Supporting Information:

Synthesis and Properties of Squaraine-Modified DNA

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TABLE OF CONTENTS

1) General \hspace{10cm} S2
2) Experimental procedures \hspace{10cm} S3
3) Synthetic procedures and NMR, MS data (Fig. S1-4) of compounds 1-4 \hspace{10cm} S5
4) Absorption and fluorescence spectra of the squaraine diol 2, Fig. S5 \hspace{10cm} S12
5) MS and HPLC data of the squaraine (Sq) modified ON1 and ON2, Fig. S6-8, Table S1 \hspace{10cm} S13
6) Example of determination of the quantum yield, Fig. S9 \hspace{10cm} S15
7) Tm experiments, Fig. S10, S11, Table S2 \hspace{10cm} S16
8) Influence of the temperature on the absorption and emission spectra, Fig. S12-14 \hspace{10cm} S18
9) Stability of the squaraine diol 2 to treatment by 30\% NH\textsubscript{4}OH solution, Fig. S15 \hspace{10cm} S19
10) CD spectra, Fig. S16, S17 \hspace{10cm} S20
1) General

Chemical reactions were monitored by TLC (Silica gel, Macherey-Nagel) or spectrophotometrically.

$^1$H-NMR and $^{31}$P-NMR spectra were measured on a Bruker avance 300 spectrometer in DMSO-d$_6$ or CDCl$_3$ using signal of remaining non-deuterium solvent as an internal standard for $^1$H-NMR spectra: 2.50 ppm in DMSO and 7.26 ppm in CDCl$_3$.

Mass spectrometry was performed with an Applied Biosystems MDS-Sciex QTRAP LC/MS/MS system and LTQ Orbitrap XL system.

Absorption spectra and extinction coefficients ($\varepsilon$) were recorded in 1-cm quartz cells at 20 °C on Varian Cary-100 Bio-UV/VIS spectrophotometer equipped with a Varian Cary-block temperature controller.

Emission spectra and quantum yields ($\Phi_F$) were measured in 1-cm quartz cells at 20 °C on Varian Cary Eclipse fluorescence spectrophotometer equipped with a Varian Cary-block temperature controller.

CD spectra were recorded on a JASCO J-715 spectropolarimeter using quartz cuvettes with an optical path of 1 cm.

HPLC separation and purity determination were performed with a Shimadzu LC system equipped with a Shimadzu-block temperature controller.

Reagents and solvents for synthesis of the compounds 1-4 were purchased from Alfa Aesar, Acros Organics, Fluka and Sigma Aldrich. UltraMILD CE phosphoramidites and other reagents for synthesis of the modified oligonucleotides ON1 and ON2 were from Glen Research and Proligo® Reagents. Unmodified oligonucleotides ON3 and ON4 were from Microsynth (Balgach, Switzerland).
2) Experimental procedures.

Oligonucleotides ON1 and ON2 were prepared via automated oligonucleotide synthesis on a 394-DNA/RNA synthesizer (Applied Biosystems). UltraMILD CE phosphoramidites, UltraMILD supports and special Cap Mix A (THF/Pyridine/Pac₂O) were used for the synthesis. Cleavage from the solid support and final deprotection were done by treatment with fresh 30% NH₄OH solution at room temperature for 2 hours. The oligonucleotides were purified by reverse phase HPLC: column LiChrospher® 100 RP-18, 250 mm × 4 mm, Merck; mobile phase A = (Et₃NH)OAc (0.1 M, pH 7.4); mobile phase B = MeCN; elution at 25 °C; gradient 0 – 40% B over 22 min, then 40-100% B over 5 min.

Measurements of spectra in aqueous solutions (except quantum yield determinations) were performed at a concentration of 1.5 µM (10 mM PB, pH = 7.4, 100 mM NaCl) for oligonucleotides and at a concentration of 1.5 µM + 1.5 µM of each strand (10 mM PB, pH = 7.4, 100 mM NaCl) for duplexes. Concentration of oligonucleotides was determined using molar absorptivities of ε₂₆₀(ON1) = 233,400 M⁻¹ cm⁻¹, ε₂₆₀(ON2) = 212,200 M⁻¹ cm⁻¹, ε₂₆₀(ON3) = 231,400 M⁻¹ cm⁻¹ and ε₂₆₀(ON4) = 216,500 M⁻¹ cm⁻¹; a value of ε₂₆₀(Sq) = 11,000 M⁻¹ cm⁻¹ was applied for calculation of ε₂₆₀(ON1) and ε₂₆₀(ON2).

Thermal denaturation experiments were carried out on a Varian Cary-100 Bio-UV/VIS spectrophotometer and data were collected at 260, 590 and 630 nm for duplexes ON1*ON2, ON1*ON4 and ON2*ON3 with internal squaraine modifications in the backbone, and at 260 and 630 nm for the non-modified duplex ON3*ON4 (cooling-heating-cooling cycles in the temperature range of 20-90 °C, temperature gradient of 0.5 °C/min). Melting temperature (Tₘ) values were determined as the maximum of the first derivative of the melting curve. Fluorescence melting curves and temperature dependent fluorescence curves of the duplex ON1*ON2 and oligonucleotides ON1 and ON2 were collected at 645 nm with excitation at 600 nm (cooling-heating-cooling cycles in the temperature range of 20-90 °C, temperature gradient of 1 °C/min). Spectra were not corrected for temperature induced absorbance changes.

For measurement of the extinction coefficient (ε) of squaraine, the diol 2 (~ 10 mg) was dissolved in 50 mL of methanol or chloroform, the stock solution (50 µL) was diluted (1:1000) and the absorbance was measured in a 1-cm standard quartz cell. The dye concentrations were in the range of 3.0 to 4.0×10⁻⁷ M. The extinction coefficients were calculated according to the Beer-Lambert law. The ε of the dye was independently measured several times and the average value was taken. The reproducibility for determining the extinction coefficients was ± 2,000 M⁻¹ cm⁻¹.

For the determination of the quantum yields, the integrated relative intensities of the samples were measured against Cy5 (quantum yield, QY_Cy5 = 27% in water [1]) as the reference. Absorbance of the solutions at the excitation wavelength (600 nm for the squaraine diol 2, ON1 and ON2 and 615 nm for the duplex ON1*ON2) was between 0.04–0.06, measured in a 1-cm cell. The emission spectra of the solutions were recorded and the quantum yields of the samples were determined as described in [2] according to formula:

\[ QY = QY_{Cy5} \cdot \left( \frac{F}{F_{Cy5}} \right) \cdot \left( \frac{A_{Cy5}}{A} \right) \cdot \left( \frac{n^2}{n_{Cy5}^2} \right), \]

where $F$ and $F_{\text{Cy5}}$ are the integrated areas of the fluorescence spectra, $A$ and $A_{\text{Cy5}}$ are the absorbance at the excitation wavelength and $n$ and $n_{\text{Cy5}}$ are the refraction indices of solvents used for the sample under examination and Cy5, respectively.

**Stability of the squaraine diol 2 to treatment by 30 % NH$_4$OH at room temperature and at 55 °C.**

Stock solution of the squaraine diol 2 in MeOH was added to 30 % NH$_4$OH until the absorbance at the maximum reached 0.9. The solution was shaken at room temperature for 6 h. Absorption spectra were measured before the beginning of shaking ($A_0$) and after 2 and 6 h ($A_1$). To examine the stability at 55 °C, 5 solutions (A-E) with absorbance at the maximum of 0.8-1.1 were treated in the same manner as described above. These solutions were shaken at 55 °C: A – 10 min, B – 30 min, C – 60 min, D – 120 min and E – 180 minutes. Absorption spectra were measured before the beginning of shaking ($A_0$) and after treatment ($A_1$). Values of the $A_1/A_0$ ratio were used for analysis.
3) Synthetic procedures

**Synthesis of 1-(6-hydroxyhexyl)-2,3,3-trimethyl-3H-indolium bromide (1):**

A mixture of 2,3,3-trimethylindolenine (1.01 g, 6.28 mmol), 6-bromo-1-hexanol (1.70 g, 9.39 mmol) and acetonitrile (10 ml) was refluxed under argon atmosphere for 30 h. The solvent was removed under reduced pressure and the residue was dissolved in methanol. The product was precipitated by diethyl ether and the solution was removed by decantation. A deep red resin was obtained after drying. \( \lambda_{\text{max}} \) (Abs) = 279 nm (MeOH). Yield: 1.51 g (65%).

1H-NMR (300 MHz, DMSO-d<sub>6</sub>, ppm): \( \delta \) 8.03-7.94 (1H, m, arom H), 7.89-7.81 (1H, m, arom H), 7.66-7.59 (2H, m, arom H), 4.46 (2H, t, \( J = 7.72 \) Hz, NCH<sub>2</sub>), 3.38 (2H, t, \( J = 5.93 \) Hz, CH<sub>2</sub>OH), 2.85 (3H, s, 2-CH<sub>3</sub>), 1.91-1.72 (2H, m, CH<sub>2</sub>), 1.54 (6H, s, [3-CH<sub>3</sub>]<sub>2</sub>), 1.49-1.29 (6H, m, CH<sub>2</sub>).

ESI-MS LR (pos, MeCN): MS(calc. exact, C<sub>17</sub>H<sub>26</sub>NO<sub>3</sub>) = 260.2014; MS(found) = 260.2007.

**Synthesis of the squaraine diol (2):**

Compound 1 (0.370 g, 1.10 mmol) was dissolved in pyridine (7 ml). Then 3,4-dihydroxy-3-cyclobutene-1,2-dione (50.01 mg, 0.438 mmol) and 1-butanol (7 ml) were added. The mixture was stirred at 110 °C for 5 hours. The solvent was removed under reduced pressure and residue was purified by column chromatography (Silica gel 60, 0–7% MeOH in CH<sub>2</sub>Cl<sub>2</sub>) to give the diol as a blue powder. \( R_f = 0.23 \). (Silica gel 60, 5% MeOH in CH<sub>2</sub>Cl<sub>2</sub>). Yield: 121 mg (46%).

1H-NMR (300 MHz, DMSO-d<sub>6</sub>, ppm): \( \delta \) 7.53 (2H, d, 7.35 Hz, arom H), 7.37-7.29 (4H, m, arom H), 7.16 (2H, t, \( J = 6.87 \) Hz, arom H), 5.79 (2H, s, CH), 4.35 (2H, t, \( J = 5.18 \) Hz, OH), 4.07 (4H, t, \( J = 6.5 \) Hz, NCH<sub>2</sub>), 3.38 (4H, t, \( J = 5.56 \) Hz, CH<sub>2</sub>OH), 1.77-1.62 (4H, m, CH<sub>2</sub>), 1.68 (12H, s, 3-CH<sub>3</sub>), 1.53-1.28 (12H, m, CH<sub>2</sub>).

ESI-MS LR (pos, MeOH): MS(calc. exact, C<sub>38</sub>H<sub>48</sub>BrN<sub>2</sub>O<sub>4</sub>) = 596.3614; MS(found) = 596.3605.
Synthesis of the mono(dimethoxytrityl)-protected derivative (3):

4,4'-Dimethoxytrityl chloride (0.101 g, 0.307 mmol) was added to a solution of the squaraine diol 2 (0.230 g, 0.385 mmol) in dry pyridine (10 ml). The mixture was stirred at room temperature under argon for 4 h. An additional portion of 4,4'-dimethoxytrityl chloride (65 mg, 0.192 mmol) was added after 2 h. The solvent was removed under reduced pressure and the residue was purified by column chromatography (Silica gel 60, 0–5% MeOH in CH$_2$Cl$_2$/EtOAc/NEt$_3$, 49/49/2, v/v/v). R$_f$ = 0.51 (Silica gel 60, 5% MeOH in CH$_2$Cl$_2$/EtOAc/NEt$_3$, 49/49/2, v/v/v). Yield: 134 mg (39%).

$^1$H-NMR (300 MHz, DMSO-d$_6$, ppm): $\delta$ 7.52 (2H, d, $J = 7.53$ Hz, arom H), 7.40-7.11 (15H, m, arom H), 6.87 (4H, d, $J = 9.04$ Hz, arom H), 5.80 (1H, s, CH), 4.35 (1H, t, $J = 5.18$ Hz, OH), 4.13-3.98 (4H, m, NCH$_2$), 3.72 (6H, s, OCH$_3$), 3.37 (2H, t, $J = 5.84$ Hz, CH$_2$), 2.93 (2H, t, $J = 6.21$ Hz, CH$_2$), 1.76-1.59 (4H, m, CH$_2$), 1.67 (6H, s, 3-CH$_3$), 1.66 (6H, s, 3-CH$_3$), 1.58-1.47 (2H, m, CH$_2$), 1.46-1.27 (10H, m, CH$_2$).

ESI-MS LR (pos, MeCN): MS (calc. exact, C$_{59}$H$_{66}$N$_2$O$_6$) = 898.4921; MS(found) = 898.49.

Synthesis of the squaraine phosphoramidite (4):

$^1$H-NMR (300 MHz, CDCl$_3$, ppm): $\delta$ 7.45-7.09 (15H, m, arom H), 6.96 (2H, t, $J = 7.82$ Hz, arom H), 6.82 (4H, d, $J = 8.85$ Hz, arom H), 5.94 (2H, s, CH), 4.04-3.90 (4H, m, NCH$_2$), 3.89-3.75 (2H, m, CH$_2$), 3.78 (6H, s, OCH$_3$), 3.70-3.51 (4H, m, CH$_2$OP), 3.03 (2H, t, $J = 6.5$ Hz, CH$_2$ODMT), 2.64 (2H, t, $J = 6.5$ Hz, CH$_2$CN), 1.87-1.74 (4H, m, CH$_2$), 1.78 (12H, s, 3-CH$_3$), 1.68-1.55 (4H, m, CH$_2$, superimposed with water), 1.51-1.37 (8H, m, CH$_2$), 1.17-1.18 (12H, m, CHCH$_3$).
$^{31}$P-NMR (300 MHz, CDCl$_3$): $\delta = 147.39$

ESI-MS LR (pos, MeCN): MS (calc. exact, C$_{68}$H$_{83}$N$_4$O$_7$P) = 1098.5999; MS(found) = 1098.60.
Figure S1. $^1$H-NMR (top) and ESI-MS (bottom) spectra of the compound 1
**Figure S2.** $^1$H-NMR (top) and ESI-MS (bottom) spectra of the squaraine diol 2.
Figure S3. $^{1}$H-NMR (top) and ESI-MS (bottom) spectra of compound 3
Figure S4. $^1$H-NMR (top), $^{31}$P-NMR (middle) and ESI-MS (bottom) spectra of the Sq phosphoramidite
4) Absorption and fluorescence spectra of the squaraine diol 2.

Figure S5. Normalized absorption and fluorescence spectra of the squaraine diol 2 measured in methanol (left) and chloroform (right) at concentration $c \sim 1 \times 10^{-6}$ M. Excitation at 600 nm.
5) Mass-spectrometry and HPLC data of the squaraine (Sq) modified ON1 and ON2.

Table S1. Mass spectrometry data of the ON1 and ON2 (ESI-MS, negative mode, MeCN/H₂O/0.5% Et₃N).

<table>
<thead>
<tr>
<th>Oligomer</th>
<th>Sequence</th>
<th>Molecular formula</th>
<th>Calc. Exact mass</th>
<th>Found mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>ON1</td>
<td>5’ AGCTCGGTCASqCGAGAGTGCA</td>
<td>C₂₃₁H₂₉₁N₇₃O₁₂₆P₂₀</td>
<td>6724.3516</td>
<td>6724.3424</td>
</tr>
<tr>
<td>ON2</td>
<td>3’ TCGAGCCAGTSqGCTCTCAGGT</td>
<td>C₂₃₁H₂₉₁N₇₃O₁₂₆P₂₀</td>
<td>6724.3516</td>
<td>6724.3424</td>
</tr>
</tbody>
</table>

Figure S6. HR-MS of ON1.
Figure S7. HR-MS of ON2.

Figure S8. HPLC data of oligonucleotides ON1 (left) and ON2 (right) recorded at 260 and 630 nm.
6) Example of determination of the quantum yield

![Graph showing absorption and fluorescence spectra](image)

**Figure S9.** Quantum yield measurement of Sq 2 and oligonucleotides ON1 and ON2 in methanol. Left: absorption spectra of the samples (Sq 2, ON1 and ON2) and reference (Cy5, in water) with the absorption at the excitation wavelength (600 nm) around 0.05. Right: emission spectra of the above mentioned solutions.
7) Tm experiments.

**Figure S10** Thermal denaturation-renaturation of hybrid ON1*ON4 (top), ON2*ON3 (middle) and ON3*ON4 (bottom) observed at 260 nm, 590 nm and 630 nm; cooling (–), heating (“”) and cooling (---) curves.
Figure S11. Example of Tm determination procedure: duplex ON3*ON4, melting curves at 260 nm (left) and first derivative curve, Tm = 70.7 °C (right).

Table S2. Hybridization data of the squaraine containing duplexes ON1*ON2, ON1*ON4 and ON2*ON3 and non-modified analogs ON3*ON4 (Sq-Sq vs. A-T base pair).

<table>
<thead>
<tr>
<th>Oligomer</th>
<th>Duplex</th>
<th>Tm, °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>ON1</td>
<td>5’ AGCTCGGTCA\text{Sq}CGAGAGTGCA 3’ TCGAGCCAGTAGCTCTCACGT</td>
<td>70.4</td>
</tr>
<tr>
<td>ON2</td>
<td>5’ AGCTCGGTCA\text{Sq}CGAGAGTGCA 3’ TCGAGCCAGTAGCTCTCACGT</td>
<td>70.4</td>
</tr>
<tr>
<td>ON1</td>
<td>3’ TCGAGCCAGTAGCTCTCACGT</td>
<td>63.4</td>
</tr>
<tr>
<td>ON4</td>
<td>3’ TCGAGCCAGTAGCTCTCACGT</td>
<td>62.7</td>
</tr>
<tr>
<td>ON2</td>
<td>5’ AGCTCGGTCA\text{Sq}CGAGAGTGCA 3’ TCGAGCCAGTAGCTCTCACGT</td>
<td>70.7</td>
</tr>
<tr>
<td>ON3</td>
<td>5’ AGCTCGGTCA\text{Sq}CGAGAGTGCA 3’ TCGAGCCAGTAGCTCTCACGT</td>
<td>70.7</td>
</tr>
</tbody>
</table>
8) Influence of the temperature on the absorption and emission spectra of Sq compounds.

**Figure S12.** Influence of the temperature on the absorption and emission spectrum of the squaraine diol 2 measured in methanol at concentration $c \sim 1.5 \times 10^{-6}$ M in the temperature range 20-60 °C.

**Figure S13.** Influence of the temperature on the absorption and emission spectrum of oligonucleotide ON2 in the temperature range 20-80 °C.

**Figure S14.** Influence of the temperature on the absorption spectrum of duplex ON1∗ON2 in the temperature range 20-80 °C.
9) Stability of the squaraine diol 2 to treatment by 30% NH₄OH solution

**Figure S15.** Decay of the long-wavelength absorption band of the squaraine diol 2 after treatment by 30% NH₄OH solution at 55 °C and at room temperature. Relative absorbance A/A₀, where A₀ is absorption intensity at starting point, and A is absorption intensity at time-point.
10) CD spectra

Figure S16. CD spectra of hybrids ON1*ON4 (top), ON2*ON3 (middle) and ON3*ON4 (bottom).
Figure S17. CD spectrum of ON2 (3 µM) at 20 °C and 80 °C (indicated by arrows).