Preparation of 1-Ethynylpyrene-Modified DNA via Sonogashira-Type Solid-Phase Couplings and Characterization of the Fluorescence Properties for Electron-Transfer Studies

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A range of 1-ethynylpyrene-modified oligonucleotides (Py–≡–dU) has been prepared by a special semi-automated strategy using Sonogashira-type cross-coupling conditions in the solid phase. The absorption and fluorescence properties of the Py–≡–dU-modified single strands and their corresponding double-stranded duplexes have been characterized. The presented spectra show that the Py–≡–dU emission is quenched in DNA duplexes bearing a thymine or cytosine adjacent to the Py–≡–dU group. This shows that the Py–≡–dU moiety is able to inject an electron into the base stack while simultaneously acting as a spectroscopic label to enable the observation of these processes. Hence, Py–≡–dU-modified oligonucleotides are promising donor systems in order to study electron injection and electron transport in DNA spectroscopically.

Introduction

The migration of charge in DNA plays a key role in understanding the routes of DNA damaging, mutations and cancer.[1] Furthermore, charge transfer in DNA has been used in DNA nanotechnology[2] and in order to obtain a highly sensitive electrochemical readout on DNA-modified electrodes and DNA chips.[3] Compared to the broad knowledge about the mechanisms of oxidative hole-transfer and -transport[4] little is known about the migration of excess electrons in DNA. Hence, the mechanisms of oxidative hole-transfer and -transport were simply transferred to the problem of reductive electron-transfer and -transport. Among the DNA bases, cytosine (C) and thymine (T) can be reduced most easily.[5] Accordingly, a hopping mechanism was proposed for the excess electron migration in DNA which involves all base pairs and the pyrimidine radicals ($C^{\cdot -}$ and $T^{\cdot -}$) as intermediate charge carriers.[6]

Most knowledge about reductive electron transfer and transport in DNA comes from γ-pulse radiolysis studies suggesting a thermally activated hopping process above 170 K.[7] Zewail et al. have reported femtosecond time-resolved studies on the reduction of T and C by photoexcited 2-aminopurine in DNA duplexes.[8] Recently, a new DNA assay was published by Carell et al. which allows the determination of the efficiency of the DNA-mediated electron migration by observing the cleavage of T-T dimers. The electron migration was photoinitiated by a flavine derivative as an artificial DNA base.[9] It was shown that the amount of T-T dimer cleavage depends rather weakly on the distance to the flavine moiety, indicating a thermally activated electron- migration process. Lewis et al. have investigated the photoinduced electron injection into DNA hairpins which have been synthetically capped by a stilbene diether derivative serving as the electron donor.[10] The electron injection rates in these systems indicate that the reduction potentials of C and T are slightly different and depend on the hydrogen-bonding pattern in different base pairs. Recently, we contributed to this field by experiments using 5-pyrenyl-2′-deoxyuridine (Py-dU) and 5-pyrenyl-2′-deoxycytidine (Py-dC) as nucleoside models for electron transport in DNA.[11] Our results showed a significant difference of the basicity of the generated pyrimidine radical anions ($C^{\cdot -}$ or $T^{\cdot -}$), which implies a significance for the understanding of electron migration in DNA. Furthermore, in Py-dU-modified DNA duplexes, an efficient photochemically induced electron transport from the Py-dU group occurs only to the adjacent T or C.[12] A structural issue of this DNA assay is the linkage of the pyrene group to the oligonucleotide via a single C−C bond. Therefore, it cannot be excluded that the pyrene moiety interacts directly with bases in the DNA duplex adjacent to the Py-dU group. As a result, the charge-transfer processes could follow this shortcut. In an attempt to reduce these structural concerns, the aim of the work presented herein was to prepare DNA duplexes containing the nucleoside base analog 5-(1-ethynylpyrenyl)-2′-deoxyuridine (Py−≡−dU). The acetylene bridge provides the necessary structural rigidity in order to separate the pyrene group from the base stack, and, additionally, a strong

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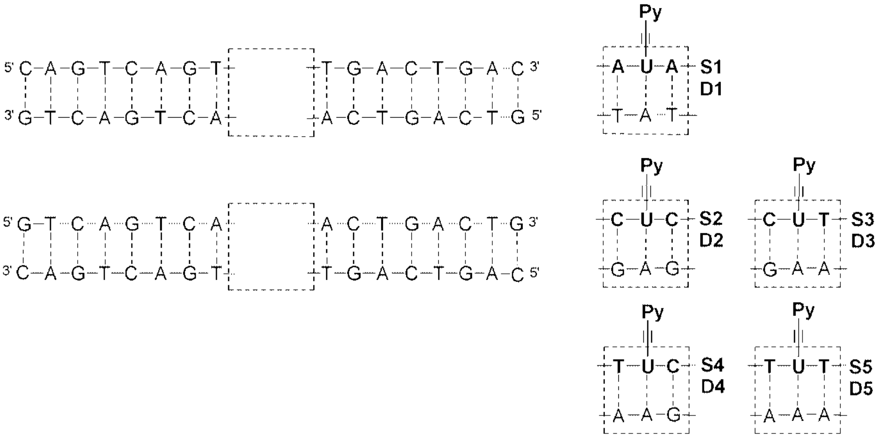
electronic coupling between the pyrene and uridine moiety. Hence, fast electron-injection rates are expected. The emission properties of the Py−≡−dU-modified DNA duplexes were investigated systematically by means of models for electron injection into DNA. Additionally, a fast synthetic approach is presented for the preparation of 1-ethynylpyrenyl-modified oligonucleotides that is not limited by the "bottleneck" of a time-consuming phosphoramidite synthesis.

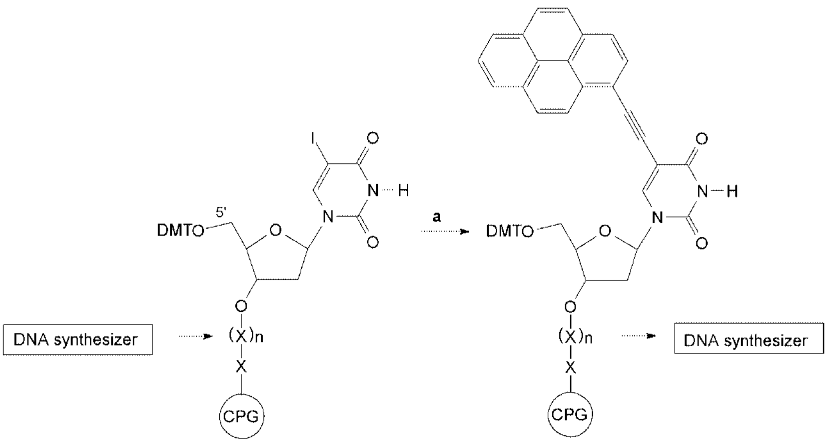
Synthesis of Py−≡−dU-Modified Oligonucleotides

Two structural features of the Py−≡−dU-modified DNA duplexes should allow us to fully control electron injection into DNA: (i) a clear separation of the pyrene moiety from the DNA base stack via an acetylene bridge, and (ii) a partial intercalation of the uracil (dU) as part of the Py−≡−dU moiety. It has been shown in various experiments that intercalation of the charge donor is crucial for a fast and efficient charge transfer.[4,13] Additionally, the Watson−Crick base pairing properties of the dU should be unchanged by attaching the 1-ethynylpyrene moiety, and the introduction of Py−≡−dU should not significantly perturb nucleic acid complexes, since the sterically rigid substituent at the 5-position of dU is directed into the major groove of the duplex. The nucleoside base analog Py−≡−dU has been synthesized previously by Berlin et al.[14] According to their results, the fluorescence intensity of the Py−≡−dU group increases in double stranded DNA compared to single strands and shows a pronounced bathochromic shift.

In the present work, a range of pyrene-modified duplexes (**D1**−**D5**) was prepared, that mainly differ by the bases flanking the Py−≡−dU unit (Scheme 1). The sequences were based on the Py-dU-modified duplexes we have used previously in electron transport experiments.[12] Guanine (G) was not used as a flanking DNA base in order to avoid oxidative hole-injection and hole-transfer processes which can be photoinitiated by pyrene-modified G derivatives.[15]



Scheme 1. Py−≡−dU-modified single-stranded oligonucleotides **S1**−**S5** and DNA duplexes **D1**−**D5**



Scheme 2. Schematic representation of the synthesis of Py−≡−dU-modified oligonucleotides in the solid phase: A DNA strand is synthesized automatically. As the last nucleotide, 5-iodo-2′-desoxyuridine is incorporated. Without deprotection of the 5′-hydroxy group, the DNA synthesizer is stopped. The CPG vials are removed from the synthesizer and a Sonogashira-coupling reagent mixture containing [$Pd(PPh_3)_4$] (60 mM), 1-ethynylpyrene (120 mM), and CuI (60 mM) in DMF/Et_3N (3.5:1.5) was added to the CPG vials under dry conditions via syringes (Step a). After a coupling time of 3 h at room temp., the CPGs were washed with different solvents and dried. The CPG vials were attached to the DNA synthesizer and the synthesis is continued automatically

The nucleoside Py−≡−dU was incorporated by a combination of standard phosphoramidite chemistry and Pd^0-catalyzed Sonogashira-type cross-coupling reactions.[16] Scheme 2 shows a schematic representation of the synthetic strategy: (i) The oligonucleotide was synthesized following standard protocols on a DNA synthesizer up to the position of the Py−≡−dU unit; (ii) 2′-deoxy-5-iodouridine was inserted automatically without the final deprotection of the terminal 5′-OH group; (iii) the CPG vials were subsequently removed from the synthesizer and a Sonogashira-coupling reagent mixture containing $[Pd(PPh_3)_4]$ (60 mM), 1-ethynylpyrene (120 mM), and CuI (60 mM) in DMF/Et_3N (3.5:1.5) was added to the CPG vials under dry conditions with syringes. After a coupling time of 3 h at room temp., the CPGs were washed with different solvents and dried; (iv) The CPG vials were attached to the DNA synthesizer and the synthesis was continued automatically. Modification of the standard procedures for deprotection and cleavage of the oligonucleotides from the solid phase, or during workup, was not necessary. The Py−≡−dU-modified oligonucleotides were purified by semipreparative HPLC on a wide-pore C18 column and identified by MALDI-TOF mass spectrometry (Table 1). The HPLC analysis of the unpurified oligonucleotides showed a nearly quantitative coupling (> 95%) of the 1-ethynylpyrene to the oligonucleotide under the previously mentioned conditions.

Table 1. Calculated and experimentally determined masses of the Py−≡−dU-modified single-stranded oligonucleotides **S1**−**S5**

DNA strand	Mass calcd.	Mass exp.
S1	6022	6025
S2	5974	5976
S3	5989	5989
S4	5989	5989
S5	6004	6004

Spectroscopic Properties of the Py−≡−dU-Modified Single-Stranded Oligonucleotides

Absorption spectra were recorded for the Py−≡−dU-labeled single-stranded oligonucleotides **S1**−**S5** in phosphate buffer (Figure 1). The spectra exhibit similar shapes, including two absorption maxima of the Py−≡−dU unit that are significantly red-shifted (378 and 402 nm) relative to the absorption of the modified nucleosides that are directly linked to the pyrene moiety, such as Py-dU (≈ 340 nm).[12] Hence, this red-shift can be assigned to the influence of the acetylene group on the pyrene moiety. DMSO is known to disrupt the secondary structure of nucleic acids by interrupting the stacking interactions.[17] Therefore UV/Vis spectra of **S1**−**S5** were recorded in DMSO to test the influence of base stacking on the Py−≡−dU absorption and to get an estimation of the extinction coefficient of Py−≡−dU at 260 nm to enable determination of the oligonucleotide concentration by measuring absorption at 260 nm. Remarkably, in DMSO the absorption spectra of all five oligonucleotides **S1**−**S5** (Figure 2) exhibit the same shape and the same absorption maxima (374 and 398 nm). This provides evidence that base-stacking interactions between the Py−≡−dU group and the adjacent bases are indeed interrupted in DMSO, since in aqueous buffer the spectra are significantly different.

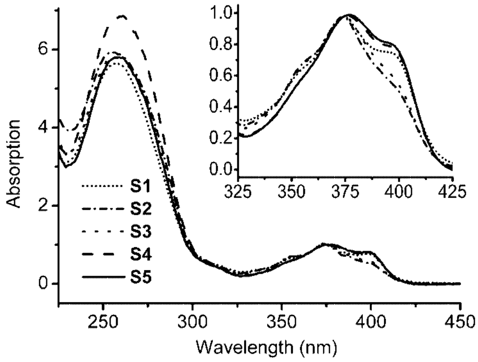


Figure 1. UV/Vis spectra of the single-stranded oligonucleotides **S1**−**S5** in phosphate buffer (10 mM, pH 7.0)

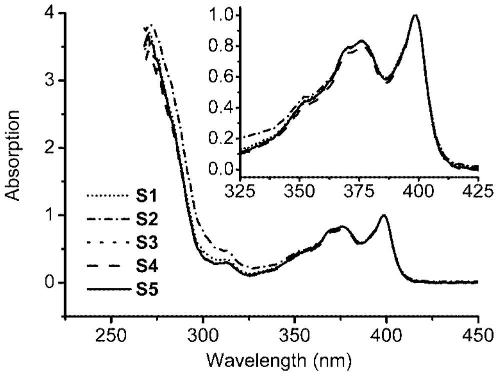


Figure 2. UV/Vis spectra of the single-stranded oligonucleotides **S1**−**S5** in DMSO

The steady-state fluorescence spectra of the single-stranded oligonucleotide samples **S1**−**S5** were recorded in buffer with identical optical densities at an excitation wave-

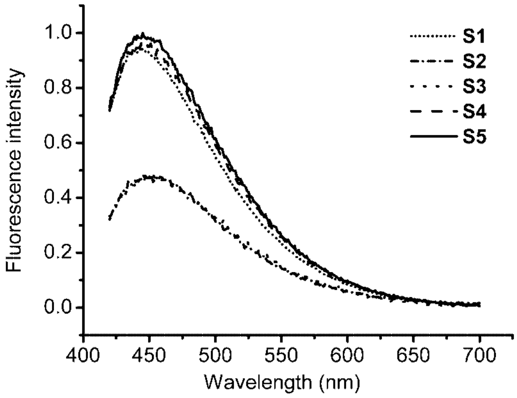


Figure 3. Steady-state fluorescence spectra of the single-stranded oligonucleotides **S1**−**S5** in phosphate buffer (10 mM, pH 7.0), excitation at 402 nm

length of 402 nm, and in DMSO with identical optical densities at an excitation wavelength of 398 nm to find out how the flanking bases effect the quantum yield of the Py−≡−dU group in duplex DNA. The fluorescence signals of the single strands **S1−S5** in aqueous buffer are significantly different (Figure 3) whereas the emission intensities in DMSO are virtually identical (Figure 4). A comparison between the emission spectra of **S1−S5** in aqueous buffer and in DMSO clearly shows that the different emission quantum yields result from stacking and other interactions between the Py−≡−dU group and other bases in the randomly folded single-strands, and that such interactions are interrupted in DMSO.

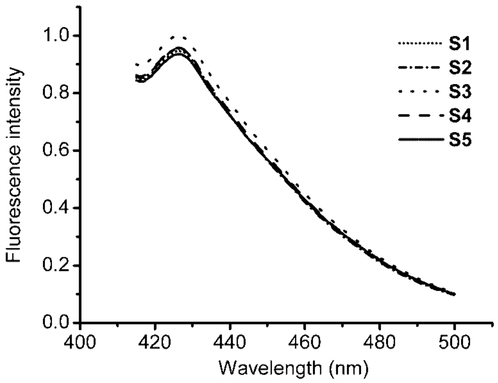


Figure 4. Steady-state fluorescence spectra of the single-stranded oligonucleotides **S1−S5** in DMSO, excitation at 398 nm

Spectroscopic Properties of the Py−≡−dU-Modified DNA Duplexes

It is crucial for the electron-transfer experiments that the pyrene group of the DNA duplexes **D1−D5** is located outside the base stack of the DNA duplex. In contrast to the Py-dU system described previously,[12] the pyrene moiety in **D1−D5** is linked covalently to dU by an acetylene bridge. Thus, the distance between the pyrene group and the bases next to dU can be expected to be too large and too rigid for direct stacking interactions. Furthermore, it should not significantly interfere with the structure and stability of the duplex. To find out if incorporation of the Py−≡−dU group perturbs the DNA duplex structure stability and conformation, the melting temperatures (T_m) and CD spectra of the duplexes **D1−D5** were recorded. The measured T_m values of **D1−D5** were compared with the calculated ones for duplexes containing T instead of the Py−≡−dU group as a reference (Table 2). In general, the melting temperatures of the Py−≡−dU-containing duplexes **D1−D5** are slightly higher than the calculated T_m of the corresponding reference duplexes. This slight increase of between 1 and 3 °C can be attributed to the stabilization of the DNA duplex structure by the acetylene group[18] and is not a result of the intercalation of the pyrene moiety. In the latter case either a significant increase or decrease of T_m would be expected as a result of the hydrophobic stabilization of the pyrene moiety inside the DNA duplex, or as a result of the replacement of the counterbase by the pyrene moiety, respectively.[19] The CD spectra of the duplexes **D1−D5** confirm the overall B−DNA conformation (Figure 5). In summary, it can be concluded that the stability and structure of the duplexes are not significantly perturbed by the incorporation of the nucleotide base analog Py−≡−dU.

Table 2. Melting temperatures (T_m) of the Py−≡−dU-modified DNA duplexes **D1−D5** in phosphate buffer (10 mM), pH 7.0, in comparison with the calculated ones for duplexes containing T instead of the Py−≡−dU group as a reference

DNA	Sequence	T_m	Sequence	T_m
D1	5′-A-Py−≡−dU-A-3′	52 °C	5′-A-T-A-3′	51 °C
D2	5′-C-Py−≡−dU-C-3′	56 °C	5′-C-T-C-3′	55 °C
D3	5′-C-Py−≡−dU-T-3′	55 °C	5′-C-T-T-3′	53 °C
D4	5′-T-Py−≡−dU-C-3′	56 °C	5′-T-T-C-3′	53 °C
D5	5′-T-Py−≡−dU-T-3′	53 °C	5′-T-T-T-3′	51 °C

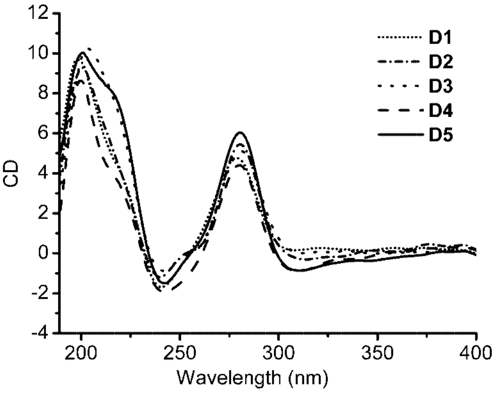


Figure 5. CD spectra of DNA duplexes **D1−D5** (2.5 µM) in phosphate buffer (10 mM, pH 7.0)

Subsequently, absorption and fluorescence emission spectra were measured for all DNA duplexes in aqueous buffer solution. The absorption spectra of the duplexes **D1−D5** with identical concentration show two major maxima in the range of the Py−≡−dU absorption with slightly varying peak positions and intensities (Figure 6). Fluorescence emission spectra were recorded at an excitation wavelength of 402 nm (Figure 7). One can clearly see that, contrary to the measurements of the single strands **S1−S5** in DMSO, the

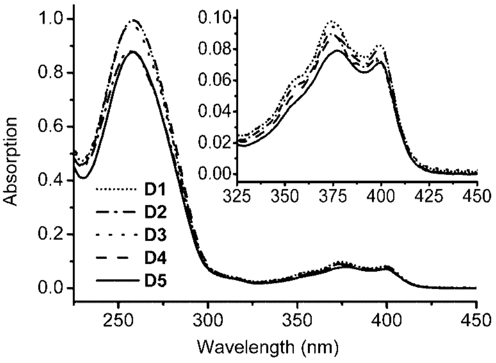


Figure 6. UV/Vis spectra of the DNA duplexes **D1−D5** (2.5 µM) in phosphate buffer (10 mM, pH 7.0)

emission intensities of the duplexes **D1**−**D5** in aqueous buffer vary significantly depending on the bases flanking the Py−≡−dU unit. The relative quantum yields decrease in the following order: **D1** > **D3** ≈ **D2** > **D4** ≈ **D5**. These fluorescence intensities are contrary to the measurements of the single strands **S1**−**S5** in aqueous buffer (Figure 3), where a completely different order was measured: **S5** ≈ **S4** ≈ **S1** > **S3** ≈ **S2**.

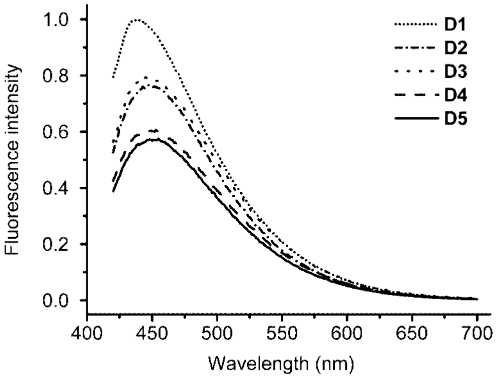
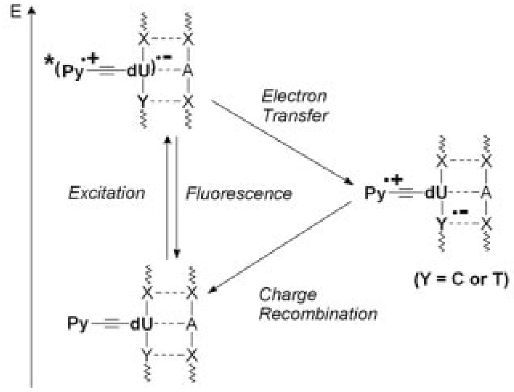


Figure 7. Steady-state fluorescence spectra of the DNA duplexes **D1**−**D5** (2.5 μM) in phosphate buffer (10 mM, pH 7.0), excitation at 402 nm; the emission intensity is corrected by the optical density at the excitation wavelength

Discussion

In the Py−≡−dU unit of the modified oligonucleotides, the two chromophores pyrene and dU are linked covalently by an acetylene bridge. Such systems bear a strong electronic coupling between them and exhibit intense, unstructured fluorescence bands with solvent-dependent maxima. These emissions show that a charge transfer takes place yielding intramolecular exciplexes containing both excited state and charge-separated state character.[20] Such an exciplex emission with a maximum at about 450 nm is observed in the fluorescence experiments with the single-strands **S1**−**S5** in buffer (Figure 3) as well as the duplexes **D1**−**D5** in buffer (Figure 7).

For an assignment of electron-transfer processes, it is important to compare the fluorescence spectra of the single strands **S1**−**S5** in DMSO (Figure 3), where base-stacking and other base-base interactions are interrupted, with the spectra of the double strands **D1**−**D5** in buffer solution (Figure 7). Despite the uncertainty related to irreversible electrochemistry, the following trend for the reducibility of the nucleobases was established: T,U ≈ C >> A > G.[5] In fact, the relative fluorescence intensities of the duplexes **D1**−**D5** follow this trend of the reducibility of DNA bases. A significant quenching of the emission can be observed when a T or C is placed adjacent to the Py−≡−dU group, as is the case, for example, in **D5**. In contrast, duplex **D1** shows the highest emission quantum yield indicating that an electron transfer from the Py−≡−dU group to the adjacent A is unfavorable. With respect to this trend, we conclude that the exciplex of the Py−≡−dU group that is formed upon photoexcitation is only able to reduce adjacent pyrimidine bases, C or T (Scheme 3). This observation can be attributed to a base-to-base electron transfer from the Py−≡−dU group to adjacent pyrimidines. Time-resolved laser spectroscopy measurements are currently being performed in order to obtain the dynamics of the photoexcited processes in the Py−≡−dU-modified oligonucleotides.



Scheme 3. Photoexcited processes in Py−≡−dU-modified DNA duplexes. An electron transfer from the photoexcited Py−≡−dU group results in fluorescence quenching and occurs if T or C is the adjacent DNA base (Y)

Conclusions

Py−≡−dU-modified oligonucleotides can be prepared by a semi-automated synthetic strategy using Sonogashira-type cross-coupling conditions in the solid phase. Using this method, a range of Py−≡−dU-modified DNA duplexes has been synthesized and characterized. The presented absorption and fluorescence spectra indicate that the Py−≡−dU moiety is able to inject an electron into the base stack when a C or T is placed adjacent to the Py−≡−dU group, while simultaneously acting as a spectroscopic label to enable observation of these processes. Thus, the trend of the emission quantum yield of the Py−≡−dU-modified duplexes **(D1** > **D3** ≈ **D2** > **D4** ≈ **D5)** is similar to that observed using the Py-dU-modified duplexes, as reported recently.[12] Hence, Py−≡−dU-modified oligonucleotides are promising donor systems in order to study electron injection and electron transport in DNA.

Experimental Section

Materials and Methods: Solvents were dried according to standard procedures. All reactions were carried out under argon. Chemicals were purchased from Fluka and used without further purification. All spectroscopic measurements were performed in quartz glass cuvettes (1 cm). Absorption spectra were recorded with a Varian Cary 100 spectrometer. Melting curves were measured with DNA duplex (2.5 μM) in Na-Pi buffer solution (10 mM) with NaCl (250 mM), pH 7.0. The temperature was controlled by a Cary temperature control unit, and was increased from 10 to 90 °C at a heating rate of 1 °C/min. The absorption was measured at 260 nm every 1 °C. The fluorescence spectra were recorded with a Fluoromax-3 fluorimeter (Jobin−Yvon). All emission spectra were recorded with a bandpass of 2 nm for both excitation and emission and are intensity corrected. CD spectra were recorded with a Jasco J-15 spectropolarimeter using a Na-Pi buffer solution (10 mM), pH 7.0. CD experiments were performed with DNA duplex (2.5 μM), a step resolution of 1 nm, scan speed of 1000 nm/min, and with 2 nm bandwidth. Five spectra were averaged, and the buffer blank spectra were subtracted.

Preparation of the Oligonucleotides: The unmodified oligonucleotides were prepared on an Expedite 8909 DNA synthesizer from Applied Biosystems by standard phosphoramidite chemistry. Phosphoramidites and CPGs (1 μmol) were purchased from Glen Research, and all other synthesizer chemicals from ABI. After preparation, the trityl-off oligonucleotide was cleaved off the resin and was deprotected by treatment with concd. NH_4OH at 60 °C for 10 h. The oligonucleotide was dried and purified by HPLC on a semipreparative RP-C18 column (300 Å, Supelco) using the following conditions: A = NH_4OAc buffer (50 mM), pH = 6.5; B = MeCN; gradient = 0−15% B over 45 min. The oligonucleotides were lyophilized and quantified by their absorbance at 260 nm.[21] Duplexes were formed by heating to 80 °C (10 min), followed by slow cooling.

Synthesis and Purification of 5-(1-Ethynylpyrenyl)-2′-deoxyuridine-Containing DNA: The solid-phase synthesis was performed in such a manner that the sequence was stopped after the incorporation of 5′-DMT-3′-cyanoethyl-*N*,*N*′-diisopropyl phosphoramidite-2′-deoxy-5-iodouridine, without deprotecting the 5′-hydroxy group or cleaving the oligonucleotide from the resin. The column was subsequently removed from the synthesizer and dried in vacuo. Then the column was attached to a syringe and a reaction solution consisting of ethynylpyrene (60 μmol), $[Pd(PPh_3)_4]$ (30 μmol) and CuI (30 μmol) in dry DMF/Et_3N (3.5:1.5) (0.5 mL) was injected into the column and into another syringe, attached to the other end of the column. The reaction solution was moved back and forth through the column between the two syringes several times to ensure even distribution of the reaction solution. After a coupling time of 3 h at room temp., the reaction solution was discarded, the column washed with DMF/Et_3N (9:1) (10 mL) and dry MeCN (40 mL), dried in vacuo, and reinstalled on the synthesizer. Solid-phase synthesis was resumed, and additional DNA bases were added. The deprotection, cleavage from resin and purification by HPLC was performed as described above, but the gradient for HPLC purification was run from 0−30% MeCN over 45 min.

The concentrations of the oligodeoxynucleotides containing Py−≡−dU were determined by measuring their absorption at 260 nm using the following extinction coefficients ε_{260}: 219.3 $\text{mM}^{-1}\,\text{cm}^{-1}$ for **S1**, 204.7 $\text{mM}^{-1}\text{cm}^{-1}$ for **S2**, 206.0 $\text{mM}^{-1}\text{cm}^{-1}$ for **S3**, 206.0 $\text{mM}^{-1}\text{cm}^{-1}$ for **S4**, 207.4 $\text{mM}^{-1}\text{cm}^{-1}$ for **S5**. The extinction coefficients were determined by measuring the absorption of **S1**−**S5** at 260 nm and 392 nm in DMSO to interrupt base stacking and therefore allow estimation of the extinction coefficient of Py−≡−dU at 260 nm, which was calculated according to the following equation: ε_{260}(Py−≡−dU) = ε_{392}(Py−≡−dU) * A_{260}(**S*n***)/A_{392}(**S*n***) − ε_{260}(DNA$_n$). ε_{260}(Py−≡−dU) is the extinction coefficient of Py−≡−dU at 260 nm, ε_{392}(Py−≡−dU) is the extinction coefficient of Py−≡−dU at 392 nm, A_{260}(**S*n***) is the absorbance at 260 nm of a Py−≡−dU-modified DNA strand **S*n*** (n = 1−5), A_{392}(**S*n***) is the absorbance at 392 nm of the same Py−≡−dU-modified DNA strand **S*n*** (n = 1−5), and ε_{260}(DNA$_n$) is the extinction coefficient of the unmodified DNA$_n$. With an ε_{392}(Py−≡−dU) value of 42 $\text{mM}^{-1}\text{cm}^{-1}$ the resulting ε_{260}(Py−≡−dU) was calculated to be 40 $\text{mM}^{-1}\text{cm}^{-1}$, which could then be used to calculate the ε_{260}(**S*n***). Masses of DNA strands **S1** to **S5** were determined by MALDI-TOF mass spectrometry to confirm the correct base sequence and the successful incorporation of the Py−≡−dU group.

Acknowledgments

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