Single base interrogation by a fluorescent nucleotide: Each of the four DNA bases identified by fluorescence spectroscopy

Pavol Cekan and Snorri Th. Sigurdsson*
University of Iceland, Science Institute, Dunhaga 3, 107 Reykjavik, Iceland

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List of abbreviations

TEN buffer	250 mM NaCl, 10 mM Tris, 1 mM Na_2EDTA, pH 7.5
PNE buffer	10 mM Na_2HPO_4, 100 mM NaCl, 0.1 mM Na_2EDTA, pH 7.0
Tris buffer	50 mM Tris, 10 mM $MgCl_2$, pH 8.0
DPAGE	Denaturing polyacrylamide gel electrophoresis
RP-HPLC	Reverse-phase high-performance liquid chromatography

Materials and methods

General. All commercial reagents were used without further purification. Water was purified on a MILLI-Q water purification system. Neutral silica gel was purchased from Silicycle. Analytical thin layer chromatography (TLC) was performed on glass plates (Merck Kieselgel 60 F_{254}). NMR spectra for all organic compounds were recorded on an Avance 400 MHz Bruker NMR spectrometer and the chemical shifts were reported parts per million (ppm) relative to the deuterated NMR solvent used [^{1}H-NMR: $CDCl_3$ (7.26 ppm), MeOH-d_4 (4.84 and 3.31 ppm); ^{13}C-NMR: $CDCl_3$ (77.00 ppm), MeOH-d_4 (49.10 ppm)]. Commercial grade $CDCl_3$ was passed over basic alumina, immediately prior to use. DNA oligomers were synthesized on an ASM 800 DNA synthesizer from Biosset (Russia). UV-VIS spectra were recorded on a PerkinElmer Lambda 25 UV/VIS spectrometer. Analytical RP-HPLC samples were run on a Beckman Coulter Gold HPLC system using Beckman Coulter Ultrasphere C18 4.6×250mm analytical column with UV detection at 254 nm. Solvent gradients for analytical RP-HPLC were run at 1.0 mL/min as follows: Solvent A, 50 mM Et_3NOAc (pH 7.0); solvent B, 100% CH_3CN; 4% B isocratic for 4 min, 10 min linear gradient from 4% B to 20% B, 10 min linear gradient to 50% B, 5 min linear gradient to 80% B, isocratic for 1 minute, then a 5-min gradient to initial conditions (4% B). Steady-state fluorescence measurements were carried out at 20 °C in a macro fluorescence cell with a path length of 0.5 cm on a SPEX FluoroMax spectrometer using an excitation wavelength of 365.5 nm. CD spectra were recorded on a JASCO J-810 spectropolarimeter at 25 °C with path length of 1 mm (Hellma), 10 scans, scanned from 500 nm to 200 nm with response of 1s, data pitch of 0.1 nm and band width of 1.0 nm. Continuous wave (CW) EPR spectra were recorded on a MiniScope MS200 (Magnettech, Germany) spectrometer (100 kHz modulation frequency, 1.0 G modulation amplitude, and 2.0 mW microwave power). Molecular weight (MW) of oligonucleotides was determined by MALDI-TOF analysis (Bruker, Autoflex III) and that of organic compounds by ESI-MS (Bruker, MicroTof-Q).

DNA synthesis and purification. Modified and unmodified oligonucleotides were synthesized by a trityl-off synthesis on 1.0 µmol (1000 Å CPG columns) scale using phosphoramidites with standard base protection. All commercial phosphoramidites, columns and solutions were purchased from ChemGenes. For spin-labeled DNA, the spin-labeled phosphoramidite[1] was site-specifically incorporated into the oligonucleotides by manual coupling. The DNA was deprotected at 55 °C for 8 h and purified by 23% DPAGE. The oligonucleotides were visualized by UV shadowing, the bands were excised from the gel, crushed and soaked in TEN buffer for 20 h. For filtration of DNA elution solutions, 0.45 µm polyethersulfone membrane (disposable filter device from Whatman) was used. The DNA elution solutions were desalted using Sep-Pak cartridge (Waters Corporation) according to manufacturer's instructions. After removing the solvent in vacuo, the DNA was dissolved in deionized and sterilized H_2O (100 µL). Concentrations of oligonucleotides were calculated from Beer´s law based on measurements of absorbance at 260 nm using extinction coefficients listed in **Table S1**. Extinction coefficients were determined by using UV WinLab oligonucleotide calculator (V2.85.04, PerkinElmer).

Table S1. DNA sequences and extinction coeffincients, ε ($M^{-1} \cdot cm^{-1}$).

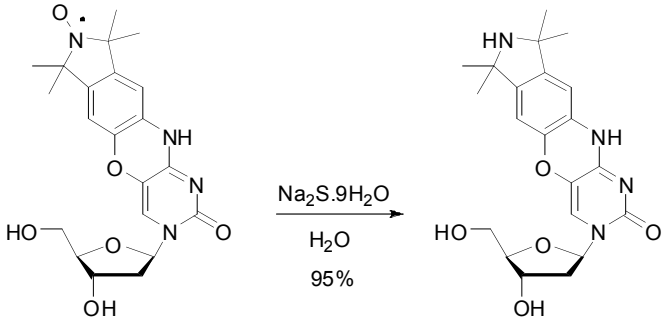
5'-d(GAC CTC G**Ç**A TCG TG)	128300
5'-d(GAC CTC G**Ç**$^{\mathbf{f}}$A TCG TG)	128300
5'-d(GAC CTC GCA TCG TG)	128300
5'-d(CAC GAT GCG AGG TC)	135100
5'-d(CAC GAT ACG AGG TC)	139200
5'-d(CAC GAT CCG AGG TC)	133400
5'-d(CAC GAT TCG AGG TC)	134300
5'-d(CCC T**Ç**C TGT CC)	85600
5'-d(CCC T**Ç**$^{\mathbf{f}}$C TGT CC)	85600
5'-d(GGA CAG GAG GG)	118100
5'-d(GGA CAG AAG GG)	120000
5'-d(GGA CAG TAG GG)	117500
5'-d(GGA CAG CAG GG)	114200
5'-d(CCC T**Ç**T TGT CC)	86500
5'-d(CCC T**Ç**$^{\mathbf{f}}$T TGT CC)	86500
5'-d(GGA CAA GAG GG)	120000
5'-d(GGA CAA AAG GG)	120700
5'-d(GGA CAA TAG GG)	118800
5'-d(GGA CAA CAG GG)	116300

UV-Monitored thermal denaturation. The DNA duplexes (ca. 2-3 nmoles) were prepared by mixing a fluorescent strand with 1.25 eq of the complementary strand in PNE buffer (100 µL) and using the following annealing protocol: 90 °C for 2 min, 60 °C for 5 min, 50 °C for 5 min, 40 °C for 5 min, 22 °C (room temperature) for 15 min. Then the samples were diluted to 1 mL with PNE buffer and degassed using argon. The samples were heated up from 20 °C to 90 °C (0.5 °C / min) and absorbance at 260 nm recorded.

Steady-state fluorescence. All DNA samples were measured at 1.25×10^{-5} M in 400 µL of PNE buffer at 20 °C using an excitation wavelength of 365.5 nm. Fluorescence spectra were averaged over five scans.

Quantum yield determination. Quantum yields were determined using anthracene as a standard[2] for all DNA samples in PNE buffer (**Table 1**). Quantum yields of all DNAs were determined using the following equation: $\Phi_{F(x)} = (A_s/A_x) \bullet (F_x/F_s) \bullet (n_x/n_s)^2 \bullet \Phi_{F(s)}$, where **S** is the standard (anthracene), **X** is the unknown (sample being measured), **A** is the absorbance at the excitation wavelength (365.5 nm), **F** is the area under the emission curve, ***n*** is the refractive index of the solvent (H_2O: 1.3439, EtOH: 1.3610) and **Φ** is the quantum yield.[3]

Synthesis procedures



1 **2**

*Phenoxazine-derived nucleoside (amine **2**).* A solution of spin-labeled nucleoside **1** (10.0 mg, 0.023 mmol) in DMF (1.00 mL) was treated with $Na_2S \cdot 9H_2O$ (56.0 mg, 0.234 mmol) and the reaction mixture stirred for 8 h at 45 °C. The reaction mixture was concentrated in vacuo and the product was purified by flash column chromatography using neutral silica gel (gradient 100:0 to 65:35, CH_2Cl_2:MeOH) to give **2** as an yellowish solid (9.0 mg, 95%).

TLC (Silica gel 15 % MeOH/CH_2Cl_2), Rf(**1**) = 0.65, Rf (**2**) = 0.05
^{1}H-NMR (MeOH-d_4): δ 1.50 (s, 12H, 4 × CH_3), 2.15 (m, 1H, H2'), 2.33 (m, 1H, H2'), 3.78 (m, 2H, H5') 3.94 (m, 1H, H3'), 4.38 (m, 1H, H4'), 6.23 (t, J = 7 Hz, 1H, H1'), 6.60 (s, 1H, ArH), 6.66 (s, 1H, ArH), 7.72 (s, 1H, C_6H).
^{13}C-NMR (MeOH-d_4): 30.77, 30.88, 41.84, 62.73, 65.44, 72.13, 87.55, 89.01, 109.63, 110.93, 123.59, 128.05, 129.56, 142.94, 143.71, 144.18, 155.71, 156.21.
ESI-MS ($M+H^+$): calcd. for $C_{21}H_{26}O_5N_4H$ 415.1981, found 415.1972

Reduction of spin-labeled DNA. An aqueous solution of the spin-labeled oligomer 5'-d(GACCTCG**Ç**ATCGTG) (20 nmoles) in 0.1 M Na_2S (w/v, 50 µL) was heated at 45 °C for 14 h. The completion of the reaction was verified by EPR spectroscopy. The DNA solution was diluted with sodium acetate (3.0 M, 10 µL, pH 5.3). The DNA was precipitated by addition of abs. ethanol (-20 °C, 940 µL) and the sample stored at -20 °C over 10 h. After centrifugation (13,000 rpm, 20 min, -10 °C), the supernatant was removed, 70% ethanol/water solution (-20 °C, 500 µL) was added and the resulting mixture allowed to stand at -20 °C for 5 h. After centrifugation, the supernatant was removed and the pellet was washed with cold ethanol (50 µL). After drying the pellet, it was dissolved in deionized and sterilized H_2O (100 µL) and the DNA analyzed by DPAGE (**Figure 2**).

Characterization of oligomers

Enzymatic digestion of DNA and HPLC analysis. A solution of unmodified DNA 14-mer (4.7 nmol) in Tris buffer (40 μL) was treated with 0.1 units of snake venom phosphodiesterase I (PDE I) and 4 units of calf intestinal alkaline phosphatase and incubated at 37 °C for 50 h. Spin-labeled and reduced spin-labeled DNA 14-mer (4.7 nmol) was digested using the same protocol, but 3 units of nuclease P1 from *Penicillium citrinum* were additionally used to efficiently cleave phosphodiester bond between neighbouring purine nucleotides and **Ç** or **Ç^{f}**. Samples were analyzed by analytical RP-HPLC (**Figure S1**). RP-HPLC analysis of the unmodified oligomer digest revealed the four expected peaks corresponding to dC, dI (from enzymatic hydrolysis of dA by an adenosine deaminase-contamination in phosphodiesterase I), dG and dT (**Figure S1A**). Analysis of the enzymatic digest of the spin-labeled oligomer (**Figure S1B**) shows the presence of a new peak corresponding to **Ç**. Similar analysis of the reduced spin-labeled oligomer (**Figure S1C**) shows the presence of a strongly retained compound that had the same retention time as **Ç^{f}** (**Figure S1D**). The presence of **Ç^{f}** in digest was confirmed by co-injection (**Figure S1E**) of the samples shown in **Figure S1C** and **D**.

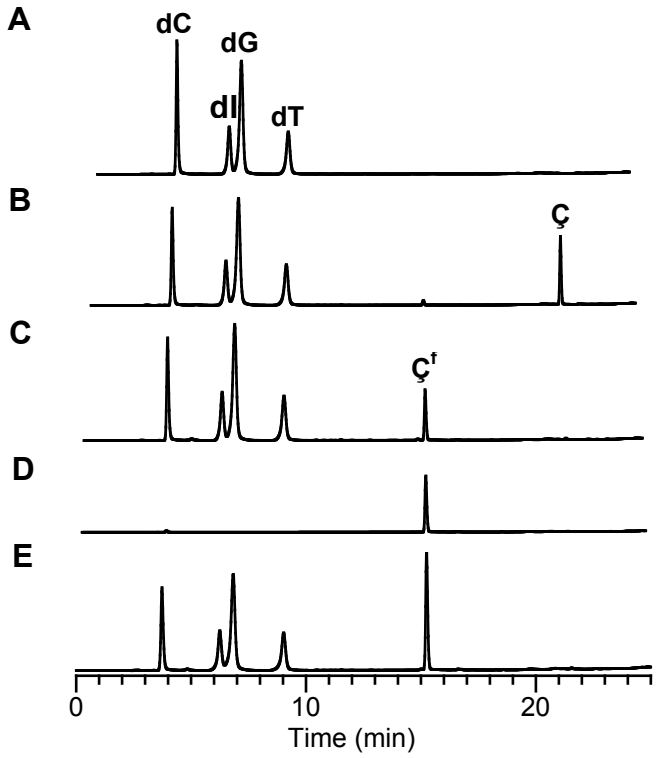


Fig. S1 HPLC diagram after the enzymatic digestion of unmodified DNA 14-mer 5'-d(GACCTCGCATCGTG) (**A**), spin-labeled DNA 14-mer 5'-d(GACCTCG**Ç**ATCGTG) (**B**), reduced DNA 14-mer 5'-d(GACCTCG**Ç^{f}**ATCGTG) (**C**), reduced spin-labeled nuceleoside **Ç^{f}** (**D**) and injection of an equimolar amount of reduced spin-labeled nuceleoside **Ç^{f}** and enzymatically digested reduced DNA 14-mer 5'-d(GACCTCG**Ç^{f}**ATCGTG) (**E**).

MALDI-TOF mass spectroscopy. The MW of spin-labeled oligonucleotide 5'-d(GACCTCG**Ç**ATCGTG) and reduced oligonucleotide 5'-d(GACCTCG**Ç**^{f}ATCGTG) was determined via MALDI-TOF MS analysis (in linear mode) (**Figure S2**). The unmodified oligonucleotide 5'-d(GACCTCGCATCGTG) was used as an internal standard (MW 4240.7). The MW (m/z) of the spin-labeled DNA and reduced DNA was observed to be 4442.4 (calcd. 4442.1) and 4427.2 (calcd. 4427.1), respectively, the latter consistent with loss of oxygen and addition of hydrogen.

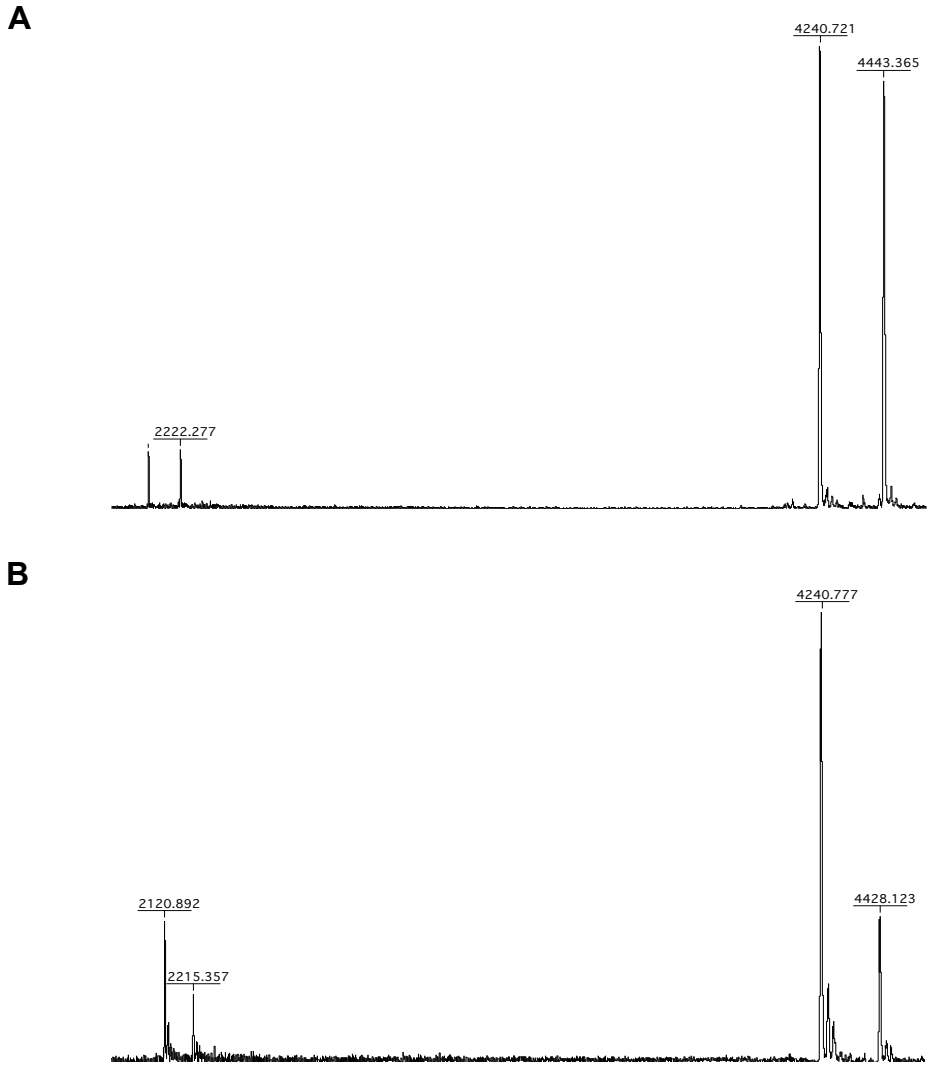


Fig. S2 MS Analysis of spin-labeled DNA 14-mer 5'-d(GACCTCG**Ç**ATCGTG) (**A**) and reduced DNA 14-mer 5'-d(GACCTCG**Ç**^{f}ATCGTG) (**B**). For both MS analyses, unmodified DNA 14-mer 5'-d(GACCTCGCATCGTG) was used as an internal standard (MW 4240.7).

CD measurements. DNA samples (2.5 nmol of duplex) (**Figure S3A**) were dissolved in PNE buffer (100 µL), annealed and diluted to 200 µL with PNE buffer. CD spectra of unmodified 14-mer duplex and reduced 14-mer duplex possessed negative and positive molar ellipticities at ca. 250 and 280 nm, respectively, characteristic of a right-handed B-DNA (**Figure S3B**). CD spectra of 14-mer duplexes containing **Ç^{f}•A**, **Ç^{f}•C** and **Ç^{f}•T** mismatches were similar, and thus consistent with B-form DNA (**Figure S3C**).These studies indicate that the reduced spin-labeled nucleoside, **Ç^{f}**, does not significantly alter the structure and conformation of either modified DNA or mismatched DNA.

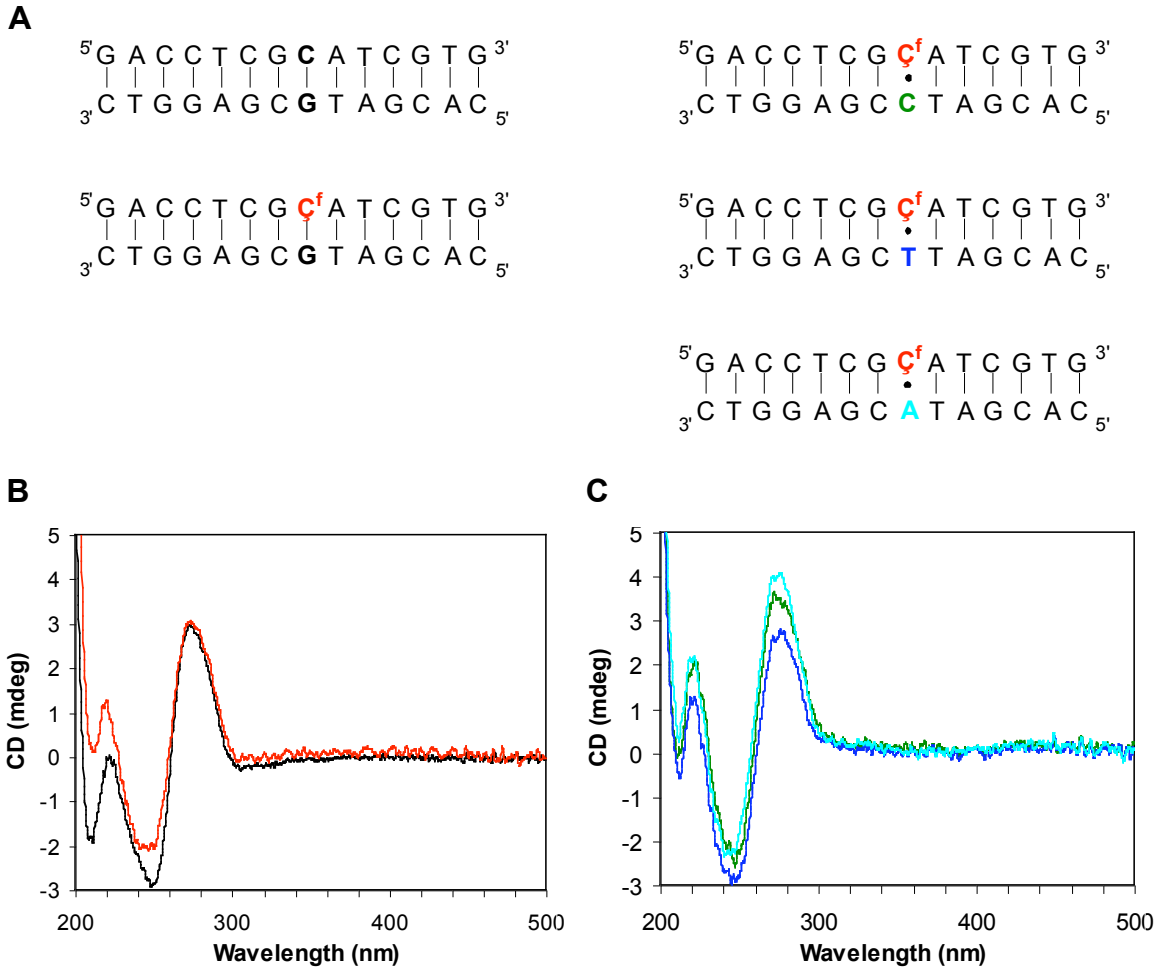


Fig. S3 A. Sequences of all duplexes used for CD measurements. **B.** CD spectra of an unmodified DNA 14-mer duplex (black) and reduced 14-mer duplex (red). **C.** CD spectra of 14-mer duplex containing **Ç^{f}•C** (green), **Ç^{f}•T** (blue) and **Ç^{f}•A** (cyan).

Concentration dependence on fluorescence. The appropriate amounts of DNA samples (**Figure S3A**) were dissolved in PNE buffer (100 µL), annealed and diluted to 400 µL with PNE buffer (5.5 – 12.5 µM of single strand or duplex). Fluorescence measurements were recorded at 20 °C using an excitation wavelength of 365.5 nm (**Figure S4**). Fluorescence spectra were averaged over five scans.

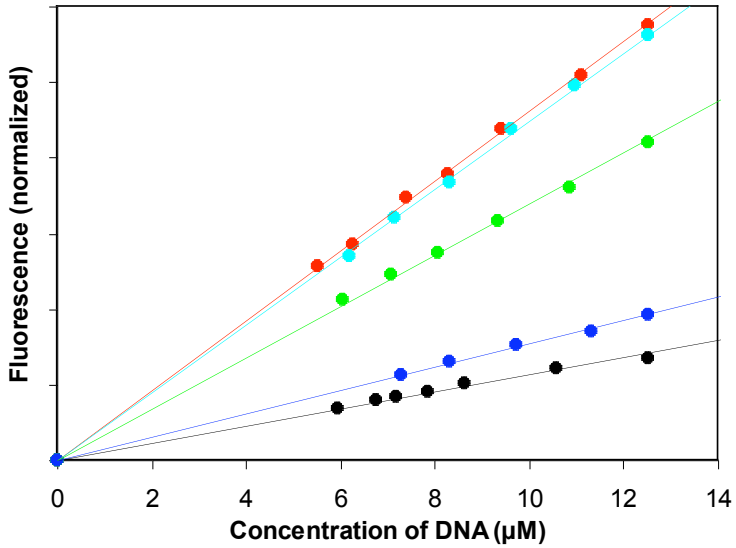


Fig. S4 Fluorescence intensity of reduced 14-mer as a single-strand (red), duplex (black), duplex containing **Ç^{f}•C** (green), **Ç^{f}•T** (blue) and **Ç^{f}•A** (cyan) at various concentrations of reduced DNA.

References

[1] N. Barhate, P. Cekan, A. P. Massey, S. T. Sigurdsson, *Angew. Chem. Int. Ed.,* 2007, **46**, 2655.
[2] H. Du, R.-C. A. Fuh, J. Li, L. A. Corkan and J. S. Lindsey, *Photochem. Photobiol.,* 1998, **68**, 141.
[3] S. Fery-Forgues and D. Lavabre, *J. Chem. Educ.,* 1999, **76**, 1260.