Supporting Information for

Synthesis, Characterization, and Cellular Uptake of DNA-Binding Rose Bengal Peptidoconjugates

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Materials

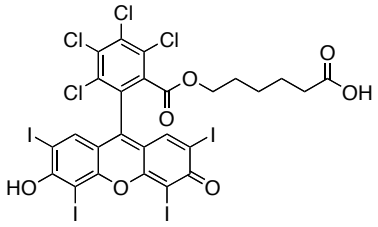
Solvents were purchased from Fisher and reagents were purchased from Aldrich Chemical Co. (Milwaukee, WI) or Acros Organics (Morris Plains, NJ). Amino acids were purchased from Advanced ChemTech (Louisville, KY). All solvents and reagents were used without further purification. HPLC grade acetonitrile and Millipore water were used for HPLC analysis. Rose bengal, dye content 95% was purchased from Aldrich. 6-carboxyfluorescein (6-FAM) was purchased from Molecular Probes (Eugene, OR). Fmoc-protected Rink amide resin 4-(2',4'-Dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy resin (100-200 mesh) was purchased from NovaBiochem (San Diego, CA) with a substitution level of 0.64 mmol/g. Molar extinction coefficient (ε) = 104, 700 M^{-1} cm^{-1} in MeOH at 558 nm for RB-peptide conjugates;[1] ε = 68, 000 M^{-1} cm^{-1} in 0.5 M Tris buffer, pH 7.4 at 494 nm for Fl-peptide conjugate.[2]

Analysis

Reverse phase HPLC was performed with an Agilent 1100 series HPLC using either an analytical column (Varian C18, 5 micron, 250 x 4.6 mm) at a flow rate of 1 mL/min or a preparative column (Hamilton PRP-1, 10 micron, 250 x 10 mm) at a flow rate of 3.5 mL/min. For analytical HPLC a linear solvent gradient from 20 to 100% B over 80 min was used (solvent A = 0.1% TFA in H_2O; solvent B = 0.1% TFA in CH_3CN). Analytical thin-layer chromatography (TLC) was performed using Silica Gel 60 Å F254 precoated plates (0.25 mm thickness). Flash column chromatography was performed using Silica Gel 60 Å (32-63 micron). Mass spectral analysis was performed by the Boston College Mass Spectrometry Facility. Samples were analyzed by accurate mass electrospray mass spectrometry (ESI-MS) operating in positive mode on a Micromass LCT mass spectrometer or MALDI-TOF MS on a Micromass TofSpec 2E using α-cyano-4-hydroxycinnamic acid matrix. UV analysis was performed on an Agilent 8453 Diode Array Spectrophotometer. Steady state fluorescence measurements were performed on a Jobin-Yvon Horiba Fluorolog®-3. For all steady state measurements the λ_{ex} = 500 nm and the λ_{em} = 520 - 650 nm. The excitation and emission slits were both set to 6.5 nm to prevent emission intensities above 4 million counts per second.

Quantum yield measurements

Quantum yields are reported relative to a 5 μM RB standard in methanol (Φ = 0.08).[3] Sample used for fluorescence measurements contained RB-conjugate concentrations of 5 μM and a CT DNA bp concentration of 50 μM to ensure that the absorbance of the sample was < 0.06. The buffer contained 10 mM sodium phosphate and 100 mM sodium chloride. Emission spectra were corrected according to absorbance values at 500 nm and were then normalized to a RB standard.



Synthesis of rose bengal 5-carboxy pentyl ester (2)

(Method adapted from *Neckers et al.*[4]) RB disodium salt (1.07 g, 1.05 mmol, 1.0 equiv.) and H_2O (10 mL) were added to a 50 mL round bottom flask. The solid was dissolved with heating to give a red solution. A solution of 6-bromohexanoic acid (0.600 g, 3.08 mmol, 2.9 equiv.) in Me_2CO (10 mL) was added to the RB solution. The resulting red solution was refluxed (80 °C) for 24 h and then cooled to room temperature. The mixture was concentrated *in vacuo* to give a red oil, and then suspended in Me_2CO (10 mL), filtered to remove NaBr salt, and washed with Me_2CO (3 x 10 mL). The filtrate was concentrated under reduced pressure. The crude material was purified via silica gel flash chromatography using EtOAc with 0-30% MeOH to give the desired product as a red solid (0.253 g, 22 %). **TLC** R_f 0.24 (EtOAc:MeOH 88:12) **^{1}H NMR** matched with literature reference;[4] **Exact mass** calculated for $[C_{26}H_{14}Cl_4I_4O_7]$ $[M + Na]^+$ requires *m/z* 1108.557 Found 1108.558 (ESI+). **HPLC retention time** 47.6 min

General procedure for the synthesis of dye-peptide conjugates

Dye-peptide conjugates were synthesized on solid support using commercially available Rink amide resin. Couplings were performed using 4 equivalents of Fmoc-protected amino acid, 4 equivalents of HBTU and 8 equivalents of *N,N*-diisopropylethylamine in DMF for 3 hours. Deprotection of the Fmoc group was achieved using 20% piperidine in DMF for 30 minutes (to minimize diketopiperazine formation, dipeptides were deprotected using 50% piperidine in DMF for 5 min). The dye moiety was attached to a resin-bound peptide as described below. To minimize byproducts resulting from the Rink amide resin under high concentrations of TFA a 2-step procedure for detachment/deprotection of the resin was followed according to the method described in the Novabiochem catalog.[5] The dye-peptide conjugates were detached from the resin by slurrying in 10% TFA:DCM [v/v] and transferred to a glass funnel with a fine sinter. The solvent was allowed to drip slowly through the resin bed and was washed with 5% TFA:DCM [v/v] and concentrated *in vacuo*. Deprotection was achieved by stirring the residue in 95:5 TFA:TIS solution at room temperature for 2 h.

The solution was concentrated *in vacuo* and purified via RP-HPLC (H_2O/CH_3CN in 0.1% TFA). The products were isolated by lyophilization and characterized by MALDI-TOF mass spectrometry. The purity of the peptides was > 95% as determined by analytical RP-HPLC (H_2O/CH_3CN in 0.1% TFA).

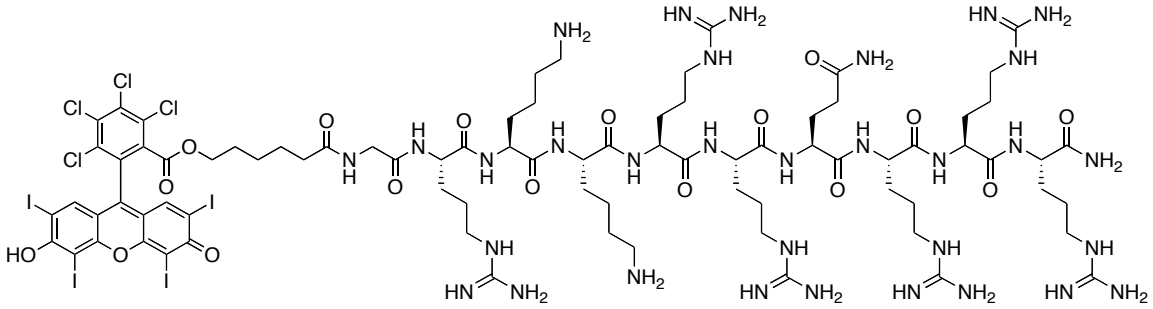
General procedure for the synthesis of RB-peptide conjugates (3a-f)

The rose bengal moiety (RB) was attached by treating a resin-bound peptide (1.0 equiv.) with RB 5-carboxy pentyl ester (3.0 equiv.), HBTU (3.0 equiv.), HOBt (3.0 equiv.) and *i*-Pr_2NEt (6.0 equiv.) in DMF for 12 hours.

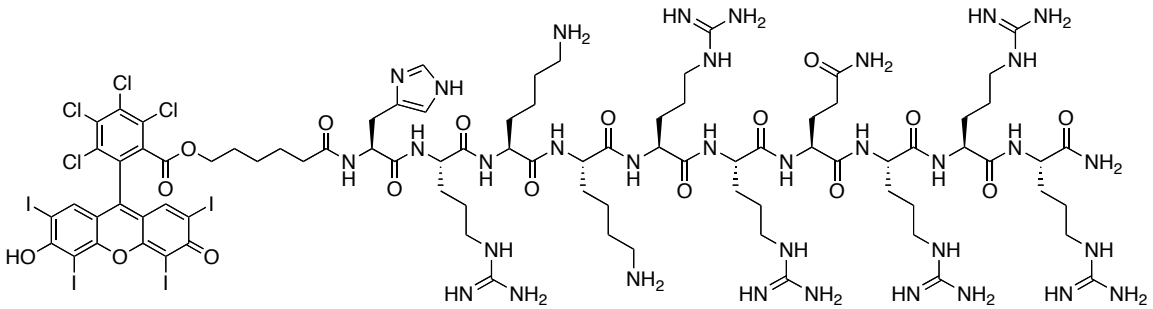
General procedure for the synthesis of Fl-peptide conjugate (4)

The fluorescein moiety (Fl) was attached via an aminohexanoic acid spacer by treating a resin-bound peptide (1.0 equiv.) with 6-carboxyfluorescein (3.0 equiv.), HBTU (3.0 equiv.), HOBt (3.0 equiv.) and *i*-Pr_2NEt (6.0 equiv.) in DMF for 12 hours.

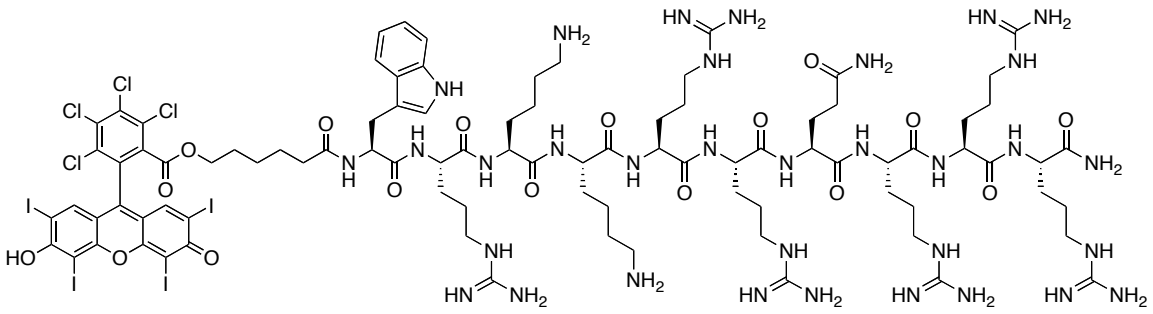
Characterization of conjugates



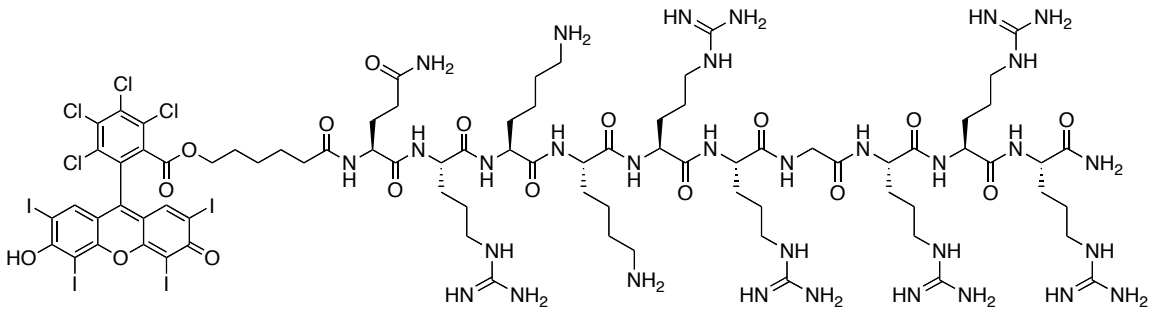
RB-GRKKRRQRRR-CONH$_2$ (3a). MALDI-TOF calculated for [$C_{81}H_{122}Cl_4I_4N_{32}O_{17}$] $[M + H]^+$ requires *m/z* 2465. Found 2466. **HPLC retention time** 26.0 min



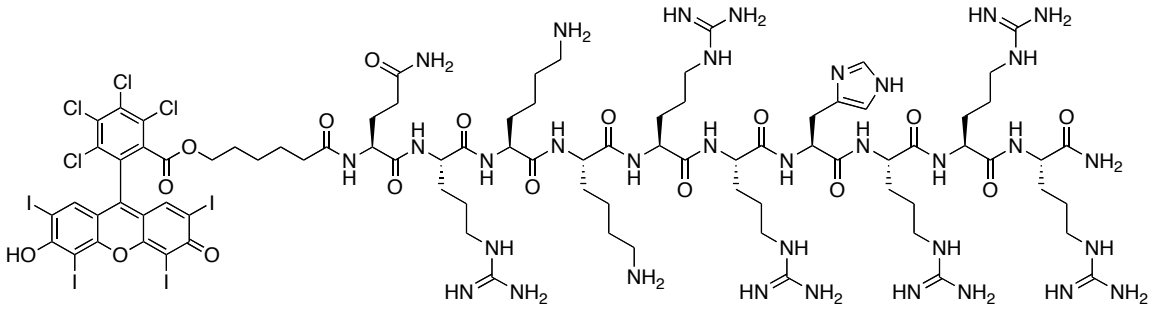
RB-HRKKRRQRRR-CONH$_2$ (3b). MALDI-TOF calculated for [$C_{85}H_{126}Cl_4I_4N_{34}O_{17}$] $[M + H]^+$ requires *m/z* 2546. Found 2547. **HPLC retention time** 25.7 min



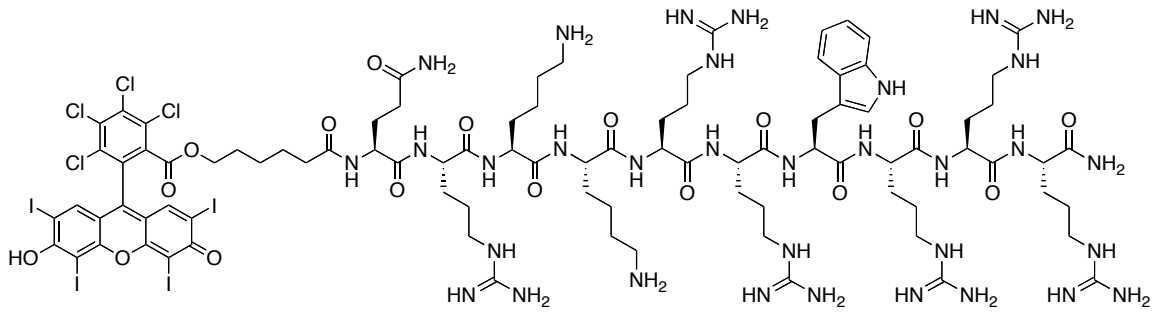
RB-WRKKRRQRRR-CONH$_2$ (3c). MALDI-TOF calculated for [$C_{90}H_{129}Cl_4I_4N_{33}O_{17}$] [M + H]$^+$ requires *m*/*z* 2595. Found 2596. **HPLC retention time** 29.3 min



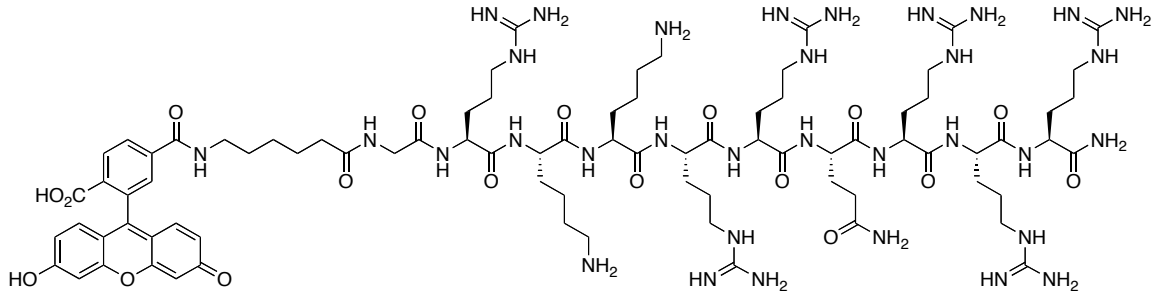
RB-QRKKRRGRRR-CONH$_2$ (3d). MALDI-TOF calculated for [$C_{81}H_{122}Cl_4I_4N_{32}O_{17}$] [M + H]$^+$ requires *m*/*z* 2465. Found 2468. **HPLC retention time** 25.9 min



RB-QRKKRRHRRR-CONH$_2$ (3e). MALDI-TOF calculated for [$C_{85}H_{126}Cl_4I_4N_{34}O_{17}$] [M + H]$^+$ requires *m*/*z* 2546. Found 2550. **HPLC retention time** 25.6 min



RB-QRKKRRWRRR-CONH$_2$ (3f). MALDI-TOF calculated for [$C_{90}H_{129}Cl_4I_4N_{33}O_{17}$] [M + H]$^+$ requires *m/z* 2595. Found 2597. **HPLC retention time** 26.1 min



Fl-GRKKRRQRRR-CONH$_2$ (4). MALDI-TOF calculated for [$C_{82}H_{131}N_{33}O_{18}$] [M + H]$^+$ requires *m/z* 1867 Found 1867. **HPLC retention time** 7.65 min

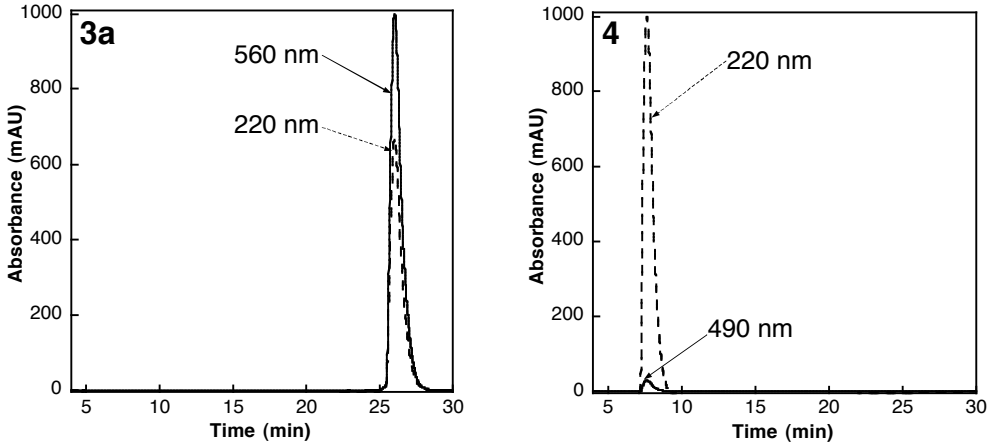


Figure S1. HPLC analysis of purified compounds **3a** and **4**. Buffer A (0.1% TFA in H_2O), Buffer B (0.1% TFA in CH_3CN); gradient, 20-100% B in 80 min. HPLC chromatogram of **3a**: 220 nm (□□), 560 nm (□) *left*; **4**: 220 nm (□□), 490 (□) nm *right*. Compounds **2**, **3a-f**, and **4** all exhibited purities of > 95%.

Cell Culture

HeLa 229 cells (ATCC) were cultured as subconfluent monolayers on 25 or 75 cm^2 cell culture plates with vent caps (Corning) in 1 x minimum essential medium medium (Gibco) supplemented with 10% (v/v) fetal bovine serum (ATCC) in a humidified incubator at 37 °C containing 5 % CO_2 (gas).

Confocal Microscopy

HeLa cells that had been grown to subconfluence were dissociated from the surface with 2 mL of 0.05 % Trypsin/0.53 mM EDTA (Cellgro) for 15 minutes at 37 °C. Aliquots of 1 x 10^5 cells were plated in four-well Lab-Tek glass bottom coverslips (Nalge Nunc Inc.) and cultured overnight to allow cell adherence. The culture media was removed and the cells were rinsed in 1 x Ca^{2+} and Mg^{2+} free PBS, pH 7.4 (Cellgro). The cells were incubated for 1.5 hours at 37 °C with 500 L of media containing 10 M peptide conjugate (**3a** and/or **4**). The cells were washed (3 x 5) minutes with 1 mL of PBS. Cells were incubated with 500 L PBS and placed on ice. Images were taken with an inverted Leica TCS SP2 scanning confocal microscope with a 40x oil immersion lens. The images were analyzed with the Leica confocal software program.

Cells incubated with a single RB- or Fl-peptide conjugate were used to identify appropriate emission collection parameters and to minimize bleed-through of the other fluorophore. The excitation wavelength for both **3a** and **4** was 488 nm. Fl emission was collected from 490-540 nm and RB emission was collected from 600-650 nm. These parameters were used in all experiments. Cells were only exposed to propidium iodide (MP Biomedicals) to determine the extent of cell death (< 5 %). Cells that brightly fluoresced in subsequent experiments were assumed to be dead and were not used in the evaluation of the conjugates

Absorption and fluorescence spectra

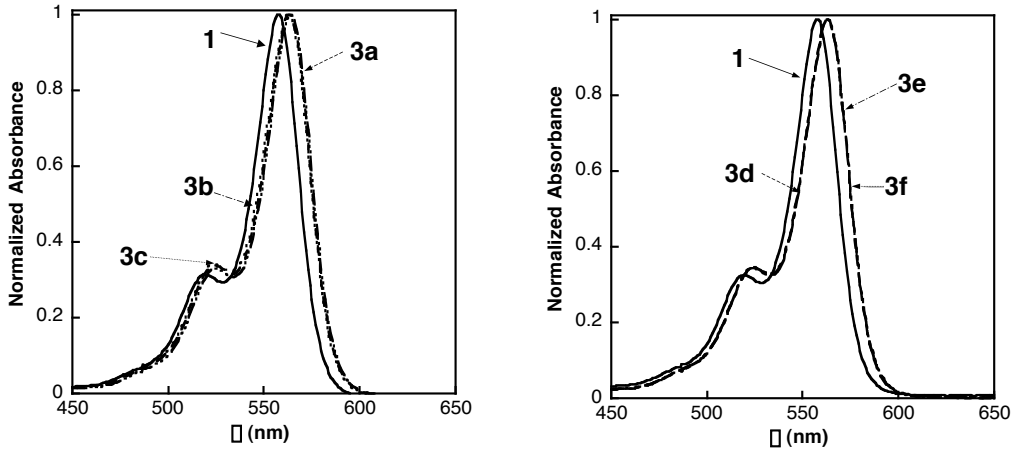


Figure S2. Absorption spectra for **1** (solid line); **3a** (long dash), **3b** (long/short dash), **3c** (short dash) *(right)*; **3d** (long dash), **3e** (long/short dash), **3f** (short dash) (*left)* in methanol.

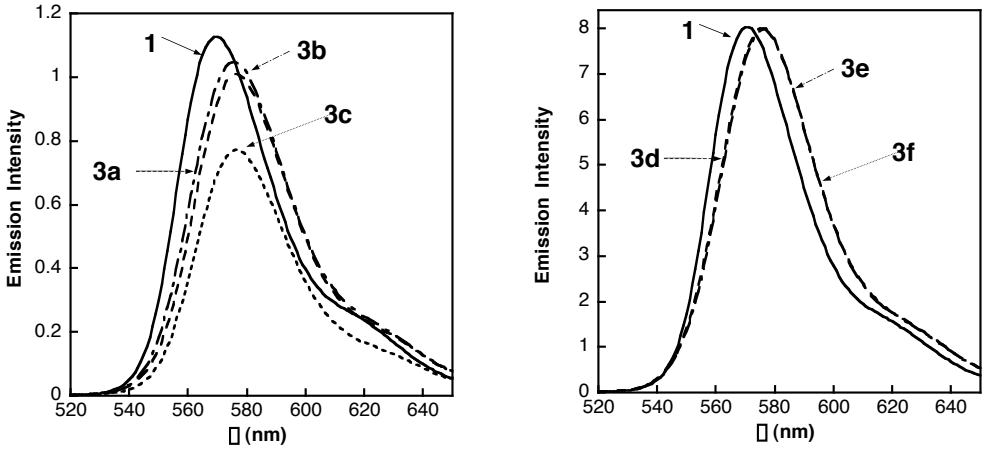


Figure S3. Fluorescence spectra for **1** (solid line); **3a** (long dash), **3b** (long/short dash), **3c** (short dash) *(right)*; **3d** (long dash), **3e** (long/short dash), **3f** (short dash) (*left)* in methanol.

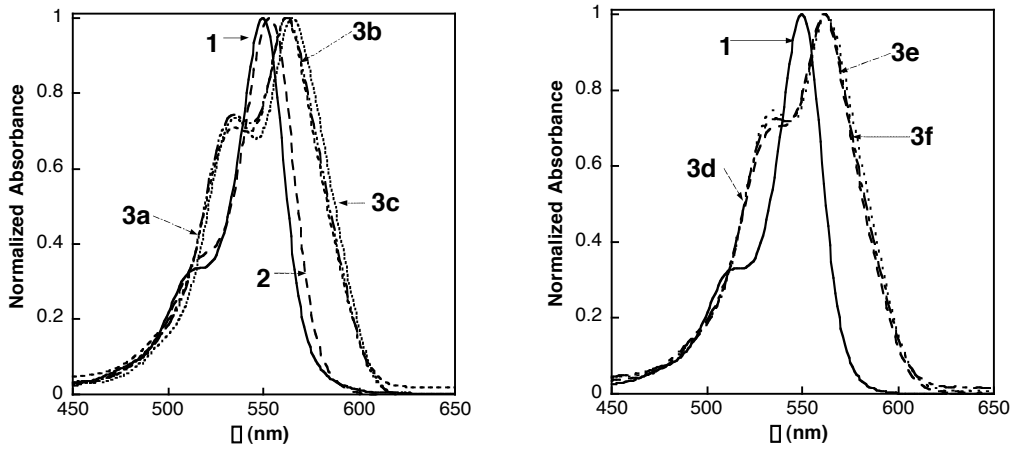


Figure S4. Absorption spectra for **1** (solid line); **3a** (long dash), **3b** (long/short dash), **3c** (short dash) *(right)*; **3d** (long dash), **3e** (long/short dash), **3f** (short dash) (*left)* in buffer.

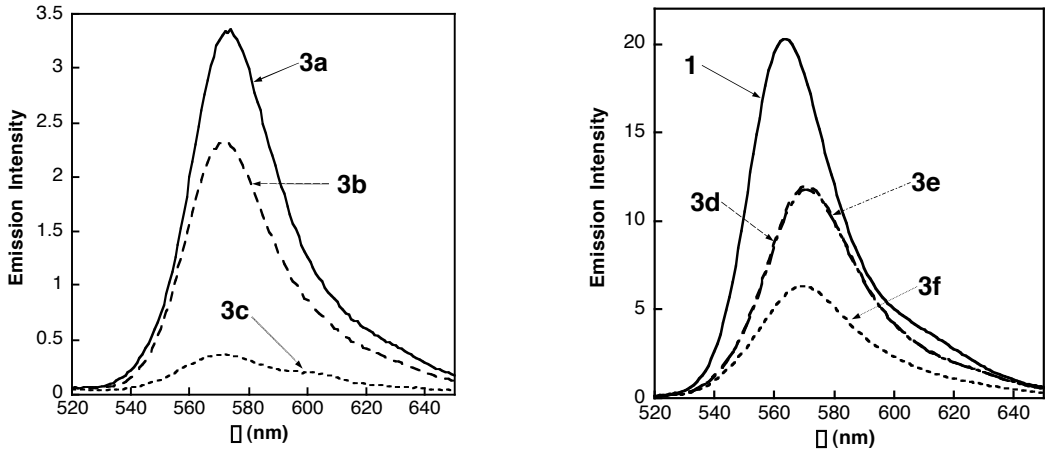


Figure S5. Fluorescence spectra for **1** (solid line); **3a** (long dash), **3b** (long/short dash), **3c** (short dash) *(right)*; **3d** (long dash), **3e** (long/short dash), **3f** (short dash) (*left)* in buffer.

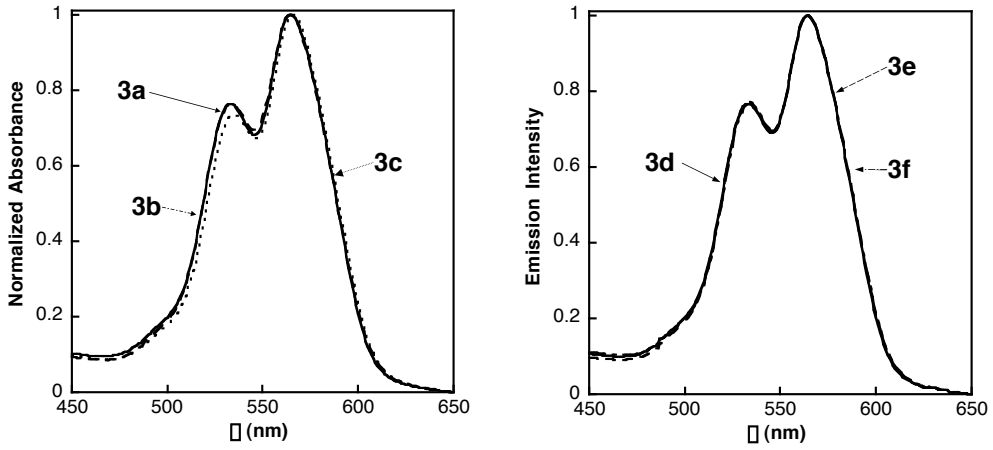


Figure S6. Absorption spectra for **1** (solid line); **3a** (long dash), **3b** (long/short dash), **3c** (short dash) *(right)*; **3d** (long dash), **3e** (long/short dash), **3f** (short dash) (*left)* in buffered solutions containing CT DNA.

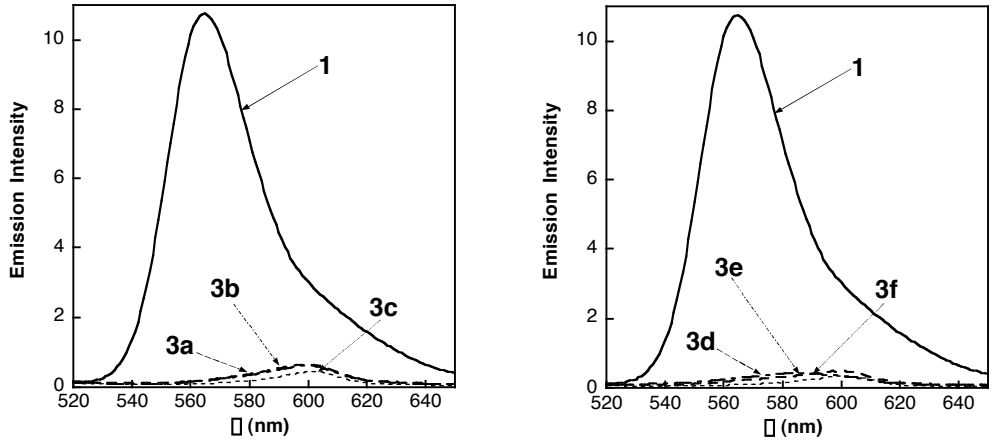


Figure S7. Fluorescence spectra for **1** (solid line); **3a** (long dash), **3b** (long/short dash), **3c** (short dash) *(right)*; **3d** (long dash), **3e** (long/short dash), **3f** (short dash) (*left)* in buffered solutions containing CT DNA.

Table S1: Absorption λ_{max} values

Compound	λ_{max} Abs (Methanol)	λ_{max} Abs (buffer)	λ_{max} Abs (DNA)
RB (**1**)	558	550	549
RB-COOH (**2**)	561	553	559
RB-**G**RKKRRQRRR (**3a**)	563	562	565
RB-**H**RKKRRQRRR (**3b**)	563	562	563
RB-**W**RKKRRQRRR (**3c**)	564	565	566
RB-QRKKRR**G**RRR (**3d**)	563	564	561
RB-QRKKRR**H**RRR (**3e**)	563	564	561
RB-QRKKRR**W**RRR (**3f**)	563	564	562

Table S2: Emission λ_{max} values

Compound	λ_{max} Em (Methanol)	Em λ_{max} (Buffer)	λ_{max} Em (DNA)
RB (**1**)	571	564	564
RB-COOH (**2**)	573	564	561
RB-**G**RKKRRQRRR (**3a**)	577	566	n/a**
RB-**H**RKKRRQRRR (**3b**)	577	567	n/a
RB-**W**RKKRRQRRR (**3c**)	578	556*	n/a
RB-QRKKRR**G**RRR (**3d**)	576	576	n/a
RB-QRKKRR**H**RRR (**3e**)	576	576	n/a
RB-QRKKRR**W**RRR (**3f**)	575	575	n/a

*The λ_{max} appears shifted because of a low intensity.

**λ_{max} values are not reported for DNA-bound conjugates because the emission is almost completely quenched.

Reductive quenching of RB

The E_{oo} of *RB was calculated to be 2.2 eV, while the reduction potential of this dye is reported to be +0.6 V in aqueous buffer.[6] This yields an excited state reduction potential of +1.6 V for the excited state of RB. Considering an oxidation potential for tryptophan of –1.0 V, we calculate a favorable driving force of –0.6 V for the reductive quenching by Trp. Likewise, considering that the oxidation potential of guanine is ~ -1.2 V, it is reasonable that *RB also reacts with this DNA base.

Photooxidation of the DNA bases by the excited state of RB

To test the sequence dependence of the quenching of *RB by DNA, samples containing 0.5 μM of **3a** were titrated with solutions of dG_{15}, dC_{15}, dA_{15} or dT_{15} and the fluorescence was measured after each addition. The data was analyzed according to the Stern-Volmer equation (**Eq. 1**). For the concentration range of 0-0.2 μM oligonucleotide, k_qτ values of 300, 26, 33, and 23 μM^{-1} were obtained for dG_{15}, dC_{15}, dA_{15} or dT_{15}. This qualitative analysis indicates that the only DNA base that efficiently quenches *RB is guanine.

$$\textbf{Eq. 1} \quad F_O / F = k_q \tau [Q] + 1$$

Measurement of dissociation constants

Dissociation constants were determined with fluorescence titrations performed in a 384 well plate with a total volume of 40 μL in each well. The concentration of RB-conjugates was kept constant and the concentration of CT DNA was increased until fluorescence signals plateaued. Each sample was run in triplicate and the values of each concentration point were averaged. Scatchard analysis was used to obtain K_d values.

Singlet oxygen monitoring and DNA damage analysis

The production of $^1O_2$ was monitored using an assay based on the peroxidation of diphenylbenzofuran (DPFB).[8] DPFB loses its visible absorbance upon oxidation and therefore provides a useful tool for the measurement of $^1O_2$. The irradiation of RB, RB-carboxylic acid (**2**), and RB-peptide conjugates produced comparable amounts of $^1O_2$ in aqueous solutions lacking DNA.

The damage of plasmid DNA was monitored using the nicking assay as described.[9] While DNA subjected to prolonged (> 15 minutes) irradiation in the presence of RB was cleaved to a significant extent, no damage was observed with RB-peptide conjugates. Gel electrophoresis analysis of a ^{32}P labeled 15-base oligonucleotide showed no G oxidation after piperidine treatment or any other type of damage, confirming that these compounds do not damage DNA.

References for supporting information

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