Uridine-Conjugated Ferrocene DNA Oligonucleotides: Unexpected Cyclization Reaction of the Uridine Base

C. J. Yu,*,† Handy Yowanto,† Yanjian Wan,†
Thomas J. Meade,‡ Yoochul Chong,† Michael Strong,†
Leslee H. Donilon,† Jon Faiz Kayyem,† Michael Gozin,† and
Gary F. Blackburn†

Clinical Micro Sensors, Inc.
101 Waverly Drive, Pasadena, California 91105
Division of Biology and the Beckman Institute
California Institute of Technology
Pasadena, California 91125

Received December 6, 1999

The study of energy and electron-transfer processes through DNA duplexes and the development of DNA hybridization probes and electrochemical sensors have resulted in the incorporation of numerous transition-metal complexes into DNA oligonucleotides. These include ruthenium,[1] osmium,[1c,2] iron,[3] rhodium,[1d,4] and copper complexes.[5] Ferrocene (Fc) and its derivatives are attractive electrochemical probes because of their stability and convenient synthetic chemistry. Fc-containing DNA oligonucleotides have been prepared by attaching ferrocenyl moieties to the 5′ termini through either solid-phase synthesis using phosphoramidites or by reacting suitable ferrocenyl derivatives with end-functionalized oligonucleotides.[3]

Our efforts have focused on ways to develop microsensors for electronically detecting nucleic acids where we are particularly interested in site-specific incorporation of ferrocenyl derivatives into DNA oligonucleotides.[6−8] Here, we report the design and synthesis of new Fc-containing phosphoramidites (**III**−**IV**) (Scheme 1) in which ferrocences are conjugated to the nucleobase of dU through an unsaturated bond. By utilizing **III** and **IV** and automated DNA/RNA synthesis techniques, Fc derivatives can be incorporated into any position of the DNA sequence.[9]

Under the Sonogashira coupling reaction conditions,[10] Fc acetylene[11] was coupled to 5′-O-DMT-5-iodo-dU,[12] as shown in Scheme 1. Surprisingly, two products were isolated from this reaction using silica gel and HPLC. The products were characterized by ^{1}H NMR, MS, and UV−vis analyses.[13] The ^{1}H NMR spectrum of **I** (DMSO-d_6) reveals a peak at 11.72 ppm that disappears after exchange with D_2O. This is indicative of the amide proton of dU. In contrast, compound **II** has a peak at 5.58 ppm consistent with a vinyl proton, and showed no peaks beyond 10 ppm. The structures of the two isomers were assigned (Scheme 1**)** on the basis of ^{1}H NMR spectra. UV−vis spectra are consistent with the assigned structures (Figure 1). For the cyclized **II**, three absorption bands with the longest wavelength at 352 nm can be explained by the enhanced conjugation between Fc and the unsaturated moiety through a conjugated five-membered ring. In contrast, **I** exhibits two absorption bands with the longest wavelength at 280 nm.

The ratio of the two isomers is dependent on the reaction time. For short reaction times, **I** was obtained with no detectable amount of **II**, while long reaction times lead to substantial formation of **II**. To rule out catalytic promoted cyclization of **I** to **II**, **I** was heated to 55 °C in DMF/TEA and was cleanly converted into **II** as monitored by HPLC. Finally, both **I** and **II** were converted into their corresponding phosphoramidites (**III** and **IV)** using standard reagents and procedures (Scheme 1).

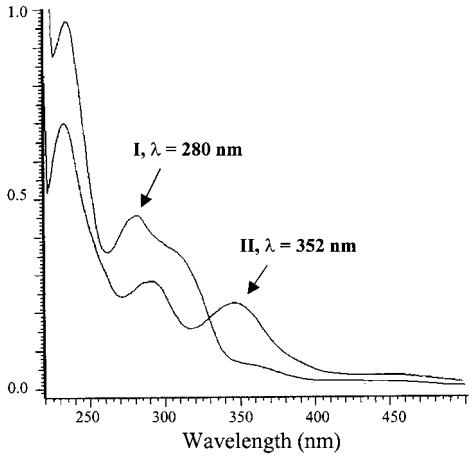
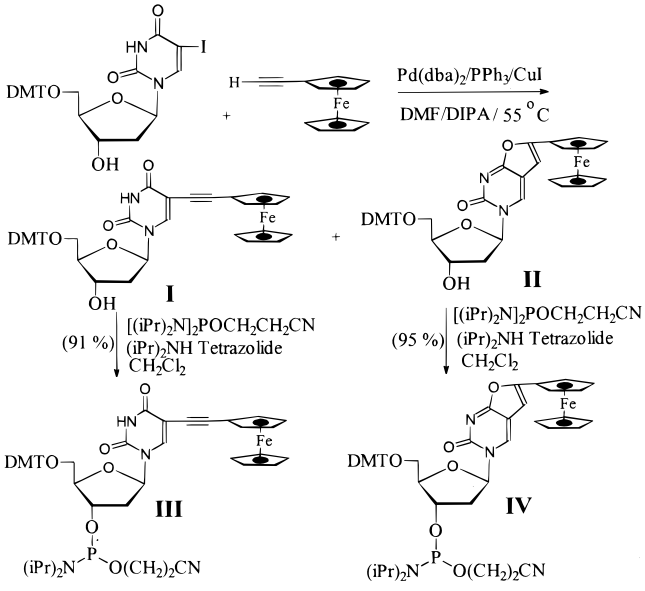


Figure 1. UV−vis spectra of compounds **I** and **II**.

Scheme 1. Synthesis of Phosphoramidites **III** and **IV**



† Clinical Micro Sensors, Inc.
‡ California Institute of Technology.

(1) (a) Meade, T. J.; Kayyem, J. F. *Angew. Chem., Int. Engl*. **1995**, *34*, 352−254. (b) Krider, E. S.; Meade, T. J. *J. Bio. Inorg. Chem*. **1998**, *3*, 222−225. (c) Hurley, D. J.; Tor, Y. *J. Am. Chem. Soc*. **1998**, *120*, 2194−2195. (d) Murphy, C. J.; Arkin, M. R.; Jenkins, Y.; Ghatlia, N. D.; Bossmann, S. H.; Turro, N. J.; Barton, J. K. *Science* **1993**, *262*, 1025−1029. (e) Meggers, E.; Kusch, D.; Gies, B. *Helv. Chim. Acta* **1997**, *80*, 640−652.

(2) Holmlin, R. E.; Yao, J. A.; Barton, J. K. *Inorg. Chem*. **1999**, *38*, 174−189.

(3) (a) Ihara, T.; Nakayama, M.; Murata, M.; Nakano, K.; Maeda, M. *Chem. Commun*. **1997**, 1609−1610. (b) Ihara, T.; Maruo, Y.; Takenaka, S.; Takagi, M. *Nucleic Acids Res.* **1996**, *24*, 4273−4280. (c) Mucic, R. C.; Herrlein, M. K.; Mirkin, C. A.; Letsinger, R. L. *Chem. Commun*. **1996**, 555−557.

(4) (a) Hall, D. B.; Barton, J. K. *J. Am. Chem. Soc*. **1997**, *119*, 5045−5046. (b) Hall, D. B.; Holmin, R. E.; Barton, J. K. *Nature* **1996**, *382*, 731−735.

(5) Bashkin, J. K.; Frolova, E. I.; Sampath, U. *J. Am. Chem. Soc*. **1994**, *116*, 5981−5982.

(6) Yu, C. J.; Chong, Y.; Kayyem, J. F.; Gozin, M. *J. Org. Chem*. **1999**, *64*, 2070−2079.

(7) Creager, S. E.; Yu, C. J.; Bamdad, C.; O'Connor, S. D.; Maclean, T.; Lam, E.; Chong, Y.; Olsen, G. T.; Luo, J. Y.; Gozin, M.; Kayyem, J. F. *J. Am. Chem. Soc*. **1999**, *121*, 1059−1064.

(8) Wilson, E. K. *Chem. Eng. News* **1998**, May 25, 47−49.

(9) The application of these Fc-containing oligonucleotides to electronic detection of nucleic acids is the subject of a forthcoming report.

(10) Sonogashira, K.; Tohda; Hagihara, N. *Tetrahedron Lett.* **1975**, 4467−4470.

(11) Doisneau, G.; Balavoine, G.; Fillebeen-Khan, T. *J. Organomet. Chem*. **1992**, *425*, 113−117.

(12) Ahmadian, M.; Zhang, P. M.; Bergstrom, D. E. *Nucleic Acids Res*. **1998**, *26*, 3127−3135.

(13) See Supporting Information for experimental details.

10.1021/ja994241m CCC: $19.00
Published on Web 06/29/2000

III and **IV** were incorporated into DNA oligonucleotides using automated DNA/RNA synthesis techniques.[13] The target DNA oligonucleotides, **D1**−**D7**, are purified by HPLC. Two Fc-modified oligonucleotides, **D3** (containing **III**; calcd MS 4774.78, found 4775.90) and **D4** (containing **IV**; calcd MS 4774.78, found 4776.50), were characterized by MALDI-TOF MS and illustrate successful incorporation of Fc into DNA.

D1	5'-ACC ATG GAC TC***A*** GAT-3'
D2	5'-ATC ***T***GA GTC CAT GGT-3'
D3	5'-ATC (**III**)GA GTC CAT GGT-3'
D4	5'-ATC (**IV**)GA GTC CAT GGT-3'
D5	5'-ATC ***A***GA GTC CAT GGT-3'
D6	5'-ACC ATG GAC TC***G*** GAT-3'
D7	5'-ATC ***C***GA GTC CAT GGT-3'

The thermal denaturation curves of four pairs of DNA hybrids **D1**:**D2** (perfect match), **D1**:**D3** (**III** at the 12th position), **D1**:**D4** (**IV** at the 12th position), **D1**:**D5** (AA mismatch at the 12th position)] were obtained (Figure S2).[13] The data derived from the thermal denaturation curves clearly indicate that the melting temperatures (T_m) of **D3** (48.0 °C) and **D4** (48.2 °C) are similar, but more than 5 deg lower than that of a perfect match (**D2**, 53.7 °C). This destabilization is very close to that observed for a single mismatch in an oligonucleotide (e.g., **D5**; T_m = 47.7 °C).

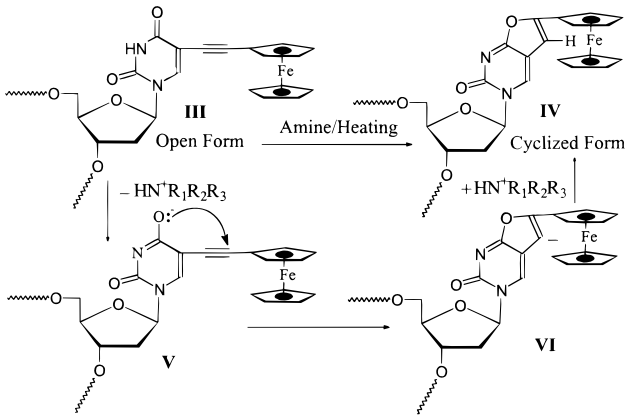
However, when **D3** or **D4** is hybridized to **D6** where the Fc-containing moiety is paired to dG at the 12th position, it is found that the T_m value of either **D3**:**D6** or **D4**:**D6** was enhanced from 48.0 °C to 56.5 °C, which is higher than that of a perfect match (**D1**:**D2**, 53.7 °C). By comparison to T_m values of the GC perfect matched hybrids (**D6**:**D7**, 60.7 °C) and the GT mismatched hybrids (**D2**:**D6**, 52.4 °C) (Figure S3),[13] we can conclude that the Fc-containing moiety behaves more like dC and prefers to hybridize to dG instead of dA.

These thermal study results prompted us to examine the favored structure of the Fc moieties when covalently attached to DNA. To address this question, enzymatic digestions of **D3** and **D4** were performed to isolate and characterize the modified nucleosides. A cocktail of enzymes including S1, P1 endonucleases, or snake venom phosphodiesterase 1, and bacterial alkaline phosphatase were found to be capable of digesting the Fc-containing oligonucleotides. HPLC and UV−vis analyses of the digest showed that standard nucleosides were cleanly separated from the modified Fc nucleosides.[13] We suspect that incomplete dephosphoration of the Fc-containing nucleotide and the poor solubility of Fc-containing nucleoside are responsible for the multiple peaks observed in the HPLC analysis.

These results do not unambiguously confirm the structural identity of the modified nucleosides. Therefore, two water-soluble dimers, 5′-(**IV**)T-3′, and 5′-(**III**)T-3′, were prepared and HPLC analysis revealed that these dimers had identical retention times and identical UV−vis spectra (Figure S6).[13] By comparing the dimers' UV−vis spectra to that of **I** and **II**, we concluded that the Fc moiety of the modified oligonucleotides existed exclusively in the cyclized forms regardless of whether **III** or **IV** was used in DNA synthesis. On the basis of this observation we conclude that compound **III** is cyclized into **IV** during the standard DNA cleavage and deprotection process.

The formation of a cyclized product is in contrast to numerous investigations where functional groups such as amines and transition-metal complexes have been incorporated into DNA oligonucleotides via Pd-catalyzed cross-coupling reactions between terminal alkynes and 5-iodo-dU in either the solution phase or the solid phase.[1c,h,14−16] For example, Tor and co-workers have reported that 5-ethynyl dU was successfully coupled to either Ru or Os complexes bearing 3-bromo-1,10-phenanthroline and the corresponding metal-conjugated DNA oligonucleotides through a triple bond was prepared.[1c] However, the formation of the cyclized products during either Sonogashira coupling reactions or DNA cleavage and deprotection has not been reported. Robins and Barr[14] observed that prolonged treatment of 5-hexynyl dU with $HgSO_4$/aqueous dioxane or treatment of the same compound with CuI/hot TEA/methanol gave the corresponding cyclized product in 36% and 82% yield, respectively. These results indicate that the cyclization reaction is catalyzed by metal ions under harsh conditions. However, our observations suggest that nucleophilic cycloaddition to the ferrocenylethynyl moiety under standard Pd-catalyzed coupling or basic conditions proceeds smoothly without catalyst in contrast to results observed by Robin and Barr.[14]

Scheme 2. Proposed Mechanism for the Cyclization Reaction



The results reported here are the first observation of cyclization of dU base under basic conditions without catalyst. We propose a mechanism for which the cyclization reaction occurs in amine-containing solution as shown in Scheme 2. An amine attracts an imide proton from dU to form an oxy-anion intermediate **V**. This oxy-anion intermediate undergoes intramolecular nucleophilic addition to the triple bond to form the carbanion **VI**. Back proton-transfer of carbanion **VI** from the ammonium salt gives the cyclized product. We postulate that other bases such as KOH and NaOH might function similarly.

The mild condition required for this cyclization can be attributed to C≡C bond activation by the ferrocenyl group, a feature that dramatically accelerates nucleophilic attack by the oxy-anion. The mechanism we propose is consistent with other substrates involving similar ring closure reactions in basic media.[17]

In summary, two new Fc-containing **III** and **IV** compounds have been synthesized and successfully incorporated into DNA oligonucleotides. Following examination of the UV−vis spectra and HPLC profiles of the two water-soluble DNA dimers, it was found that **III** was cyclized into **IV** during DNA cleavage and deprotection and an unexpected cyclization reaction of dU in basic media without catalyst was observed. The thermal studies on these Fc-containing DNA oligonucleotides indicated that the cyclized Fc-containing moiety has a preference to hybridize to dG instead of dA.

Acknowledgment. We thank Yitzahk Tor and Robert Umek for helpful discussions. We want to thank Joseph C. Kim at CMS for obtaining ^{31}P NMR spectra of **III** and **IV**.

Supporting Information Available: Synthetic procedures and analytical data for new derivatives, including experimental procedures for DNA synthesis and purification, thermal denaturation curves, and HPLC profiles (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA994241M

(14) Robins, M. J.; Barr, P. J. *J. Org. Chem.* **1983**, *48*, 1854−1862.
(15) Tong, G.; Lawlor, J. M.; Tregear, G. W.; Harambidis, J. *J. Org. Chem.* **1983**, *48*, 1854−1862.
(16) Hashimoto, H.; Nelson, M. G.; Switzer, C. *J. Am. Chem. Soc.* **1993**, *115*, 7128−7134.
(17) (a) Kondo, Y.; Watanabe, R.; Sakamoto, T.; Yamanaka, H. *Chem. Pharm. Bull. Jpn.* **1989**, *37*, 2933−2936. (b) Sakamoto, T.; Kondo, Y.; Yamanaka, H. *Chem. Pharm. Bull. Jpn.* **1982**, *30*, 2410−2416.