Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry

Supporting Information:

Synthesis and Properties of Squaraine-Modified DNA

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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 (ε) 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 (Φ_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/Pac_2O) were used for the synthesis. Cleavage from the solid support and final deprotection were done by treatment with fresh 30% NH_4OH 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 = $(Et_3NH)OAc$ (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 absorbtivities of ε_{260}(ON1) = 233,400 M^{-1} cm^{-1}, ε_{260}(ON2) = 212,200 M^{-1} cm^{-1}, ε_{260}(ON3) = 231,400 M^{-1} cm^{-1} and ε_{260}(ON4) = 216,500 M^{-1} cm^{-1}; a value of ε_{260}(Sq) = 11,000 M^{-1} cm^{-1} was applied for calculation of ε_{260}(ON1) and ε_{260}(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 (T_m) 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:

$$QY = QY_{Cy5} \cdot (F/F_{Cy5}) \cdot (A_{Cy5}/A) \cdot (n^2/n_{Cy5}^2),$$

1 R.B. Mujumdar, L.A. Ernst, S.R. Mujumdar, C.J. Lewis, A.S. Waggoner, Cyanine dye labeling reagents: sulfoindocyanine succinimidyl esters. Bioconjugate Chem., Vol. 4, No. 2, 1993, 105–111.
2 C.A. Parker, Photoluminescence of Solutions, Elsevier Publishing Company, Amsterdam, 1968.

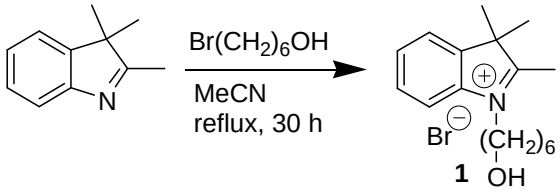
where F and F_{Cy5} are the integrated areas of the fluorescence spectra, A and A_{Cy5} are the absorbance at the excitation wavelength and n and n_{Cy5} are the refraction indices of solvents used for the sample under examination and Cy5, respectively.

Stability of the squaraine diol 2 to treatment by 30 % NH_4OH at room temperature and at 55 °C.

Stock solution of the squaraine diol **2** in MeOH was added to 30 % NH_4OH 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.

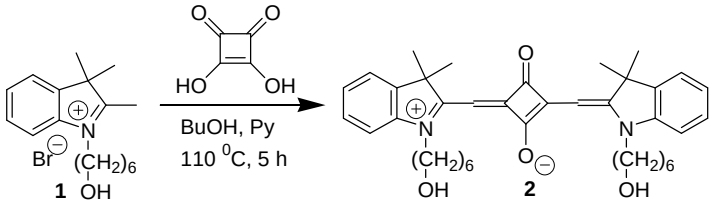
3) Synthetic procedures

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



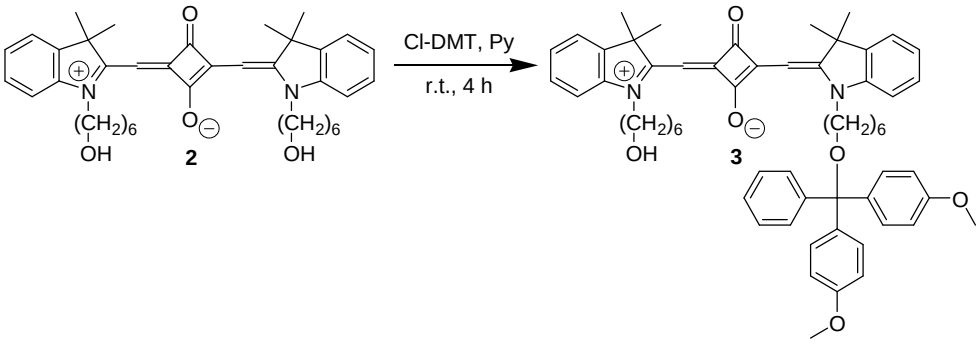
A mixture of 2,3,3-trimethylindolenine (1.01 g, 6.28 mmol), 6-bromo-1-hexanol (1.70 g, 9.39 mmol) and acetonitrile (10 ml) was refluxed under argon atmosphere for 30 h. The solvent was removed under reduced pressure and the residue was dissolved in methanol. The product was precipitated by diethyl ether and the solution was removed by decantation. A deep red resin was obtained after drying. λ_{max} (Abs) = 279 nm (MeOH). Yield: 1.51 g (65%).
^{1}H-NMR (300 MHz, DMSO-d_6, ppm): δ 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_2), 3.38 (2H, t, *J* = 5.93 Hz, $C\underline{H}_2OH$), 2.85 (3H, s, 2-CH_3), 1.91-1.72 (2H, m, CH_2), 1.54 (6H, s, [3-CH_3]$_2$), 1.49-1.29 (6H, m, CH_2).
ESI-MS LR (pos, MeCN): MS(calc. exact, $C_{17}H_{26}NO^+$) = 260.2014; MS(found) = 260.2007.

Synthesis of the squaraine diol (2):



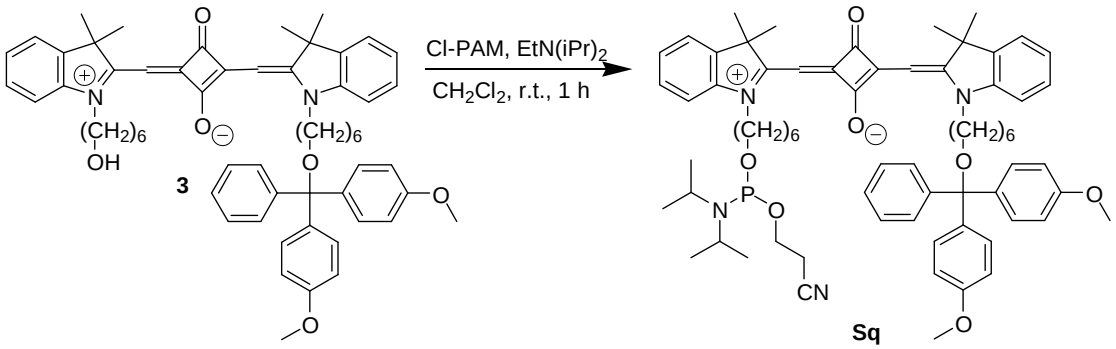
Compound **1** (0.370 g, 1.10 mmol) was dissolved in pyridine (7 ml). Then 3,4-dihydroxy-3-cyclobutene-1,2-dione (50.01 mg, 0.438 mmol) and 1-butanol (7 ml) were added. The mixture was stirred at 110 °C for 5 hours. The solvent was removed under reduced pressure and residue was purified by column chromatography (Silica gel 60, 0–7% MeOH in CH_2Cl_2) to give the diol as a blue powder. R_f = 0.23. (Silica gel 60, 5% MeOH in CH_2Cl_2). Yield: 121 mg (46%).
^{1}H-NMR (300 MHz, DMSO-d_6, ppm): δ 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_2), 3.38 (4H, t, *J* = 5.56 Hz, $C\underline{H}_2OH$), 1.77-1.62 (4H, m, CH_2), 1.68 (12H, s, 3-CH_3), 1.53-1.28 (12H, m, CH_2).
ESI-MS LR (pos, MeOH): MS calc. exact, $C_{38}H_{48}BrN_2O_4$) = 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_2Cl_2/EtOAc/NEt_3, 49/49/2, v/v/v). R_f = 0.51 (Silica gel 60, 5% MeOH in CH_2Cl_2/EtOAc/NEt_3, 49/49/2, v/v/v). Yield: 134 mg (39%).
^{1}H-NMR (300 MHz, DMSO-d_6, ppm): δ 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), 5.78 (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, $C\underline{H}_2OH$), 2.93 (2H, t, *J* = 6.21 Hz, CH_2DMT), 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, $C_{59}H_{66}N_2O_6$) = 898.4921; MS(found) = 898.49.

Synthesis of the squaraine phosphoramidite (4):



2-Cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (38 µL, 0.170 mmol) was added under argon to a solution of compound **3** (0.134 g, 0.149 mmol) and diisopropylethylamine (78 µL, 0.447 mmol) in dry dichloromethane (7 ml). The mixture was stirred at room temperature for 1 h. The mixture was partially concentrated under reduced pressure and purified by column chromatography (Silica gel 60, CH_2Cl_2/EtOAc/Et_3N, 49/49/2, v/v/v). The first blue fraction yielded compound 4. R_f = 0.76 (Silica gel 60, 5% MeOH in CH_2Cl_2/EtOAc/Et_3N, 49/49/2, v/v/v). Yield: 100 mg (61%).
^{1}H-NMR (300 MHz, $CDCl_3$, ppm): δ 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, $C\underline{H}CH_3$), 3.78 (6H, s, OCH_3), 3.70-3.51 (4H, m, CH_2OP), 3.03 (2H, t, *J* = 6.5 HZ, CH_2ODMT), 2.64 (2H, t, *J* = 6.5 Hz, CH_2CN), 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, $CHC\underline{H}_3$).

^{31}P-NMR (300 MHz, $CDCl_3$): δ = 147.39
ESI-MS LR (pos, MeCN): MS (calc. exact, $C_{68}H_{83}N_4O_7P$) = 1098.5999; MS(found) = 1098.60.

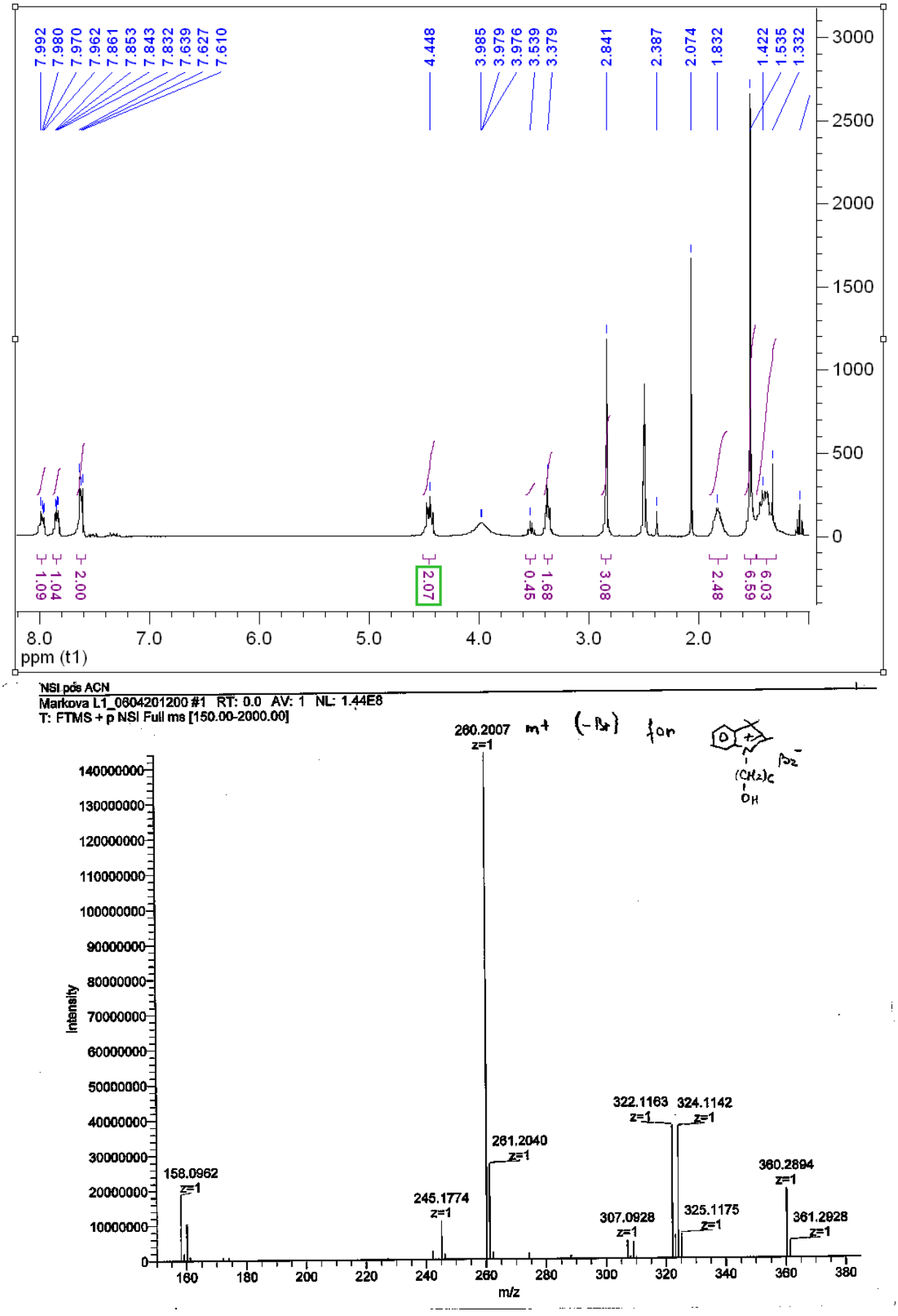


Figure S1. ^{1}H-NMR (top) and ESI-MS (bottom) spectra of the compound **1**

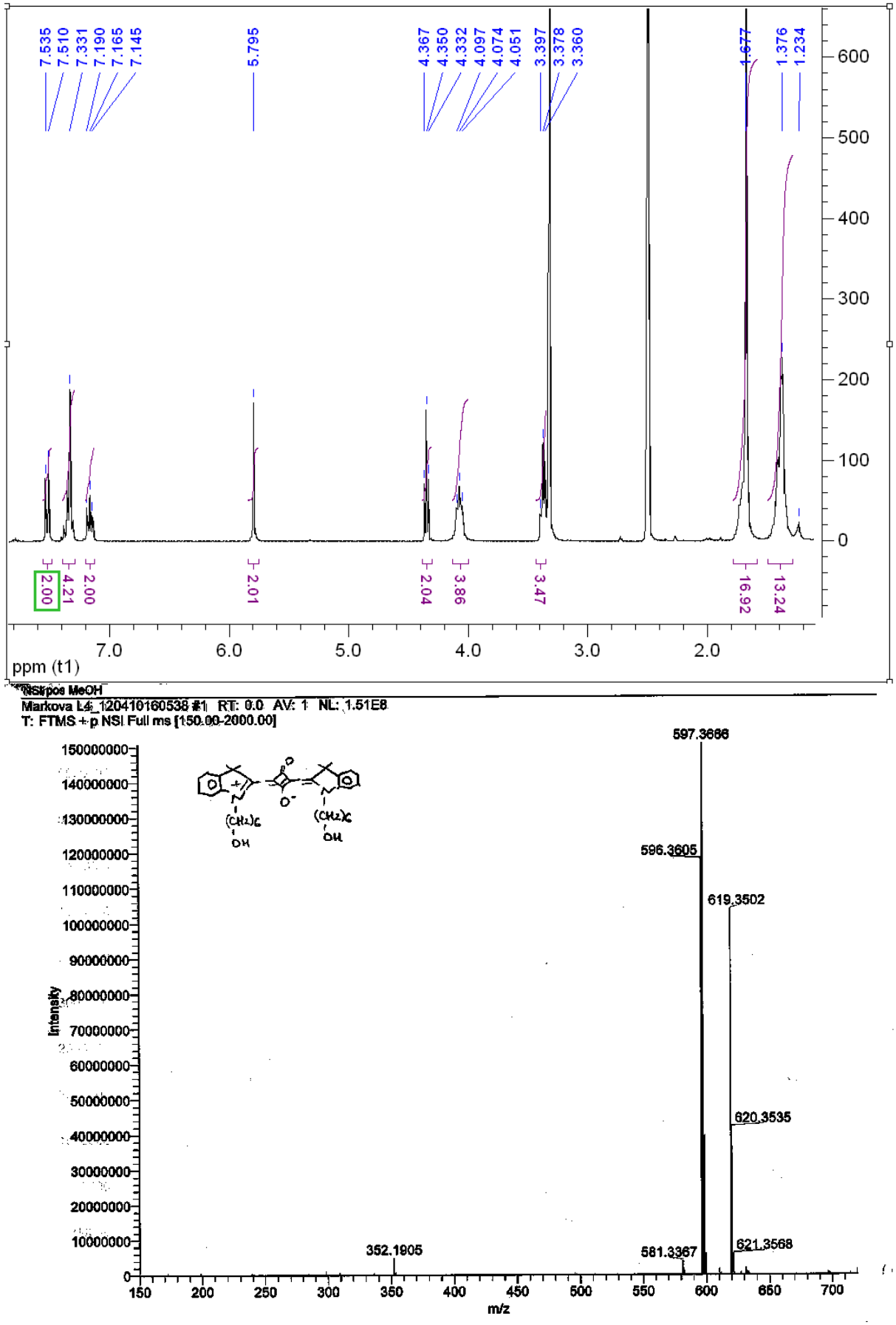


Figure S2. ^{1}H-NMR (top) and ESI-MS (bottom) spectra of the squaraine diol **2**

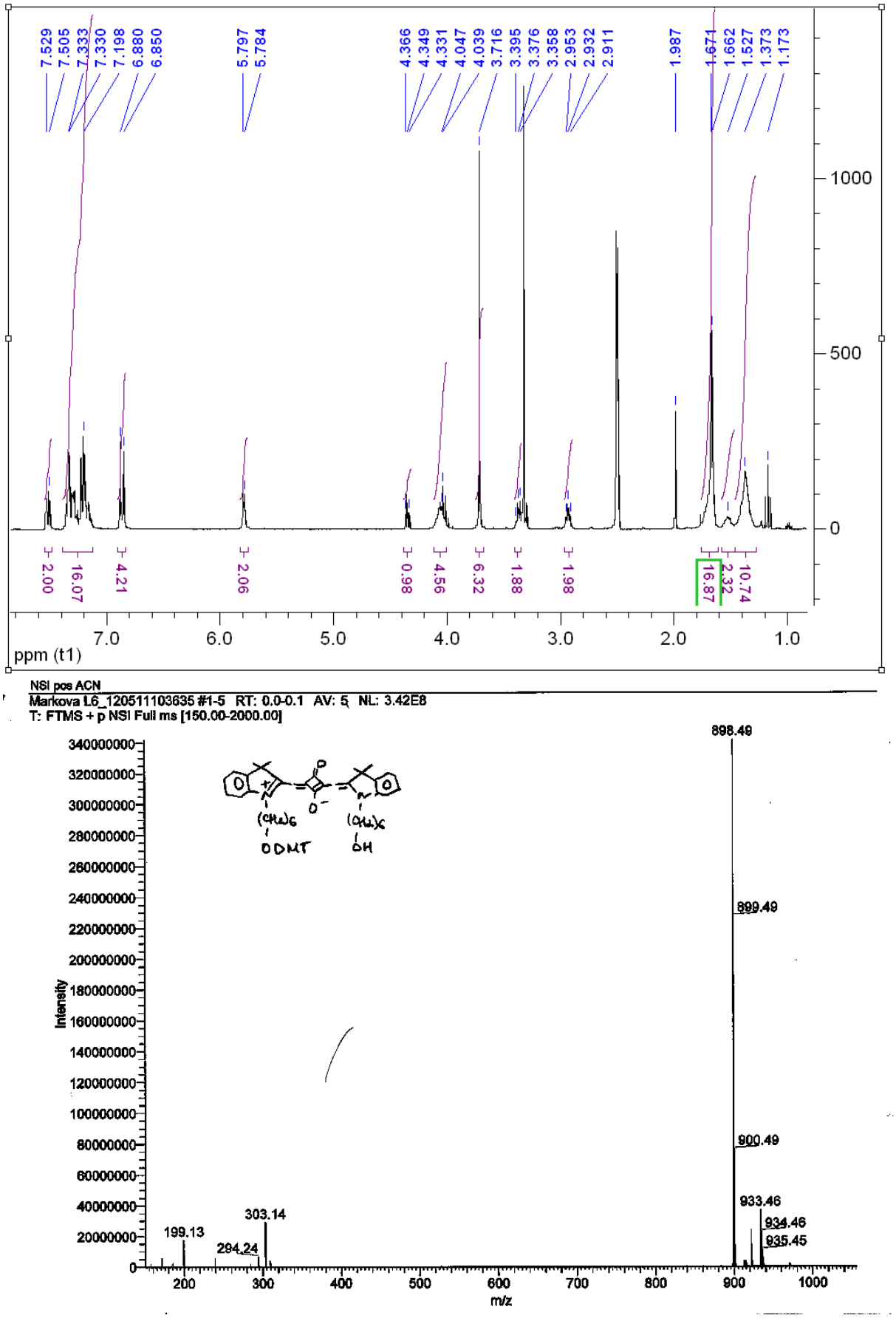


Figure S3. [1]H-NMR (top) and ESI-MS (bottom) spectra of compound **3**

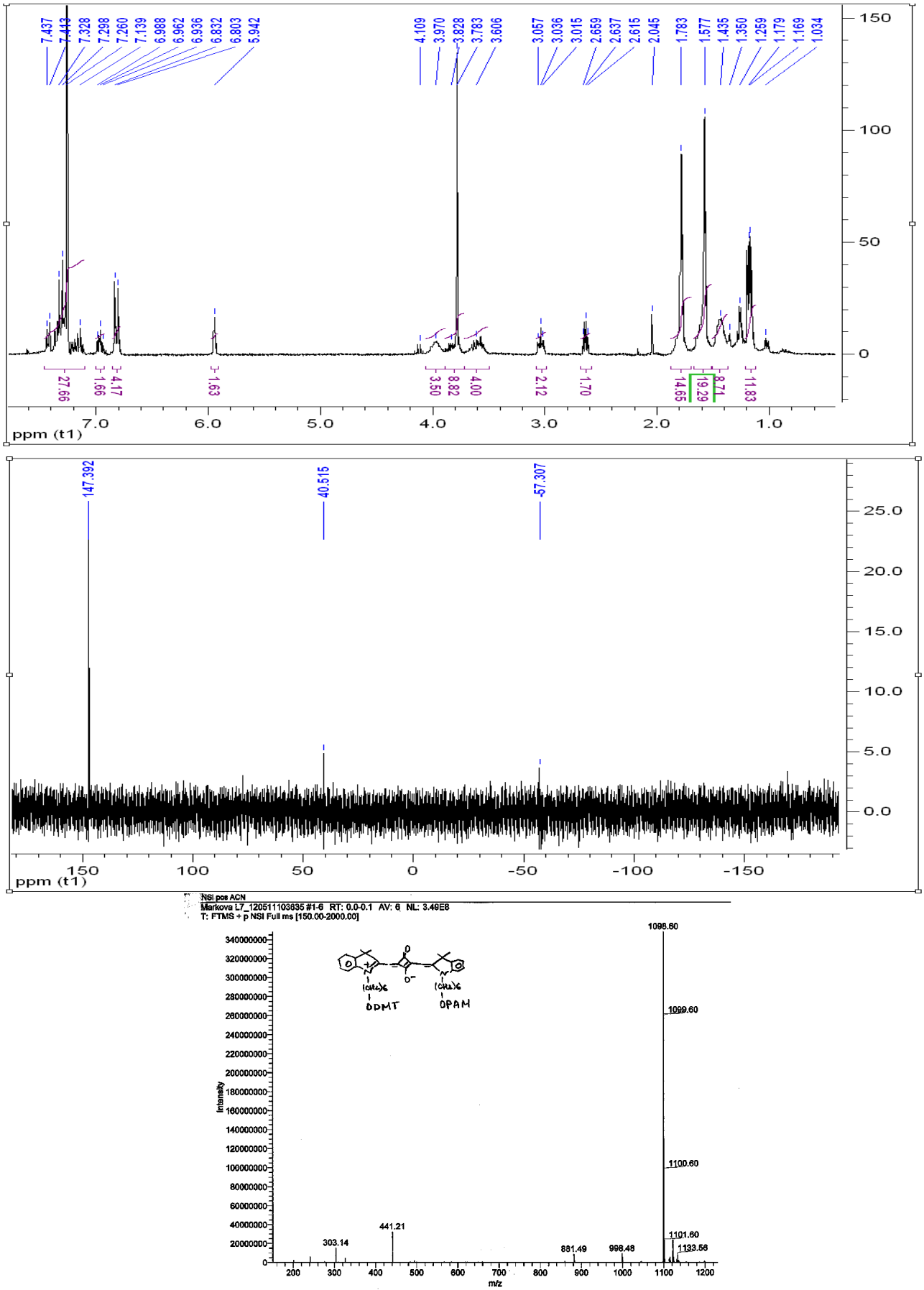


Figure S4. ^{1}H-NMR (top), ^{31}P-NMR (middle) and ESI-MS (bottom) spectra of the **Sq** phosphoramidite

4) Absorption and fluorescence spectra of the squaraine diol 2.

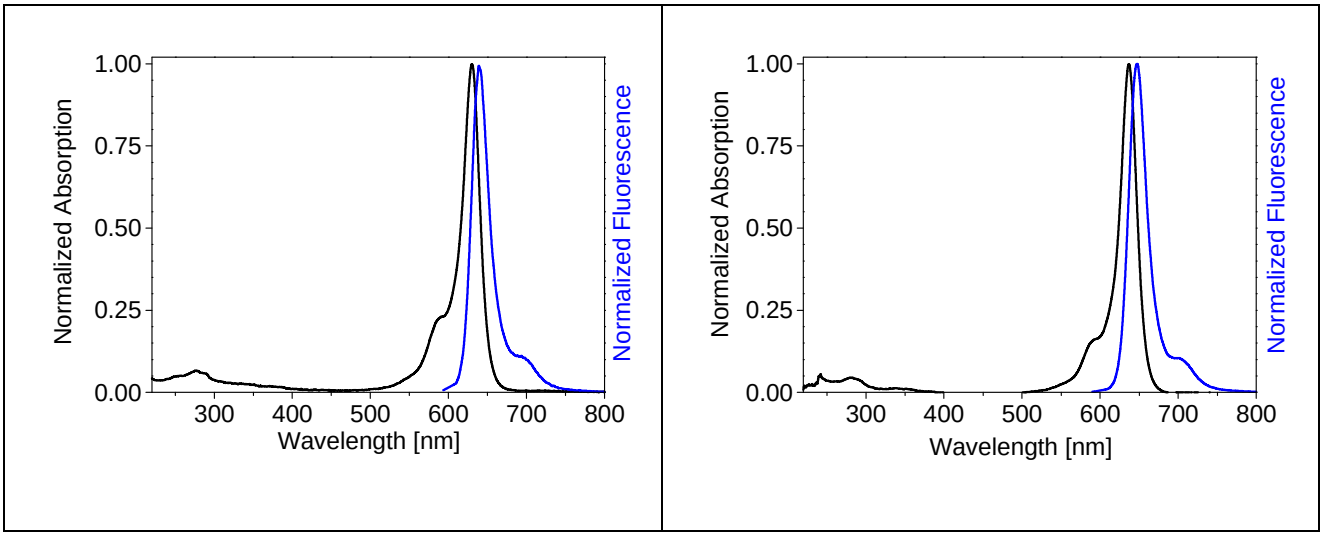


Figure S5. Normalized absorption and fluorescence spectra of the squaraine diol **2** measured in methanol (left) and chloroform (right) at concentration $c \sim 1\times10^{-6}$ M. Excitation at 600 nm.

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/H_2O/0.5% Et_3N).

Oligo-mer	Sequence	Molecular formula	Calc. Exact mass	Found mass
ON1	5' AGCTCGGTCA**Sq**CGAGAGTGCA	$C_{233}H_{291}N_{83}O_{122}P_{20}$	6822.3876	6822.3856
ON2	3' TCGAGCCAGT**Sq**GCTCTCACGT	$C_{231}H_{293}N_{73}O_{126}P_{20}$	6724.3516	6724.3424

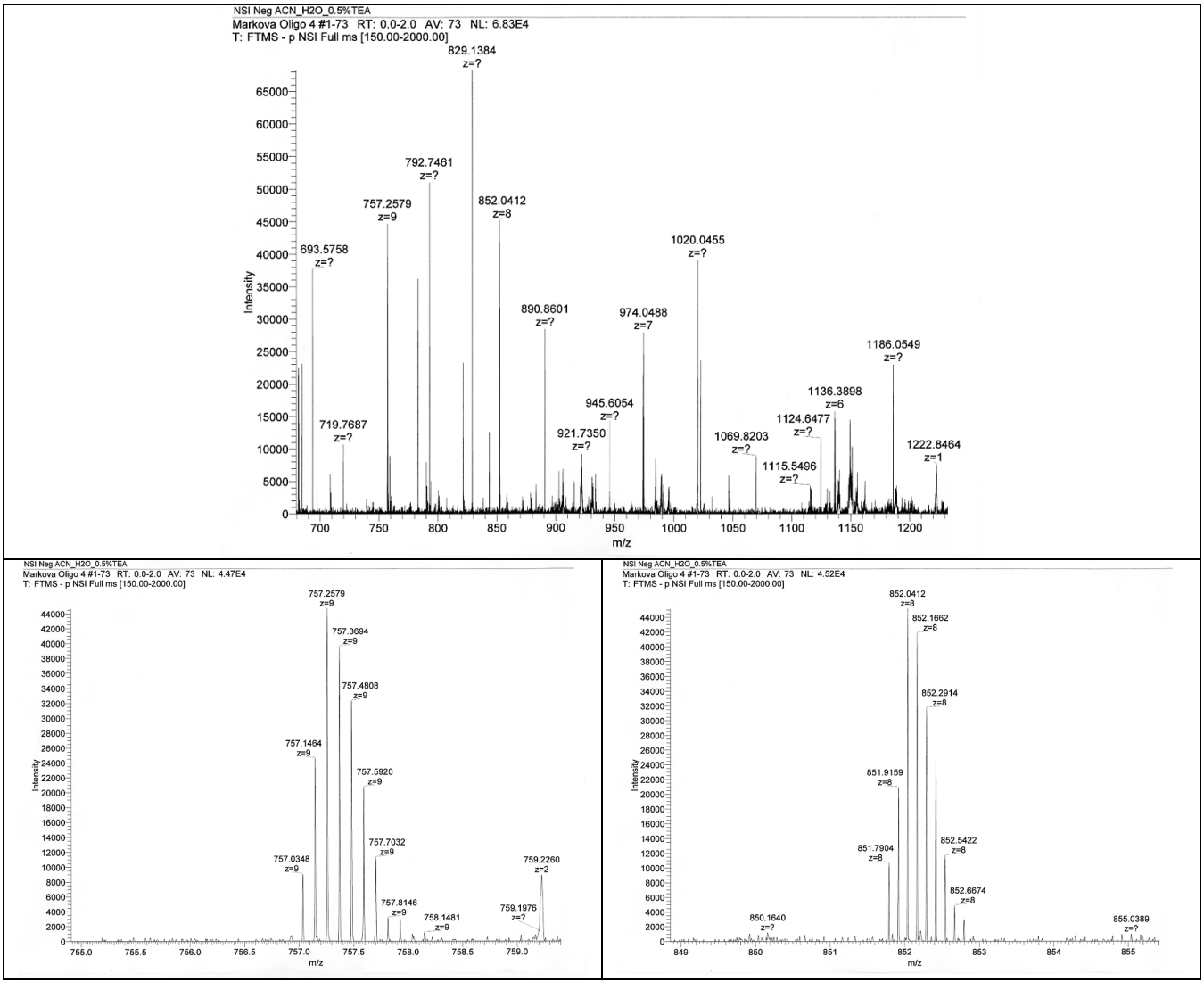


Figure S6. HR-MS of **ON1.**

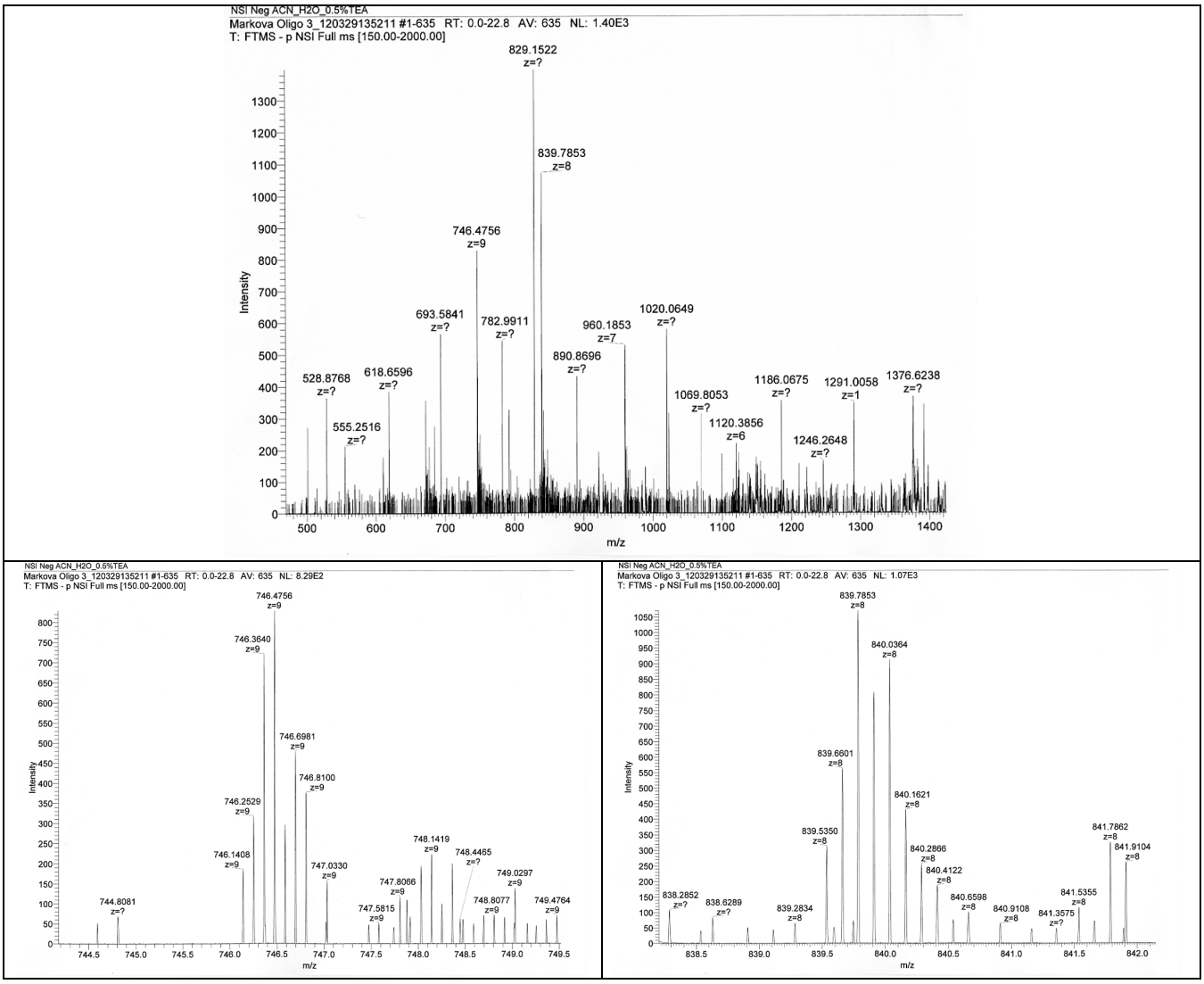


Figure S7. HR-MS of **ON2.**

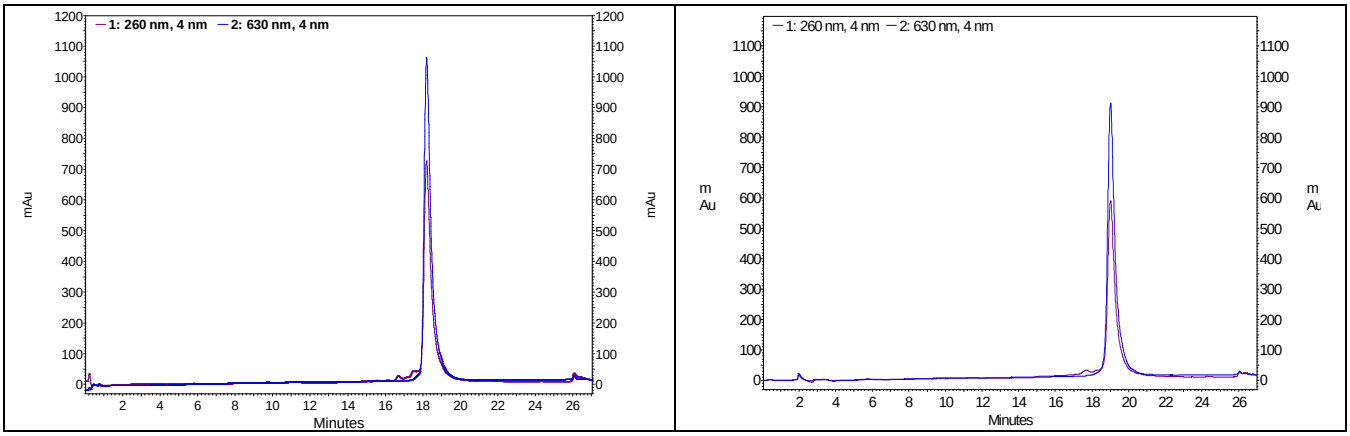


Figure S8. HPLC data of oligonucleotides **ON1** (left) and **ON2** (right) recorded at 260 and 630 nm.

6) Example of determination of the quantum yield

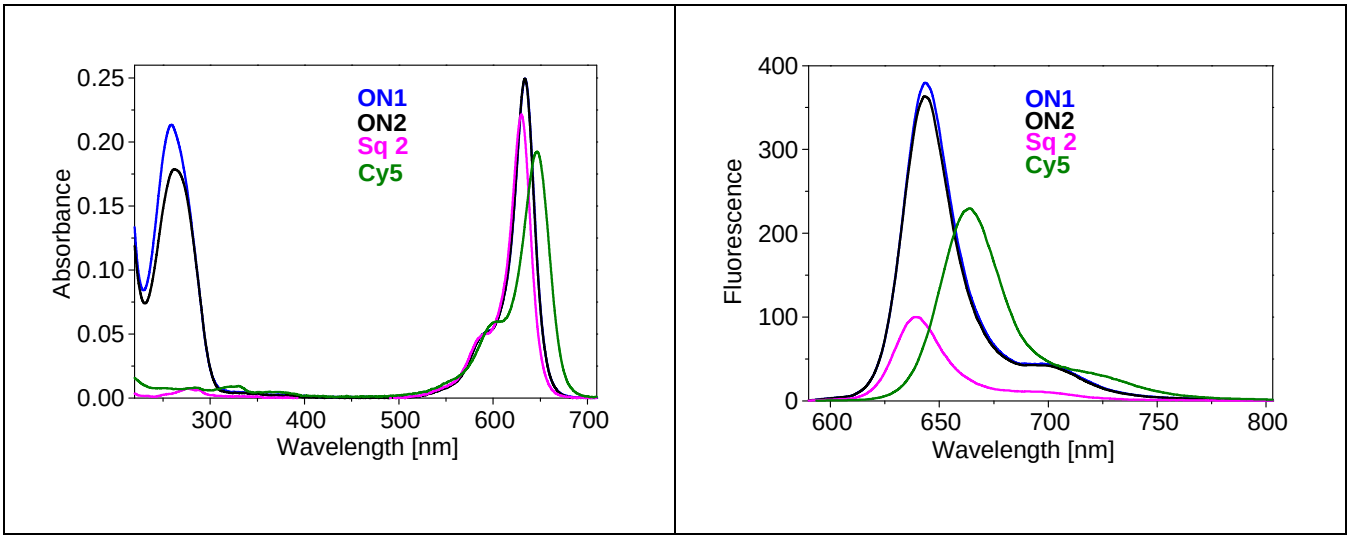


Figure S9. Quantum yield measurement of Sq **2** and oligonucleotides **ON1** and **ON2** in methanol. Left: absorption spectra of the samples (Sq **2**, **ON1** and **ON2**) and reference (**Cy5**, in water) with the absorption at the excitation wavelength (600 nm) around 0.05. Right: emission spectra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

7) Tm experiments.

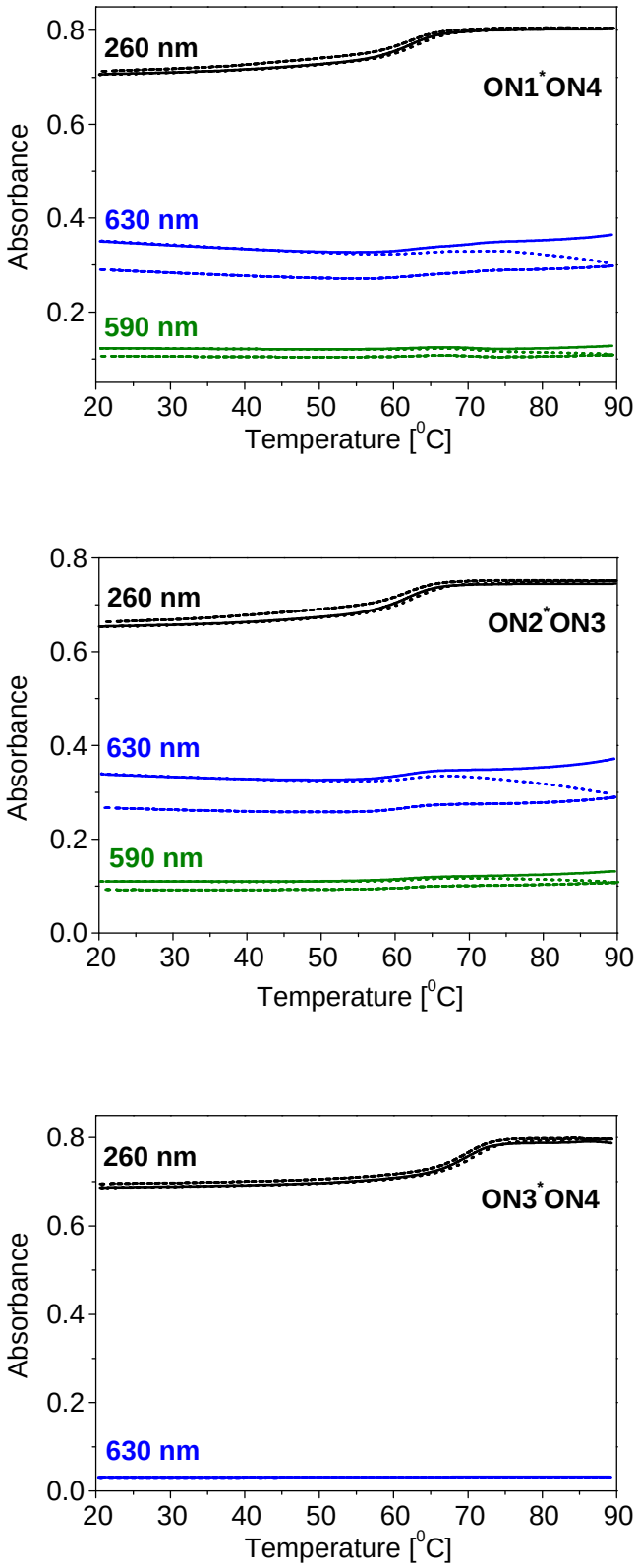


Figure S10 Thermal denaturation-renaturation of hybrid **ON1*ON4** (top), **ON2*ON3** (middle) and **ON3*ON4** (bottom) observed at 260 nm, 590 nm and 630 nm; cooling (–), heating (¨) and cooling (---) curves.

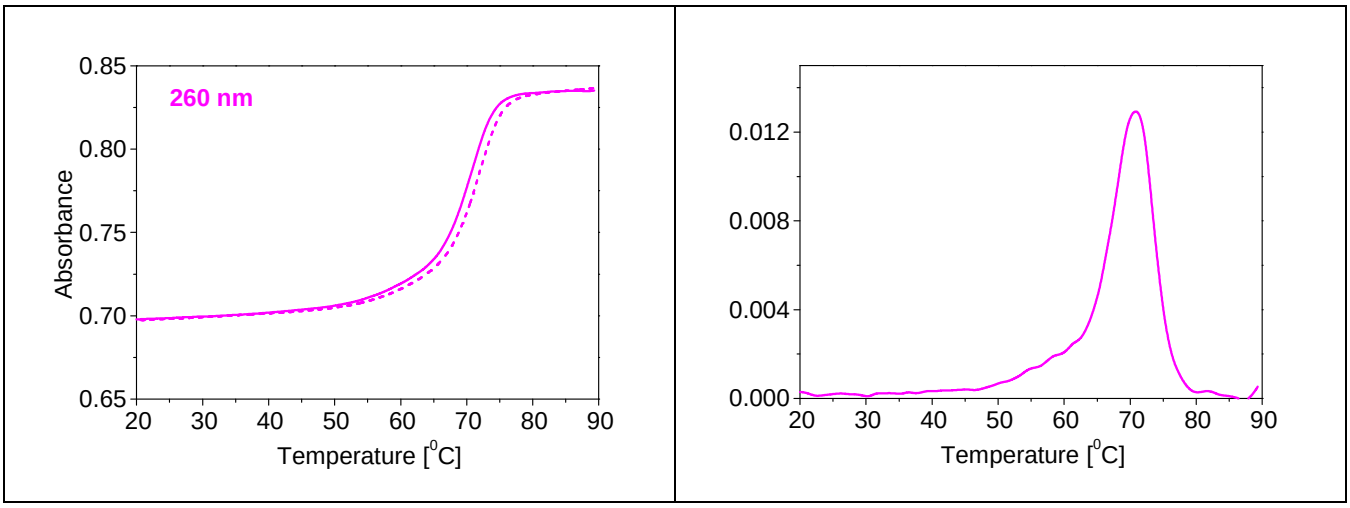


Figure S11. Example of Tm determination procedure: duplex **ON3*ON4**, melting curves at 260 nm (left) and first derivative curve, Tm = 70.7 °C (right).

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).

Oligomer	Duplex	Tm, °C
ON1 **ON2**	5‘ AGCTCGGTCA**Sq**CGAGAGTGCA 3’ TCGAGCCAGT**Sq**GCTCTCACGT	70.4
ON1 **ON4**	5‘ AGCTCGGTCA**Sq**CGAGAGTGCA 3’ TCGAGCCAGT**A**GCTCTCACGT	63.4
ON2 **ON3**	3’ TCGAGCCAGT**Sq**GCTCTCACGT 5’ AGCTCGGTCA**T**CGAGAGTGCA	62.7
ON3 **ON4**	5’ AGCTCGGTCA**T**CGAGAGTGCA 3’ TCGAGCCAGT**A**GCTCTCACGT	70.7

8) Influence of the temperature on the absorption and emission spectra of Sq compounds.

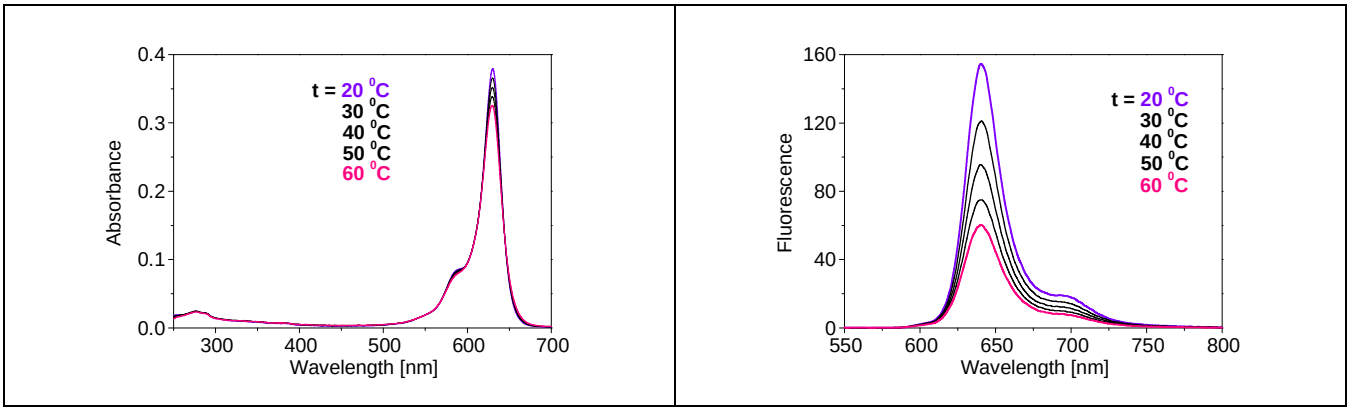


Figure S12. Influence of the temperature on the absorption and emission spectrum of the squaraine diol **2** measured in methanol at concentration $c \sim 1.5\times10^{-6}$ M in the temperature range 20-60 °C.

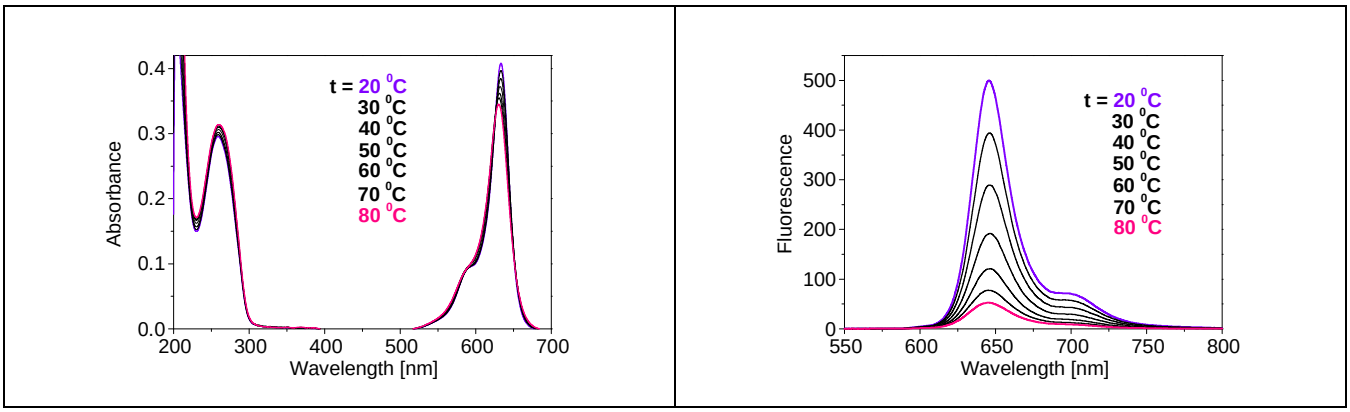


Figure S13. Influence of the temperature on the absorption and emission spectrum of oligonucleotide **ON2** in the temperature range 20-80 °C.

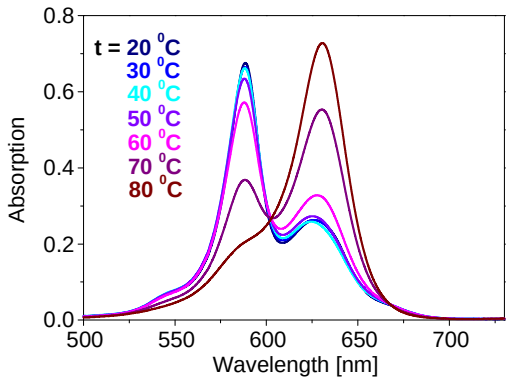


Figure S14. Influence of the temperature on the absorption spectrum of duplex **ON1*ON2** in the temperature range 20-80 °C.

9) Stability of the squaraine diol 2 to treatment by 30% NH_4OH solution

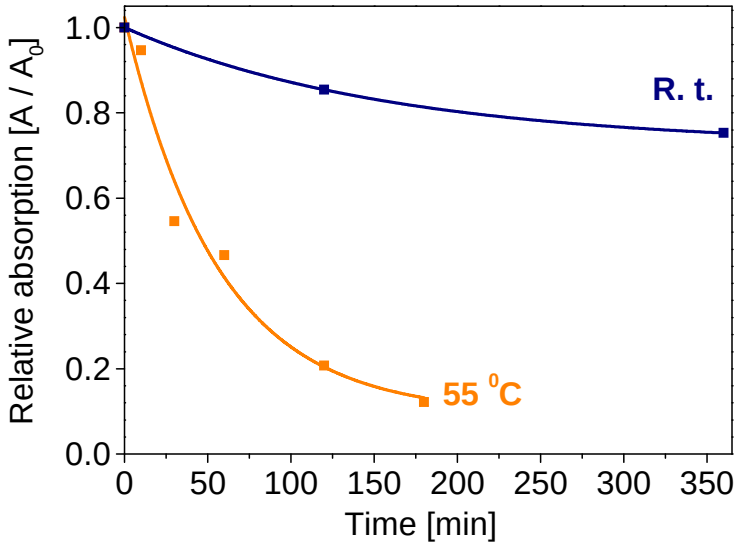


Figure S15. Decay of the long-wavelength absorption band of the squaraine diol **2** after treatment by 30% NH_4OH solution at 55 °C and at room temperature. Relative absorbance A/A_0, where A_0 is absorption intensity at starting point, and A_0 is absorption intensity at time-point.

10) CD spectra

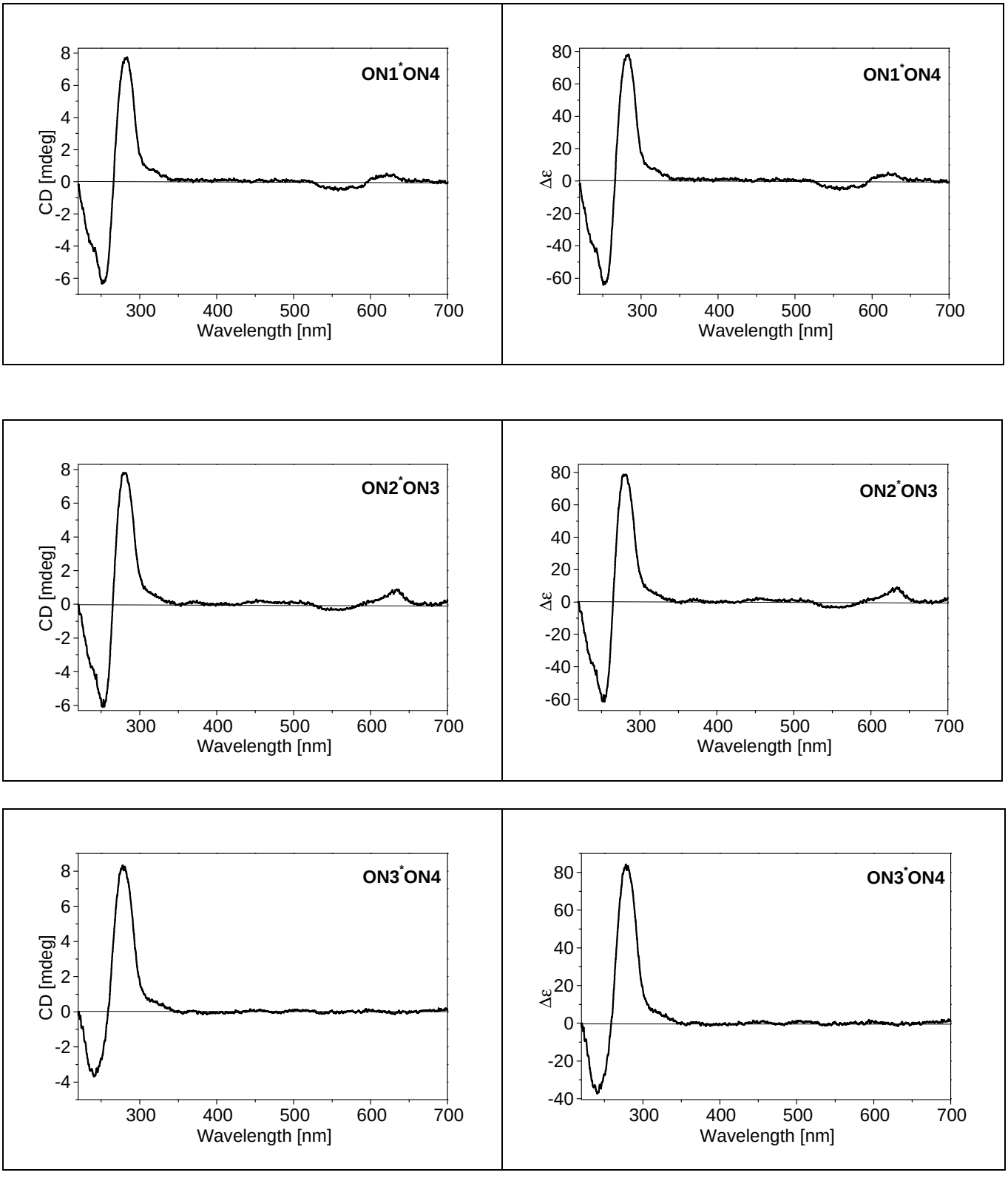


Figure S16. CD spectra of hybrids **ON1*ON4** (top), **ON2*ON3** (middle) and **ON3*ON4** (bottom).

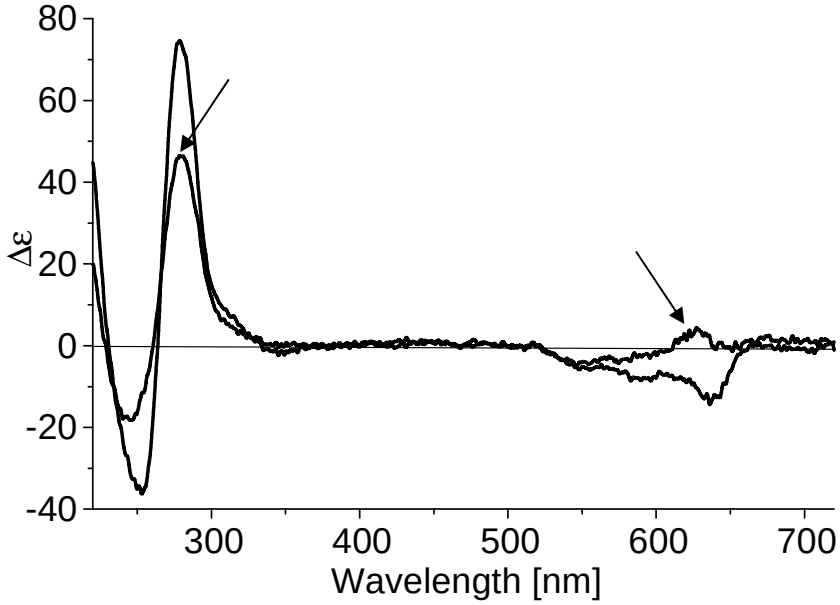


Figure S17. CD spectrum of **ON2** (3 μM) at 20 °C and 80 °C (indicated by arrows).