SUPPORTING INFORMATION Part 1

Synthesis of New Asymmetric Xanthene Dyes *via* Catalyst free S_NAr with Sulfur Nucleophiles

Michaela Kotaskova‡, Okan Osman Oglou‡ and Mark Helm*

Institute of Pharmacy and Biochemistry, Johannes Gutenberg-University Mainz, Staudingerweg 5, D-55128 Mainz, Germany

Email: mhelm@uni-mainz.de

Contents Part 1

General Methods

All chemicals were purchased from reputable suppliers Sigma Aldrich, Acros Organics, Fischer Scientific, Fluka (Steinheim, Nidderau, Deisenhofen, Germany) and used without further purification. Boc-cysteamine was from Alfa Aeser GmbH & Co KG (Karlsruhe, Germany). Deuterized solvents were purchased from Deutero (Kastellaun, Germany). DMF was distilled from calcium hydride. All reactions were sealed with septa through which an argon atmosphere was introduced unless otherwise noted.

Reactions were monitored by thin layer chromatography (TLC) using pre-coated silica gel plates Polygram Sil G/UV$_{254}$ (40 x 80 mm) from Macherey-Nagel (Düren, Germany). Compounds were made visible by UV illumination. Preparative thin layer chromatography (pTLC) was performed on pre-coated siliga gel glass plates, Silica gel 60 F_{254} (layer thickness 1 mm) from Merck (Darmstadt, Germany). Column chromatography (CC) was performed on silica gel 60 (230-400 mesh) from Merck.

FD-MS spectra were recorded on a Finnigan MAT 95 mass spectrometer, and ESI-MS on a Micromass LCT mass spectrometer. MALDI-TOF (positive mode) mass spectra were recorded on a Bruker BIFLEX III spectrometer.

IR spectra were recorded on a Nicolet Avatar 330 FT-IR.

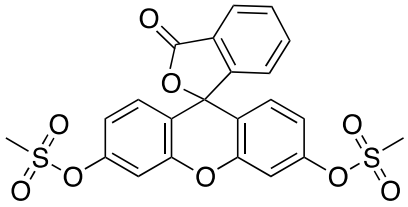
NMR-spectra were recorded on a Bruker AC 300 MHz (^{1}H: 300 MHz, ^{13}C: 75 MHz) and on a Bruker 400 MHz (^{1}H: 400 MHz, ^{13}C: 100 MHz).

^{1}H and ^{13}C NMR spectra were referenced to TMS or residual solvent peaks. Chemical shifts (δ) are in ppm and abbreviations used are as follows: s = singlet, d = doublet, t = triplet, m= multiplet, coupling constant (Hz), integration. Data for ^{13}C NMR spectra are reported by chemical shift (δ ppm).

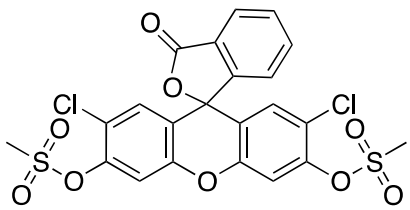
Ultraviolet absorption spectra were recorded on a Jasco V-6500 spectrophotometer. Fluorescence spectroscopic studies were performed on Jasco FP-6500 fluorescence spectrometer equipped with a Peltier element for temperature control.

General procedure for formation of dimesylated fluorescein and its derivatives:

The following procedure for **1a** is representative. Fluorescein (0.68 g, 2 mmol) was dissolved under an argon atmosphere in 10 mL of dry pyridine. The mixture was cooled to 0 °C and stirred vigorously. Methanesulfonyl chloride (620 µL, 8 mmol, 4 equiv) was diluted in 5 mL of dry pyridine and added dropwise to the cooled solution of fluorescein. The ice bath was removed and the mixture was stirred for 4 h at rt. The solvent was evaporated *in vacuo*. The mixture was separated by column chromatography over silica gel, where the product was eluted with dichloromethane and unreacted fluorescein remained on the column. The solvent was evaporated and the resulting product was dried *in vacuo*. The product was obtained as a white solid with the yield of 90% (0.88 g, 1.8 mmol).



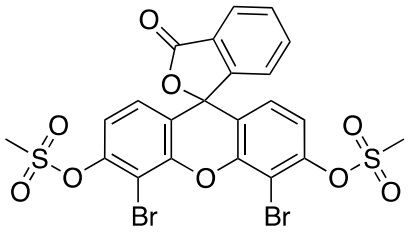
3-Oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-3',6'-diyl dimethanesulfonate (1a): (90%, white solid) ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.09 (d, ^{3}J = 8.3 Hz, 1H), 7.80 (m, 2H), 7.52 (d, ^{4}J = 2.6 Hz, 2H), 7.43 (d, ^{3}J = 8.3 Hz, 1H), 7.17 (dd, ^{3}J = 9.6, ^{4}J = 2.6 Hz, 2H), 7.01 (d, ^{3}J = 9.6 Hz, 2H), 3.48 (s, 6H) ppm; ^{13}C NMR (75 MHz, DMSO-d_6) δ 168.5, 152.3, 151.1 (2C), 150.5 (2C), 136.4, 131.0, 130.2 (3C), 125.5, 124.4, 119.2 (2C), 118.0 (2C), 111.2 (2C), 80.5, 37.9 (2C) ppm; MALDI-TOF-MS: 489.23 $[M + H]^+$, HRMS (ESI) calcd for $C_{22}H_{16}O_9NaS_2$ $[M + Na]^+$ 511.0133, found 511.0119; FT-IR $\tilde{V}$ (cm^{-1}) 3039 ν (C-H, arom.), 2937 ν (CH_3, aliph.), 1772 ν (C=O, lactone), 1607 ν (C=C, arom.), 1488, 1423, 1353 ν (S=O), 1233, 1133, 1106



2',7'-Dichloro-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-3',6'-diyl dimethanesulfonate (1b): This compound was prepared from 2',7'-dichlorofluorescein according to general procedure described above (82%, white solid). ^{1}H NMR (400 MHz,

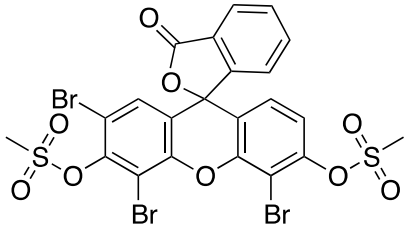
DMSO-d_6) δ 8.07 (d, ^3J = 7.6 Hz, 1H), 7.82 (m, 2H), 7.72 (s, 2H), 7.47 (d, ^3J = 7.6 Hz, 1H), 7.18 (s, 2H), 3.61 (s, 6H) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ 167.9, 151.1, 149.2 (2C), 146.2 (2C), 136.2, 130.9, 129.4 (2C), 125.7, 125.3, 124.1, 122.4 (2C), 118.8 (2C), 113.1 (2C), 79.3, 39.5 (2C) ppm; FD-MS: m/z (%): 556.1 (100), 557.0 (28), 558.0 (82); HRMS (ESI) calcd for $C_{22}H_{15}Cl_2O_9S_2$ $[M + H]^+$ 556.9535, found 556.9537; FT-IR $\tilde{V}$ (cm^{-1}) 3015 ν (C-H, arom.), 2931 ν (CH_3, aliph.), 1768 ν (C=O, lactone), 1608 ν (C=C, arom.), 1476, 1407, 1370 ν (S=O), 1187, 1159, 1077, 965

General procedure for formation of dimesylated brominated fluorescein: The following procedure for mesylation of brominated fluoresceins was performed from commercially available 4',5'-dibromofluorescein, which contained also 2',4',5'-tribromofluorescein. The mixture of 4',5'-dibromofluorescein and 2',4',5'-tribromofluorescein (2 g, 4.08 mmol) was dissolved under an argon atmosphere in 30 mL of dry pyridine. The reaction mixture was cooled to 0 °C and stirred vigorously. Methanesulfonyl chloride (1.26 mL, 16.3 mmol, 4.0 equiv) in 5 mL of dry pyridine was added dropwise. The cooling was removed after 30 min and the reaction mixture was stirred at room temperature for 5 h. Pyridine was evaporated *in vacuo* and the residue was dried under reduced pressure. Purification by gradient column chromatography over silica gel using CH_2Cl_2, CH_2Cl_2/CH_3OH (99:1) as elution mixture allowed separation of two products namely dimesylated dibrominated derivative and dimesylated tribrominated derivative as white solids with the yield of 22% (590 mg, 0.91 mmol) and 14% (410 mg, 0.56 mmol).



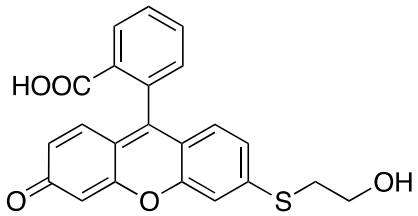
4',5'-Dibromo-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-3',6'-diyl dimethanesulfonate (1c): (22%, white solid) ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.10 (d, ^3J = 7.6 Hz, 1H), 7.97 – 7.67 (m, 2H), 7.55 (d, ^3J = 7.6 Hz, 1H), 7.36 (d, ^3J = 8.8 Hz, 2H), 7.03 (d, ^3J = 8.8 Hz, 2H), 3.59 (s, 6H) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ 168, 151.3, 148.4 (2C), 148.2 (2C), 136.1, 131.3, 128.2 (2C), 125.3, 125.1, 124.5, 119.8 (2C), 118.9 (2C), 106.1 (2C), 80.4, 55.0 (2C) ppm; FD-MS: m/z (%): 644.5 (44), 645.4 (12), 646.5 (100), 647.5 (28), 648.4 (68); HRMS (ESI) calcd for $C_{22}H_{15}Br_2O_9S_2$ $[M + H]^+$ 644.8524, found 644.8548; FT-IR

$\tilde{V}$ (cm^{-1}) 3039 ν (C-H, arom.), 2941 ν (CH_3, aliph.), 1750 ν (C=O, lactone), 1585 ν (C=C, arom.), 1413, 1360 ν (S=O)

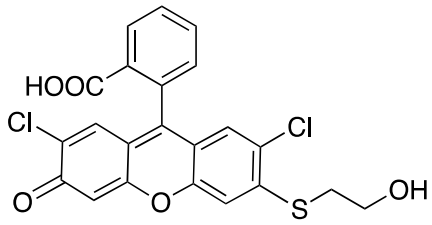


2',4',5'-Tribromo-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-3',6'-diyl dimethanesulfonate (1d): (14%, white solid) ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.09 (d, ^3J = 7.2 Hz, 1H), 7.81 – 7.65 (m, 2H), 7.25 (d, ^3J = 8.8 Hz, 1H), 7.20 (d, ^3J = 7.2 Hz, 1H), 7.10 (s, 1H), 6.87 (d, ^3J = 8.8 Hz, 1H), 3.57 (s, 3H), 3.33 (s, 3H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 151.47, 148.56, 136.2 (2C), 131.2 (2C), 130.9 (2C), 127.4 (2C), 126.1 (2C), 125.5, 124.0 (2C), 121.2, 120.2 (2C), 119.2, 113.4, 42.2, 39.5 ppm; FD-MS: m/z (%): 722.3 (26), 723.3 (15), 724.3 (99), 725.3 (38), 726.3 (100), 727.3 (26); HRMS (ESI) calcd for $C_{22}H_{14}Br_3O_9S_2$ $[M + H]^+$ 722.7629, found 722.7619; FT-IR $\tilde{V}$ (cm^{-1}) ν 3039 ν (C-H, arom.), 2937 ν (CH_3, aliph.), 1764 ν (C=O, lactone), 1591 ν (C=C, arom.), 1418, 1367 ν (S=O), 1178, 1048, 832

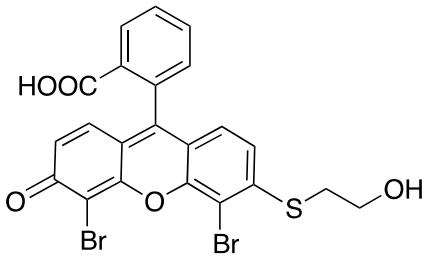
General procedure for the preparation of ethylthio-modified xanthene dyes from 2-mercaptoethanol: The following procedure for 2-(6-((2-hydroxyethyl)thio)-3-oxo-3H-xanthen-9-yl)benzoic acid (**2a**) is representative. 3-Oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-3',6'-diyl dimethanesulfonate (**1a**) (2 g, 4.1 mmol) was dissolved under an argon atmosphere in 24 mL of dry DMF. DBU (0.61 ml, 4.1 mmol, 1.0 equiv) was added into 2-mercaptoethanol (1.15 ml, 16.4 mmol, 4.0 equiv) in 2 mL of dry DMF and the mixture was stirred for 30 min. The reaction mixture of starting material was cooled to 0 °C and the mixture with activated 2-mercaptoethanol was added dropwise. Then the reaction was allowed to warm up to room temperature. The pH of the reaction mixture after adding the reagents was 8. The reaction was monitored by TLC on silica gel (CH_2Cl_2/CH_3OH, 95:5) over a period of 4 h. After 4 hours the starting material was not completely converted but fluorescein started to appear on TLC. The mixture was stirred additionally for 4 h, and then neutralized with acetic acid and the solvent was evaporated *in vacuo*. The residue was purified by column chromatography on silica gel using the gradient elution mixture from 100% CH_2Cl_2 up to 95:5 CH_2Cl_2/CH_3OH. The product **2a** was obtained as a yellow solid with the yield of 45% (0.720 g, 1.8 mmol).



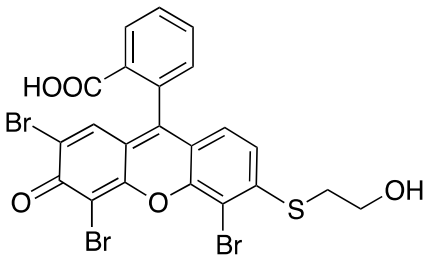
2-(6-((2-Hydroxyethyl)thio)-3-oxo-3H-xanthen-9-yl)benzoic acid (2a): (45%, yellow solid); ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.18 (s, 1H), 8.01 (d, ^{3}J = 7.6 Hz, 1H), 7.79 (t, ^{3}J = 7.6 Hz, 1H), 7.72 (t, ^{3}J = 7.6 Hz, 1H), 7.34 – 7.23 (m, 2H), 7.02 (dd, ^{3}J = 8.4 Hz, ^{4}J = 1.6 Hz, 2H), 6.71 (s, 1H), 6.65 (d, ^{3}J = 8.4 Hz, 1H), 6.59 (m, 2H), 3.61 (t, ^{3}J = 6.8 Hz, 2H), 3.13 (t, ^{3}J = 6.8 Hz, 2H) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ 168.6, 159.6, 152.3, 151.5, 151.0, 140.8, 135.7, 130.2, 129.1, 128.2, 125.8, 124.7, 124.0, 122.7, 115.5, 113.8, 112.9, 109.2, 102.3, 82.3, 59.6, 34.0 ppm; FD-MS: m/z (%): 392.1 (100); HRMS (ESI) calcd for $C_{22}H_{17}O_5S$ $[M + H]^+$ 393.0797, found 393.0790; FT-IR $\tilde{V}$ (cm^{-1}) 3520 ν (OH), 3260 ν (OH), 1731 ν (C=O), 1603 ν (C=C, arom.), 1405, 1235, 1113, 844



2-(2,7-dichloro-6-((2-hydroxyethyl)thio)-3-oxo-3*H*-xanthen-9-yl)benzoic acid (2b): This compound was prepared from 2',7'-dichloro-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-3',6'-diyl dimethanesulfonate (**1a**) according to the general procedure described for **2a** (41%, orange solid). ^{1}H NMR (400 MHz, DMSO-*d6*) δ 11.13 (s, 1H), 8.03 (d, 1H, ^{3}J = 7.6 Hz), 7.82 (td, ^{3}J = 7.6 Hz, ^{4}J = 1.2 Hz, 1H), 7.76 (td, ^{3}J = 7.6 Hz, ^{4}J = 1.2 Hz, 1H), 7.41 (s, 1H), 7.36 (d, ^{3}J = 7.6 Hz, 1H), 6.95 (s, 1H), 6.79 (s, 1H), 6.70 (s, 1H), 5.11 (t, ^{3}J = 5.6 Hz, 1H), 3.69 (t, ^{3}J = 6.4 Hz, 2H), 3.22 (t, ^{3}J = 6.4 Hz, 2H) ppm; ^{13}C (75 MHz, DMSO-*d6*) δ 168.2, 155.3, 151.4, 149.9, 149.6, 140.7, 136.0, 130.7, 128.3, 127.6, 125.7, 124.9, 123.8, 116.5 (2C), 115.9, 114.00, 110.2, 103.7, 80.9, 59.1, 33.8 ppm; FD-MS: m/z (%): 460.5 (100), 461.5 (29.5), 462.5 (57.5); HRMS (ESI) calcd for $C_{22}H_{14}Cl_2O_5NaS$ $[M + Na]^+$ 482.9837, found 482.9849;
FT-IR $\tilde{V}$ (cm^{-1}) 3536 ν (OH), 3160 ν (OH), 2923 ν (CH_2, aliph.), 1767 ν (C=O), 1593 ν (C=C, arom.), 1439, 1387, 1284, 860

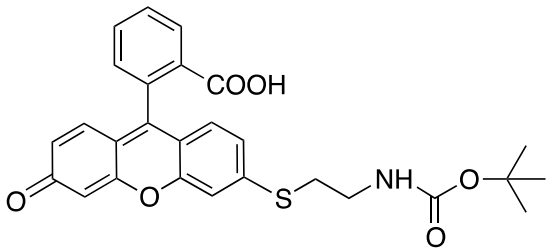


2-(4,5-dibromo-6-((2-hydroxyethyl)thio)-3-oxo-3*H*-xanthen-9-yl)benzoic acid (2c): This compound was prepared from 4',5'-dibromo-3-oxo-3H-spiro [isobenzofuran-1,9'-xanthene]-3',6'-diyl dimethanesulfonate (**1c**) according to the general procedure described above (53%, orange solid). ^{1}H NMR (400 MHz, CD_3OD) δ 8.06 (d, ^{3}J = 7.2 Hz, 1H), 7.83 – 7.70 (td, ^{3}J = 7.2 Hz, ^{4}J = 1.2 Hz, 2H), 7.28 (d, ^{3}J = 7.2 Hz, 1H), 7.15 (d, ^{3}J = 8.8 Hz, 1H), 6.81 (d, ^{3}J = 8.4 Hz, 1H), 6.72 (d, ^{3}J = 8.8 Hz, 1H), 6.67 (d, ^{3}J = 8.8 Hz, 1H), 3.79 (t, ^{3}J = 6.6 Hz, 2H), 3.17 (t, ^{3}J = 6.6 Hz, 2H) ppm; ^{13}C NMR (75 MHz, CD_3OD) δ 168.4, 157, 151.8, 148.5, 142.6, 135.9, 130.5 (2C), 127.5, 127.0 (2C), 125.7, 124.9, 124.3, 121.2, 116.3 (2C), 113, 110.6, 97.6, 59.1, 34.4 ppm; FD-MS: m/z (%): 548.1 (100), 550.2 (50); HRMS (ESI) calcd for $C_{22}H_{14}Br_2O_5NaS$ $[M + Na]^+$ 570.8826, found 570.8808; FT-IR $\tilde{V}$ (cm^{-1}) 3500-3100 ν (OH), 2930 ν (CH_2, aliph.), 1748 ν (C=O, lactone), 1599 ν (C=C, arom.), 1435, 1399, 1286, 1107



2-(2,4,5-tribromo-6-((2-hydroxyethyl)thio)-3-oxo-3*H*-xanthen-9-yl)benzoic acid (2d): This compound was prepared from 2',4',5'-tribromo-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-3',6'-diyl dimethanesulfonate (**1d**) according to the general procedure described above (39%, red solid). 1H NMR (400 MHz, DMSO-d6) δ 8.02 (d, 3J = 7.6 Hz, 1H), 7.83 (t, 3J = 7.6 Hz, 1H), 7.75 (t, 3J = 7.6 Hz, 1H), 7.42 (d, 3J = 7.6 Hz, 1H), 7.10 (d, 3J = 8.6 Hz, 1H), 6.85 (d, 3J = 8.6 Hz, 1H), 6.67 (d, 3J = 8.6 Hz, 2H), 3.63 (t, 3J = 6.4 Hz, 2H), 3.10 (t, 3J = 6.4 Hz, 2H) ppm; ^{13}C NMR (75 MHz, DMSO-*d6*) δ 168.3 (2C), 148.7, 147.9, 142.4, 135.7 (2C), 130.5 (2C), 128.4, 126.7 (2C), 126.5, 125.0, 124.5, 120.9 (2C), 116.7, 108.9, 100.1, 59.1, 34.4 ppm; FD-MS: m/z (%): 629.1 (100), 629.99 (42), 631.0 (39), 633.0 (11); HRMS (ESI) calcd for $C_{22}H_{13}Br_3O_5NaS$ $[M + Na]^+$ 648.7931, found 648.7950; FT-IR $\tilde{V}$ (cm^{-1}) 3391 ν (OH), 3105 ν (arom.), 2941 ν (CH_2, aliph.), 1708 ν (C=O, lactone), 1593 ν (C=C, arom.), 1429, 1397, 1282

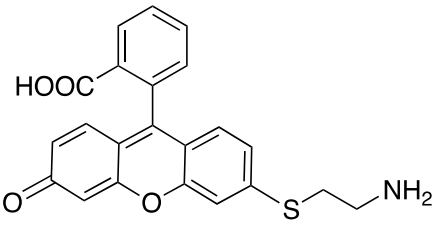
Procedure for the preparation of ethylthio-modified xanthene dye from Boc-cysteamine: 3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-3',6'-diyl dimethanesulfonate (**1a**) (1 g, 2 mmol) was dissolved in 30 mL of dry DMF. The mixture of Boc-cysteamine (1.38 mL, 8.2 mmol, 4.0 equiv) and DBU (306 µL, 2 mmol, 1.0 equiv) were added to 5 mL of dry DMF and agitated for 30 min. The initial solution was cooled to 0 °C and the separately prepared mixture was added slowly in a dropwise manner. The color of reaction mixture changed from yellow to red-orange clear solution. After 30 min the cooling was removed and the solution was stirred at rt over night. DMF was evaporated *in vacuo* and the reaction mixture was dried under reduced pressure. The residue was purified by the gradient column chromatography over silica gel with CH_2Cl_2 to CH_2Cl_2/CH_3OH, 99:1-97:3. The product (**5a**) was obtained as a yellow solid with the yield of 38% (373 mg, 0.76 mmol).



2-(6-((2-((*Tert*-butoxycarbonyl)amino)ethyl)thio)-3-oxo-3H-xanthen-9-yl)benzoic acid (5a): (yellow solid, 38%) ^{1}H NMR (400 MHz, DMSO-*d6*) δ 8.01 (d, ^3J = 7.6 Hz, 1H), 7.77 (td, ^3J = 7.6 Hz, ^4J = 1.1 Hz, 1H), 7.70 (td, ^3J = 7.6 Hz, ^4J = 1.1 Hz, 1H), 7.33 (d, ^4J = 2Hz, 1H), 7.28 (d, ^3J = 7.6 Hz, 1H), 7.09 (t, ^3J = 5.5 Hz, 1H), 7.03 (dd, ^3J = 8.4, ^4J =2.0 Hz, 1H), 6.67 (d, ^3J = 8.4 Hz, 1H), 6.63 (d, ^4J = 2 Hz, 1H), 6.60 (d, ^3J = 8.8 Hz, 1H), 6.53 (dd, ^3J = 8.8 Hz, ^4J = 2.2 Hz, 1H), 3.15 (t, ^3J = 6.4 Hz, 2H), 3.08 (t, ^3J = 6.4 Hz, 2H), 1.37 (s, 9H) ppm; ^{13}C NMR (75 MHz, DMSO-*d6*) δ 168.5, 155.5, 152.3, 151.2, 140.5, 134.9, 130.1 (2C), 129.3 (2C), 128.3 (2C), 125.3, 124.5, 122.6, 116.1, 113.9, 109.6, 102.4 (2C), 99.5, 77.9, 39.5, 30.9, 28.2 (3C) ppm; FD-MS: m/z (%): 491.7 (100); HRMS (ESI) calcd for $C_{27}H_{26}NO_6S$ $[M + H]^+$ 492.1481, found 492.1470; FT-IR $\tilde{V}$ (cm^{-1}) 3325 ν (OH), 2970 ν (CH_3, aliph.), 2925 ν (CH_2, aliph.), 1760 ν (C=O, lactone), 1658 δ (N-H), 1597 ν (C=C, arom.), 1560, 1462, 1242, 1106

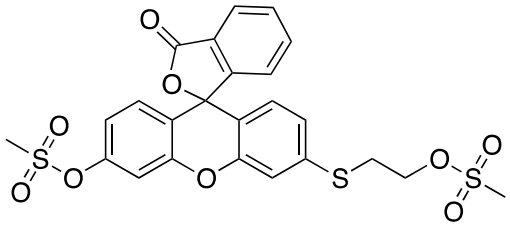
Deprotection of Boc-protective group: 2-(6-((2-((tert-butoxycarbonyl) amino)ethyl)thio)-3-oxo-3H-xanthen-9-yl)benzoic acid (**5a**) (50 mg, 0.102 mmol) was taken up in 2.5 mL of CH_2Cl_2 and TFA (0.5 mL) to cleave Boc-protective group. The reaction mixture was stirred at room temperature for 2 h monitored by TLC on silica gel (CH_2Cl_2/CH_3OH, 95:5). After 2 h starting material was fully converted and the spot on the start of TLC plate presumable new

product (not moving because of formation of TFA salt) was presented. Toluene (3 mL) was added; the reaction mixture was concentrated to dryness and then azeotroped with CH_3OH three times. The red residue was triturated with CH_2Cl_2 and filtered off. Then washed with CH_2Cl_2 and ether and dried *in vacuo*. The lyophilization from water afforded the product (**6a**) as an orange-gold solid with the yield of 95% (38 mg, 0.097 mmol).



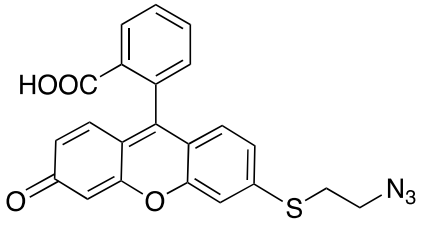
2-(6-((2-Aminoethyl)thio)-3-oxo-3H-xanthen-9-yl)benzoic acid (6a): (95%, orange-gold solid) ^{1}H NMR (300 MHz, DMSO-*d6*) δ 10.30 (s, 1H), 8.03 (d, 1H, ^3J = 7.3 Hz), 7.87 (s, 2H), 7.84 – 7.64 (m, 2H), 7.41 (d, ^4J = 1.9 Hz, 1H), 7.30 (d, ^3J = 7.3 Hz, 1H), 7.06 (dd, ^3J = 8.4 Hz, ^4J =1.2 Hz, 1H), 6.70 (d, ^3J = 8.1 Hz, 2H), 6.60 (m, 2H), 3.27 (t, ^3J = 6.3 Hz, 2H), 3.02 (t, ^3J = 6.3 Hz, 2H) ppm; ^{13}C NMR (75 MHz, DMSO-*d6*) δ 168.6, 159.8, 152.3, 151.5, 151.2, 138.4, 135.8, 130.3, 129.2, 128.6, 125.9, 124.8, 124.0, 123.3, 116.4, 114.6, 113.1, 109.1, 102.2, 82.1, 37.9, 28.4 ppm; FD-MS: m/z (%): 392.4 (100); HRMS (ESI) calcd for $C_{22}H_{18}NO_4S$ $[M + H]^+$ 392.0957, found 392.0964; FT-IR $\tilde{V}$ (cm^{-1}) 3056 ν (C-H, arom.), 1736 ν (C=O), 1666 δ (N-H), 1597 ν (C=C, arom.), 1401, 1196, 1106

General procedure for mesylation of the hydroxyethyl group: 2-(6-((2-hydroxyethyl)thio)-3-oxo-3H-xanthen-9-yl)benzoic acid (**2a**) (200 mg, 0.51 mmol) and DMAP (374 mg, 3.06 mmol, 6.0 equiv) were suspended in 10 mL of dry CH_2Cl_2 under an argon atmosphere. The turbid mixture was stirred for 30 min. The reaction mixture was cooled to 0 °C and methanesulfonyl chloride (158 µL, 2.04 mmol, 4.0 equiv) in 1 mL of dry CH_2Cl_2 was added dropwise. The suspension was stirred for 30 min at 0 °C, warmed to room temperature and then stirred for 14 h. Additional 10 mL of CH_2Cl_2 was added and the solution was washed with 15 mL of water. The water phase was extracted twice with CH_2Cl_2 (15 mL). The organic phases were combined, dried over anhydrous Na_2SO_4 and evaporated *in vacuo*. The residue was separated by gradient column chromatography over silica gel (100% CH_2Cl_2 up to CH_2Cl_2/CH_3OH, 99:1). The product (**3a**) was obtained as a white-beige fluffy solid with the yield of 77.5% (217 mg, 0.39 mmol).



2-(3'-(Methylsulfonyloxy)-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-6'-ylthio)ethyl methanesulfonate (3a): (77.5%, white-beige solid) ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.08 – 8.02 (m, 1H), 7.73 – 7.63 (m, 2H), 7.29 (d, ^{4}J = 2.4 Hz, 2H), 7.17 (m, 1H), 7.04 (dd, ^{3}J = 8.4 Hz, ^{4}J = 1.9 Hz, 1H), 7.00 (dd, ^{3}J = 8.7 Hz, ^{4}J = 2.4 Hz, 1H), 6.88 (d, ^{3}J = 8.7 Hz, 1H), 6.77 (d, ^{3}J = 8.4 Hz, 1H), 4.37 (t, ^{3}J = 7.2 Hz, 2H), 3.31 (t, ^{3}J = 7.2 Hz, 2H), 3.20 (s, 3H), 3.02 (s, 3H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.1, 152.7, 151.8, 151.3, 150.3, 138.7, 135.7, 130.5, 129.8 (2C), 128.8, 126.2, 125.6, 124.6, 124.0, 118.4, 117.9, 117.1, 116.7, 111.2, 67.3, 38.0, 37.9, 32.0 ppm; ESI-MS: m/z (%): 549.06 (100); FT-IR $\tilde{V}$ (cm^{-1}) 1764 ν (C=O, lactone), 1598 ν (C=C, arom.), 1405, 1352 ν (S=O), 1171, 1106, 967

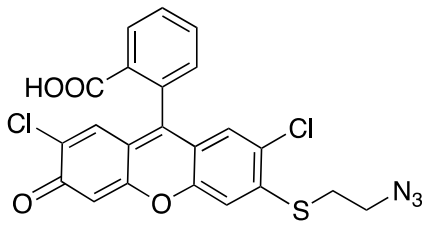
Procedure for the conversion of mesylate to azide: Under an argon atmosphere, 2-(3'-(methylsulfonyloxy)-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-6'-ylthio)ethyl methanesulfonate (**3a**) (76 mg, 0.138 mmol) and sodium azide (45 mg, 0.69 mmol, 5.0 equiv) were dissolved in 10 mL of dry DMF. The reaction mixture was warmed to 60 °C and stirred for 52 h. According to TLC on silica gel (CH_2Cl_2/CH_3OH, 95:5) the starting material was not completely converted. Silica gel for chromatography was deactivated using 3% Et_3N in CH_2Cl_2. Column chromatography on silica gel using a gradient elution system of 100% CH_2Cl_2 up to CH_2Cl_2/CH_3OH, 97:3. The product was re-purified by preparative TLC on silica gel (CH_2Cl_2/CH_3OH, 95:5). The product 2-(6-(2-azidoethylthio)-3-oxo-3H-xanthen-9-yl)benzoic acid (**4a**) was obtained as an orange fluffy solid with the yield of 33% (19 mg, 0.0455 mmol).



2-(6-(2-azidoethylthio)-3-oxo-3H-xanthen-9-yl)benzoic acid (4a): (33%, orange fluffy solid) ^{1}H NMR (400 MHz, CD_3OD) δ 8.04 (dd, ^{3}J = 7.6 Hz, ^{4}J = 0.8 Hz, 1H), 7.79 (td, ^{3}J = 7.6 Hz, 1.2 Hz, 1H), 7.73 (td, ^{3}J = 7.6 Hz, ^{4}J = 1.2 Hz, 1H), 7.33 (d, ^{4}J = 1.9 Hz, 1H), 7.23 (d,

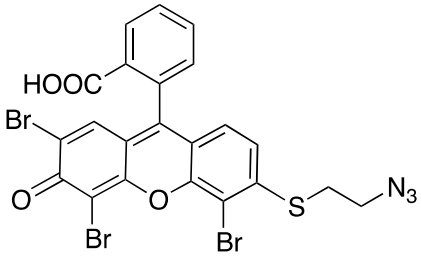
^{3}J = 7.6 Hz, 1H), 7.07 (dd, ^{3}J = 8.4 Hz, ^{4}J = 1.9 Hz, 1H), 6.73 (m, 2H), 6.62 (d, ^{3}J = 8.8 Hz, 1H), 6.57 (dd, ^{3}J = 8.8, Hz, ^{4}J = 2.4 Hz, 1H), 3.54 (t, ^{3}J = 6.6 Hz, 2H), 3.24 (t, ^{3}J = 6.6 Hz, 2H) ppm; ^{13}C NMR (100 MHz, CD_3OD) δ 171.3, 154.4, 153.6, 153.0, 140.8, 136.7, 131.3, 130.1, 129.6 (2C), 127.8, 125.9, 125.2, 124.8, 118.1, 117.1, 113.8, 110.9, 103.6, 51.3, 49.0, 33.3 ppm; ESI-MS: m/z (%): 418.10 (100); HRMS (ESI) calcd for $C_{22}H_{16}N_3O_4S$ $[M + H]^+$ 418.0862, found 418.0879; FT-IR $\tilde{V}$ (cm^{-1}) ν 3258 (OH), 2933 (CH_2, aliph.), 2102 ν (N_3), 1730 ν (C=O), 1630, 1601 ν (C=C, arom.), 1457, 1405, 1221, 1112

General procedure for the conversion of hydroxylxanthenes to xanthene azides without intermediate purification: The following procedure is representative. In a 2-neck flask (100 mL) under an argon atmosphere 2-(2,7-dichloro-6-((2-hydroxyethyl)thio)-3-oxo-3H-xanthen-9-yl)benzoic acid (**2b**) (100 mg, 0.217 mmol) was suspended in 8 mL of dry CH_2Cl_2. DMAP (160 mg, 1.3 mmol, 6.0 equiv) was added and the suspension was stirred for 30 min. The turbid mixture was cooled to 0 °C and methanesulfonyl chloride (67 µL, 0.87 mmol, 4.0 equiv) in 2 mL of dry CH_2Cl_2 was added in a dropwise manner. The suspension was stirred for 30 min at 0 °C, warmed to room temperature and then stirred for 20 h. Additional 10 mL of CH_2Cl_2 was added and the reaction mixture was extracted with 10 mL of water. The organic phase was dry over anhydrous Na_2SO_4 and the solvent was evaporated *in vacuo*. This mixture was used further without any purification. The residue was dissolved in 10 mL of dry DMF and NaN_3 (70 mg, 1.09 mmol, 5.0 equiv) was added. The reaction mixture was heated up to 60 °C and stirred for 52 h. Then the solvent was evaporated *in vacuo* and the residue was dried under the reduced pressure. The crude reaction mixture was purified by column chromatography over silica gel using CH_2Cl_2/CH_3OH, 99:1. The product was obtained as an orange solid with the yield of 31% (20 mg, 0.04 mmol).



2-(6-((2-Azidoethyl)thio)-2,7-dichloro-3-oxo-3H-xanthen-9-yl)benzoic acid (4b): (31%, orange solid) ^{1}H NMR (400 MHz, DMSO-*d6*) δ 11.16 (s, 1H), 8.03 (d, ^{3}J = 7.6 Hz, 1H), 7.82 (td, ^{3}J = 7.6 Hz, ^{4}J = 1.2 Hz, 1H), 7.76 (td, ^{3}J = 7.6 Hz, ^{4}J = 1.2 Hz, 1H), 7.46 (s, 1H), 7.36 (d, ^{3}J = 7.6 Hz, 1H), 6.95 (s, 1H), 6.83 (s, 1H), 6.70 (s, 1H), 3.90 (t, ^{3}J = 7.2 Hz, 2H), 3.58 (t, ^{3}J = 7.2 Hz, 2H) ppm; ^{13}C NMR (75 MHz, DMSO-*d6*) δ 168.2, 155.4, 151.4, 149.8, 149.7,

138.9, 136.0, 130.7, 128.3, 127.9, 125.7, 125.5, 125.3, 123.9, 116.6 (2C), 114.9, 110.1, 103.7, 80.8, 42.4, 33.1 ppm; FD-MS: m/z (%): 485.4 (94), 486.4 (13), 487.4 (100), 488.4 (29); HRMS (ESI) calcd for $C_{22}H_{14}Cl_2N_3O_4S$ $[M + H]^+$ 486.0082, found 486.0060; FT-IR $\tilde{V}$ (cm^{-1}) 3412 ν (OH), 2922 ν (CH_2, aliph.), 2105 ν (N_3), 1768 ν (C=O, lactone), 1601 ν (C=C, arom.), 1481, 1384, 1184



2-(6-((2-azidoethyl)thio)-2,4,5-tribromo-3-oxo-3*H*-xanthen-9-yl)benzoic acid (4d): (19%, red solid) ^{1}H NMR (400 MHz, CD_3OD) δ 8.10 (d, ^3J = 7.6 Hz, 1H), 7.92 – 7.68 (m, 2H), 7.32 (d, ^3J = 7.6 Hz, 1H), 7.19 (d, ^3J = 8.4 Hz, 1H), 6.94 (s, 1H), 6.85 (d, ^3J = 8.4 Hz, 1H), 3.60 (t, ^3J = 6.8 Hz, 2H), 3.30 – 3.22 (t, ^3J = 6.8 Hz, 2H) ppm; ^{13}C NMR (75 MHz, CD_3OD) δ 170.5, 150.3, 149.7, 143.6, 136.7, 131.8 (2C), 131.1, 130.4, 128.3, 128 (2C), 126.7, 125.6, 123.1 (2C), 118.5, 114, 111.6, 77.1, 51, 32.7 ppm; FD-MS: m/z (%): 653.2 (100), 654.3 (39), 655.2 (97); HRMS (ESI) calcd for $C_{22}H_{13}Br_3N_3O_4S$ $[M + H]^+$ 651.8177, found 651.8199; FT-IR $\tilde{V}$ (cm^{-1}) 3077 ν (C-H, arom.), 2928 ν (CH_2, aliph.), 2101 ν (N_3), 1760 ν (C=O, lactone), 1600 ν (C=C, arom.), 1403, 1193

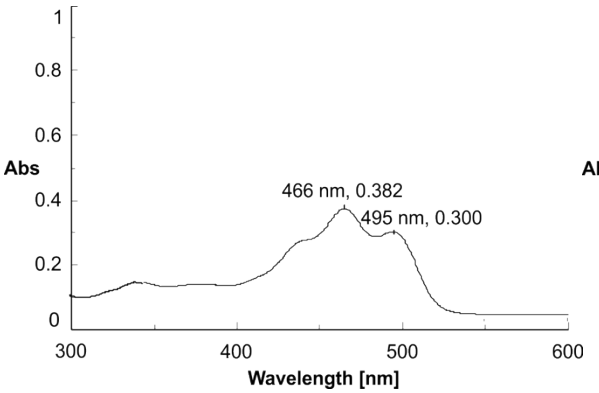
Characterization of Photophysical Properties of Xanthene Sulfides

The photophysical properties of new xanthene dyes including absorption, extinction coefficient at the maximum absorption wavelength (λ_{max}), excitation (λ_{ex}), emission (λ_{em}), quantum yield (Φ), and photostability were characterized.

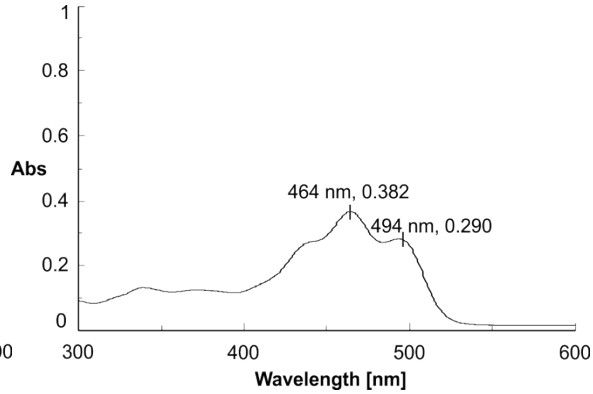
Stock solutions of the fluorophores were prepared by accurate weighing and dissolving 2-8 mg of the fluorophore in ethanol (HPLC grade) to obtain a concentration of 5 mM.

Absorption spectra were measured in Suprasil quartz glass cuvettes (Hellma, Müllheim, Germany) with 0.1 cm path length in basic medium (0.1 M NaOH) at 25 °C. The samples were prepared as stock solutions in ethanol and diluted such that the ethanol concentration did not exceed 1% v/v. Absorption spectra were recorded from 300 to 600 nm to determine the maximum absorption wavelength (λ_{max}). 10 μM solutions of dyes were prepared. Global and local absorption maxima are indicated in the graphs. The dye samples produced two absorption maxima of similar values within the difference of 30 nm.

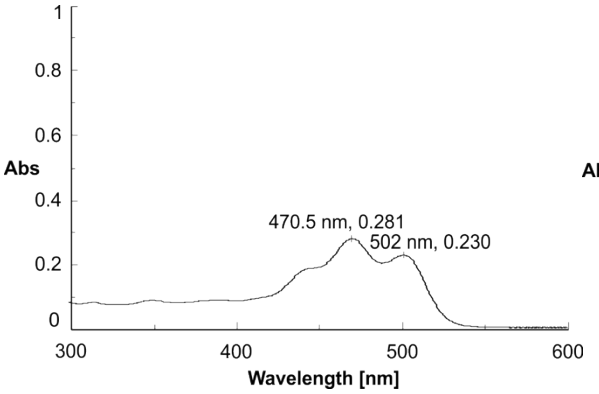
2a



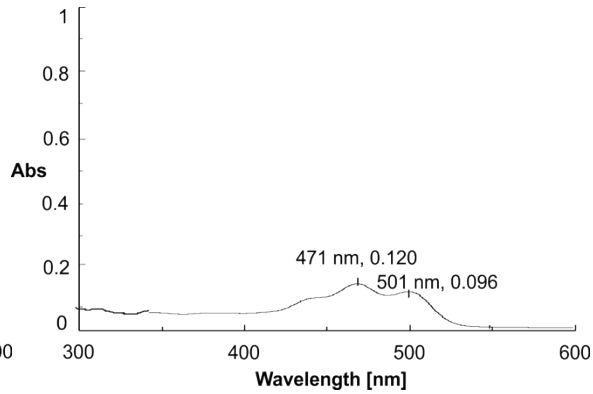
4a



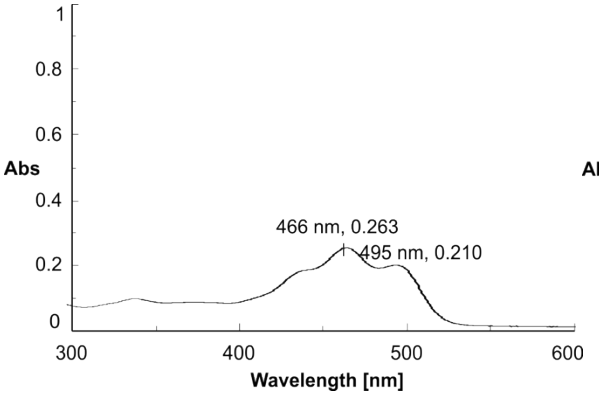
2b



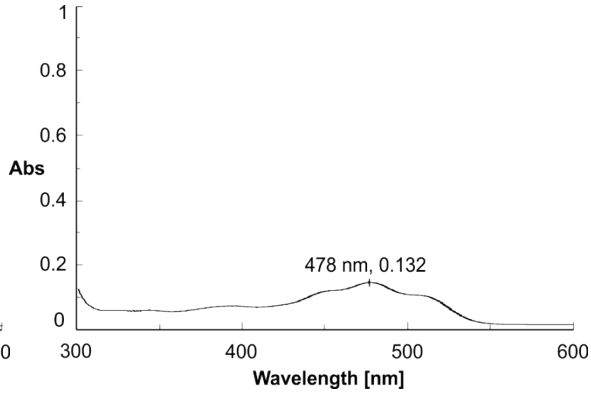
4b



6a



2c



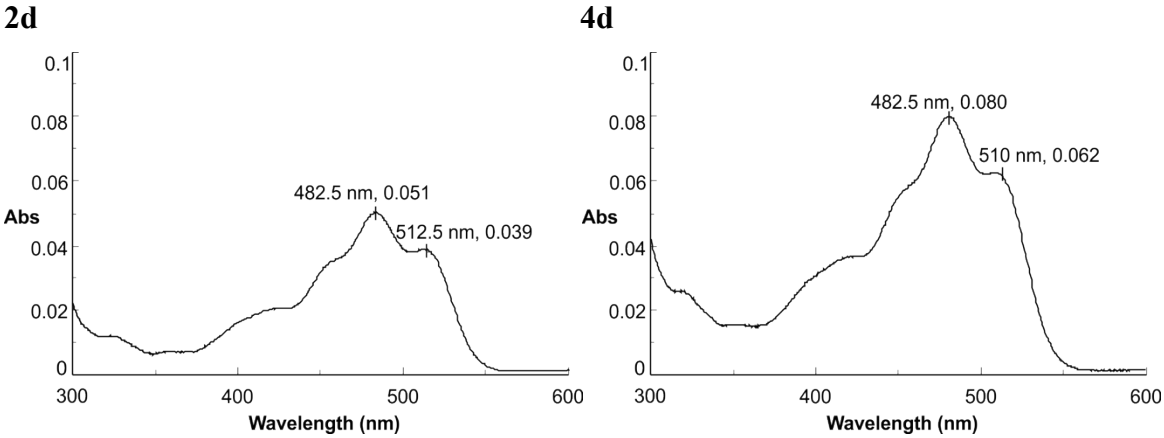
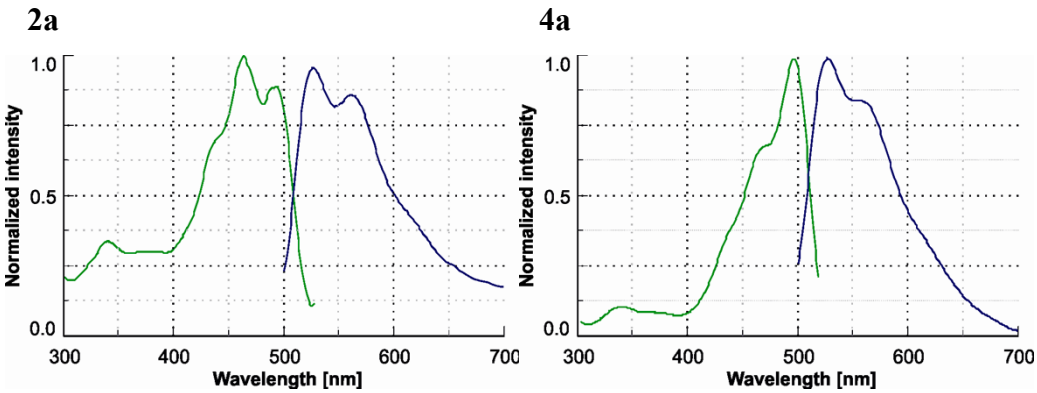


Figure S1. Absorption graphs of asymmetric xanthene dyes in basic medium.

For fluorescent measurements, samples were diluted to final concentration of 5 μM in basic medium (0.1 M NaOH) and were analyzed in Suprasil quartz glass cuvettes with 3 mm path length. Excitation and emission scans were performed at 25 °C with the following parameters: 3 nm bandwidth of excitation and emission, data pitch of 1 nm, response time of 0.5 s, scanning speed of 1000 nm min, and spectral correction of excitation and emission. The normalized excitation and emission spectra of selected xanthene dyes are illustrated in figure S2.



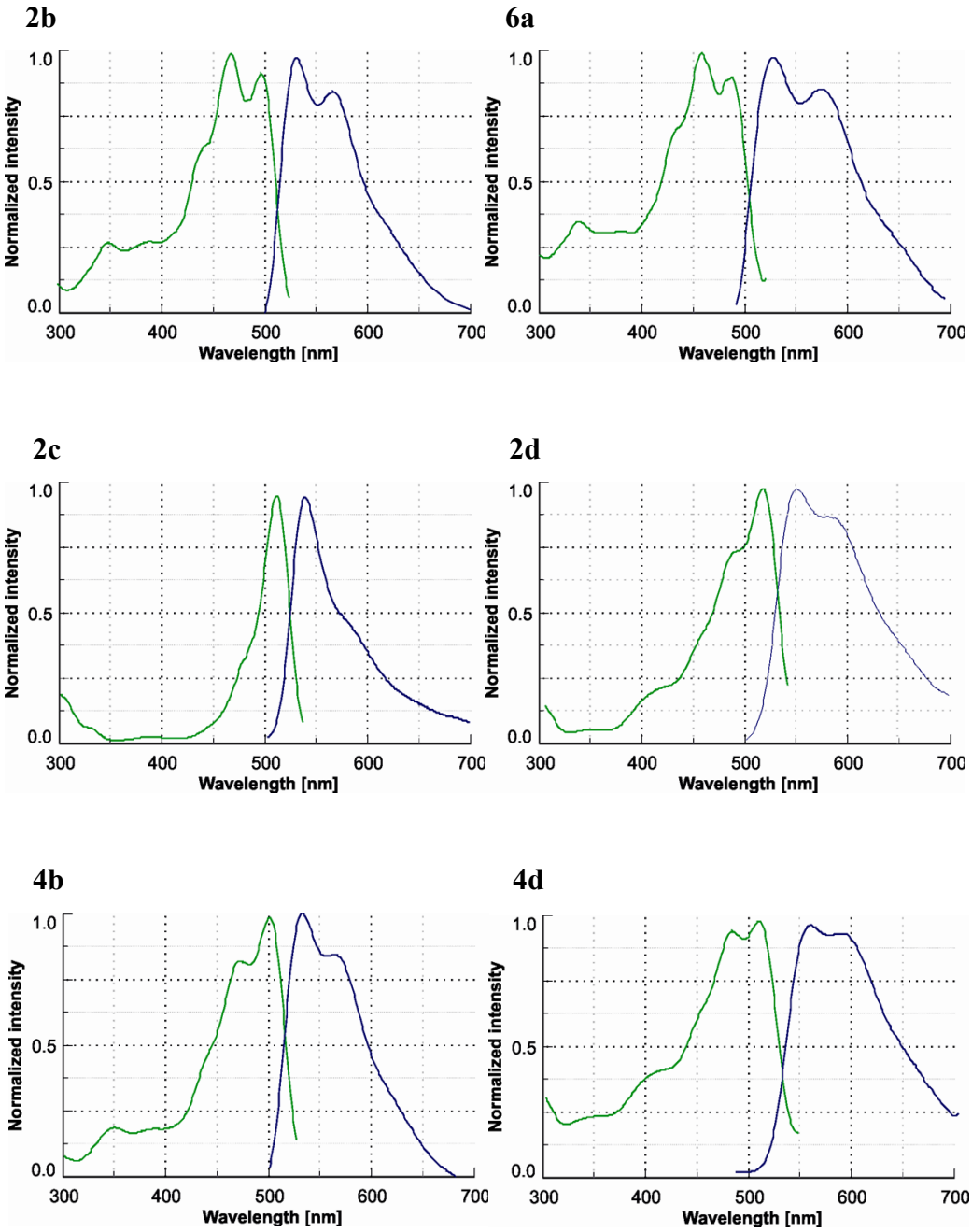


Figure S2. Normalized excitation and emission spectra of selected dyes in basic medium.

The Beer-Lambert law ($A = \varepsilon \cdot c \cdot l$) was used to determine the extinction coefficients of samples by measuring the absorbance of solutions at four known concentrations. The absorbances (y-axis) were plotted towards the corresponding concentrations (x-axis) and the extinction coefficients were calculated by linear regression using Beer's law. The absorption spectra were measured in 1-cm path length cuvettes in 1 mM NaOH. The samples were prepared as stock solutions in ethanol and diluted such as the ethanol concentration did not exceed 1% v/v. Extinction coefficients were determined from the absorptions with higher values.

Absorption spectra were measured as described above. The quantum yields were determined by using diluted samples (A < 0.1) in 0.1 M NaOH in water. Emission scans were performed at 25 °C with the following parameters: 3 nm bandwidth of excitation and emission, data pitch of 1 nm, response of 0.5 s, scanning speed of 1000 nm min, and spectral correction of multiplier and illumination lamp. Samples were excited at 488 nm and the emission was recorded from at least 5 nm above the excitation wavelength to 700 nm (emission profile). These quantum yields were obtained by comparison of the integrated area of the emission spectra ($\int F_{em,\ sample}$) of the samples with the integrated area of emission spectra of standard sample ($\int F_{em,\ standard}$), fluorescein in 0.1 M NaOH in water, which has a quantum efficiency 0.95 ± 0.03.[1,2] The integrated area of the emission spectrum was calculated in the section between 500 - 600 nm. The quantum yield of a sample was related to that of the standard, and determined by the equation 1[3]

$$\Phi_{sample} = (A_{standard}/A_{sample}) \left(\int F_{em,\ sample}/\int F_{em,\ standard}\right) (\eta_{sample}/\eta_{standard})^2 \Phi_{standard} \quad \textbf{(Equation 1)}$$

wherein Φ is the fluorescent quantum yield, A is the absorbance at the excitation wavelength, $\int F$ is the area under the emission curve, η is the refractive index of the solvent.
The concentrations of the test samples were adjusted to match the absorbance of the standard at the excitation wavelength so that the absorbance ratio is equal to 1. The refractive index ratio is also equal 1 since water was used for both the standard and the sample solutions. Under these conditions the quantum yield (Φ) was calculated with the following equation 2[1]

$$\Phi_{sample} = \Phi_{standard} \left(\int F_{em,\ sample}/\int F_{em,\ standard}\right) \quad \textbf{(Equation 2)}$$

Integrated fluorescent intensity of four different concentrations was plotted against corresponding absorbance at this concentration. The quantum yields of brominated fluorescent dyes **2c**, **2d**, **4d** were not determined because their fluorescence at such a low concentrations was too weak to be detected. The determined quantum yields are included in table S1.

Compound	λ_{max} (nm)	ε at λ_{max} (M^{-1} cm^{-1})	λ_{ex} (nm)	λ_{em} (nm)	Φ
2a	466, 495	34 000	465, 495	526, 560	0.22
2b	471, 502	27 000	472, 502	531, 568	0.17
2c	473	13 100	514	538	- [a]
2d	481, 511	4 500	516	554, 588	- [a]
4a	464, 494	38 000	497	527, 556	0.24
4b	470, 502	12 000	474, 504	533, 565	0.16
4d	480, 511	6 300	485, 511	554, 584	- [a]
6a	466, 495	25 000	465, 496	526, 562	0.20

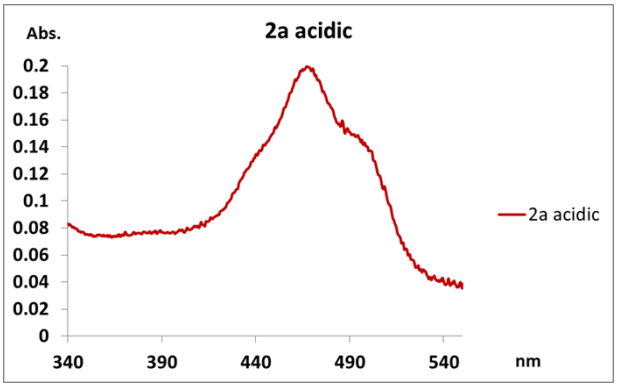
[a] Fluorescence too weak to be detected

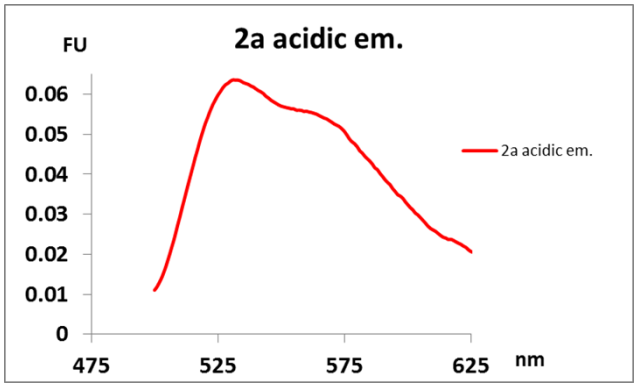
Table S1. Spectral data for selected xanthene dyes.

pH depend absorbance and emission spectra of thioxanthene dyes

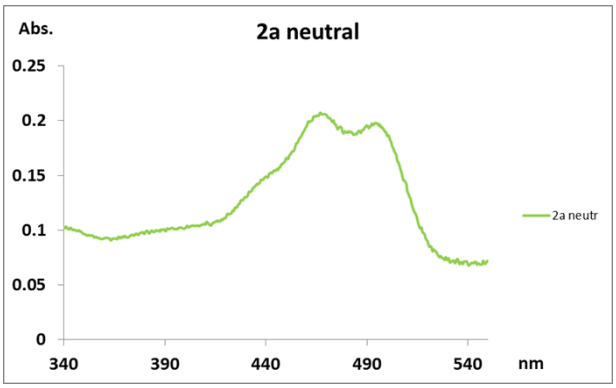
Compound **2a** (250 µM)

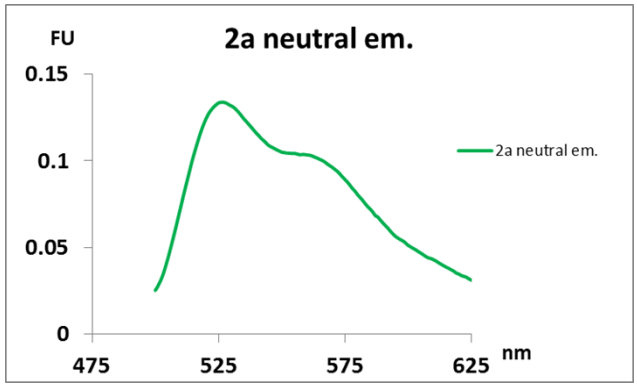
pH **3** (1 mM HCl sol.)



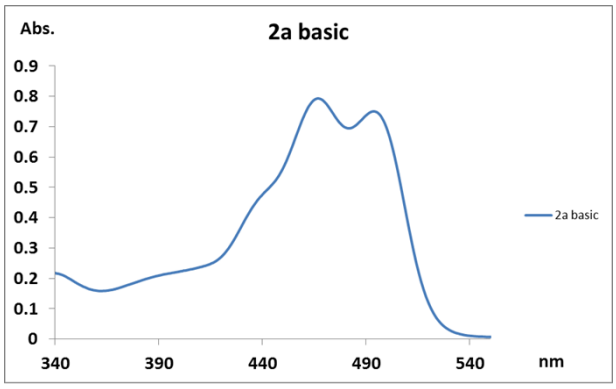


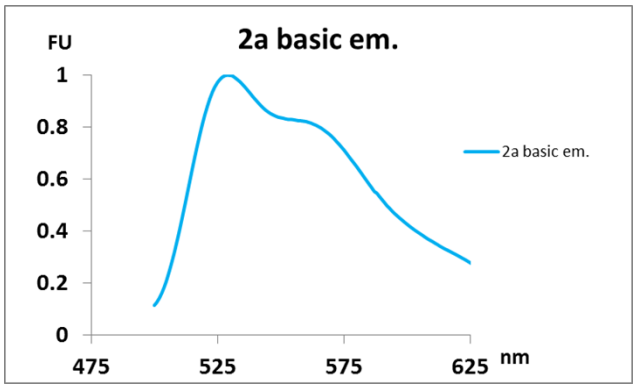
pH **7,3** (Et_3NHOAc buffer)





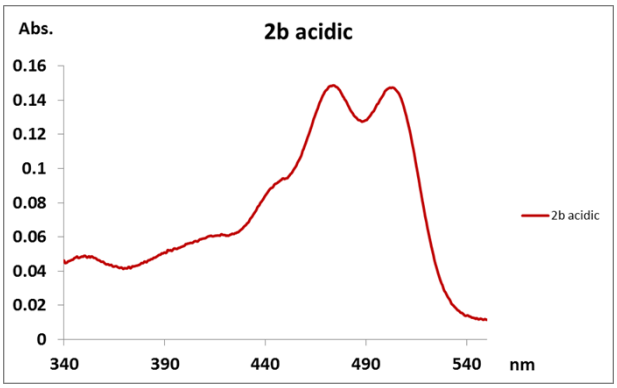
pH **11** (1 mM NaOH sol.)

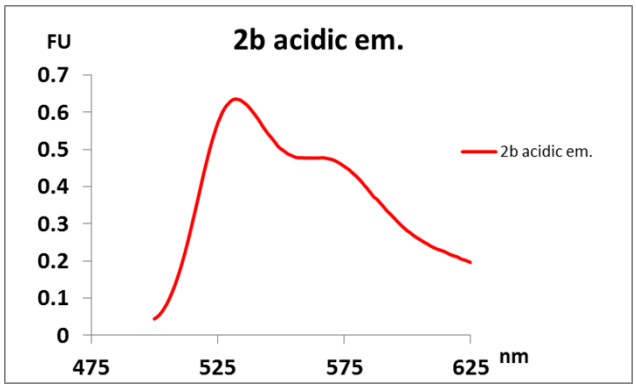




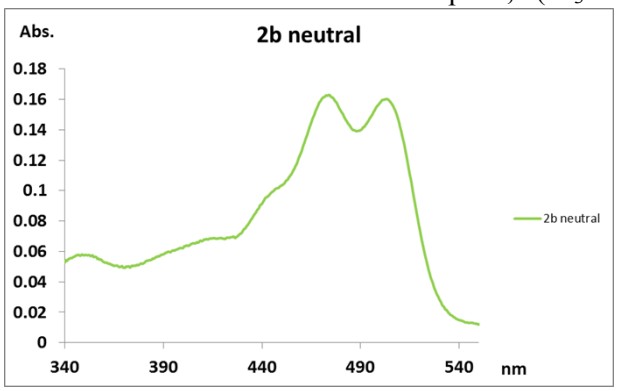
Compound **2b** (200 μM)

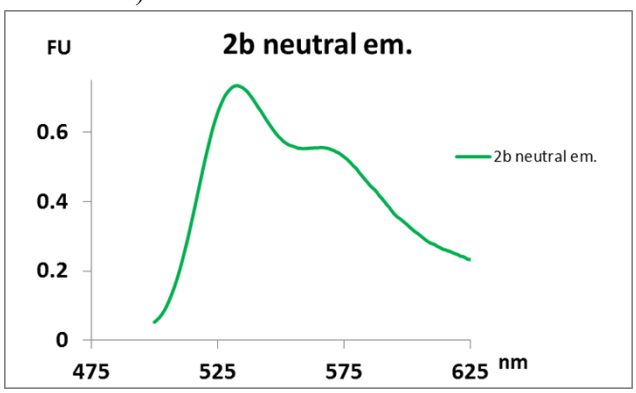
pH **3** (1 mM HCl sol.)



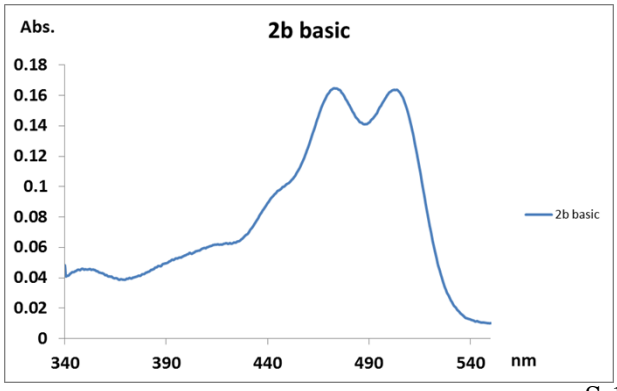


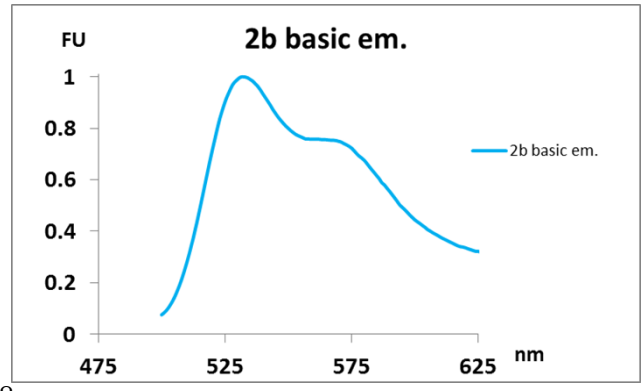
pH **7,3** (Et_3NHOAc buffer)





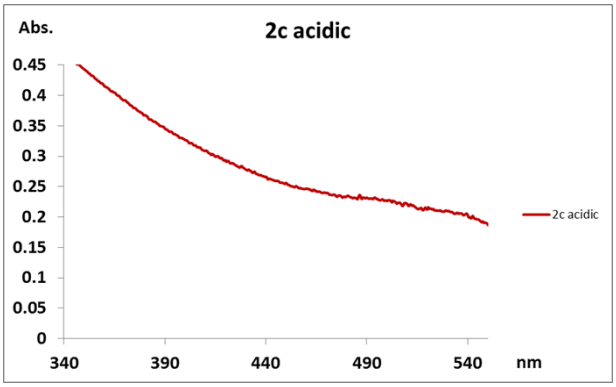
pH **11** (1 mM NaOH sol.)

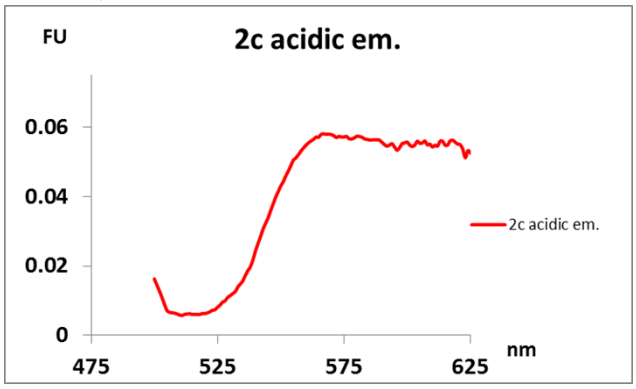




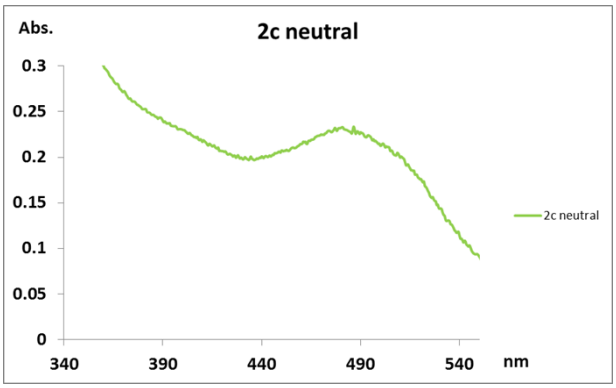
Compound **2c** (200 µM)

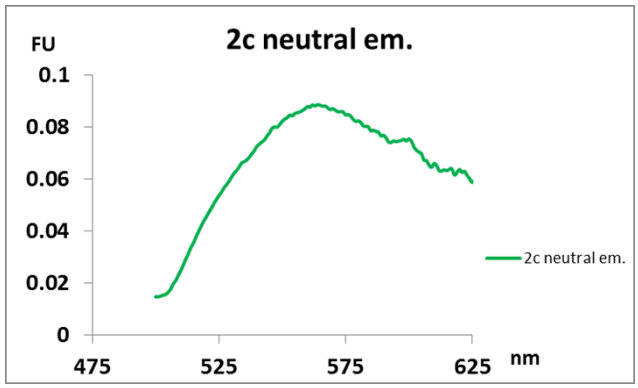
pH **3** (1 mM HCl sol.)



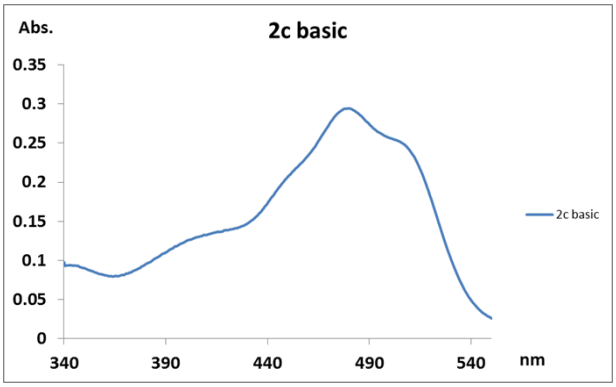


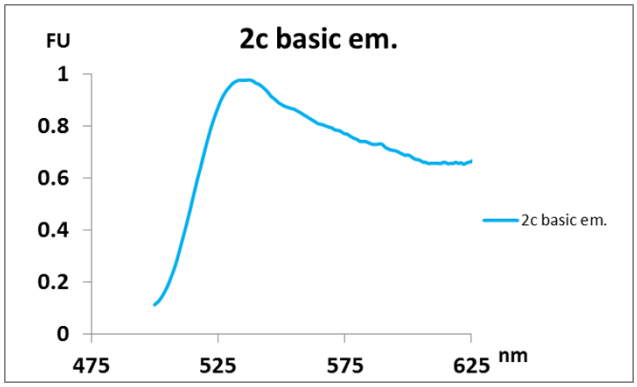
pH **7,3** (Et_3NHOAc buffer)





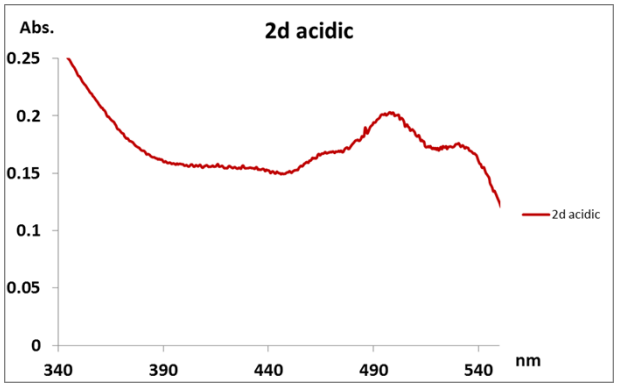
pH **11** (1 mM NaOH sol.)

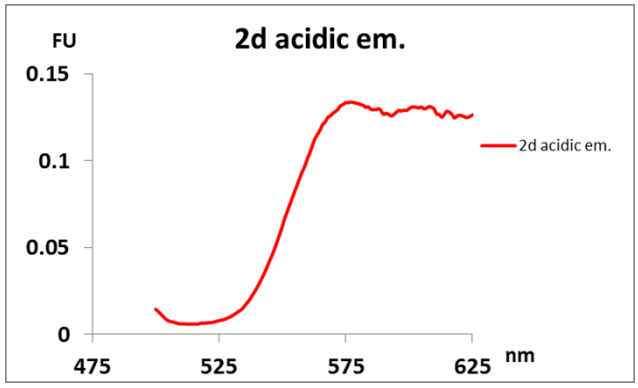




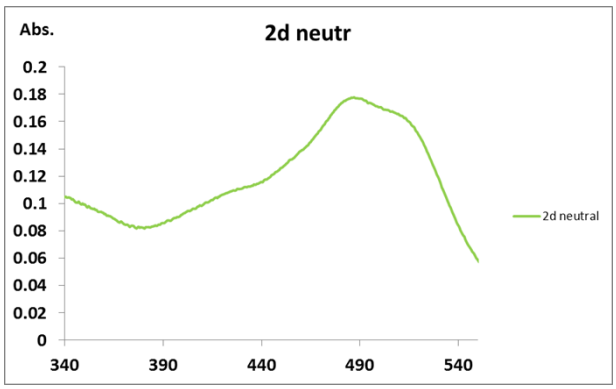
Compound **2d** (250 µM)

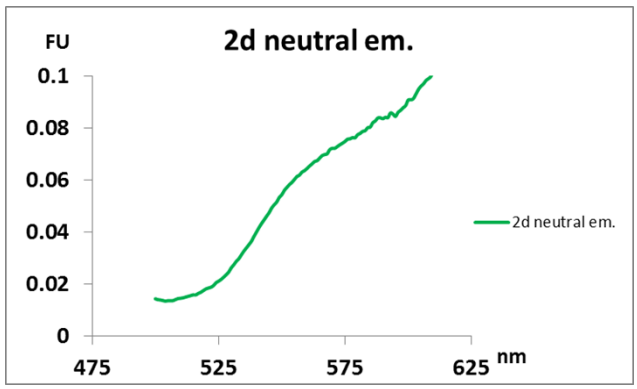
pH **3** (1 mM HCl sol.)



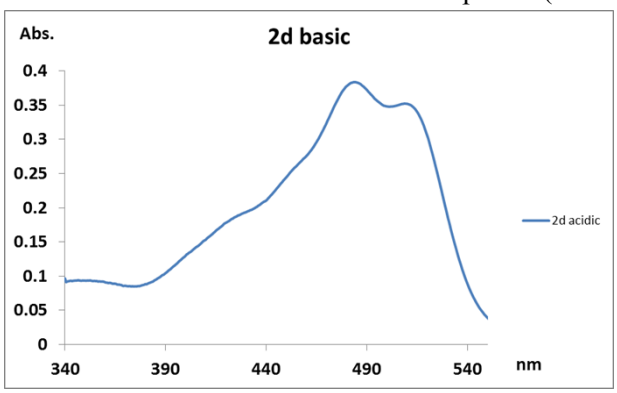


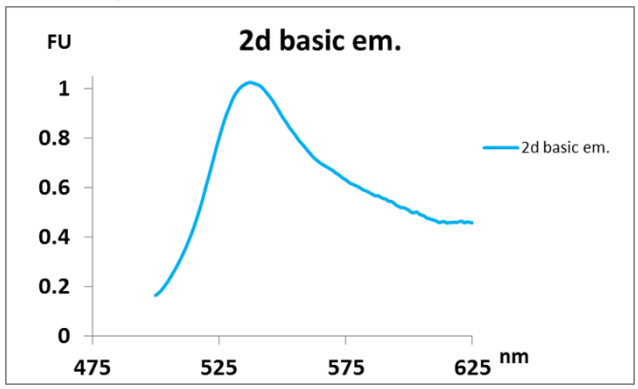
pH **7,3** (NH_4Ac buffer)





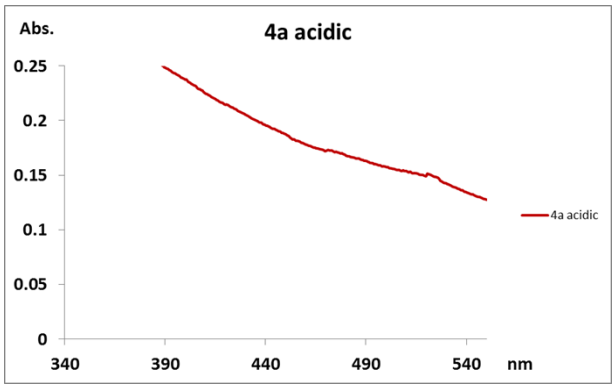
pH **11** (1 mM NaOH sol.)

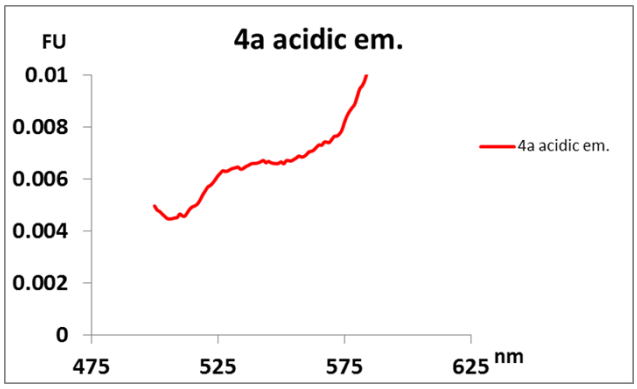




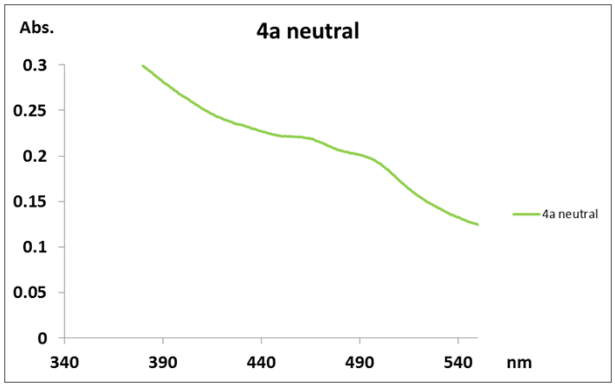
Compound **4a** (200 µM)

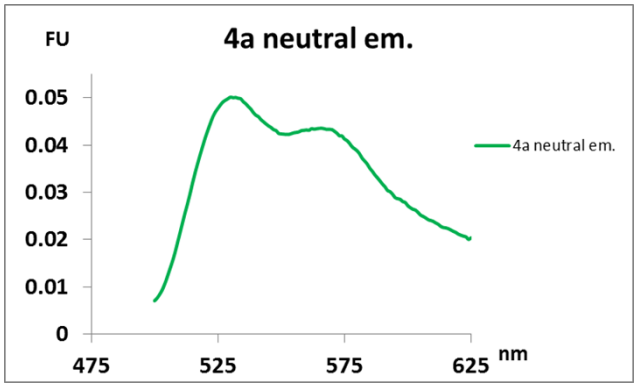
pH **3** (1 mM HCl sol.)



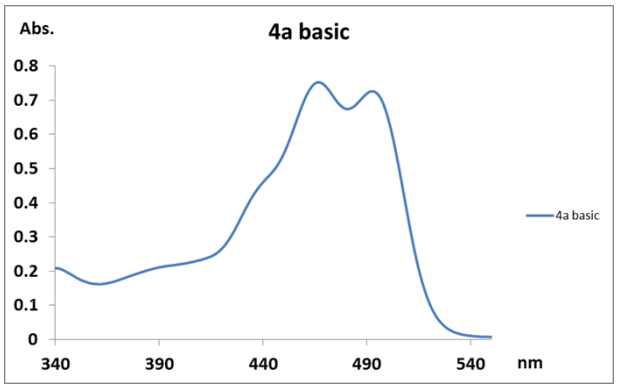


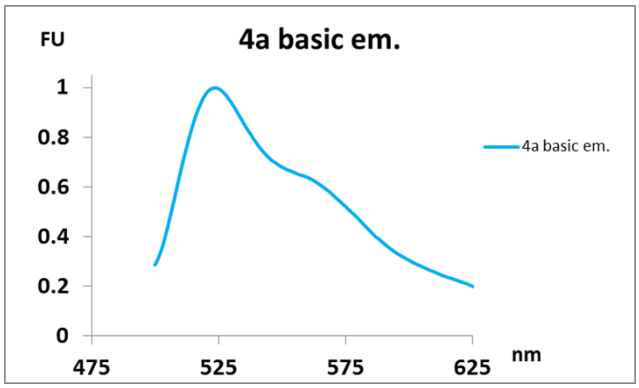
pH **7,3** (Et_3NHOAc buffer)





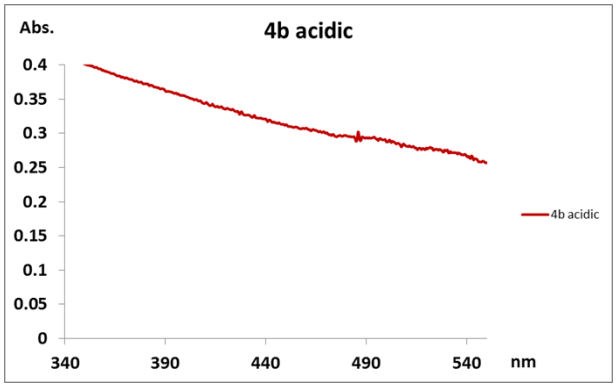
pH **11** (1 mM NaOH sol.)

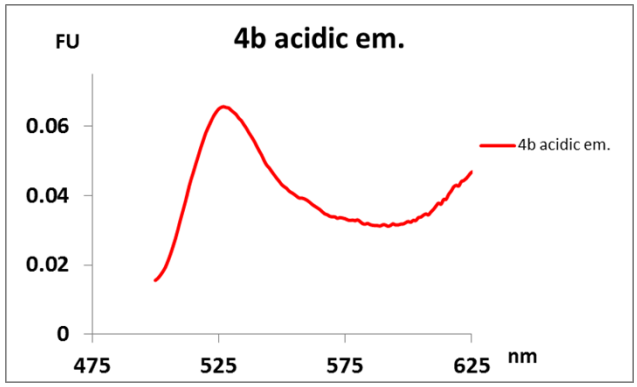




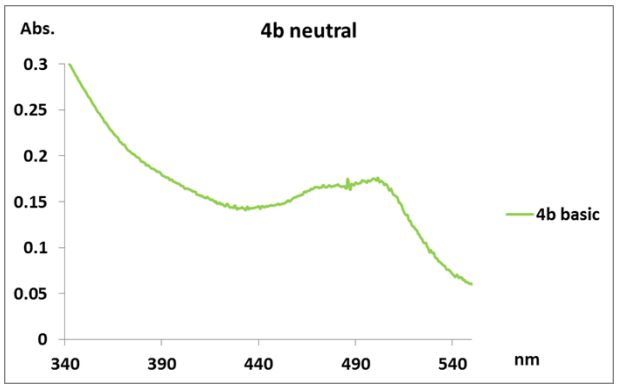
Compound **4b** (200 µM)

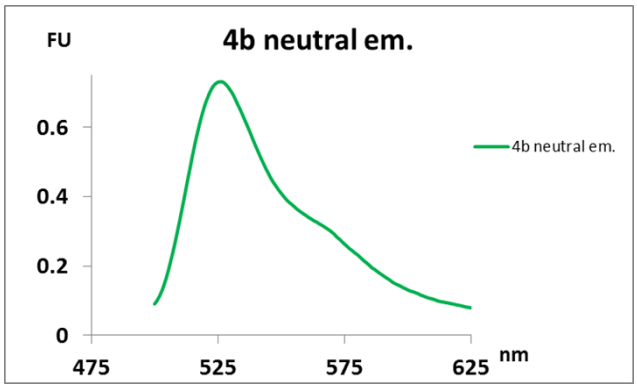
pH **3** (1 mM HCl sol.)



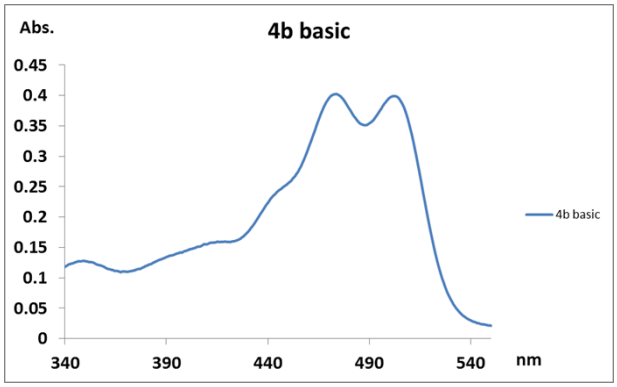


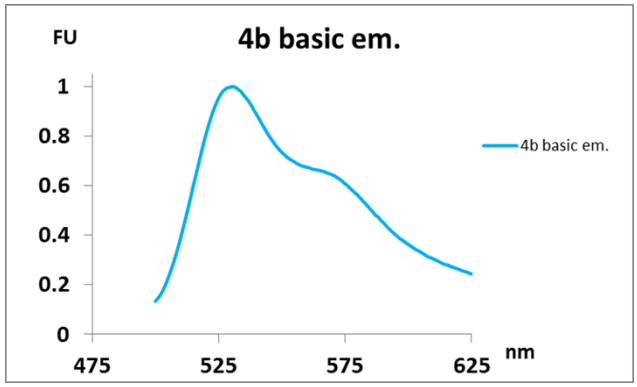
pH **7,3** (Et_3NHOAc buffer)





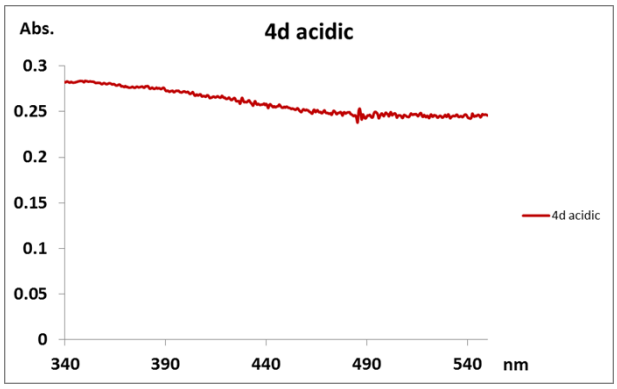
pH **11** (1 mM NaOH sol.)

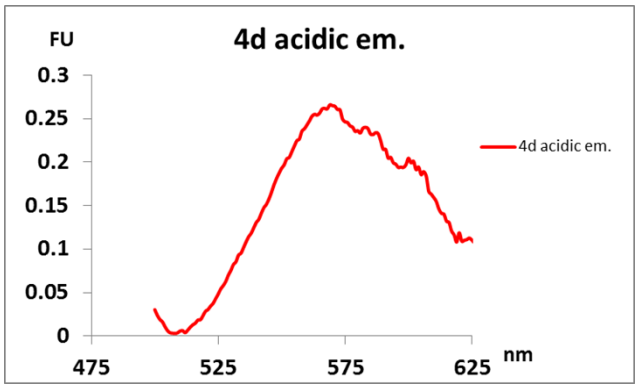




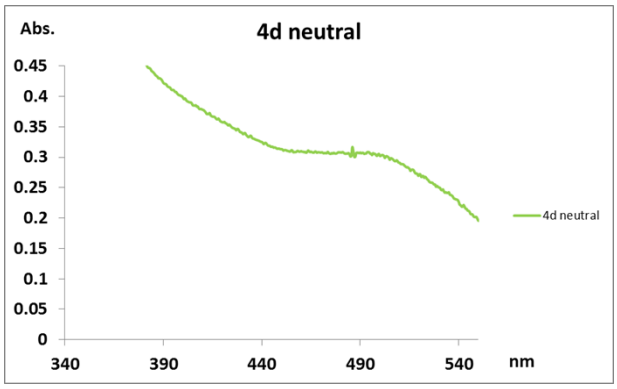
Compound **4d** (250 µM)

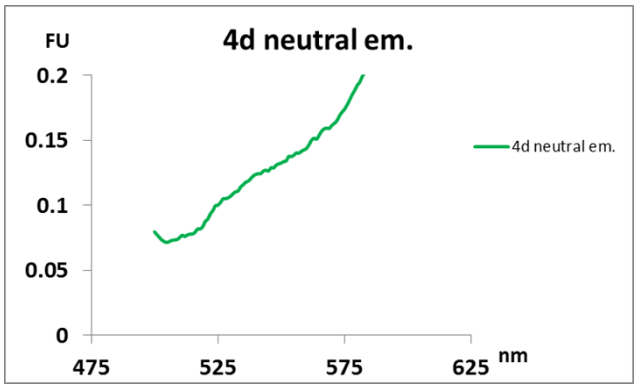
pH **3** (1 mM HCl sol.)



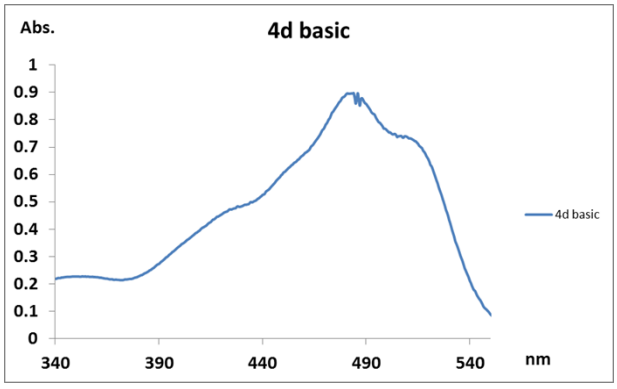


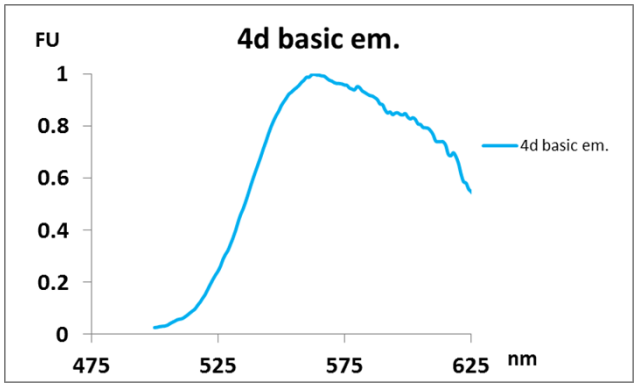
pH **7,3** (Et_3NHOAc buffer)





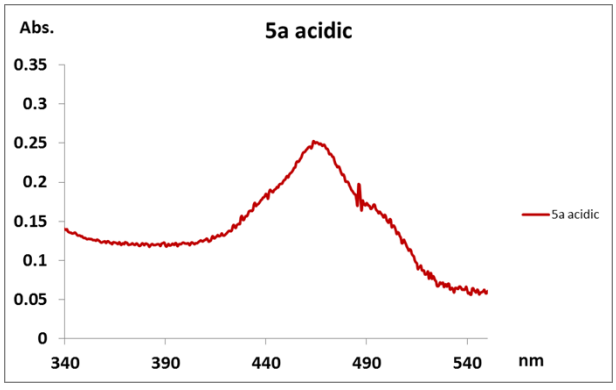
pH **11** (1 mM NaOH sol.)

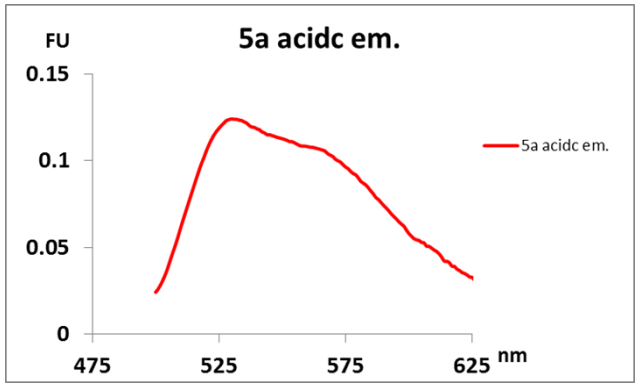




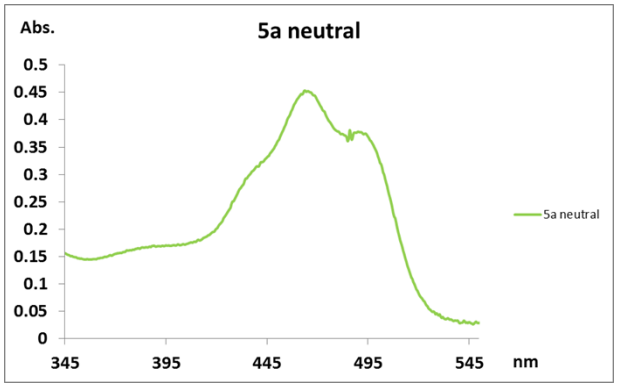
Compound **5a** (200 µM)

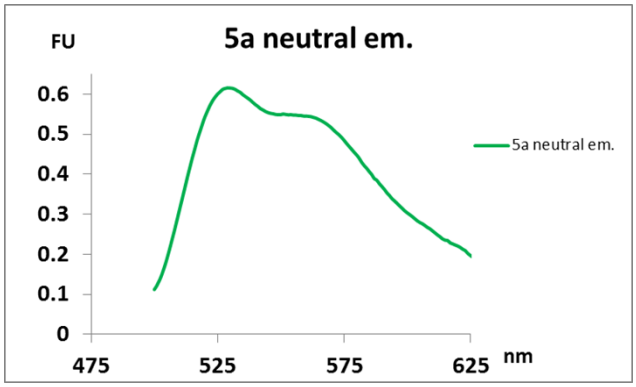
pH **3** (1 mM HCl sol.)



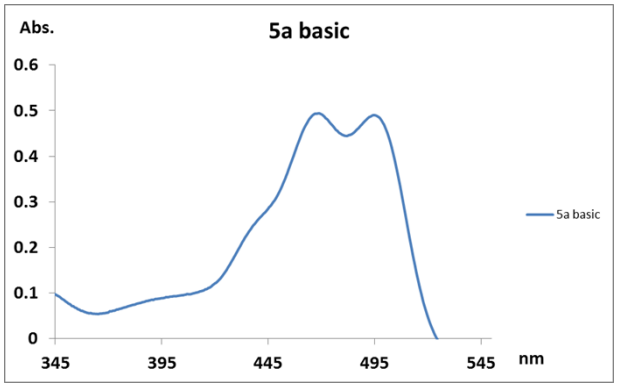


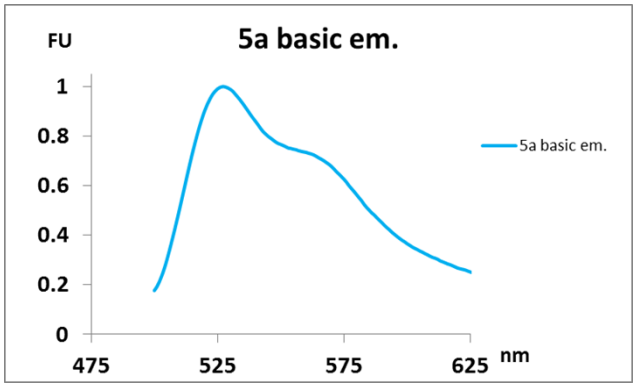
pH **7,3** (Et_3NHOAc buffer)





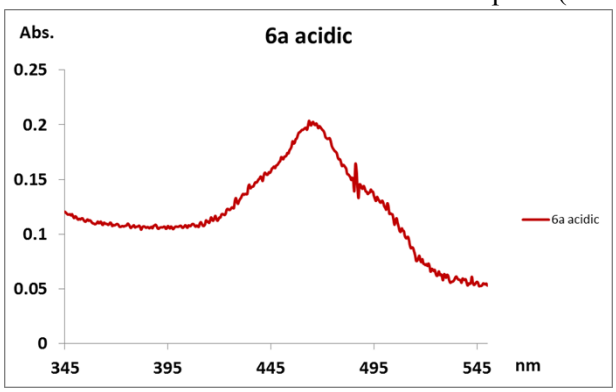
pH **11** (1 mM NaOH sol.)

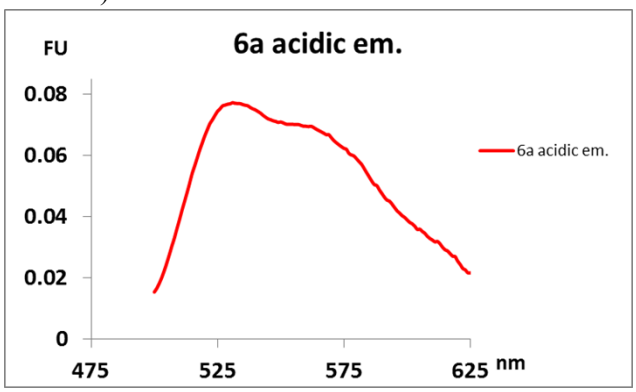




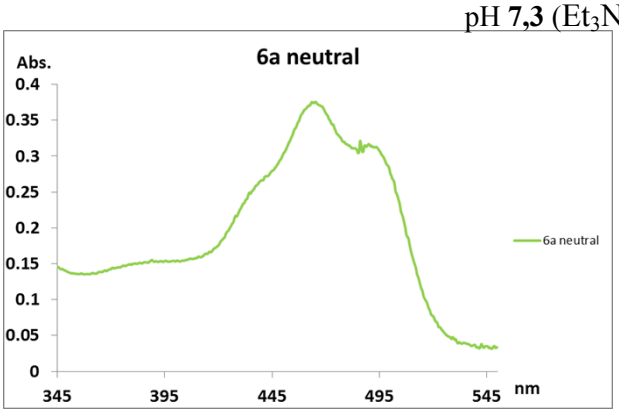
Compound **6a** (200 µM)

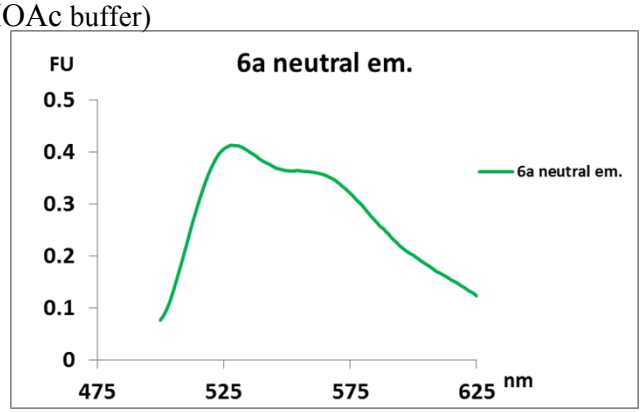
pH **3** (1 mM HCl sol.)





pH **7,3** (Et_3NHOAc buffer)





pH **11** (1 mM NaOH sol.)

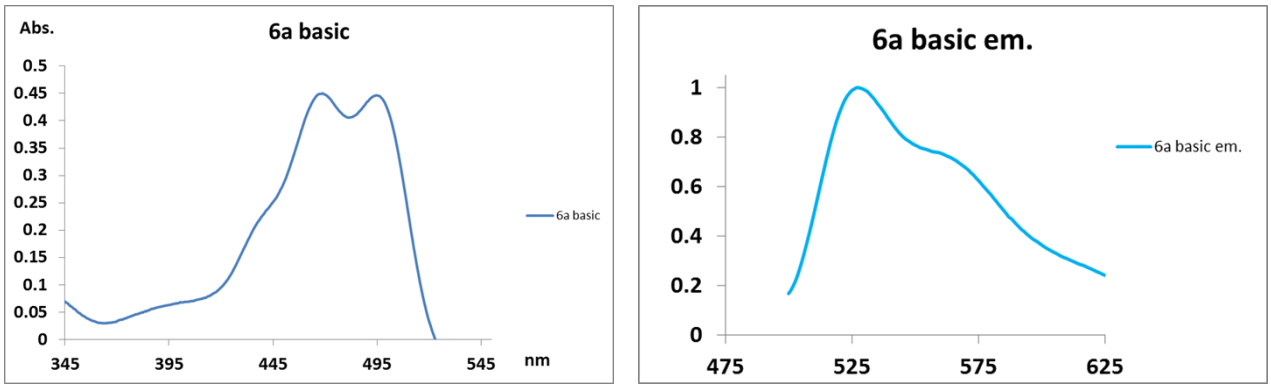


Figure S3. absorbance and emission spectra of thioxanthene dyes with pH 3, 7.3 and 11

Photobleaching studies

Photobleaching of asymmetric xanthene dyes was performed in 3 mm path length Suprasil quartz glass cuvette on a fluorimeter (light source: Xenon lamp, power: 150 Watt). For the analysis of photostability of dyes, samples were diluted to equiabsorbing solutions in Et_3NHOAc buffer (pH 7.3), homogenized in an ultrasonic bad for 15 minutes and stored for 30 minutes in RT. Additionally, the parameters were adjusted to 10 nm bandwidth excitation to guarantee sufficient bleaching. Samples were constantly excited for 2 h at 488 ± 3 nm recording the emission spectra in 10 seconds intervals. The photostability of dyes was analyzed towards fluorescein (Fl) and 2',7'-dichlorofluorescein (DCFl). Percentage of initial intensities of xanthene dyes upon continuous excitation for 2 h is included in table S2.

Dye	% of initial intensity (2h)
FL	32 +/- 7,2
DCFL	36 +/- 3,1
2a	83 +/- 2,5
2b	82 +/- 4,9
4a	78 +/- 1,9
4b	70 +/- 2,4
6a	82 +/- 2,7

Table S2. Percentage of initial intensities of xanthene dyes upon excitation for 2 h.

Photobleaching studies

Photobleaching of asymmetric xanthene dyes was performed in 3 mm path length Suprasil quartz glass cuvette on a fluorimeter (light source: Xenon lamp, power: 150 Watt). For the analysis of photostability of dyes, samples were diluted to equiabsorbing solutions in Et3NHOAc buffer (pH 7.3), homogenized in an ultrasonic bad for 15 minutes and stored for 30 minutes in RT. Additionally, the parameters were adjusted to 10 nm bandwidth excitation to guarantee sufficient bleaching. Samples were constantly excited for 2 h at 488 ± 3 nm recording the emission spectra in 10 seconds intervals. The photostability of dyes was analyzed towards fluorescein (Fl) and 2',7'-dichlorofluorescein (DCFl). Percentage of initial intensities of xanthene dyes upon continuous excitation for 2 h is included in table S2.

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4a	78 +/- 1,9
4b	70 +/- 2,4
6a	82 +/- 2,7

Table S2. Percentage of initial intensities of xanthene dyes upon excitation for 2 h.

Mathematical analysis of photobleaching studies

Used formula for the analysis with Qti-Plot® software:

monoexponential curves	$f(x) = A \cdot e^{-t / \lambda}$
biexponential curves	$f(x) = A_1 \cdot e^{-t / \lambda 1} + A_2 \cdot e^{-t / \lambda 2}$
half-life	$tx_{1/2} = \tau_x \cdot \ln(2)$
mean lifetime	$\tau_x = 1 / \lambda_x$

remarks: λ is given in hour

Table S3. Exponential functions, half-life and calculated mean lifetime τ

compound	f(t)	$t_{1/2}$ in h	calc. τ in h
FL	$0{,}898 \cdot e^{-0{,}58\ t}$	1,19	$\tau = 1{,}72$
DCFL	$0{,}998 \cdot e^{-0{,}51\ t}$	1,36	$\tau = 1{,}96$
2a	$0{,}980 \cdot e^{-0{,}076\ t}$	9,12	$\tau = 13{,}16$
2b	$0{,}931 \cdot e^{-0{,}064\ t1} + 0{,}057 \cdot e^{-3{,}45\ t2}$	$t1_{1/2} = 10{,}83$ $t2_{1/2} = 0{,}20$	$\tau_1 = 15{,}63$ $\tau_2 = 0{,}29$
4a	$0{,}701 \cdot e^{-4 \cdot 10\text{^}-8\ t1} + 0{,}295 \cdot e^{-0{,}64\ t2}$	no fit	no fit
4b	$0{,}869 \cdot e^{-0{,}122\ t1} + 0{,}125 \cdot e^{-1{,}13\ t2}$	$t1_{1/2} = 5{,}68$ $t2_{1/2} = 0{,}61$	$\tau_1 = 8{,}20$ $\tau_2 = 0{,}88$
6a	$0{,}979 \cdot e^{-0{,}092\ t}$	7,53	10,87

Click reaction and purification of ODN

Azido-functionalized xanthene dyes 4a, 4b & 4d were tested for labeling of oligonucleotides. The dyes were conjugated *via* Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC)[5] with a commercially available DNA primer containing a terminal alkyne, 5'-C8-Alkyne-CGC GCG AAG CTT AAT ACG ACT CAC TAT A (Glen Research No. 10-1543, supplied by IBA). The click reaction was performed under exclusion of light in a benchtop thermomixer (550 rpm) for 2 h at 25 °C. The following pipetting scheme was used for each dye.

Compound	c_{stock} (mM)	C_{final} (mM)	V (µL)
Buffer NaH_2PO_4	1000	100	20
THPTA	50	2.5	10
Na ascorbate	50	5	20
$CuSO_4$. $5H_2O$	5	0.5	20
Dye	1	0.05	10
ODN	0,1	0.01	20
H_2O			100
			200

The reaction mixture was precipitated by centrifugation for 40 min 13000 xg after addition of 10 volumes of a solution of lithium perchlorate in acetone (2% w/v).

LC-MS method and analysis

Sample preparation

500 pmol of 4a labeled ODN was digested with Nuclease P1, Snake Venom Phosphodiesterase and Shrimp Alkaline Phosphatase, as previously described[4].

LC-MS and LC-MS/MS analysis

The digested DNA was analyzed on an Agilent 1260 series equipped with a diode array detector (DAD) and Triple Quadrupole mass spectrometer Agilent 6460. A Synergy Fusion RP column (4 µm particle size, 80 Å pore size, 250 mm length, 2 mm inner diameter) from Phenomenex (Aschaffenburg, Germany) was used at 35 °C. The solvents consisted of 5 mM ammonium acetate buffer adjusted to pH 5.3 using acetic acid (solvent A) and pure acetonitrile (solvent B). The elution started with 100% solvent A with a linear gradient to 8% solvent B at 10 min. For complete click product elution solvent B was increased to 75% at 20 minutes. Initial conditions were regenerated by rinsing with 100% solvent A for 10 minutes. The flow rate was 0.5 mL/min.

The effluent from the column was first measured photometrical at 254 nm by the DAD, followed by the mass spectrometer equipped with an electrospray ion source (Agilent Jet Stream). ESI parameters were as follows: gas temperature 350 °C, gas flow 5 L/min, nebulizer pressure 50 psi, sheath gas temperature 350 °C, sheath gas flow 12 L/min and capillary voltage 3000 V. The MS was setup in the Neutral Loss scan mode to scan all masses that display a neutral fragment loss of 116 Da (mass of deoxyribose) in the CID hexapole for deoxynucleoside identification. The parameters used were as follows: fragmentor voltage 50 V, CID 7 eV, cell accelerators voltage 2V, mass range of 200 to 1000 Da in positive ion. For a more detailed analysis, the sample was analyzed in product ion scan mode. Therefore, quadrupole 1 was adjusted to filter the detected mass of 749 (see Figure S4), followed by

fragmentation at 10 eV collision energy in the collision cell and final mass fragment analysis in quadrupole 2 (see Figure S5).

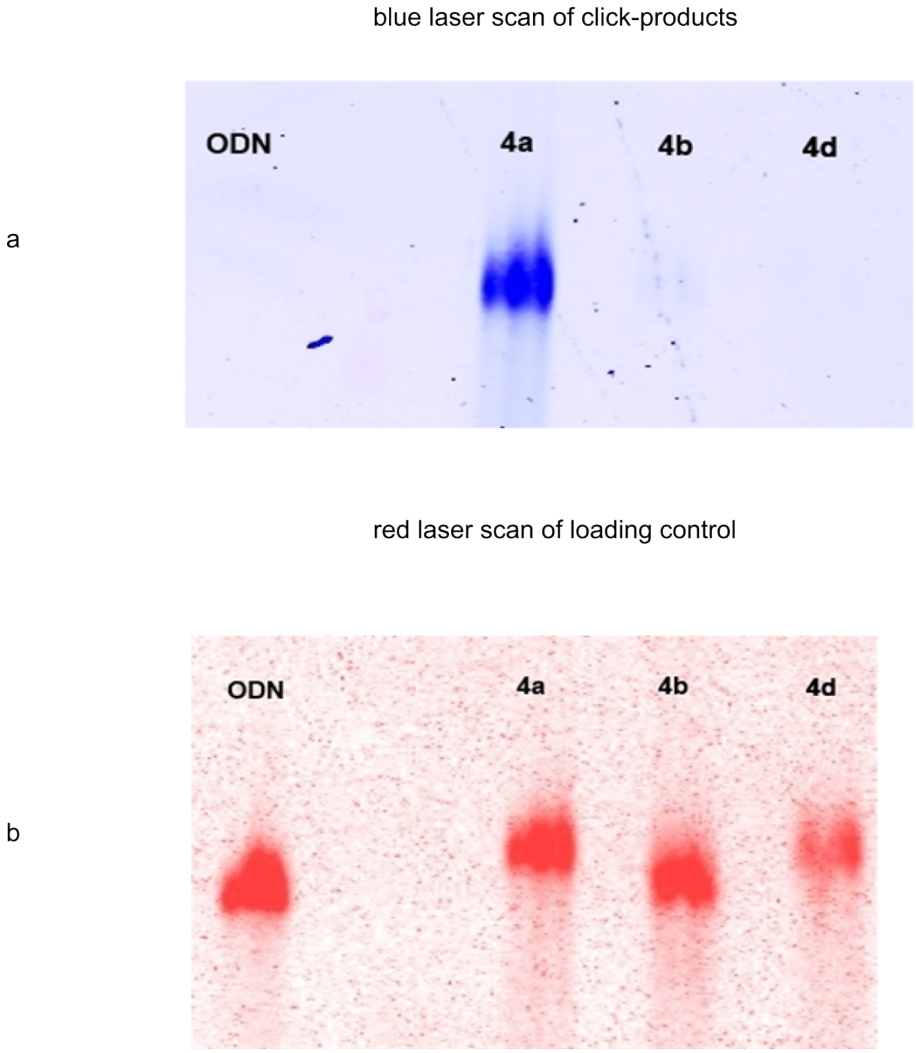

Figure S4. Denaturing 15% PAGE analysis of oligodeoxynucleotide click products. LD loading dye; ODN alkyne carrying oligodeoxynucleotide; 4a, 4b, 4d denote lanes loaded with click reaction mixtures of ODN with the respective azide compound. The upper panel shows a fluorescence emission scan (520 BP filter, ±30 nm) upon excitation at 488 nm detecting xanthenes conjugates, the lower panel shows fluorescence emission scan (670 BP filter, ±30 nm) upon excitation at 632 nm after staining all nucleic acids with stains all as a loading control.

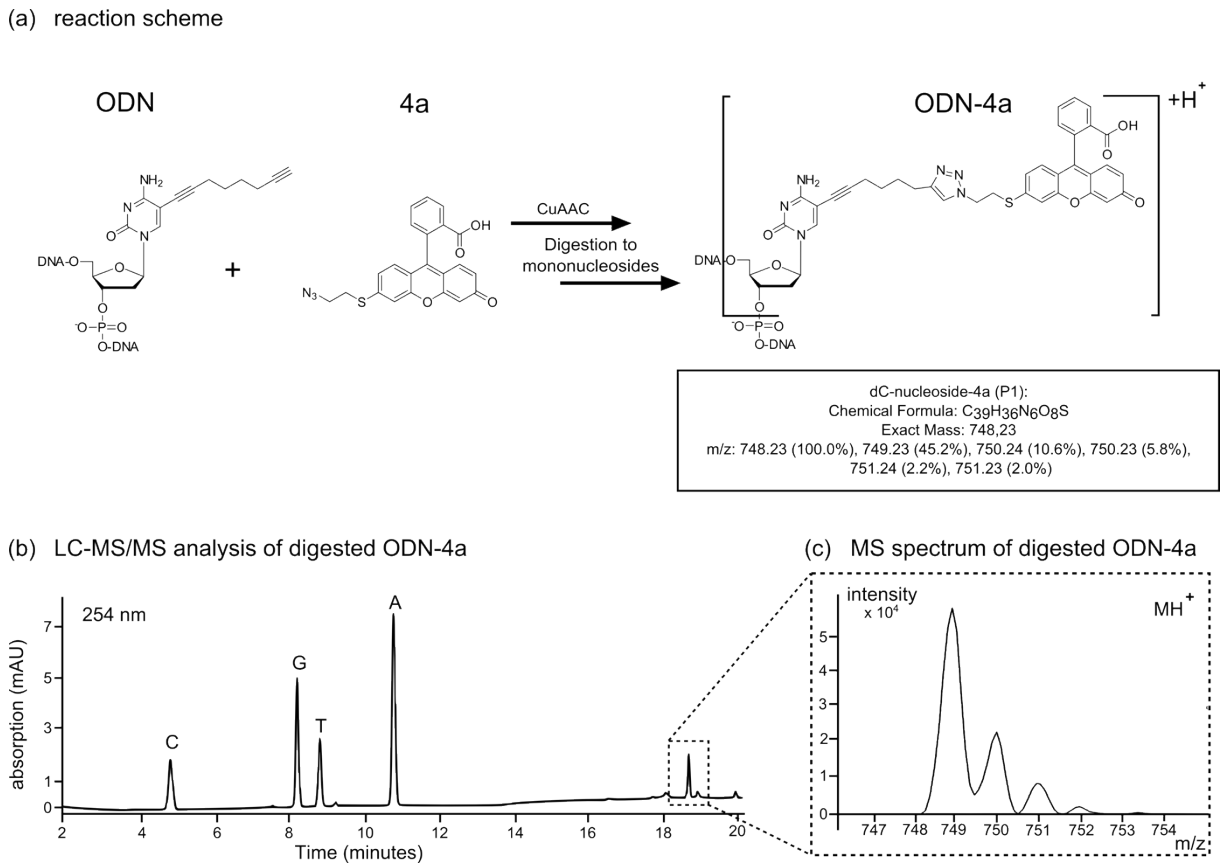

Figure S4. LC-Ms analysis of nucleoside click conjugate of 4a. The CuAAC reaction of the relevant alkyne-bearing nucleoside in the ODN with 4a is shown in (a). The ODN is enzymatically hydolysed to the composing nucleosides. The right part of (a) shows the protonated form of the of nucleoside conjugate as generated during electrospray ionization (ESI). Panel (b) shows the UV trace of an HPLC separation of the nucleoside mixture during an LC-MS/Ms run, and the mass spectrum corresponding to the peak of the conjugate eluting at 18.6 minutes is shown in (c). Note that the measured mass of the protonated form deviates by exactly one unit from the calculated m/z ratios of the unprotonated form.

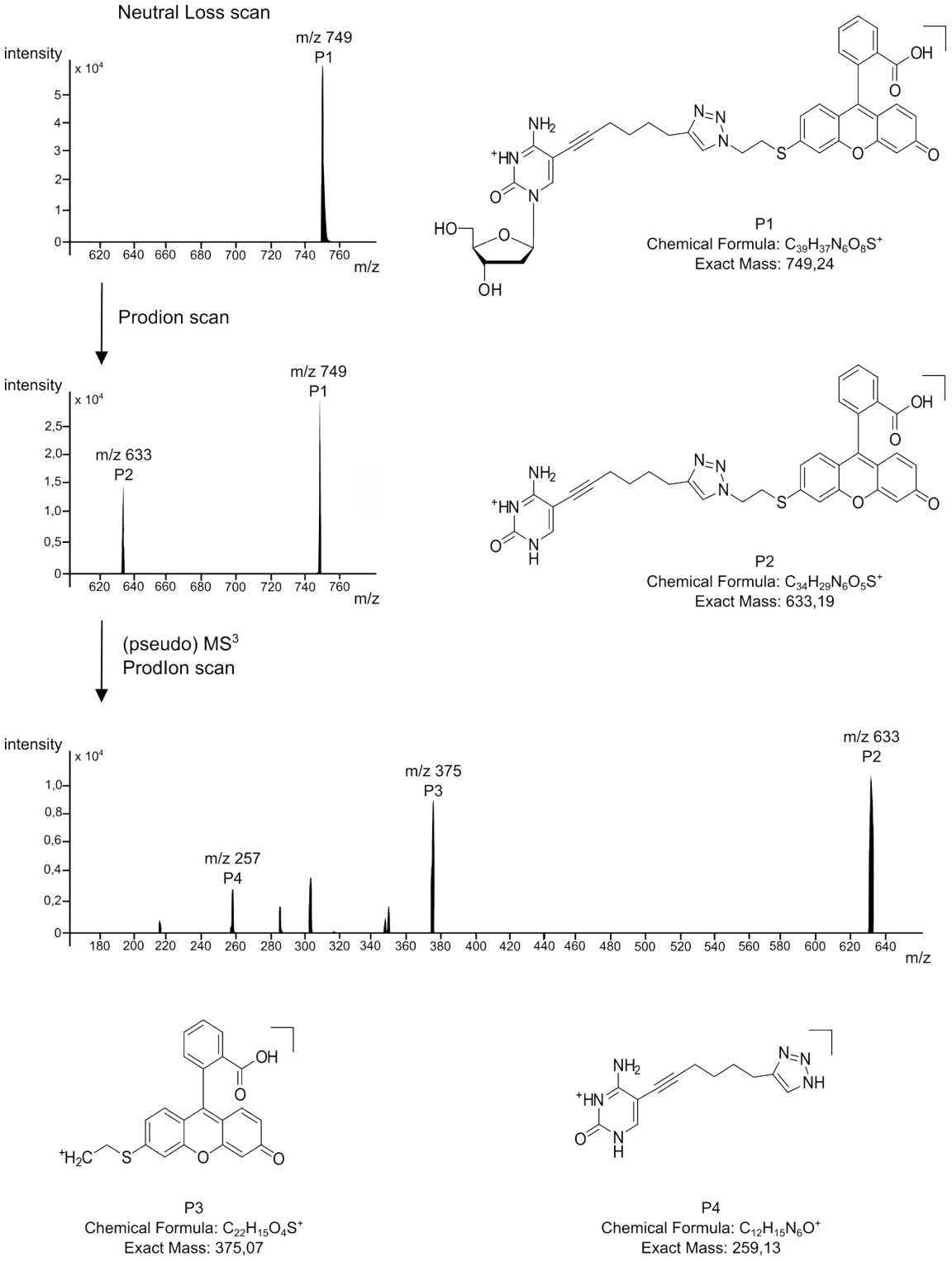

Figure S6. LC-MS/MS analysis of nucleoside-4a conjugate. The peak form Figure S4(c) was submitted to successive fragmentation, the first one resulting in cleavage of the *N*-glycosidic bond, which was the further fragmented to yield the displayed signals, whose corresponding structural interpretations are depicted as P2 through P4.

References

1. Lavis, L. D.; Rutkoski, T. J.; Raines, R. T., Tuning the pKa of Fluorescein to Optimize Binding Assays. *Analytical Chemistry* **2007,** *79* (17), 6775-6782.
2. Grimm, J.; Lavis, D. L., Synthesis of rhodamines from fluoresceins using Pd-catalyzed C-N cross-coupling. *Org. Lett.* **2011,** *13*, 6354-6357.
3. Peng, T.; Yang, D., Construction of a library of rhodol fluorophores for developing new fluorescent probes. *Org. Lett.* **2010,** *12*, 496-499.
4. Kellner, S.; Seidu-Larry, S.; Burhenne, J.; Motorin, Y.; Helm, M., A multifunctional bioconjugate module for versatile photoaffinity labeling and click chemistry of RNA. *Nucleic Acids Research* **2011,** *39* (16), 7348-7360.
5. Kolb, H. C.; Finn, M. G.; Sharpless, K. B., Click Chemistry: Diverse chemical function from a few good reactions. *Angew. Chem. Int. Ed.* **2001,** *40*, 2004–2021.