homogeneous reaction using $(nBu)_4NBH_4$/$ZnCl_2$ in dichloromethane.[17] Compound **1**, 4-bromobutyrylferrocene, prepared from acylation of ferrocene was reduced to **2** using method a (55%) or method b (65%) by modifying reagent ratios and reaction times (see Experimental Section).

(ii) Preparation of Ferrocene-Containing Phosphoramidites. To introduce functional groups such as amines into the ribose ring, a base-catalyzed alkylation method has been used.[18−20] The regioselectivity (2′- vs 3′-substitution) of the alkylation reactions is dependent on the nucleosides. By adopting literature procedures, adenosine was reacted with sodium hydride in DMF followed by addition of **2** to afford **3** in good yield (Scheme 2). The minor 3-substituted isomer obtained can be separated by silica gel chromatography. The selectivity of this reaction can be attributed to the higher acidity of the 2′-hydroxy proton of the purine nucleoside.[19] The exocyclic amino group of the adenosine base, and the 5′-OH group were sequentially protected by PAC and DMT, respectively, in a one-pot reaction to give **4** in good yield. Compound **4** is converted to the phosphoramidite **I** by employing standard reagents.[21]

The same synthetic strategy is applied to the preparation of the cytidine analogue (Scheme 3). The reaction of cytidine, a pyrimidine nucleoside, with **2** gave an inseparable mixture of **5** and **6** in good yield (76%). Protection of the amino group of the cytidine base by reaction of **5** and **6** with isobutyryl chloride resulted in formation of a mixture of **7** and **8** that was used without purification in the next step. Once the 5′-hydroxy groups were protected by DMT, two products **9** and **10** were generated and separated on silica gel in 34% and 61% yield, respectively. The similarity of the 2′- and the 3′-hydroxyl groups of the pyrimidine nucleosides is responsible for the poor selectivity of substitution using cytidine.[19,22] The structural assignments of **9** and **10** (Scheme 3) are based on ^1H and ^{13}C NMR spectra of **9** and **10**, in comparison with that of the reported analogues.[22] Although the ^1H NMR spectra of **9** and **10** in DMSO-d_6 show subtle differences due to the spectral overlapping (for **10**, H5 at 7.05 ppm and H6 at 8.30 ppm; for **9**, H5 at 7.02 ppm and H6 at 8.39 ppm), their ^{13}C NMR spectra provide direct evidence for their assignments. Upon examining the ^{13}C NMR spectra of **9** and **10** it was found that the C2′ of **10** at 82.26 ppm is different from the C2′ of **9** at 76.29 ppm and the C3′ of **10** at 67.86 is different from the C3′ of **9** at 73.66 ppm. The downfield ^{13}C shifts of alkyl-substituted carbons are consistent with that observed for another substrates, methyl- and allyl-substituted products.[22,23] The structural assignments were further supported by the thermal stability studies of the isomeric DNA oligonucleotides (section iv). In addition, **7** was obtained from the acid treatment of **9** and was spectrally characterized. Finally, **9** and **10** were converted into the corresponding phosphoramidites **III** and **II**, respectively, employing standard techniques.

(iii) Incorporation of Ferrocenyl Derivatives into DNA Oligonucleotides. Phosphoramidites **I**, **II**, and **III** were incorporated into oligonucleotides using an automated DNA/RNA synthesizer with average coupling efficiencies greater than 96%. Standard reaction protocols were employed except for prolonged coupling times (15 min). All oligonucleotides were purified by HPLC on either a C6 reversed-phase column or an Oligo R3 (polystyrene-based) column. Table 1 lists the sequences and numberings of the synthesized oligonucleotides. Three modified oligonucleotides, **D12** (containing **I**, calcd MS 5113.95, found 5113.90), **D15** (containing **II**, calcd MS 5055.86, found 5058.17), and **D17** (containing **III**, calcd MS 5055.86, found 5058.35), were characterized by MALDI-TOF mass spectral analyses and illustrate the successful incorporation of ferrocenyl moieties into the oligonucleotides.

(iv) Thermal Denaturation Studies of Ferrocene-Modified Oligonucleotides. Two nucleotide sequences (15mers) were prepared for this study and hybridized with their complementary sequences. Table 2 lists the

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Scheme 3

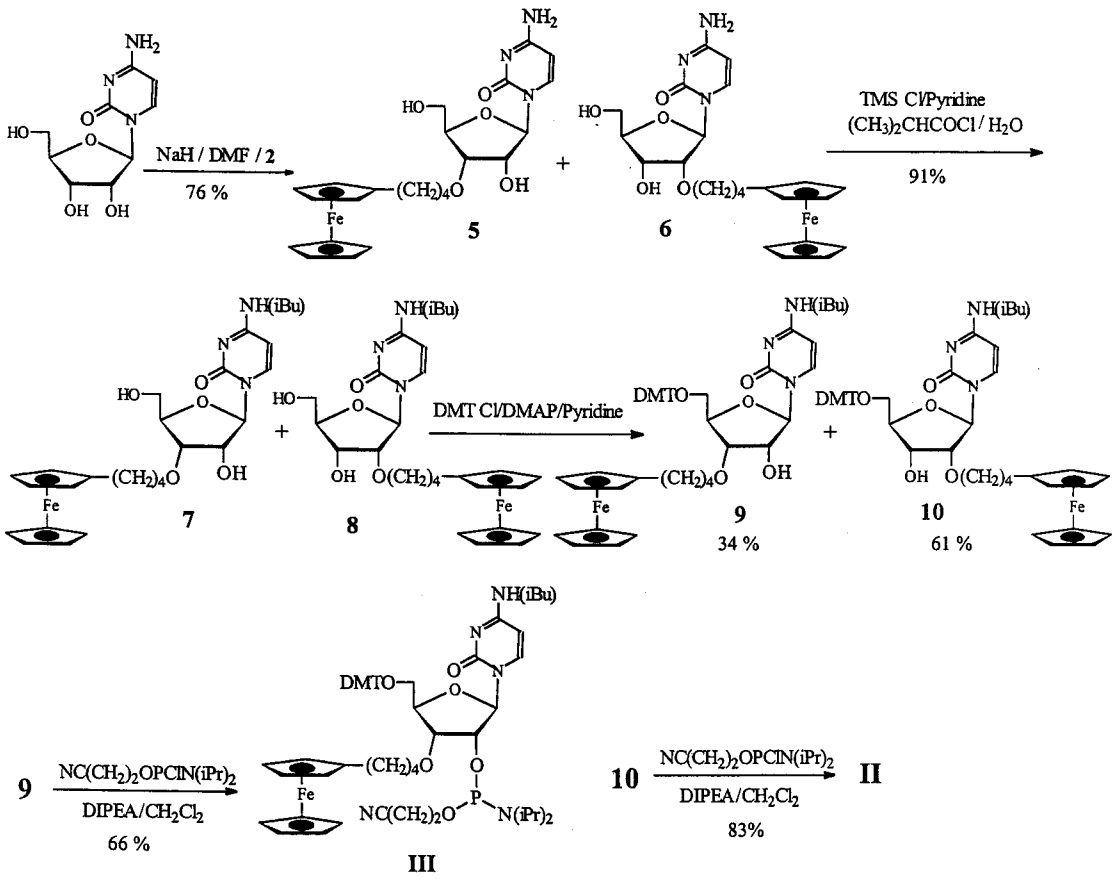


Table 1. DNA Oligonucleotides and Their Sequences

DNA entry	DNA oligonucleotides sequences (15mers)
D10	**5′-ACC ATG GAC TCT GTT-3′** (sequence I)
D11	5′-AAC AGA GTC CAT GGT-3′
D12	5′-A(**I**)C (**I**)GA GTC CAT GGT-3′
D13	**5′-AGC TGG CTC TAG TGT-3′** (sequence II)
D14	5′-ACA CTA GAG CCA GCT-3′
D15	5′-A(**II**)A (**II**)TA GAG CCA GCT-3′
D16	5′-ACA CTA GAG (**II**)CA GCT-3′
D17	5′-A(**III**)A (**III**)TA GAG CCA GCT-3′
D18	5′-ACA CTA GAG (**III**)CA GCT-3′
D19	5′-ACA CTA GAG *G*CA GCT-3′ (6th GG mismatch)

Table 2. T_m Values of DNA Oligonucleotides

	DNA pairs				
	D10:D11	**D10:D12**	**D13:D14**	**D13:D15**	**D13:D16**
T_m (°C)	51.2	51.3	58.3	57.4	58.7

melting temperatures (T_m) derived from the thermal denaturation curves of the DNA duplexes. Data from Table 2 show that a DNA duplex in which two ferrocene-containing purine bases were incorporated at the12th and 14th positions (**D10:D12**) have a T_m value of 51.2 °C, identical to the nonmodified DNA hybrid (**D10:D11**). For **II** (pyrimidine base), T_m values of oligonucleotides bearing either mono modification at the sixth position (**D16**) or dual modifications at the 12th and 14th positions (**D15**) are essentially the same as that of a perfect match (**D14**) (58 °C). Clearly, incorporation of either **I** or **II** into oligonucleotides has no significant effect on DNA duplex stability, indicating that **I** or **II** do not substantially disrupt the DNA helix.

To confirm the structural assignments of **9** and **10**, the thermal stability of the oligonucleotidtes containing **III** were also investigated. Figure 2 shows the thermal

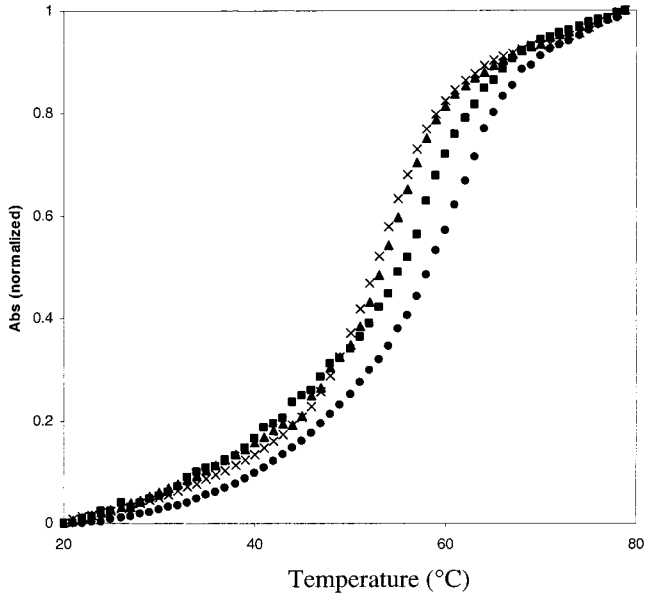
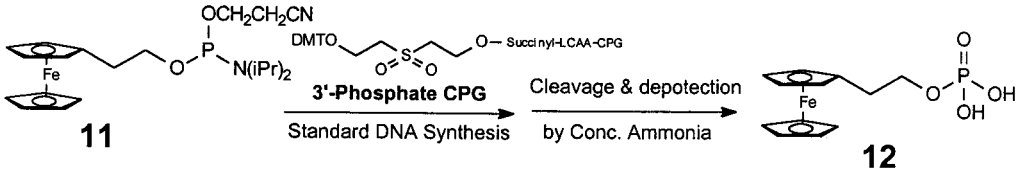


Figure 2. Thermal denaturation curves for **D13:D14** duplex (●, T_m = 58.3 °C), **D13:D19** duplex (■, T_m = 55.5 °C), **D13:D18** duplex (▲, T_m = 54. 2 °C), **D13:D17** duplex (×, T_m = 53.8 °C).

denaturation curves of four pairs of oligonucleotides, **D13:D14** (perfect match), **D13:D19** (GG mismatch at the sixth position), **D13:D18** (**III** at the sixth position), and **D13:D17** (**III** at the 12th and 14th positions). The T_m values of **D17** and **D18** (53.7 and 54.2 °C, respectively) are similar but 4° lower than that of **D14**, a perfect match (58.3 °). Furthermore, the destabilization observed for **D18** is higher than that observed for a single GG

Scheme 4



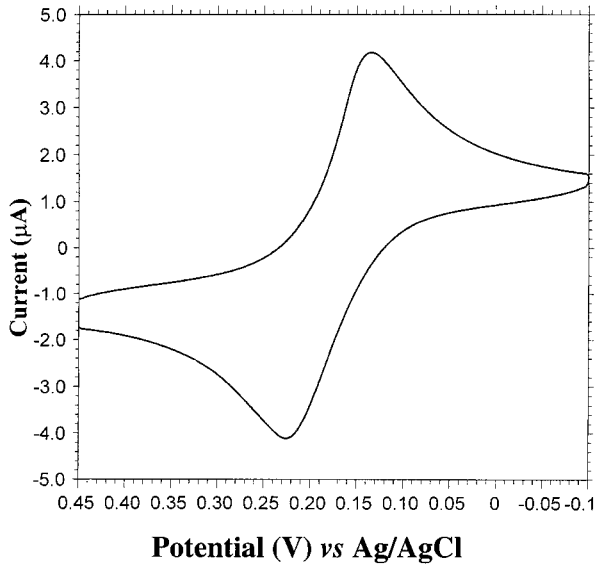


Figure 3. Cyclic voltammogram of **D12** in an aqueous buffer solution.

mismatch (**D19**, 55.5 °C) at the same position. These data unveiled that when ferrocenyl moieties at the wrong position (3′-substituted) were incorporated into DNA oligonucleotides, the corresponding duplexes showed substantially lower thermal stability. In addition, study on the thermal stability of the DNA oligonucleotides containing **III** provided further evidence that the structural assignments on compounds **9** and **10** are unambiguous. The lower T_m values of duplexes containing **III** can be explained by the incorporation of a 3′-subsitituted isomer **III** that alters the hybridization geometry of the DNA duplexes leading to destabilization.

(v) Electrochemistry of Ferrocene-Modified Oligonucleotides in Solution. To test the application of ferrocene-modified oligonucleotides as signaling probes in the electronic detection of nucleic acids, we have investigated the electrochemical behavior of **I**, **II**, and **III** using cyclic voltammetry (CV). The CV of **D12** (containing **I**) in buffered aqueous solution is shown in Figure 3. A reversible wave associated with oxidation and subsequent reduction of the pendant ferrocenyl moiety is observed ($E_{1/2}$ = 0.181 V vs Ag/AgCl).

To compare the electrochemical behaviors of these ferrocene-containing oligonucleotides (**I** or **II** or **III**) to that of monosubstituted ferrocenyl derivatives in the aqueous buffer, the water-soluble complex **12** was prepared from the compound **11**[24] using standard DNA synthesis techniques (Scheme 4) and was characterized by MS (calcd MS 310.01, found 310). The electrochemical analysis (CV) of complex **12** in the same buffer solution gave the identical cyclic voltammogram ($E_{1/2}$ = 0.188 V vs Ag/AgCl, data not shown). The experimental data indicated that the electrochemical characteristics of the ferrocene-containing DNA oligucleotides are very similar to those of the simple ferrocenyl derivatives at the identical conditions.

This result also demonstrates that the ferrocene-modified DNA oligonucleotides are electrochemically detectable and can be used as signaling probes for hybridization reactions.[25]

Conclusion

Two new ferrocene-containing phosphoramidites (**I** and **II)** have been synthesized and characterized. By employing automated DNA/RNA synthesis techniques, these modified phosphoramidites can be inserted at various positions along an oligonucleotide. The thermal denaturation characteristics of these metal-modified DNA duplexes were investigated. It was found that two modifications with either **I** or **II** at varying positions have no effect on the thermal stability of the DNA, but the incorporation of ferrocenyl moieties at the 3′-position would lead the significant destabilization. Further, electrochemical measurements of the ferrocene-modified DNA were studied by CV, indicating that these complexes are electrochemically active and can be used as the signaling probes in electronic detection of nucleic acids on bioelectronic sensors.[24]

Experimental Section

Materials. Sodium hydride, *N*,*N*-dimethylaminopyridine (DMAP), triethylamine (TEA), trimethylchlorosilane (TMSCl), phenoxyacetyl chloride (PAC Cl), 1.0 M $ZnCl_2$ ether solution, tetrabutylammonium borohydride [$(nBu)_4NBH_4$], diisopropylethylamine (DIPEA), ferrocene, 4-bromobutyryl chloride, and isobutyryl chloride were purchased from Aldrich and used as received. DMF (anhydrous), pyridine (anhydrous), dichloromethane, silica gel (240−400 mesh), acetonitrile (MeCN, HPLC grade), ethyl acetate, sodium bicarbonate, hexane and methanol were purchased from EM Science and used as received. Acetonitrile (MeCN, DNA synthesis grade) and dichloromethane (DNA synthesis grade) were purchased from Burdick & Jackson. 2-Cyanoethyl *N*,*N*-diisopropyl chlorophosphane, 4,4′-dimethoxytrityl chloride (DMT Cl), andenosine, and cytidine were purchased from Chemgenes and used as received. All unmodified phosphoramidites and ancillary reagents were purchased from Glen Research and used as received.

Instrumentation. GC/MS was performed at 70 eV with a 50 m × 0.2 mm capillary column programmed at 140 °C for 1 min and 280 °C at 10 °C min^{-1}. ^1H NMR spectra were recorded on 300 or 400 MHz machines, ^{31}P NMR spectra were recorded on a 400 MHz spectrometer, and UV spectra were recorded on a HP 845X UV−vis system. Oligo R3 HPLC columns were purchased from PerSeptive Biosystems, and C6 columns were purchased from Keystone Scientific.

(24) Unpublished results.

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Mass spectra for organic compounds were obtained from Mass Consortium at San Diego using HP 1100 MSD for electrospray, and high-resolution FAB MS were obtained from Mass Spectrometry Laboratory for Biotechnology at North Carolina State University. MALDI-TOF mass spectra for DNA oligonucleotides were obtained from Caltech Protein/Peptide Micro Analysis Lab.

All DNA oligonucleotides were synthesized using either an ABI 394 or an ABI 392 RNA/DNA synthesizer. HPLC analyses were performed on Hitachi D7000 systems equipped with a diode array; using a Betasil C_6 reversed-phase column (25 cm × 4.6 mm i.d.) for modified DNA oligonucleotides and an Oligo R3 polystyrene column (10 cm × 4.6 mm i.d.) for nonmodified DNA oligonucleotides. For all ferrocene-modified DNA oligonucleotides, the gradient system is 10−35% MeCN over 32 min and 35−100% MeCN over 10 min in 100 mM TEAA (pH = 6.5). For unmodified DNA, the gradient system is 0−25% MeCN over 32 min and 25−100% MeCN over 8 min in 100 mM TEAA (pH = 6.5).

All T_m values of DNA duplexes were measured under the following conditions: [DNA strand] = 2.0 μM; buffer, 1X SSC, with temperature ramped from 20 to 80 °C at a rate of 1 °C per min.

CV data were acquired using a computer-based CHI instrument (model 660) electrochemical workstation with a scan rate of 10 mV/s and the following conditions: [**D12**], 0.75 mM; [complex **12**], 2.9 mM; buffer, 50 mM NaCl, 50 mM $MgCl_2$, 50 mM TRIS-HCl, pH 7.0; working electrode, gold wire; reference electrode, Ag/AgCl.

Synthesis of Compound 1. To a solution of 40.0 g (0.22 mol.) of ferrocene and 30.1 g of alummiunm chloride (0.22 mol) in 750 mL of dichloromethane was added dropwise a solution of 24.9 mL (0.22 mmol) of bromobutyryl chloride in 150 mL of dichoromethane. The mixture was stirred at room temperature for 1 h and poured into 300 mL of ice water. The organic layer was washed with 5% sodium bicarbonate solution (3 × 500 mL), dried over sodium sulfate, and concentrated. The crude product was purified on a 300-g silica gel column, packing with hexane and eluting with 0−80% dichloromethane in hexane. The desired fractions were pooled and concentrated to afford 54.1 g (75%) of the title compound: ^{1}H NMR (300 MHz, DMSO-d_6) δ 4.79 (d, J = 3.0 Hz, 2H, Fc), 4.56 (d, J = 3.0 Hz, 2H, Fc), 4.23 (s, 5H, Fc), 3.61 (t, J = 14.2 Hz, 2H, CH_2Br), 2.90 (t, J = 14.0 Hz, 2H, COCH_2), 2.10 (m, 2H, $CH_2CH_2CH_2$).

Synthesis of Compound 2. Method A. To a solution of 6.0 g (18.0 mmol) of compound **1** in 120 mL of toluene was added 35.9 g (0.55 mol) of zinc, 3.3 g (12.0 mmol) of mercuric chloride, and 100 mL of water. While the mixture was stirred vigorously, 80 mL of concentrated HCl aqueous solution (12 M) was added slowly. The reaction mixture was stirred at room temperature for 16 h. The organic layer was separated, washed with water (2 × 100 mL), dried over sodium sulfate, and concentrated. The crude product was purified on a 90-g silica gel column, packing and eluting with hexane. The desired fractions were pooled and concentrated to afford 3.13 g (55%) of the title product.

Method B. To a solution of 51.2 g (0.15 mol) of compound **1** and 39.4 g (0.15 mol) of $(nBu)_4NBH_4$ in 500 mL of dichloromethane cooled in an ice−water bath was added 306 mL (0.31 mol) of 1.0 M $ZnCl_2$ ether solution slowly. The mixture was stirred at room temperature for 35 min and poured into 700 mL of water. The organic layer was separated, dried over sodium sulfate, and concentrated. The crude product was purified on a 350-g silica gel column, packing with 1% TEA/hexane and eluting with 0−10% dichloromethane/hexane. Fractions were pooled and concentrated to afford 31.8 g (65%) of the product: ^{1}H NMR (300 MHz, DMSO-d_6) δ 4.04−4.11 (m, 9H, Fc), 3.55 (t, J = 14.3 Hz, 2H, CH_2Br), 2.32 (t, J = 14.0 Hz, 2H, Fc-CH_2), 1.83 (m, 2H, CH_2CH_2Br), 1.60 (m, 2H, Fc-CH_2CH_2). Anal. Calcd for $C_{14}H_{17}BrFe$: 321. Found: 321. GC-MS *m*/*z* (relative intensity) 322 (96), 320 (100).

Synthesis of Compound 3. To a solution of 43.1 g (0.16 mol) of adenosine in 2.5 L of DMF cooled in an ice−water bath was added 7.1 g (0.18 mol) of NaH (60% in mineral oil). The resulting suspension was warmed to room temperature, stirred for an additional 1 h, and recooled into the same ice−water bath. To this cold reaction mixture was added a solution of 25.9 g (0.08 mol) of compound **2** in 300 mL of DMF. The reaction mixture was brought to room temperature and stirred overnight at 35 °C. The reaction was quenched by slow addition of 200 mL of water, and the mixture was concentrated in vacuo. The resulting residue was dissolved in a mixture of 600 mL of water and 600 mL of ethyl acetate. After separation of the organic layer, the aqueous layer was extracted with ethyl acetate (3 × 300 mL). The combined extractions were dried over sodium sulfate and concentrated. The crude product was purified on a 350-g silica gel column, packing with 1% TEA/50% hexane/dichloromethane and eluting with 1−2% methanol/ethyl acetate. The desired fractions were pooled and concentrated to afford 18.6 g (45%) of the title product: ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.40 (s, 1H, 2-H), 8.15 (s, 1H, 8-H), 7.40 (s, 2H, $-NH_2$), 6.01 (d, J = 6.3 Hz, 1H, 1′-H), 5.45 (dd, $J1$ = 6.9 Hz, $J2$ = 6.6 Hz, 1H, 5′-OH), 5.20 (d, J = 5.1 Hz, 1H, 3′-OH), 4.50 (t, J = 5.1 Hz, 1H, 2′-H), 4.27−4.36 (m, 1H, 3′-H), 3.95−4.04 (m, 10H, 4′- H + 9Fc), 3.55−3.66 (m, 4H, 5′-H + OCH_2−), 2.15 (t, J = 7.4 Hz, 2H, Fc-CH_2), 1.36 (m, 4H, $-CH_2CH_2-$). Anal. Calcd for $(C_{24}H_{29}N_5O_4Fe + Na)^+$: 530. Found: 530.

Synthesis of Compound 4. To a solution of 15.7 g (30.9 mmol) of **3** in 650 mL of anhydrous pyridine cooled in an ice−water bath was added 10.7 mL (77.3 mmol) of TMS Cl. After the mixture stirred for 1 h at the same temperature, 10.7 mL (77.2 mmol) of PAC Cl was added. The mixture was stirred at 0 °C for 1.5 h and brought to room temperature for 30 min. The solution was diluted by adding 800 mL of dichloromethane and was washed with 5% sodium bicarbonate (2 × 600 mL). After removal of dichloromethane by aspirator, the resulting pyridine solution was diluted by addition of 150 mL of water. The reaction mixture was stirred at room temperature for 1.5 h and concentrated to dryness. The residue was coevaporated twice with anhydrous pyridine and was redissolved in 350 mL of dry pyridine. While the resulting solution was cooled to 0 °C, into this cold solution were added 12.6 g (37.1 mmol) of DMT Cl and 0.2 g of DMAP. The mixture was stirred at room temperature overnight and diluted by addition of 800 mL of dichloromethane. The reaction mixture was washed with 5% sodium bicarbonate (2 × 600 mL), dried over sodium sulfate and concentrated. The product was purified on a 350-g silica gel column, packing with 1% TEA/hexane and eluting by 1% TEA/0−80% CH_2Cl_2/hexane. The desired fractions were pooled and concentrated to afford 14.0 g (48%) of the title product: ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.10 (s, 1H, amide), 8.65 (s, 1H, 2-H), 8.61 (s, 1H, 8-H), 6.81−7.35 (m, 18H, aromatic protons), 6.16 (d, J = 5.1 Hz, 1H, 1′-H), 5.31 (d, J = 6.0 Hz, 1H, 3′-OH), 5.04 (s, 2H, CO-CH_2O), 4.68 (t, J = 5.1 Hz, 1H, 2′-H), 4.38 (q, J = 5.1 Hz, 1H, 3′-H), 4.11−4.16 (m, 1H, 4′-H), 3.97−4.04 (m, 9H, Fc), 3.61−3.72 (m, 8H, $-OCH_2-$ + 2 × OCH_3), 3.26−3.47 (m, 2H, 5′-H), 2.18 (t, J = 7.5 Hz, 2H, Fc-CH_2CH_2), 1.39−1.47 (m, 4H, Fc-$CH_2CH_2CH_2$). Anal. Calcd for $C_{53}H_{53}N_5O_8Fe$: 944. Found: 944.

Synthesis of Compounds 5 and **6.** A solution of 14.9 g (61.4 mmol) of cytidine in 350 mL of anhydrous DMF was prepared by heating to 35 °C for 0.5 h and cooling in an ice−water bath. To this solution was added 2.5 g (65.2 mmol) of NaH [dispensed in mineral oil (60%)] portion by portion under argon. The reaction suspension was warmed to room temperature, stirred for 1 h, and then cooled in the same ice−water bath. Into this cold suspension was added a solution of 19.7 g (61.2 mmol) of **2** in 85 mL of DMF, and the resulting reaction mixture was slowly warmed to room temperature and then stirred at 30 °C for 16 h. After the reaction was quenched by adding 10 mL of H_2O, solvents were removed in vacuo and the residue was dissolved in a mixture of 200 mL of water and 600 mL of ethyl acetate. Upon shaking well, the organic layer was separated ,and the aqueous phase was extracted twice with ethyl acetate (2 × 300 mL). The combined extractions were dried over sodium sulfate and concentrated. The crude product was purified on a 300-g silica gel column, packing with 1% TEA/CH_2Cl_2 and eluting with 0−10% CH_3-OH/ethyl acetate. The desired fractions were pooled and concentrated to afford 15.1 g of a mixture of isomers **5** and **6**

that was not characterized by spectral analyses. On the basis of 6.5 g of the recovered starting material **2**, total yield of **5** and **6** is 76.0%.

Synthesis of Compounds 7 and 8. To a solution of 11.6 g (24.0 mmol) of **5** and **6** in 400 mL of dry pyridine cooled in an ice−water bath was added 14.0 mL of TMSCl under argon. The mixture was allowed to warm to room temperature, stirred for 40 min, and then cooled in the same bath. To this cold solution was added 10.0 mL (95 mmol) of isobutyryl chloride. The solution mixture was stirred at 0 °C for 30 min and at room temperature for 2.5 h. After recooling of the mixture to 0 °C, 150 mL of H_2O and 25 mL of concentrated ammonia solution were added. After stirring for 15 min, solvents were removed in vacuo, and the residue was dissolved in a mixture of 400 mL of ethyl acetate and 800 mL of water. The organic layer was separated, and the aqueous layer was extracted once with 400 mL of ethyl acetate. The combined organic extractions were washed with 5% $NaHCO_3$ solution (2 × 200 mL), dried over sodium sulfate, and concentrated. The crude residue was purified on a 200-g silica gel column, packing with 1% TEA/CH_2Cl_2, eluting with 0−4% CH_3OH/CH_2Cl_2. The desired fractions were pooled and concentrated to give 2.1 g (91.0%) of a mixture of **7** and **8** that is reddish and was not further characterized by spectral analyses.

Preparation of Pure Compound 7. By reaction of pure compound **9** with 3% TCA in CH_2Cl_2, followed by purification on a silica gel column, pure compound **7** was obtained: ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.91 (s, 1H, amide), 8.46 (d, J = 7.7 Hz, 1H, 6-H), 7.23 (d, J = 7.3 Hz, 1H, 5-H), 5.76 (br.s, 1H, 1′-H), 5.45 (d, J = 5.5 Hz, 1H, 2′-OH), 5.25 (t, J = 4.8 Hz, 1H, 5′-OH), 4.17−4.19 (m, 1H, 2′-H), 3.99−4.12 (m, 11H, 3′-H + 4′-H + 9Fc), 3.76−3.79 (m, 2H, $-CH_2O$), 3.54−3.61 (m, 2H, 5′-H), 2.72 [septet, J = 7.0 Hz, 1H, C*H*$(CH_3)_2$], 2.29 (t, J = 7.3 Hz, 2H, $-CH_2Fc$), 1.47−1.57 (m, 4H, $-CH_2CH_2-$), 1.06 (d, J = 6.6 Hz, 6H, 2 × CH_3); ^{13}C (75 MHz, DMSO-d_6) δ 177.80, 162.64, 154.69, 145.25,95.27, 90.84, 88.88, 82.40, 75.91, 72.81, 69.39, 68.34, 67.85, 66.86, 59.79, 34.98, 29.21, 28.77, 27.15, 19.17, 18.95. Anal. Calcd for $(C_{27}H_{35}FeN_3O_6 + Na)^+$ and $(C_{27}H_{35}FeN_3O_6 - H)^+$: 576.18 and 552.18. Found: 576 and 552.

Synthesis of Compounds 9 and 10. To a solution of 12.1 g (21.7 mmol) of a mixture **7** and **8** in 220 mL of dry pyridine under argon were added 11.0 g (32.5 mmol) of DMT Cl and 0.2 g of DMAP. The resulting solution was stirred at room temperature for 1 h, followed by addition of another 3.7 g (10.9 mmol) of DMT Cl and 0.1 g of DMAP. After stirring for an additional 2 h, the reaction was quenched by adding 10 mL of H_2O, and the reaction mixture was concentrated in vacuo. The crude products were purified on a 240-g silica gel column, packing with 1% TEA/CH_2Cl_2, eluting with 1% TEA/50% CH_2Cl_2/ethyl acetate to give 11.3 g (61%) of compound **10**, and then eluting with %1% TEA/0−4% CH_3OH/CH_2Cl_2 to give 6.4 g (34%) of compound **9**. Compound **9**: ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.88 (s, 1H, amide), 8.39 (d, J = 7.6 Hz, 1H, 6-H), 7.25−7.39 (m, 9H, DMT), 7.07 (d, J = 7.3 Hz, 1H, 5-H), 6.89−6.92 (m, 4H, DMT), 5.73 (br. s, 1H, 1′-H), 5.56 (d, J = 5.1 Hz, 1H, 3′-OH), 4.24 (t, J = 5.0 Hz, 1H, 2′-H), 4.02−4.10 (m, 11H, 3′-H + 4′-H + 9Fc), 3.71−3.78 (m, 8H, 2x OCH_3 + $-OCH_2-$) 3.54−3.59 (m, 1H, 5′-H), 3.27−3.38 (m, 1H, 5′-H), 2.72 [septet, J = 7.0 Hz, 1H, C*H*$(CH_3)_2$], 2.27 (t, J = 7.3 Hz, 2H, CH_2-Fc), 1.39−1.54 (m, 4H, $-CH_2CH_2-$), 1.08 (dd, $J1$ = $J2$ = 7.0 Hz, 6H, 2 × CH_3); ^{13}C NMR (75 MHz, $CDCl_3$) δ 177.04, 162.64, 158.36, 155.15, 144.24, 143.70, 135.33, 135.12, 129.63, 129.50, 127.85, 127.50, 126.64, 112.97, 96.38, 91.85, 88.36, 86.58, 81.19, 76.29 (2′-C), 73.67 (3′-C), 70.33, 67.99, 67.63, 66.63, 59.72, 54.71, 35.67, 29.17, 28.93, 27.07, 18.64. Anal. Calcd for $(C_{48}H_{53}FeN_3O_8 + Na)^+$ and $(C_{48}H_{53}FeN_3O_8 - H)^+$: 878.29 and 854.29. Found: 878 and 854. Compound **10**: ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.95 (s, 1H, amide), 8.30 (d, J = 7.3 Hz, 6-H), 7.22−7.41 (m, 9H, DMT), 7.05 (d, J = 7.3 Hz, 1H, 5-H), 6.89−6.92 (m, 4H, DMT), 5.83 (br. s, 1H, 1′-H), 5.12 (d, J = 7.4 Hz, 1H, 3′-OH), 4.22−4.28 (m, 1H, 2′-H), 4.02−4.10 (m, 11H, 3′-H + 4′-H + 9Fc), 3.64−3.84 (m, 9H, 5′-H + $-CH_2O-$ + 2 × OCH_3), 3.29−3.37 (m, 1H, 5′-H), 2.70 [septet, J = 6.6 Hz, 1H, C*H*$(CH_3)_2$], 2.31(t, J = 7.4 Hz, 2H, $-CH_2Fc$), 1.51−1.60 (m, 4H, $-CH_2CH_2$-), 1.06 (dd, $J1$ = $J2$ = 6.6 Hz, 6H, 2 × CH_3); ^{13}C NMR (75 MHz, $CDCl_3$) δ 176.91, 162.80, 158.61, 154.82, 144.39, 143.91, 135.62, 135.37, 129.94, 129.81, 128.20, 127.76, 126.91, 113.21, 96.47, 88.76, 88.65, 86.97, 83.09 (2′-C), 82.27, 70.68, 68.24, 67.86 (3′-C + Fc-C), 66.88, 61.12, 54.99, 36.09, 29.37, 29.16, 27.34, 18.92, 18.86. Anal. Calcd for $(C_{48}H_{53}FeN_3O_8 + Na)^+$ and $(C_{48}H_{53}FeN_3O_8 - H)^+$: 878.29 and 854.29. Found: 878 and 854.

Synthesis of Phosphoramidite I. To a solution of 14.0 g (14.8 mmol) of **4** in 500 mL of dichloromethane was added 20.0 mL of DIPEA and 0.4 g of DMAP. While the solution was cooled at 0 °C, 10 mL (44.4 mmol) of 2-Cyanoethyl N, *N*-diisopropyl chlorophosphane was added. The reaction mixture was stirred at room temperature for 2 h and was diluted by addition of 400 mL of dichloromethane. The resulting solution was washed with 5% sodium bicarbonate solution (2 × 300 mL), dried over sodium sulfate, and concentrated. The product was purified on a 150-g silica gel column, packing with 1% TEA/hexane and eluting with 1% TEA/0−50% CH_2Cl_2/hexane. The fractions were pooled and concentrated to afford 12.0 g (71%) of the title compound. The purified product was further precipitated from hexane: ^{31}P NMR (162 MHz, $CDCl_3$) 151.73, 151.03. HRMS calcd for $C_{62}H_{70}N_7O_9FeP$: 1143.4322. Found: 1143.4354.

Synthesis of Phosphoramidite II. To a solution of 1.2 g (1.40 mmol) of compound **10** in 50 mL of dichloromethane and 5 mL of DIPEA was added 0.7 g (2.8 mmol) of 2′-cyanothyl-*N*,*N*-diisopropylaminochlorophosphane under argon. The reaction mixture was stirred at room temperature overnight and diluted by addition of 70 mL of dichleomethane. The solution was washed once with 5% $NaHCO_3$ solution, dried over sodium sulfate, and concentrated. The crude product was purified on a 20-g silica gel column, packing with 3% TEA/hexane and eluting with 1% TEA/0−100% CH_2Cl_2/hexane and 1% TEA/0−50% ethyl acetate/CH_2Cl_2. The right fractions were pooled and concentrated, followed by the precipitation from hexane to afford 1.3 g (83%) of the title compound: ^{31}P NMR (164 MHz, CDCl3) δ 150.82, 150.50. HRMS calcd for $C_{57}H_{70}FeN_5O_9P$: 1055.4262. Found: 1055.4296.

Synthesis of Phosphoramidite III. To a solution of 1.1 g (1.26 mmol) of compound **9** in 50 mL of dichloromethane and 5 mL of DIPEA was added 0.6 g (2.6 mmol) of 2′-cyanothyl-*N*,*N*-diisopropylaminochlorophosphane under argon. The reaction mixture was stirred at room temperature overnight and diluted by addition of 70 mL of dichlomethane. The solution was washed once with 5% $NaHCO_3$ solution, dried over sodium sulfate, and concentrated. The crude product was purified on a 20-g silica gel column, packing with 3% TEA/hexane and eluting with 1% TEA/0−100% CH_2Cl_2/hexane and 1% TEA/0−50% ethyl acetate/CH_2Cl_2. The right fractions were pooled and concentrated, followed by the precipitation from hexane to afford 0.9 g (66%) of the title compound: ^{31}P NMR (164 MHz, $CDCl_3$) δ 152.49, 150.10. HRMS calcd for $C_{57}H_{70}FeN_5O_9P$: 1055.4262. Found: 1055.4296.

Preparation of Water Soluble Complex 12. A 0.2 M solution of compound **11**,[24] made from addition of 0.5 mL of anhydrous acetonitrile and 0.5 mL of dichloromethane into 90 mg of compound **11**, was coupled to 1 μmol 3′-phosphate CPG columns, purchased from Glen Research, on the DNA/RNA synthesizer with a coupling time of 15 min. The synthesized CPG columns were cleaved and deprotected in concentrated ammonia for 1.5 h. After removal of CPG support, the resulting yellow solution was dried on a speed-vac. The final yellow powder, complex **12**, was dissolved into appropriate buffer solutions for MS, UV−vis, and electrochemical analyses.

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