Excess Electron Transfer in Flavin-Capped DNA-Hairpins

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DNA hairpins with an electron-acceptor molecule in the head region have recently allowed Lewis and Wasielewski to gain important insight into the hole conductivity of double helical DNA. In light of our current interest in deciphering the excess electron transport properties of double helical DNA, we evaluate in this report three different flavin molecules for their ability to form a stable DNA hairpin cap. The intention was to construct novel DNA hairpins in which the flavin chromophore is perfectly stacked on top of the final base pair. A solid-phase synthesis protocol was devised for the incorporation of the three flavin-*H*-phosphonates into oligonucleotides using a mixed phosphoramidite/*H*-phosphonate/phosphoramidite protocol. We describe here that all three flavin-cap molecules yield stable DNA hairpins. UV, fluorescence and melting temperature studies showed that one of the three flavins is stacked in the intended fashion. Initial studies show that the flavin in the reduced and deprotonated state can indeed inject an electron into the hairpin. This flavin-capped DNA hairpin allows a detailed investigation of the excess electron transfer capabilities of double helical DNA.

Introduction

Hairpin-shaped oligonucleotides are DNA strands that possess a self-complementary stem and a loop region, known as the "head" of the hairpin.[1−3] DNA hairpins are stably folded oligonucleotides with a sharp, concentration-independent melting point. This feature has made DNA hairpins ideal model systems for oligonucleotide structure and function. As such, hairpin DNA molecules containing a non-nucleotide electron donor or acceptor in the "head" region have been used as model systems to investigate fundamental questions related to the electron-transfer capabilities of DNA. Lewis and Wasielewski have used hairpins with a stilbene electron acceptor "head" to measure the critical β-value of electron transfer from guanines to the stilbene head. This β-value describes the distance dependence of the superexchange electron transfer through DNA.[4−8] The DNA hairpin experiments revealed a β-value of 0.7, which was at that time in sharp contrast to other reports placing the β-value well below 0.1.[9] The hairpin model study therefore laid the foundation for our current understanding of hole transfer through DNA. It is now well accepted that positive holes do not move through DNA by superexchange alone. Giese and Schuster were among the first to propose that long-range hole movement through a DNA duplex is best described by a hopping mechanism, which is far less distance-dependent than a superexchange transfer.[10,11]

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We are currently investigating how excess electrons as negative charges move through DNA.[12,13] In this context, γ-irradiation studies suggest that excess electrons might hop through DNA as well.[14−18] Sevilla and co-workers proposed a thermally activated excess electron hopping mechanism at temperatures above −70 °C, while below this temperature superexchange is believed to dominate.

Studies by us with defined donor-DNA-acceptor model compounds at room temperature showed recently that an excess electron can move surprisingly efficiently over a distance of up to 25 Å.[12] These studies were possible because a flavin in its reduced and deprotonated redox state could be inserted into DNA, where it functions as a strong electron donor. This unusual donor possesses a very negative reduction potential of at least −2.6 V,[19,20] which is low enough to achieve efficient single-electron reduction of pyrimidines and cyclobutane pyrimidine dimers. These dimer acceptors undergo rapid cycloreversion upon single-electron reduction, which allows rapid monitoring of the electron capture event.[21−23] The same flavin donor used in our experiments is also found in nature. The enzyme DNA photolyase repairs UV-induced mutagenic cyclobutane pyrimidine dimer DNA lesions by single electron donation. This process rescues many organisms from UV-induced cell death.[24,25]

In order to gain further insight into the mechanism of long-range excess electron transfer, the synthesis of defined DNA hairpins with a flavin donor in the "head" region and a cyclobutane pyrimidine dimer electron acceptor in the stem is required. These hairpins are structurally much better defined then the double strands used in our previous studies. This is important for precise studies, because elec-

tron-transfer rates depend not only on the distance between the donor and the acceptor but are also influenced by the orientation of the chromophores relative to each other. In structurally better defined hairpin donor-DNA-acceptor systems, the electron-transfer processes can be studied with much higher accuracy and without any interference because of incomplete duplex formation.

Herein we report the design and the synthesis of stable flavin-capped DNA hairpins in which the flavin stacks on top of the last base pair in the hairpin. We also report an initial electron injection study. These novel hairpins open the door for a deeper mechanistic investigation of excess electron transfer processes in DNA.

Results and Discussion

Design and Synthesis of Flavin-Capped DNA Hairpins

The critical point for the synthesis of a stably folded flavin-capped DNA hairpin is the length of the two chains connecting the flavin with the DNA. These chains have to be long enough to avoid any disruption of the B-form stem structure. If, on the other hand, the chains are too long then the strictly required stacking of the flavin on top of the final base pair, needed for efficient charge injection into the base stack, may not occur. In order to find the optimal chain length, we first performed detailed molecular modeling studies using the SYBYL (Tripos version 6.8) software package and the force field MMFF94s. All calculations were performed in the gas phase and also in water. From these studies we could narrow down the number of possible flavin cap molecules that fulfil the above requirements. The three most promising calculated hairpin molecules **1**−**3**, together with the structures of the flavin-cap molecules, are depicted in Figure 1. The flavin cap of hairpin **1** contains two C6-chains at *N*(3) and *N*(10) of the flavin. From our modeling, depicted in Figure 1, we concluded that the hairpin is formed but the chain length is clearly on the long side, resulting in a slightly too-flexible connection, which would then disturb the efficient stacking of the flavin. The flavin-cap molecule in **3** has two C3 chains at *N*(3) and *N*(10) and, in our series, is the smallest solution. The calculations predict perfect stacking. Slight disturbance of the hairpin helix structure was, however, also noted. A shorter chain length resulted in a marked increase in duplex destabilization. The flavin-cap molecule used to build up hairpin **2** features one C6 chain at *N*(10) and one C3 chain at *N*(3). This flavin gave the best modeling results. We calculated almost perfect stacking of the flavin on top of the final base pair and almost no helix disturbance.

Based on the modeling results we rejected all other possible flavin capping molecules and concentrated on the synthesis of the three most promising hairpin molecules **1**−**3**, using the synthetic strategy depicted in Scheme 1. The starting point for the synthesis was dimethyldinitrobenzene (**4**), which was reacted with either 1-aminopropan-3-ol (**5**) or 1-amino-hexan-6-ol (**6**). The nitro groups of the products were subsequently hydrogenated and the resulting amines were added, without prior isolation, to a solution containing alloxan and boric acid in acetic acid following a

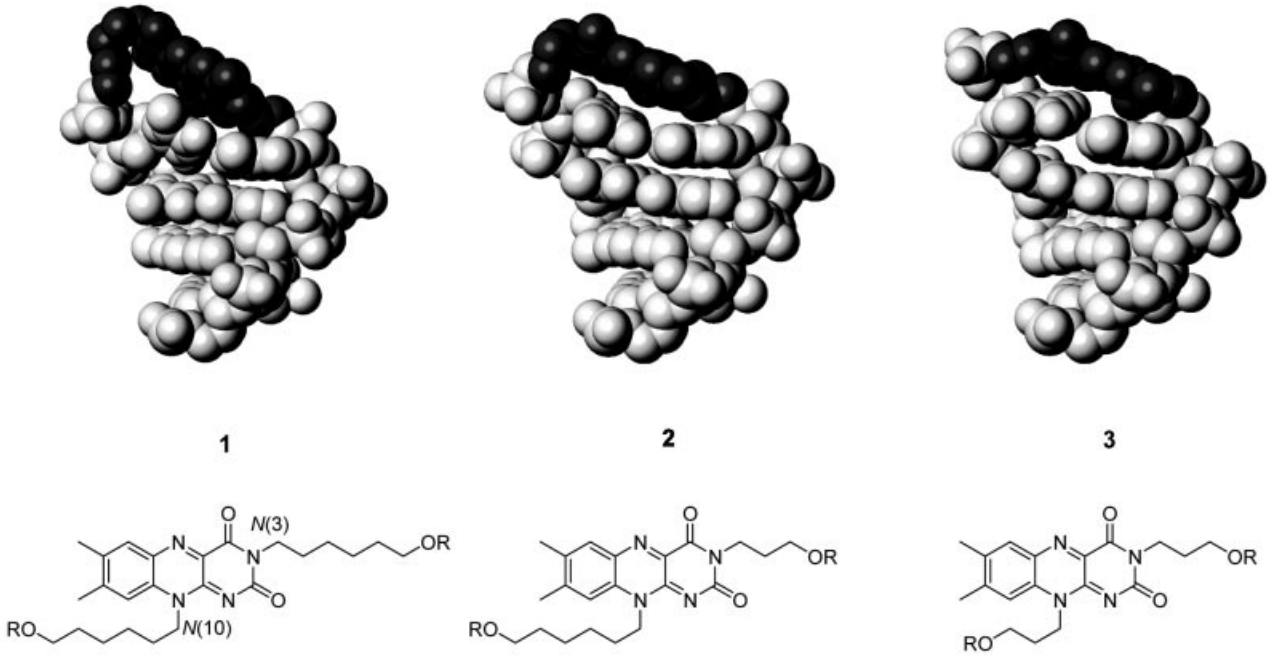
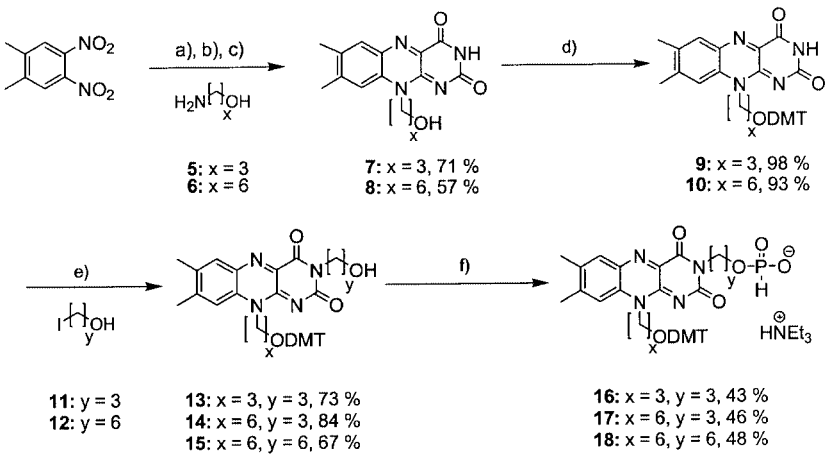


Figure 1. Calculated (SYBYL, Tripos software version 6.8, force field MMFF94s in the gas phase) structures of the three flavin-capped DNA hairpins **1**−**3** and of the three flavin capping molecules



Scheme 1. Synthesis of the flavin *H*-phosphonates **16−18** used for the preparation of the flavin capped DNA hairpins **1−3**; a) pyridine, 19 h reflux; b) H_2, Pd/C; c) HOAc, $B(OH)_3$, alloxan, room temp.; d) DMTrCl, pyridine, room temp. 93−98%; e) Cs_2CO_3, DMF, 16 h, 67−84%; f) PCl_3, triazole, *N*-methylmorpholine, 43−48%

flavin synthesis protocol developed by Kuhn and coworkers.[26,27] Subsequent protection of the hydroxyl groups of the flavin products **7** and **8** with dimethoxytrityl chloride provided the flavins **9** and **10** as yellow powders. These flavins possess excellent solubility in most organic solvents. Subsequent alkylation of both flavins **9** and **10** at *N*(3) with either 1-iodopropan-3-ol (**11**) or 1-iodo-hexan-6-ol (**12**), using Cs_2CO_3 as the base in DMF, provided the three flavin products **13−15**. Final conversion of the hydroxyl groups into the *H*-phosphonates gave the three flavin *H*-phosphonate target compounds **16−18**, ready for the incorporation into oligonucleotides.

The synthesis of the oligonucleotide hairpins **1−3** was performed on an Expedite 8900 DNA synthesizer using a mixed phosphoramidite/*H*-phosphonate/phosphoramidite coupling protocol.[28−31] This complicated protocol is required because flavin phosphoramidites are efficiently oxidized under aerobic conditions.[32,33] A complete *H*-phosphonate protocol is also not applicable because only *H*-phosphonates with standard protecting groups are commercially available. These require harsh deprotection conditions, which were found to be incompatible with the base-labile flavin molecule. Deprotection of flavin-containing oligonucleotides requires mild conditions, which forced us to use very base-labile nucleotide protecting groups. These are only available for phosphoramidite chemistry.

Synthesis of the DNA hairpins **1−3** starts with standard phosphoramidite chemistry. After cleavage of the DMT-protecting group with dichloroacetic acid at the 3′ end to the flavin, the synthesizer was programmed to pump simultaneously the flavin *H*-phosphonate and adamantoyl acid chloride. Coupling of the flavin building blocks **16−18** was extended to about 10 min (as opposed to 2 min for standard phosphoramidite chemistry). After coupling of the flavin we oxidized the *H*-phosphonate to the phosphate using iodine in H_2O/MeCN/lutidine. The DMT group of the flavin was subsequently cleaved and the DNA synthesis was continued using phosphoramidite chemistry, even in the presence of the unprotected phosphate. Cleavage of the DNA from the solid support material and of all nucleobase protecting groups was performed at the end of the synthesis by shaking of the solid support material in a solution made up of three parts ammonia (25%) in water and one part ethanol at 20 °C for 16 h. All oligonucleotides were subsequently purified by reversed-phase HPLC on a C18 Nucleosil reversed-phase column and characterized by MALDI-Tof mass spectrometry.[13,31] We frequently observed two DNA strands, corresponding to oligonucleotides obtained by cleavage of the phosphate backbone at the 5′ position to the flavin base, after deprotection rather than the desired oligonucleotide. We reasoned that this cleavage is caused by incomplete oxidation of the *H*-phosphonate on the solid support followed by cleavage of the *H*-phosphonate under the basic deprotection conditions. In all future syntheses of flavin-containing DNA we therefore pumped two different oxidizing solution through the cartridge for an additional 15 min after completion of the DNA synthesis. We first pumped a solution made up of 0.1 M I_2 in MeCN/H_2O with *N*-methylmorpholine as the base. As a second oxidizing solution 0.1 M I_2 in MeCN/H_2O with NEt_3 as the base was used. This two step oxidation protocol fully converted the *H*-phosphonate into the phosphate, which then allowed us to get the flavin-containing oligonucleotides in good to excellent yields without any observable cleavage at the original *H*-phosphonate side.

Characterization and Stability Studies of the Flavin-Capped Hairpins 1−3

In order to prove that the flavin chromophore survived the complicated synthesis and deprotection chemistry we measured UV/Vis and fluorescence spectra of the obtained DNA strands **1−3**. Two representative spectra are depicted in Figure 2; they were measured with DNA strand **2**. Similar data, however, were recorded for all three DNA strands **1−3**. All spectra are very similar to that of riboflavin itself, showing that the chromophore system of the flavin is not

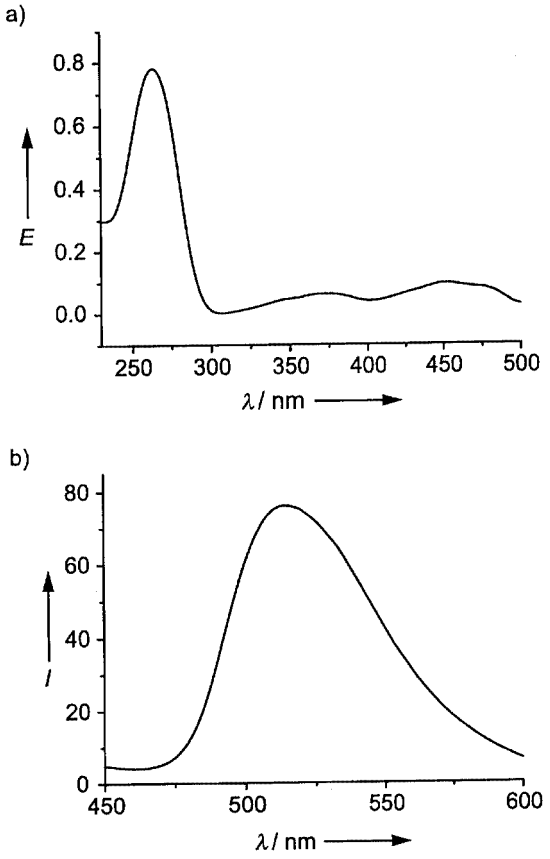


Figure 2. a) UV/Vis spectrum of the flavin-capped hairpin **2** ($c_{DNA} = 3 \times 10^{-6}$ M in 0.01 M Tris-buffer, 150 mmol NaCl, pH 7.4); b) fluorescence spectrum of the flavin-capped hairpin **2** ($c_{DNA} = 3 \times 10^{-6}$ M in 0.01 M Tris-buffer, 150 mM NaCl, pH 7.4), excitation at 366 nm; similar spectra were recorded for the hairpins **1** and **3** as well.

modified during DNA synthesis, oxidation and deprotection.

The purity of the final oligonucleotides was confirmed by HPLC showing a single sharp peak without shoulders (data not shown). All three mass spectra are in full agreement with the calculated molecular masses of the oligonucleotides **1**–**3** (see Exp. Sect.). All these data together prove that this mixed protocol allows embedding of a flavin cofactor into oligonucleotides.

In order to investigate if the three DNA strands **1**–**3** form stable hairpins at room temperature and to investigate potential stability differences, we measured all three melting points. In agreement with the calculations, which predicted that all three molecules **1**–**3** should form a stable hairpin, we observe for all three oligonucleotides a concentration-independent melting point (Figure 3), although the absolute values were different. The highest melting point was measured for the hairpin **2** (T_m = 43 °C) containing the flavin with one C3 and one C6 chain. The hairpin **3** featuring the flavin with two C3 chains is strongly destabilized (T_m = 31 °C) indicating that the chain length is much too short to bridge the two oligonucleotide strands efficiently. Hairpin **1**, which is capped with the flavin having two C6 chains is slightly less stable than **2** possibly because the chain flexibility is too high, which inhibits perfect stacking (T_m = 41 °C). All these results are in full agreement with our calculations, which predicted that the flavin with one C3 and one C6 chain would be the best cap of an oligonucleotide structure.

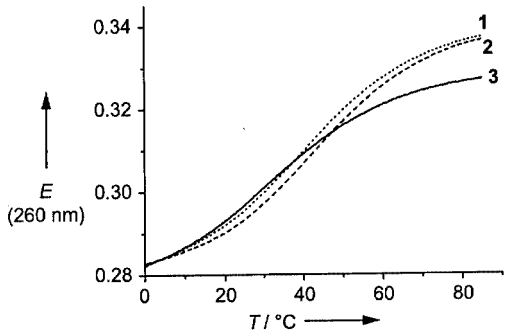


Figure 3. UV/Vis melting curve of the three flavin-capped DNA hairpins **1**–**3** ($c_{DNA} = 3 \times 10^{-6}$ M in 0.01 M Tris-buffer, 150 mM NaCl, pH 7.4), measured at 260 nm

This DNA hairpin **2** was further investigated by CD spectroscopy. The obtained CD spectrum is depicted in Figure 4. It shows the typical curve for a B duplex, which underpins once more that the flavin cap does not disturb the B conformation of the oligonucleotide stem. The data prove, furthermore, that the oligonucleotide **2** is stably folded.

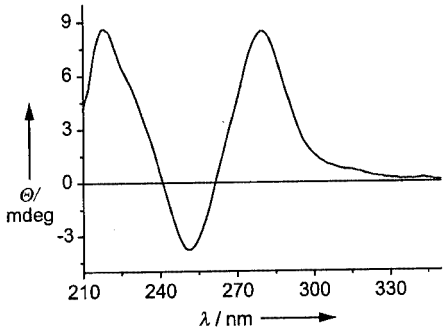


Figure 4. CD spectrum (averaged from 10 independent measurements) of the flavin-capped molecular hairpin **2** ($c_{DNA} = 3 \times 10^{-6}$ M in 0.01 M Tris-buffer, 150 mM NaCl, pH 7.4)

If the flavin is stacking on top of the final base pair one would expect the fluorescence intensity of the flavin chromophore to be modulated. In fact, both fluorescence reductions and fluorescence increases are frequently observed if fluorescent aromatic ring systems stack in or on top of DNA base pairs.[34] In order to investigate this question further we measured a fluorescence melting curve for hairpin **2** (Figure 5). The obtained fluorescence melting curve is fascinating and clearly proves that the flavin in hairpin **2** stacks on top of the final A-T base pair. The fluorescence intensity of a flavin alone decreases with increasing temperature. At the melting temperature of the hairpin, however, we observe against this trend a strong fluorescence increase. After complete melting the temperature-dependent fluorescence intensity decrease continues. From the spectrum it is clearly evident that the flavin fluorescence is reduced upon folding

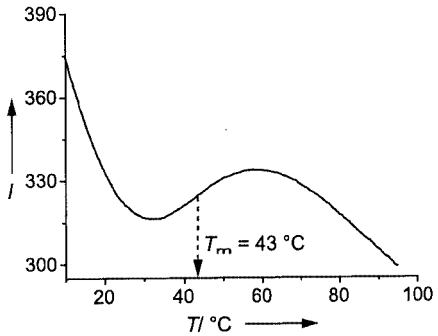


Figure 5. Fluorescence melting curve of the flavin-capped DNA hairpin **2** ($c_{DNA} = 3 \times 10^{-6}$ M in 0.01 M Tris-buffer, 150 mM NaCl, pH 7.4); excitation at 366 nm, emission observed at 520 nm

of the oligonucleotide into the hairpin structure. This result, together with our calculations, are, for us, a clear proof that the flavin stacks on top of the final base pair. This stacking reduces the fluorescence intensity.

Excess Electron Transfer Studies

In order to investigate if the flavin is able to inject electrons into the DNA hairpin we prepared for an initial study the hairpin molecule **19** (Figure 6). Compound **19** contains a pyrimidine dimer building block with an opened backbone next to the flavin cap. Upon single-electron reduction, this dimer will induce a strand break as recently described by us.[12,35] Hairpin **19** is stable as long as the flavin molecule exists in the oxidized state. For the electron injection experiment, we prepared a solution of hairpin **19** in Tris-buffer (10 mM Tris, c_{DNA} = 20 μM, pH = 7.4) and added 50 μL of this solution into four small glass tubes stoppered with a rubber septum. After purging the solutions for 10 min with nitrogen, a sodium dithionite solution was added into each vial to achieve flavin reduction. The yellow color immediately vanished confirming successful reduction of the flavin. These four vials were finally irradiated (366 nm) at T = 5 °C for 0 min, 15 min, 30 min and 60 min, respectively. The assay solutions were finally exposed to air and shaken to rapidly re-oxidise the flavin. The solutions were then analysed by ion-exchange chromatography (Nucleogel SAX column, Gradiant: 0.2 M NaCl to 1 M NaCl over 35 min). The HPL-chromatograms obtained are shown in Figure 6. It is clearly evident that at 0 min of irradiation just the flavin hairpin **19** is eluting, with a retention time of 23 min. Further irradiation causes a clear decrease of the DNA hairpin **19** and the appearance of a peak for the DNA strand **20**, which elutes at about 16 min. This DNA strand is the hairpin cleaved at the thymine dimer site. This experiment clearly proves that the flavin cap can inject a single electron into the hairpin, which triggers the cycloreversion reaction due to capturing of the electron by the dimer acceptor.

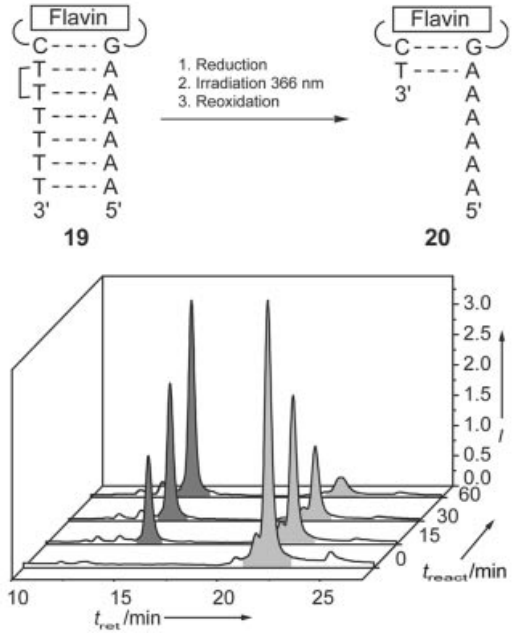


Figure 6. Flavin-capped cyclobutane pyrimidine dimer containing DNA hairpin **19** and cleavage into **20** upon electron injection into the DNA strand by the flavin in the reduced state; depiction of the HPL chromatograms obtained during an irradiation study

Conclusions

Our goal was the synthesis of flavin *H*-phosphonate, which can be used to create a flavin cap for the construction of DNA hairpin molecules. Particular emphasis was placed in our design on the ability of the flavin cap to stack perfectly on top of the final base pair. This is required if the flavin is to act as an efficient charge injector into DNA, as required for electron-transfer studies through DNA. Computer modeling was performed first of all and three promising flavin *H*-phosphonates **16**−**18** were selected that were calculated to give stably folded hairpin molecules (**1**−**3**). These three flavin molecules, one with two C3 chains (**16**), one with two C6 chains (**18**) and one with one C3 and one C6 chain (**17**) were synthesized and incorporated into oligonucleotides using a mixed phosphoramidite/*H*-phosphonate/phosphoramidite coupling protocol. The structure and stability of the three flavin-containing oligonucleotides **1**−**3** were investigated. It was found that all three flavins allow the formation of oligonucleotide hairpins. The C3/C6 chain flavin *H*-phosphonate **17**, however, gives the most stable hairpin. Further experiments prove that this flavin stacks perfectly on top of the final base pair. The flavin *H*-phosphonate **17** is therefore perfectly suited for the construction of a series of flavin-containing DNA hairpins for excess electron transfer studies through DNA. In order to study if the flavin cap is indeed injecting excess electrons into DNA, we synthesized the flavin-capped hairpin **19**, containing in addition a cyclobutane thymine dimer, which undergoes rapid cycloreversion upon electron capture resulting in a strand break. Irradiation of this hairpin containing the flavin in the reduced, and hence electron donating, state indeed gave rapid strand cleavage. This experiment showed that flavin-capped hairpins are perfectly suited to

study excess electron transfer in DNA. Further synthesis of other flavin-capped, dimer-containing hairpins and investigation of the excess electron transfer process are now under way.

Experimental Section

General: Reagents and solvents were reagent grade and used without further purification. Anhydrous Na_2SO_4 was used as the drying agent after aqueous workup. Evaporation and concentration in vacuo was done at H_2O aspirator pressure. All reactions were performed in standard glassware. Degassing of solvents was accomplished by N_2 purging for at least 4 min. Column chromatography (CC): Silica gel-*H* from Fluka. TLC: glass or aluminium sheets covered with silica gel 60 F_{254} from Merck, visualization by UV light. M.p.: Büchi SMP-20 apparatus; uncorrected. UV/Vis spectra: Varian Bio100 spectrophotometer at room temperature; λ_{max} in nm (ε in $M^{-1}cm^{-1}$). Melting points were measured using a Cary temperature controller and a sample transport accessory with a multicell block and a temp. gradient of 0.5 °C/min. Oligonucleotides were dissolved in a Tris buffer (10 mM Tris, 150 mM NaCl, pH 7.4). CD spectra were recorded on a JASCO J-810 at 10 °C. IR spectra (cm^{-1}) were recorded in KBr and measured in cm^{-1}, with a Bruker IFS 25 Fourier transform infrared spectrophotometer. Fluorescence spectra were measured on a JASCO FP750 Fluorimeter, single monochromator with ±5 nm, 150 Hg/Xe lamp, in 1 cm quartz cuvettes at room temperature. Melting points were measured using a JASCO temperature controller. Temp. gradient 0.5 °C/min. Mass spectra were recorded with a Bruker Flex III Maldi-Tof mass spectrometer (matrix: 2,4,6-trihydroxyacetophenone, 0.5 M in H_2O/diammonium citrate/0.1 M in H_2O) and on a Finnigan TSQ 700. Oligonucleotide synthesis was performed using an Expedite 8900 Gene synthesizer. Pac amidites were purchased from Pharmacia or Glen Research. A controlled-pore glass bead support was purchased from Perseptive. Acetonitrile for the oligonucleotide synthesis was obtained from Roth. All solvents were stored for 12 h over 4 Å molecular sieves prior to oligonucleotide assembly. HPLC was performed with a Merck-Hitachi Lachrome HPLC system with a flow of 1 mL/min using a Nucleosil RP18 (250 mm × 4 mm, 100 Å /5 μm or 250 mm × 4 mm, 120 Å/3 μm) column from Macherey−Nagel. HPLC-grade solvents were purchased from Riedel de Haen. All oligonucleotides were detected at 260 nm. For analytical oligonucleotide HPLC chromatography a linear gradient was used. Preparative HPLC was performed with a Nucleosil RP C18 column (250 mm × 10 mm, 100 Å/7 μm). Solvent system: A = 0.1 M NEt_3/HOAc in H_2O, B = 0.1 M NEt_3/HOAc in H_2O:CH_3CN (1:4).

10-{3-[Bis(4-methoxyphenyl)phenylmethoxy]propyl}-7,8-dimethyl-10*H*-benzo[*g*]pteridin-2,4-dione (9): For the analytical data of compounds **5**−**8** see refs.[33,36] The flavin alcohol **7** (0.194 g, 0.65 mmol) was dissolved in pyridine (15 mL) and stirred with molecular sieves (4 Å) for 1 h. 4,4′-Dimethoxytrityl chloride (372 mg, 1.10 mmol) was added and the reaction mixture was stirred at room temp. for 16 h. Methanol (2.0 mL) was added and the reaction mixture was stirred for a further hour. The solvent was evaporated and the solid residue was dissolved in chloroform and filtered. The organic phase was washed once with satd. $NaHCO_3$ solution and twice with H_2O. After drying with Na_2SO_4 and filtration the solvent was evaporated and the crude product was purified by flash chromatography with $CHCl_3$/MeOH/NEt_3 (20:1:0.2) as eluent. Compound **9** (0.398 g, 0.64 mmol, 98%) was obtained as a yellow solid. R_f ($CHCl_3$/MeOH/NEt_3, 10:1:0.2) = 0.61. M.p. 233 °C (decomp.). IR (KBr): $\tilde{\nu}$ = 3447 w cm^{-1}, 3160 w, 3107 w, 3031 m, 2954 m, 2835 w, 1706 m, 1652 s, 1609 m, 1576 s, 1516 s, 1502 s, 1464 m, 1250 s, 1174 m, 1155 s, 1075 m, 1031 m, 825 m. ^{1}H NMR (200 MHz, $CDCl_3$): δ = 2.10 (m, 2 H, CH_2), 2.40 (s, 3 H, CH_3), 2.42 (s, 3 H, CH_3), 3.27 (d, *J* = 5.6 Hz, 2 H,CH_2O), 3.71 (s, 6 H, OCH_3), 4.75 (t, *J* = 8.0 Hz, 2 H, CH_2N), 6.75 (d, *J* = 8.4 Hz, 4 H, $CH_{aromatic}$), 7.15−7.45 (m, 9 H, $CH_{aromatic}$), 7.65 (s, 1 H, CH_{flavin}), 7.99 (s, 1 H, CH_{flavin}) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ = 19.5, 21.5, 27.9, 43.6, 55.6 (2 C), 60.8, 86.5, 113.6 (4 C), 116.1, 127.4, 128.3 (4 C), 130.3 (4 C), 131.2, 133.1, 135.4, 136.2 (2 C), 136.3, 137.5, 145.3, 148.8, 150.1, 155.6, 158.9 (2 C), 159.9 ppm. MS (FD): *m*/*z* = 602 [M^+], 303 [DMT^+]. HR-MS ESI calcd. for $C_{36}H_{34}N_4O_5$ + Na^+: 625.2427; found 625.2421.

10-{6-[Bis(4-methoxyphenyl)phenylmethoxy]hexyl}-7,8-dimethyl-10*H*-benzo[*g*]pteridine-2,4-dione (10): The flavin alcohol **8** (0.100 g, 0.29 mmol) was dissolved in pyridine (9 mL) and stirred with molecular sieves (4 Å) for 1 h. 4,4′-Dimethoxytrityl chloride (168 mg, 0.50 mmol) was added and the reaction mixture was stirred at room temp. for 16 h. Methanol (1.0 mL) was added and the reaction mixture was further stirred for 1 h. The solvent was evaporated and the solid residue was dissolved in chloroform and filtered. The organic phase was washed with a satd. $NaHCO_3$ solution and twice with H_2O. After drying with Na_2SO_4 and filtration the solvent was evaporated and the crude product was purified by flash chromatography with $CHCl_3$/MeOH/NEt_3 (20:1:0.2) as eluent. Compound **10** (0.176 g, 0.27 mmol, 93%) was obtained as a yellow solid. R_f ($CHCl_3$/MeOH 20:1): 0.40. M.p. 155−156 °C. IR (NaCl): $\tilde{\nu}$ = 3481 w cm^{-1}, 3030 m, 2933 m, 2862 w, 2834 w, 1772 m, 1718 s, 1684 s, 1576 s, 1507 s, 1458 m, 1247 s, 1173 m, 1031 m, 826 m, 735 m. ^{1}H NMR (200 MHz, $CDCl_3$): δ = 1.35−1.55 (m, 4 H, CH_2), 1.58 (m, *J* = 6.4 Hz, 2 H, CH_2), 1.7−1.9 (m, 2 H, CH_2), 2.37 (s, 3 H, CH_3), 2.46 (s, 3 H, CH_3), 2.98 (t, *J* = 6.2 Hz, 2 H,CH_2O), 3.71 (s, 6 H, OCH_3), 4.59 (t, *J* = 7.6 Hz, 2 H, CH_2N), 6.74 (d, *J* = 7.0 Hz, 4 H, $CH_{aromatic}$), 7.1−7.4 (m, 10 H, $CH_{aromatic}$), 7.99 (s, 1 H, CH_{flavin}) ppm. ^{13}C NMR (50 MHz, $CDCl_3$): δ = 19.3, 21.4, 25.9, 26.5, 26.9, 29.7, 45.0, 54.9 (2 C), 62.9, 85.4, 112.9 (4 C), 115.0, 126.3, 127.4 (2 C), 127.9 (2 C),128.9, 129.7 (4 C), 130.8, 132.6, 134.7, 136.0, 136.3 (2 C), 145.1, 148.0, 149.6, 155.1, 158.1 (2 C), 159.4 ppm. MS (FD): *m*/*z* = 644 [M^+], 342 [M^+ − DMT], 303 [DMT^+]. HR-MS ESI calcd. for $C_{39}H_{40}N_4O_5$ + Na^+: 667.2896; found 667.2863.

10-{3-[Bis(4-methoxyphenyl)phenylmethoxy]propyl}-3-(3-hydroxypropyl)-7,8-dimethyl-10*H*-benzo[*g*]pteridine-2,4-dione (13): 1-Iodopropan-3-ol was prepared according to the literature.[37] The flavin **9** (150 mg, 0.25 mmol) was dissolved in dry DMF (5.3 mL) and stirred with molecular sieves (4 Å) for 2 h. To this solution, Cs_2CO_3 (117 mg, 0.36 mmol) was added and the mixture was stirred for 15 min at room temp. 1-Iodopropan-3-ol (**11**; 0.15 mL, 1.56 mmol) was added dropwise and the mixture was stirred for 16 h at room temp. The reaction mixture was then filtered, diluted with chloroform (150 mL) and the organic phase was washed once with saturated $NaHCO_3$ solution and twice with water. After drying over Na_2SO_4 the solvent was evaporated and the crude product was purified by flash chromatography with $CHCl_3$/MeOH/NEt_3 (30:1:0.2) as eluent. Compound **13** (0.120 g, 0.18 mmol, 73%) was isolated as a yellow oil. R_f ($CHCl_3$/MeOH/NEt_3, 15:1:0.2): 0.61. IR (NaCl): $\tilde{\nu}$ = 3471 m, 3057 w, 3034 w cm^{-1}, 2954 m, 2932 m, 2876 w, 2837 w, 1703 s, 1657 s, 1607 m, 1584 s, 1548 s, 1510 s, 1462 m, 1251 s, 1200 s, 1069 m, 1032 s, 911 s, 828 s, 727 s. ^{1}H NMR (200 MHz, $CDCl_3$): δ = 1.93 (m, 2 H, $CH_2CH_2CH_2OH$), 2.15 (m, 2 H, $CH_2CH_2CH_2O$), 2.44 (s, 3 H, CH_3), 2.47 (s, 3 H, CH_3), 3.32 (t, *J* = 5.0 Hz, 2 H, CH_2O), 3.52 (m, 3 H, CH_2OH + OH), 3.77

(s, 6 H, OCH_3), 4.23 (t, J = 6.2 Hz, 2 H, CH_2N), 4.81 (t, J = 7.2 Hz, 2 H, CH_2N), 6.80 (d, J = 9.0 Hz, 4 H, $CH_{aromatic}$), 7.20−7.35 (m, 7 H, $CH_{aromatic}$), 7.40 (d, J = 8.0 Hz, 2 H, $CH_{aromatic}$). 7.71 (s, 1 H, CH_{flavin}), 8.05 (s, 1 H, CH_{flavin}) ppm. ^{13}C NMR (50 MHz, $CDCl_3$): δ = 19.9, 21.9, 28.0, 31.2, 38.8, 43.2, 55.6 (2 C), 59.0, 60.8, 86.9, 113.6 (4 C), 116.0, 124.1, 127.3 (2 C), 128.3, 130.3 (2 C), 131.5 (4 C), 133.1, 135.6, 136.3 (2 C), 137.3, 145.3, 148.6, 149.0, 150.2, 156.7, 158.9 (2 C), 161.0 ppm. HR-MS ESI calcd. for $C_{39}H_{40}N_4O_6 + Na^+$: 683.2846; found 683.2852.

10-{6-[Bis(4-methoxyphenyl)phenylmethoxy]hexyl}-3-(3-hydroxypropyl)-7,8-dimethyl-10*H*-benzo[*g*]pteridine-2,4-dione (14): The flavin **10** (200 mg, 0.31 mmol) was dissolved in dry DMF (7.0 mL) and stirred with molecular sieves (4 Å) for 2 h. Cs_2CO_3 (156 mg, 0.48 mmol) was added to this solution and the mixture was stirred for 15 min at room temp. 1-Iodopropan-3-ol (**11**; 0.20 mL, 2.09 mmol) was added dropwise and the mixture was stirred for 16 h at room temp. The reaction mixture was then filtered, diluted with chloroform (200 mL) and the organic phase was washed once with saturated $NaHCO_3$ solution and twice with water. After drying over Na_2SO_4 the solvent was evaporated and the crude product was purified by flash chromatography with $CHCl_3$/MeOH/NEt_3 (30:1:0.2) as eluent. Compound **14** (0.185 g, 0.26 mmol, 84%) was isolated as a yellow oil. R_f ($CHCl_3$/MeOH/NEt_3, 20:1:0.2): 0.49. IR (NaCl): $\tilde{\nu}$ = 3463 m cm^{-1}, 3057 w, 3034 w,2935s, 2864 m, 2837 m, 1745 m, 1702s, 1656 s, 1607 m, 1584 s, 1555 s, 1510 s, 1462 m, 1445 m, 1249 s, 1178s, 1082 m, 1033 s, 911 m, 828 m, 728 s. ^1H NMR (200 MHz, $CDCl_3$): δ = 1.4−1.5 (m, 4 H, CH_2), 1.58 (m, J = 6.0 Hz, 2 H, CH_2), 1.78 (m, 2 H, CH_2), 1.90 (m, J = 6.0 Hz, 2 H, CH_2), 2.37 (s, 3 H, CH_3), 2.47 (s, 3 H, CH_3), 2.99 (t, J = 6.0 Hz, 2 H, CH_2O), 3.37 (t, J = 6.5 Hz, 1 H, OH), 3.50 (q, J = 6.0 Hz, 2 H, CH_2OH), 3.70 (s, 6 H, OCH_3), 4.20 (t, J = 6.0 Hz, 2 H, CH_2N), 4.59 (t, J = 8.0 Hz, 2 H, CH_2N), 6.73 (d, J = 9.0 Hz, 4 H, $CH_{aromatic}$), 7.1−7.3 (m, 7 H, $CH_{aromatic}$), 7.31 (s, 1 H, CH_{flavin}), 7.35 (d, J = 8.0 Hz, 2 H, $CH_{aromatic}$), 8.00 (s, 1 H, CH_{flavin}) ppm. ^{13}C NMR (50 MHz, $CDCl_3$): δ = 19.5, 21.7, 26.2, 26.8, 27.1, 29.9, 30.7, 38.3, 44.8, 55.1 (2 C), 58.5, 63.1, 85.7, 112.9 (4 C), 115.1, 123.7, 126.5, 127.6 (2 C), 128.1 (2 C), 129.9 (4 C), 130.9, 132.7, 135.1, 136.5 (2 C), 136.8, 145.2, 148.1, 149.8, 156.3, 158.2 (2 C), 160.6 ppm. MS (ESI): m/z = 741 [M^+ + K], 439 [M^+ + K − DMT], 303 [DMT^+]. HR-MS ESI calcd. for $C_{42}H_{46}N_4O_6$ + Na^+: 725.3315; found 725.3318.

10-{6-[Bis(4-methoxyphenyl)phenylmethoxy]hexyl}-3-(6-hydroxyhexyl)-7,8-dimethyl-10*H*-benzo[*g*]pteridine-2,4-dione (15): 1-Iodohexan-6-ol was prepared according to ref.[38] The flavin **10** (250 mg, 0.39 mmol) was dissolved in dry DMF (8.0 mL) and stirred with molecular sieves (4 Å) for 2 h. Cs_2CO_3 (209 mg, 0.64 mmol) was added to the solution and the mixture was stirred for 15 min at room temp. 1-Iodohexan-6-ol (**12**; 0.25 mL, 1.96 mmol) was added dropwise and the mixture was stirred for 16 h at room temp. The reaction mixture was then filtered, diluted with chloroform (200 mL) and the organic phase was washed once with saturated $NaHCO_3$ solution and twice with water. After drying over Na_2SO_4 the solvent was evaporated and the crude product was purified by flash chromatography with $CHCl_3$/MeOH/NEt_3 (50:1:0.2) as eluent. Compound **14** (0.194 g, 0.26 mmol, 67%) was isolated as a yellow oil. R_f ($CHCl_3$/MeOH, 15:1): 0.58. IR (NaCl): $\tilde{\nu}$ = 3471 m cm^{-1}, 3057 s, 3034 s, 2999 m, 2935 s, 2861 s, 1704 m, 1660 s, 1607 m, 1585 s, 1545 s, 1510 s, 1462 m, 1250 s, 1178 m, 1069 m, 1033 s, 911 m, 829 m, 732 s. ^1H NMR (200 MHz, $CDCl_3$): δ =1.30−1.90 (m, 16 H, CH_2), 2.37 (s, 3 H, CH_3), 2.45 (s, 3 H, CH_3), 2.99 (t, J = 6.2 Hz, 2 H, CH_2), 3.57 (t, J = 6.4 Hz, 2 H, CH_2), 3.72 (s, 6 H, OCH_3), 4.04 (t, J = 6.4 Hz, 2 H, CH_2OH), 4.54 (t, J = 7.6 Hz, 2 H, NCH_2), 6.75 (d, J = 9.0 Hz, 4 H, $CH_{aromatic}$), 7.1−7.4 (m, 10 H, $CH_{aromatic}$), 7.99 (s, 1 H, CH_{flavin}) ppm. ^{13}C NMR (50 MHz, $CDCl_3$): δ = 19.4, 21.5, 25.2, 26.0, 26.5, 26.7, 27.0, 27.5, 29.9, 32.5, 41.7, 44.6, 55.1 (2 C), 62.7, 63.1, 85.6, 112.9 (4 C), 115.0, 126.5, 127.6 (2 C), 128.0 (2 C), 130.0 (4 C), 130.9, 132.6, 134.9, 135.6, 136.4, 136.5 (2 C), 145.2, 147.5, 148.4, 155.7, 158.2 (2 C), 159.9 ppm. MS (ESI): m/z = 783 [M^+ + K], 767 [M^+ + Na], 745 [M^+ + H]. HR-MS ESI calcd. for $C_{45}H_{52}N_4O_6$ + Na^+: 767.3785; found 767.3756.

***H*-Phosphonate 16:** Dichloromethane (4.8 mL), *N*-methylmorpholine (1.76 mL, 18.9 mmol) and triazole (371 mg, 5.4 mmol) were added to a solution (2 M) of PCl_3 in dichloromethane (0.8 mL). After stirring for 30 min at room temp. the solution was cooled to 0 °C and a solution of the flavin **13** (120 mg, 0.18 mmol) dissolved in dichloromethane (3.6 mL) was added dropwise. After stirring for 30 min the mixture was allowed to warm to room temp. and triethylammonium bicarbonate (TEAB) buffer (1 M, 22 mL) was added. Stirring was continued for 30 min, then the organic phase was diluted with dichloromethane (100 mL) and the salts were extracted with TEAB buffer (100 mL, 1 mol/L). The organic phase was dried with Na_2SO_4 and the solvent was evaporated. The crude product was purified by flash chromatography with $CHCl_3$/MeOH/NEt_3 (10:1:0.2) as eluent. To remove additional salts, the product was again dissolved in dichloromethane and the solution extracted with TEAB buffer. The *H*-phosphonate **16** (64 mg 0.08 mmol, 43%) was obtained as a yellow oil. R_f ($CHCl_3$/MeOH/NEt_3, 10:1:0.2) = 0.27. IR (NaCl): $\tilde{\nu}$ = 3054 s cm^{-1}, 3033 s, 2955 m, 2935 m, 2864 s, 2838 s, 2238 m, 1704 m, 1660 s, 1651 s, 1585 s, 1552 s, 1505 m, 1455 m, 1250 s, 1203 m, 1178 m, 1063 m, 991 m, 829 s, 727 m. ^1H NMR (200 MHz, $CDCl_3$): δ = 1.11 [t, J = 7.2 Hz, 9 H, $CH_3(NEt_3)$], 1.95−2.10 (m, 4 H, CH_2), 2.38 (s, 3 H, CH_3), 2.41 (s, 3 H, CH_3), 2.72 [q, J = 7.2 Hz, 6 H, $CH_2(NEt_3)$], 3.26 (t, J = 4.8 Hz, 2 H, CH_2), 3.73 (s, 6 H, OCH_3), 3.91 (q, J = 7.0 Hz, 2 H, CH_2), 4.10 (t, J = 7.6 Hz, 2 H, OCH_2), 4.69 (t, J = 7.2 Hz, 2 H, NCH_2), 6.75 (d, J = 9.0 Hz, 4 H, $CH_{aromatic}$), 6.80 (d, J = 614 Hz, 1 H, PH), 7.1−7.4 (m, 9 H, $CH_{aromatic}$), 7.64 (s, 1 H, CH_{flavin}), 7.98 (s, 1 H, CH_{flavin}) ppm. ^{13}C NMR (50 MHz, $CDCl_3$): δ = 9.5 (3 C), 19.3, 21.2, 27.3, 29.0, 39.4, 42.3, 45.5 (3 C), 55.0 (2 C), 60.2, 61.6, 86.4, 113.0 (4 C), 115.4, 126.7, 127.8 (4 C), 129.7 (4 C), 130.9, 132.4, 134.8, 135.5, 135.7 (2 C), 136.3, 144.7, 147.5, 148.4, 155.4, 158.4 (2 C), 159.6 ppm. ^{31}P NMR (81 MHz, $CDCl_3$): δ = 5.19 ppm. MS (ESI, negative): m/z = 825 [M^-], 723 [M^- − $HNEt_3$], 421[M^- − $HNEt_3$ − DMT].

***H*-Phosphonate 17:** Dichloromethane (7.2 mL), *N*-methylmorpholine (2.78 mL, 29.8 mmol) and triazole (588 mg, 8.6 mmol) were added to a solution (2 M) of PCl_3 in dichloromethane (1.26 mL). After stirring for 30 min at room temp. the solution was cooled to 0 °C and a solution of the flavin **14** (190 mg, 0.27 mmol) dissolved in dichloromethane (3.6 mL) was added dropwise. After stirring for 30 min the mixture was allowed to warm to room temp. and TEAB buffer (22 mL, 1 mol/L) was added. Stirring was continued for 30 min, then the organic phase was diluted with dichloromethane (100 mL) and extracted with TEAB buffer (100 mL, 1 mol/L). The organic phase was dried with Na_2SO_4 and the solvent was evaporated. The crude product **17** was purified by flash chromatography with $CHCl_3$/MeOH/NEt_3 (10:1:0.2) as eluent. To remove surplus salts, the product was again dissolved in dichloromethane and the salts were extracted with TEAB buffer. The *H*-phosphonate **17** (108 mg 0.08 mmol, 46%) was obtained as a yellow oil. R_f ($CHCl_3$/MeOH/NEt_3, 10:1:0.2): 0.34. IR (NaCl): $\tilde{\nu}$ = 3054 w cm^{-1}, 3033 w, 2935 m, 2864 m, 2838 w, 2238 m, 1741 w, 1704 s, 1660 s, 1651 s, 1607 m, 1584 s, 1552 s, 1510 m, 1455 m, 1299 m, 1250 s, 1211 s,

1050 s, 921 m, 831 m, 727 s. ^{1}H NMR (200 MHz, $CDCl_3$): δ = 1.26 [t, *J* = 7.0 Hz, 9 H, $CH_3(NEt_3)$], 1.35−1.50 (m, 4 H, CH_2), 1.55 (m, *J* = 7.0 Hz, 2 H, CH_2), 1.7−1.85 (m, 2 H, CH_2), 1.99 (m, *J* = 7.6 Hz, 2 H, CH_2), 2.37 (s, 3 H, CH_3), 2.46 (s, 3 H, CH_3), 2.97 [q, *J* = 7.2 Hz, 8 H, $CH_2(NEt_3)$ + CH_2], 3.72 (s, 6 H, OCH_3), 3.93 (q, *J* = 6.8 Hz, 2 H, OCH_2), 4.15 (t, *J* = 6.6 Hz, 2 H, NCH_2), 4.58 (br. t, 2 H, NCH_2), 6.74 (d, *J* = 8.8 Hz, 4 H, $CH_{aromatic}$), 6.80 (d, *J* = 608 Hz, 1 H, PH), 7.1−7.4 (m, 9 H, $CH_{aromatic}$), 7.98 (s, 1 H, CH_{flavin}), 8.08 (s, 1 H, CH_{flavin}) ppm. ^{13}C NMR (50 MHz, $CDCl_3$): δ = 8.9 (3 C), 19.3, 21.5, 26.0, 26.7, 27.0, 29.1, 29.9, 39.5, 44.6, 45.8 (3 C), 55.1 (2 C), 61.8, 63.1, 85.6, 112.9 (4 C), 115.0, 126.5, 127.5 (2 C), 128.1 (2 C), 129.9 (4 C), 130.9, 132.7, 134.9, 136.4, 136.5 (2 C), 145.2, 146.7, 147.6, 148.4, 155.5, 158.2 (2 C), 159.8 ppm. ^{31}P NMR (81 MHz, $CDCl_3$): δ = 4.7 ppm. MS (ESI, negative): *m*/*z* = 765 [M^- − $HNEt_3$], 565 [M^- − DMT], 463 [M^- − $HNEt_3$ − DMT].

***H*-Phosphonate 18:** Dichloromethane (6.6 mL), *N*-methylmorpholine (0.95 mL, 10.2 mmol) and triazole (201 mg, 2.9 mmol) were added to a solution (2 M) of PCl_3 in dichloromethane (0.43 mL). After stirring for 30 min at room temp. the solution was cooled to 0 °C and a solution of the flavin **15** (65 mg, 0.09 mmol) dissolved in dichloromethane (2.0 mL) was added dropwise. After stirring for 30 min the mixture was allowed to warm to room temp. and TEAB buffer (7 mL, 1 mol/L) was added. Stirring was continued for 30 min, then the organic phase was diluted with dichloromethane (100 mL) and the salts were extracted with TEAB buffer (100 mL, 1 mol/L). The organic phase was dried with Na_2SO_4 and the solvent was evaporated. The crude product was purified by flash chromatography with $CHCl_3$/MeOH/NEt_3 (10:1:0.2) as eluent. To remove surplus salts, the product was again dissolved in dichloromethane and the organic phase was extracted with TEAB buffer. The *H*-phosphonate **18** (40 mg, 0.04 mmol, 48%) was obtained as a yellow oil. R_f ($CHCl_3$/MeOH/NEt_3, 10:1:0.2): 0.27. IR (NaCl): ṽ = 3054 w cm^{-1}, 3034 w, 2933 m, 2859 m, 2237 w, 1734 m, 1705 s, 1649 m, 1607 s, 1583 s, 1508 s, 1460 m, 1298 m, 1248 s, 1212 s, 1187 m, 1056 s, 1032 s, 988 m, 829 m, 727 s. ^{1}H NMR (200 MHz, $CDCl_3$): δ = 1.14 [t, *J* = 7.0 Hz, 9 H, $CH_3(NEt_3)$], 1.3−1.9 (m, 16 H, CH_2), 2.36 (s, 3 H, CH_3), 2.45 (s, 3 H, CH_3), 2.77 [q, *J* = 7.3 Hz, 6 H, $CH_2(NEt_3)$], 2.99 (t, *J* = 6.3 Hz, 2 H, OCH_2), 3.71 (s, 6 H, OCH_3), 3.76 (q, *J* = 7.3 Hz, 2 H, OCH_2), 4.01 (t, *J* = 6.6 Hz, 2 H, NCH_2), 4.65 (br. t, 2 H, NCH_2), 6.74 (d, *J* = 8.6 Hz, 4 H, $CH_{aromatic}$), 6.76 (d, *J* = 610 Hz, 1 H, PH), 7.1−7.4 (m, 10 H, $CH_{aromatic}$), 7.97 (s, 1 H, CH_{flavin}) ppm. ^{13}C NMR (50 MHz, $CDCl_3$): δ = 9.7 (3 C), 19.4, 21.6, 25.7, 26.1, 26.7, 26.8, 27.1, 27.8, 29.9, 30.7, 41.9, 44.6, 45.6 (3 C), 55.2 (2 C), 63.2, 63.6, 84.5, 112.9 (4 C), 115.0, 126.6, 127.6 (2 C), 128.1 (2 C), 129.9 (4 C), 130.9, 132.7, 135.0, 136.3, 136.6 (2 C), 145.3, 147.0, 147.4, 148.4, 155.7, 158.3 (2 C), 159.9 ppm. ^{31}P NMR (81 MHz, $CDCl_3$): δ = 5.1 ppm. MS (ESI): *m*/*z* = 807 [M^- − $HNEt_3$], 505 [M^- − $HNEt_3$ − DMT].

Oligonucleotide Synthesis: Oligonucleotide synthesis was performed on an Expedite 8900 DNA Synthesizer, connected to an IBM compatible PC. Synthesis of the oligonucleotides was performed by using a modified 1.0 μmol cycle and Pac-amidites. Solvents and solutions were made up according to the manufacturer's protocol. The phosphoramidite (0.1 M in MeCN) and 1*H*-tetrazole (0.5 M in MeCN) solutions were equal in concentration to those used for the synthesis of natural oligodeoxynucleotides. Average coupling yields monitored by on-line trityl assay were generally in the range of 95−99%. All syntheses were run in the trityl-off mode.

The concentrations used for *H*-phosphonate chemistry were 0.13 M in MeCN/pyridine (1:1) for the *H*-phosphonate and 0.17 M in MeCN/pyridine for the activator (adamantoylcarbonyl chloride) respectively. The cartridge was washed with MeCN/pyridine (1:1) prior to the coupling. The coupling time was 10−15 min. Capping, oxidation and detritylation were the same as for standard phosphoramidite chemistry.

DNA Purification: For the deprotection and purification of the oligonucleotides, the solid support was suspended in concentrated 25% NH_3 in H_2O/EtOH (3:1; 1 mL) and shaken for 16 h at 20 °C in an Eppendorf thermo shaker to effect deprotection and cleavage. The solvent was evaporated and the residue suspended in H_2O and purified by HPLC (C_{18}-RP, 0−40% B in 35 min, A = 0.1 M Triethylammonium acetate in H_2O, B = 0.1 M Triethylammonium acetate in H_2O/80% AcCN). DNA-containing fractions were evaporated to dryness and redissolved in H_2O. Laser desorption mass spectra (MALDI) (positive ions detected): calcd. for **1**: 2908.9; found 2912.0; calcd. for **2** 2866.9; found 2870.7; calcd. for **3**: 2824.8; found 2826.6; calcd. for **19**: 4657.2; found 4654.6.

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