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Supporting Information

Far-Red Emitting Fluorescent Dyes for Optical Nanoscopy: Fluorinated Silicon–Rhodamines (SiRF Dyes) and Phosphorylated Oxazines

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SUPPORTING INFORMATION

General Remarks

UV-visible absorption spectra were recorded on a Varian Cary 4000 UV-Vis spectrophotometer, and fluorescence spectra on a Varian Cary Eclipse fluorescence spectrophotometer. The MICROTOF spectrometer equipped with ESI ion source Apollo and direct injector with LC autosampler Agilent RR 1200 was used for obtaining high resolution mass spectra (ESI-HRMS). ESI-HRMS were obtained also on APEX IV spectrometer (Bruker). HPLC system (Knauer): Smartline pump 1000 (2×), UV detector 2500, column thermostat 4000 (25 °C), mixing chamber, injection valve with 20 and 100 µL loop for the analytical and preparative columns, respectively; 6-port-3-channel switching valve; solid (reversed) phase: Eurosphere-100 C18, 5 µm; analytical column: 250×4 mm; preparative column: 250×8 mm.

The standard analytical program for HPLC analyses was run using a binary A/B system as the mobile phase: A – water + 0.1% (v/v) TFA (trifluoroacetic acid), B – CH_3CN + 0.1% (v/v) TFA, with gradient A/B 70:30→0:100 in 25 min, flow rate 1.2 mL/min and detection at 254 nm. This program was used for all the compounds with very few exceptions, where the analysis details are specified. For all isolated compounds the HPLC area was at least 97%, unless otherwise stated.

Analytical TLC was performed on MERCK ready-to-use plates with regular silica gel 60 (F_{254}) and an UV-luminophore. Preparative column chromatography performed on regular silica gel with the particle size 40 - 63 µm, unless otherwise stated.

Immunofluorescence: labeling, preparation and mounting of the samples

For the preparation of cell samples Vero cells were grown on cover slips. Cells were fixed with anhydrous methanol for 5 min at –20°C and blocked with 5% (w/v) BSA in PBS. Then the cells were incubated with a monoclonal mouse antibodies directed against alpha-tubulin (Sigma-Aldrich, St. Louis, MO, USA), vimentin (Sigma-Aldrich) or *nuclear pore complex* subunits (in the central channel of the complex) (NUP153, Abcam, Cambridge, UK). The primary antibodies were detected with secondary antibodies (sheep anti-mouse; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) custom labeled with the fluorescent dyes. Usually, the corresponding *N*-hydroxysuccinimidyl dye esters (0.2 mg) are dissolved in ca. 20–40 µL of dry DMF and slowly added to the stirred solution of secondary antibodies (ca.1 mg of a protein) in 1 mL of the buffer solution (pH = 8–8.5) at ambient temperature. Dye-labelled antibodies were isolated by gel filtration (see ref. [1a] for the standard labeling protocol). After several washing steps with

PBS buffer the samples were mounted in Mowiol (for STED microscopy), PBS (for GSDIM microscopy), or GSDIM buffer (10% (w/v) glucose, 0.5 mg/mL glucose oxidase and 40 µg/mL catalase, 10 mM cysteamine (β-mercaptoethylamine, MEA), 10 mM Tris, pH 7.4) (for GSDIM microscopy). Sample preparation was carried out according to the standard protocols described in detail by *C. A. Wurm* and co-workers [1b].

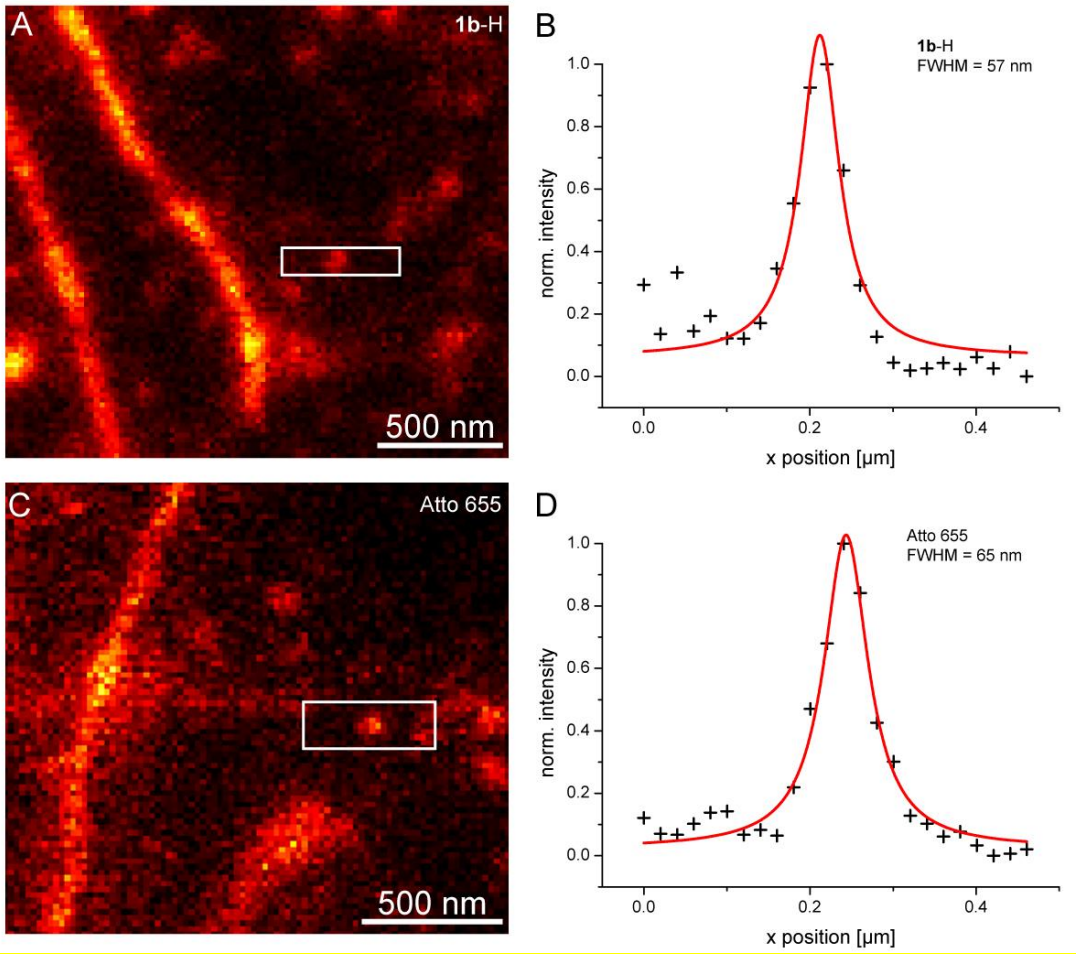


Fig. S01
Determination of the optical resolution in STED microscopy, exemplified for two dyes: **2**-H and **ATTO 655**. Both dyes have the oxazine core, but are decorated with different polar groups (see Scheme 5 in the main text and the discussion therein)**.** To determine the lateral resolution that may be achieved using the different dyes, antibody clusters within immunolabelled samples were measured. To this end line profiles averaged in the Y direction within regions encompassing the whole structure (see white insets in subfigure A and **C**). The resulting signal intensity distribution was normalized to its respective measured maximum and then fitted with a Lorentzian function. The FWHM of the Lorentzian fit was found to be (56.8 ± 3.3) nm for dye compound **1b**-H and (64.7 ± 3.1) nm for Atto 655. For the resolution achieved with other dyes see Table 1 in the main text.

STED microscopy

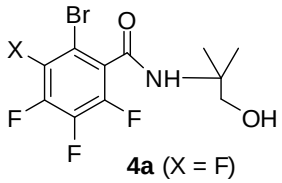
The dyes were tested in a custom built STED microscopy setup which is described in more detail elsewhere [2]. For our studies we used an excitation laser at a wavelength of 640 nm and a depletion laser at a wavelength of 800 nm. Both lasers were operated in pulsed mode. Fluorescence was detected in an emission window of (675/60) nm. Image acquisition was performed by stage-scanning in one and beam scanning in the other spatial direction in a region of interest (ROI) of (9x9) µm² and a pixel size of 20 nm with a pixel dwell time of 50 µs. To evaluate depletion efficiency and improvement of image resolution confocal images of the same sample spots were subsequently recorded under equal conditions. Sample illumination intensities were optimized for each tested dye to yield best image quality but did not differ significantly between dyes thus enabling direct comparison of imaging performance. For the investigation of each dye's bleaching behavior 10 subsequent frames per sample spot were obtained under the same STED imaging conditions as described above. Evaluation of the image resolution and bleaching behavior was performed as described in the main text and in the figure caption (Fig S01) above.

GSDIM microscopy

Nuclear pore complex subunits were labeled (in the central channel of the complex) by indirect immunofluorescence staining using primary antibodies and secondary antibodies coupled with compounds **2**-H and ATTO 655. Then the labeled samples were mounted in GSDIM buffer with Glucose Oxidase (Glox) enzyme or in PBS solution (without additives of reducing agents) and observed in GSDIM microscope. The GSDIM was performed on a Leica SR GSD 3D setup (Leica Microsystems, Mannheim, Germany) equipped with a 160x GSD objective, a SUMO stage, a 642 nm laser and a SCMOS camera (PCO edge, PCO, Kelheim, Germany). Imaging was performed at 37 °C. 50,000 frames were recorded with a dwell time of 5 ms per frame. The laser power was set to ~500 mW, i.e. 10 kW/cm^2 during pumping of the fluorophores to the dark state and during imaging. No backpumping was applied. Image evaluation was performed using the LAS AF software together with the implemented standard evaluation routines using pixels sizes of 20 nm and a detection threshold of 25 photons per pixel for GSDIM buffer and 15 photons per pixel for PBS.

Syntheses of far-red-emitting dyes and their precursors

General procedure for amidation of poly-fluorine-substituted 2-bromobenzoic acids with 2-amino-2-methyl propanol.



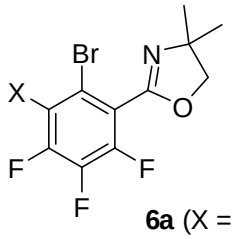
4a (X = F)
4b (X = H)

The benzoic acids **3a** or **3b** (5 mmol; for the preparation see ref [3a] and [3b], respectively) were dissolved in CH_2Cl_2 (10 ml) in a 100 mL flask, and the solution was chilled to 0 °C under an argon atmosphere. Oxalyl chloride (9 mmol, 1.8 equiv) and DMF (20 μL, catalyst) were added upon stirring, which continued for 4 h at RT Some internal pressure, which developed in the first 30 min of stirring, was being periodically relieved through a needle. The acid chloride solution was added in one portion via a syringe to a 250-mL flask containing a stirred solution of 2-amino-2-methyl propanol (20 mmol, 4 equiv) and Et_3N (15 mmol, 3 equiv) in CH_2Cl_2 (100 mL) at 0 – 5 °C under argon. The mixture with a precipitate was stirred for >30 minutes at this temperature, mixed up with an ice-cold 5 wt.% solution of $KHSO_4$ (300 mL), and well shaken. The organic solution (lower phase) was separated, washed with water (100 mL), a 2 wt.% solution of Na_2CO_3 (100 mL) and evaporated. The crude products were purified by means of column chromatography over 50 g of regular silica gel using EtOAc/CH_2Cl_2 (1:5) as the mobile phase to furnish the amides **4a**,**b** with yields of ca. 85–90% as colorless crystalline solids.

Analytical data for **4b**: t_R = 8.3 min (HPLC, *standard analytical program; see General Remarks*); TLC: R_f = 0.70 (regular silica gel plates $CHCl_3$/EtOAc 3:1). MS (ESI) m/z (positive mode, %) = 326 (90%) $[M+H]^+$. HRMS ($C_{11}H_{11}BrF_3NO_2^+$): 325.9923 (found), 325.9925 (calc.). ^{1}H-NMR (400 MHz, $CDCl_3$) δ = 1.38 (s, 6 H, CH_3), 3.65 (s, 2 H, CH_2), 6.17 (br. s. 1H, NH), 7.23 (m, 1H) ppm; ^{13}C NMR (100.6 MHz, $CDCl_3$) δ = 24.0 (CH_3), 57.3 (C), 69.4 (CH_2), 117.1 (C), 161.8 (C=O) ppm. ^{19}F NMR (376.4 MHz, $CDCl_3$): δ = –158.7 (m, 1 F), –133.1 (m, 1 F), –130.4(m, 1 F) ppm.

Analytical data for **4a**: t_R = 9.5 min (HPLC, *standard analytical program; see General Remarks*); TLC: R_f = 0.80 (regular silica gel plates $CHCl_3$/EtOAc 3:1). MS (ESI) *m/z* (positive mode,%) = 344 (90%) $[M+H]^+$. HRMS ($C_{11}H_{10}BrF_4NO_2^+$): 343.9824 (found), 343.9831 (calc.). ^{1}H-NMR (400 MHz, $CDCl_3$) δ = 1.48 (s, 6 H, CH_3), 3.72 (s, 2 H, CH_2), 6.20 (br. s. 1H, NH) ppm; ^{13}C NMR (100.6 MHz, $CDCl_3$) δ = 24.8 (CH_3), 56.2 (C), 69.8 (CH_2), 163.4 (C=O) ppm. ^{19}F NMR (376.4 MHz, $CDCl_3$): δ = –153.5 (m, 1 F), –151.1 (m, 1 F), –139.5 (m, 1 F), –127.9(m, 1 F) ppm.

Oxazoline derivatives **6a**,**b** (dye precursors with a protected acid function).

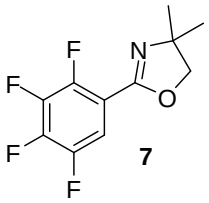


6a (X = F)
6b (X = H)

Amides **4a** or **4b** (4 mmol) were heated upon stirring for 3 – 4h at 185 – 188°C in 1,2 dichlorobenzene (8 mL) in the presence of finely powdered $KHSO_4$ (600 mg). A thick-walled test tube with a screw cup and a magnetic stirring bar was used as a reactor. Before use the solid dehydrating agent was heated in the test tube at 130 °C for few min by means of a fan. The reaction mixture was diluted with CH_2Cl_2 (100 mL) and treated with an excess of aqueous $NaHCO_3$. The organic layer evaporated and purified by column chromatography over regular silica gel (120 g) with hexane/CH_2Cl_2 (3:1) until all the dichlorobenzene is separated. After that, the oxazolines **6a**,**b** were isolated upon elution with EtOAc/CH_2Cl_2 (1:7) as colorless viscous oils with a yield of ca. 70%. Analytical data for **6b**: t_R = 16 min (HPLC, *standard analytical program; see General Remarks*). TLC: R_f = 0.30 (regular silica gel plates, $CHCl_3$/EtOAc 3:1). MS (ESI) *m*/*z* (positive mode,%) = 308 (100%) $[M+H]^+$. HRMS ($C_{11}H_9BrF_3NO^+$): 307.9822 (found), 307.9820 (calc.). ^{1}H-NMR (400 MHz, $CDCl_3$) δ = 1.43 (s, 6 H, CH_3), 4.14 (s, 2 H, CH_2), 7.26 (ddd, *J* = 9.0, 6.4, 2.4 Hz, 1H) ppm; ^{13}C NMR (100.6 MHz, $CDCl_3$) δ = 28.1 (CH_3), 69.7 (CH_2), 79.5 (C), 116.9 (C) ppm. ^{19}F NMR (376.4 MHz, $CDCl_3$): δ = –159.1 (m, 1 F), –130.1 (m, 1 F), –129.4(m, 1 F) ppm. Analytical data for **6a**: t_R = 19 min (HPLC, *standard analytical program; see General Remarks*); TLC: R_f = 0.30 (regular silica gel plates, $CHCl_3$/EtOAc 3:1). MS (ESI) *m*/*z* (positive mode,%) = 326 (100%) $[M+H]^+$. HRMS ($C_{11}H_8BrF_4NO^+$): 325.9804 (found), 326.9832 (calc.). ^{1}H-NMR (400 MHz, acetone-d_6) δ = 1.38 (s, 6 H, CH_3), 4.19 (s, 2 H, CH_2) ppm; ^{13}C NMR (100.6 MHz, acetone-d_6) δ = 27.3 (CH_3), 68.9 (CH_2), 79.3 (C) ppm. ^{19}F NMR (376.4 MHz, acetone-d_6): δ = –156.9 (m, 1 F), –152.7 (m, 1 F), –139.3 (m, 1 F), –130.6 (m, 1 F) ppm.

For further use in the metal organic syntheses (e. g., in the preparation of the Si-rhodamine dye **11**; see below) it is important to thoroughly remove the rest of the solvents (EtOAc and CH_2Cl_2) that react with alkyl (aryl) lithium reagents. To this end, the oxazoline derivatives **6a**,**b** and **7** (see below) were dissolved in a 10 – 20-fold excess of *n*-heptane, and the solution was evaporated to the constant weight of the residue by means of a rotary evaporator under vacuum of 1 – 5 mbar at 30 – 40°C (note that these oxazoline compounds are slightly volatile under higher vacuum). This procedure, when done twice, leaves no traces of potentially reactive residual solvents, as established by NMR spectroscopy.

Compound **7**. (The bromine-free oxazoline derivative).

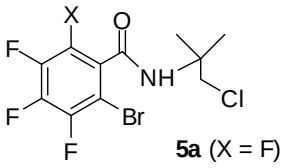


7

Compound **7** was obtained in a good yield from the commercially available 2,3,4,5-tetrafluorobenzoic acid (Aldrich) by the conventional method for protection of carboxylic acids with an oxazoline moiety. That involves amidification with 2-amino-2-methyl propanol, followed by cyclization in the presence of thionyl chloride. The details of the recipe one can find, for example, in ref. [6].

Analytical data for compound **7**: t_R = 14 min (HPLC, *standard analytical program; see General Remarks*); TLC: R_f = 0.25 (regular silica gel plates, $CHCl_3$/EtOAc 3:1). MS (ESI) *m/z* (positive mode,%) = 248 (100%) $[M+H]^+$. HRMS ($C_{11}H_9rF_4NO^+$): 248.0663 (found), 248.0620 (calc.). ^{1}H-NMR (400 MHz, $CDCl_3$) δ = 1.39 (s, 6 H, CH_3), 4.12 (s, 2 H, CH_2), 7.56 (dddd, *J* = 10, 8, 6, and 2.5 Hz, 1H) ppm; ^{13}C NMR (100.6 MHz, $CDCl_3$) δ = 28.3 (CH_3), 67.7 (CH_2), 79.8 (C), 112.0 (C) ppm. ^{19}F NMR (376.4 MHz, $CDCl_3$): δ = –154.3 (tt, *J* = 19, 3 Hz, 1 F), –150.4 (t, *J* = 20 Hz, 1 F), –138.6 (dt, *J* = 22, 12 Hz, 1 F), –134.7(ddt, *J* = 20, 13, and 7Hz, 1 F) ppm. The same compound (**7**) was isolated as the main product after the attempted lithiation of the ketone substrate **10**, when the oxazoline derivative **6a** (containing four fluorine atoms and one bromine; see structure above and the discussion in the main text). The purity and identity of compound **7** – the debromination product – was confirmed by analytical methods.

γ-Chloromethyl amide **5a**

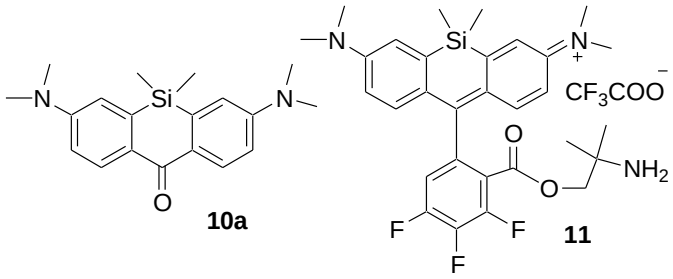


5a (X = F)

Amide **4a** was treated with a large excess of thionyl chloride at room temperature according to ref. [4]. However, it formed compound **5a** instead of the desired cyclization product -- oxazine **6a**. After evaporation of the thionyl chloride the reaction product was dissolved in CH_2Cl_2, treated with aqueous $HaHCO_3$, and purified by column chromatography over silica gel with EtOAc/CH_2Cl_2 (1:7) to furnish some 90% of the title compound (γ-chloromethyl amide **5a**). Analytical data: t_R = 9 min (HPLC, *standard analytical program; see General Remarks*); TLC: R_f = 0.30 (regular silica gel plates, hexane/EtOAc 6:1).

MS (ESI) *m*/*z* (positive mode,%) = 366 (10%), 364 (50%), 362 (40%) $[M+H]^+$. HRMS ($C_{11}H_{10}BrF_4NO_2^+$): 362.9486 (found), 362.9492 (calc.). ^{1}H-NMR (400 MHz, $CDCl_3$) δ = 1.52 (s, 6 H, CH_3), 3.86 (s, 2 H, CH_2), 5.91 (br. s. 1H, NH) ppm; ^{13}C NMR (100.6 MHz, $CDCl_3$) δ = 24.3 (CH_3), 50.5 (CH_2), 55.7 (C), 102.4 (C), 159.7 (C=O) ppm. ^{19}F NMR (376.4 MHz, $CDCl_3$): δ = –153.6 (m, 1 F), –151.2 (m, 1 F), –139.1 (m, 1 F), –128.0 (m, 1 F) ppm.

Synthesis of Si-rhodamine dye **11** by a one-pot lithiation.



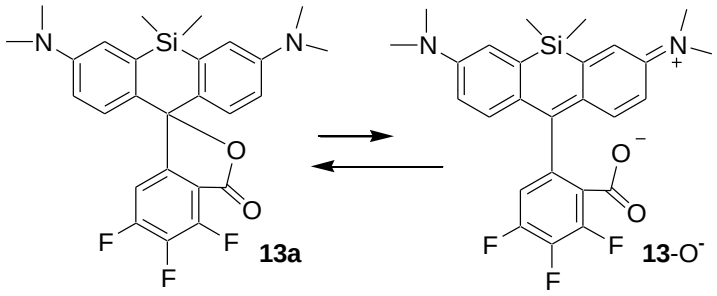
A flame-dried and argon-flushed Schlenck flask was loaded through a septum with a solution of the oxazoline precursor **6b** (210 mg, 0.72 mmol; for preparation and special treatment – see above) in a freshly distilled THF (4 mL), chilled to –78 °C (dry ice bath), and the commercial 1.7 M *t*-BuLi solution (0.70 mL, 1.2 mmol) was introduced within 1 min. upon stirring. The reaction solution was kept for 3.5 h at this temperature and a solution of the ketone substrate (structure **10a**, 40 mg, 0.123 mmol) in dry THF (4 mL) was added within 2 min upon vigorous stirring. The stirring was continued for 30 min at this temperature and for 8 more h in an ice-water bath (0 °C) under an argon atmosphere. The reaction mixture was quickly poured into a beaker containing a freshly prepared solution of glacial acetic acid (1 mL) in methanol (15 mL), which was chilled to –10 °C beforehand. The flask was rinsed with a portion of the resulting solution, which was all combined, warmed up to RT, mixed with CH_2Cl_2 (20 mL), water (20 mL), and brine (20 mL). The mixture was well-shaken, the organic layer separated, the aqueous layer extracted with 20 mL of CH_2Cl_2, and the combined organic extracts evaporated at RT almost to dryness, using a rotary evaporator. The residue was purified by column chromatography over regular silica gel (50 g) with a mixture of CH_3CN/H_2O (40:1→10:1) until all the yellow and pale brown compounds are completely washed off. Then the mobile phase was switched to CH_3CN/H_2O (6:1 + 0.1 vol.% TFA) and the fraction containing a dark blue compound was collected and concentrated to the volume of ca. 50 mL at temperatures not exceeding 35 °C. The residue was mixed up with CH_2Cl_2 (80 mL) and an equal volume of a 10% wt. aqueous NaCl solution. The organic layer was separated, dried (Na_2SO_4), filtered through a paper, and evaporated to dryness *in vacuo* to furnish 72 mg (yield 82%) of the amino ester **11** (trifluoroacetate) as a dark blue crystalline powder.

Analytical data: t_R = 8.5 min (HPLC, *standard analytical program; see General Remarks*); TLC: R_f = 0.20 (regular silica gel plates CH_2Cl_2/MeOH 5:1). MS (ESI) *m/z* (positive mode,%) = 554 (100%) [M^+]. HRMS ($C_{30}H_{35}F_3N_3O_2Si^+$): 554.2443 (found), 554.2445 (calc.). ^{1}H-NMR (400 MHz, $CDCl_3$) δ = 0.46, 0.55 (s, 6 H, $SiCH_3$), 1.09 (s, 6 H, CH_3), 2.98 (s, 12 H, NCH_3), 3.31 (br. s, 2 H, NH_2), 3.59 (s, 2 H, CH_2), 6.09 (ddd, *J* = 8.3, 5.5, 1.5 Hz, 1H), 6.68 (dd, *J* = 9.0, 3.0 Hz, 2H), 6.83 (d, *J* = 2.9 Hz), 6.89 (d, *J* = 9.0 Hz, 2H) ppm; ^{13}C NMR (100.6 MHz, $CDCl_3$) δ = -1.3 (CH_3), 0.6 (CH_3), 23.5 (CH_3), 40.1 (CH_3), 63.1 (CH_2), 73.0 (C), 114.5 (CH), 115.6 (CH), 128.8 (CH), 130.6 (C), 133.3 (C), 148.7 (C), 154.2 (C), 162.6 (C=O) ppm. ^{19}F NMR (376.4 MHz, $CDCl_3$): δ = –159.1 (m, 1 F), –139.5 (m, 1 F), –124.7(m, 1 F), –75.4 (s, 3F) ppm.

UV/Vis spectral data for compound **11**: λ_{max} abs. = 662 nm, λ_{max} fl.= 680 nm; Φ_{fl} = 45% (in H_2O, with Atto AZ 237 as a reference), $\varepsilon \times 10^{-5}$ = 1.1 $M^{-1}cm^{-1}$. For the spectral data on the most important far-red-emitting dyes (i. e., **1a**-H, **1b**-H, **13a**, and **2-H**) see Table 1 in the main text.

Under the same lithiation reaction conditions the oxazoline derivative **6a** (containing *four fluorine atoms* and one bromine) undergoes debromination to form compound **7** which was identified by analytical methods (see the structure and analytical data above and the discussion in the main text).

Si-rhodamine dye **13a** (free acid lactone).



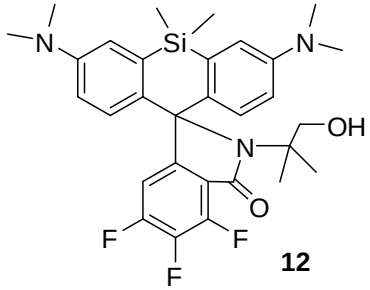
Amino ester **11** (70 mg, 0.09 mmol) was dissolved in a mixture of conc. HCl (4 mL) and water (2 mL) and heated for 3 h upon stirring in a test tube at 80 °C (the oil bath temperature). A 250-mL Erlenmeyer flask was loaded with water (50 mL), $NaHCO_3$ (5 g), EtOAc (60 mL), and CH_2Cl_2 (20 mL). The acidic reaction solution was carefully (CO_2 evolution!) added dropwise to the stirred suspension in the flask. After stirring for >20 min., the organic upper layer was separated and the aqueous phase extracted with CH_2Cl_2 (2 x 30 mL). The extracts were combined, evaporated, and purified by column chromatography over regular silica gel (35 g) with EtOAc/CH_2Cl_2 (1:7) as the mobile phase to furnish 40 mg (yield 94%) of compound **13a**. In the course of the chromatographic isolation the compound forms a visible blue zone on silica gel. Properties: a colorless crystalline solid (see photo above), very slightly soluble in water, forming a blue solution (the opened form **13-O**$^-$); well-soluble in most organic solvents. Solutions in non-polar solvents are colorless. HPLC: t_R = 14 min (A/B 50:50→0:100 in 25 min; HPLC area 99%, detection at 254 nm).

TLC: R_f = 0.60 (regular silica gel plates CH_2Cl_2/EtOAc 7:1; seen by a naked eye as a bright blue spot, which is developed as the plate gets dry in air). MS (ESI) *m/z* (positive mode,%) = 483 (100%) [M^+]. HRMS ($C_{26}H_{26}F_3N_2O_2Si^+$): 483.1713 (found), 483.1710 (calc.). ^{1}H-NMR (400 MHz, $CDCl_3$) δ = 0.56, 0.63 (s, 6 H, $SiCH_3$), 2.99 (s, 12 H, NCH_3), 6.63 (d, J = 8.8 Hz, 2H), 6.82 (m, 1H), 6.84 (d, J = 8.9 Hz, 2H), 6.97 (s, 2H) ppm; ^{13}C NMR (100.6 MHz, $CDCl_3$) δ = – 0.9 (CH_3), 0.2 (CH_3), 0.3 (CH_3), 1.1 (CH_3), 40.2 (CH_3), 40.5 (CH_3), 108.1 (CH), 108.2 (CH), 108.3 (CH), 111.3 (C), 114.5 (CH), 117.5 (CH), 127.7 (CH), 136.3 (C), 138.8 (C), 146.6 (C), 154.4 (C), 156.5 (C), 165.3 (C=O) ppm. ^{19}F NMR (376.4 MHz, $CDCl_3$): δ = –158.1 (s, 1 F), –134.1 (s, 1 F), –120.7(s, 1 F) ppm.

Fig. S02

Solid Si-rhodamine dye **13a** in the colorless lactone form.

Cyclic amide (lactam) **12**.

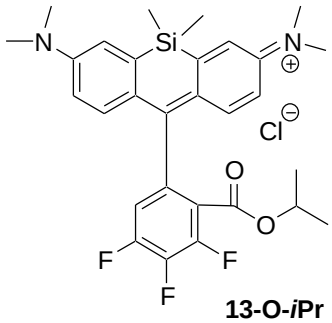


12

Amino ester **11**, when treated with bases, e. g., $NaHCO_3$, undergoes cyclization. The cyclic lactam so formed (**12**) proved to be a lot more stable toward acid hydrolysis than amino ester **11**. Therefore, bases should be avoided in the course of the lithiation reaction workup (see above). The cyclization of **11** proceeded as follows: a solution of compound **11** (16 mg, 0.024 mmol) in CH_2Cl_2 (20 mL) and a saturated aqueous $NaHCO_3$ solution (10 mL) were vigorously stirred for 30 min at RT The evaporation of the organic phase, followed by column chromatography over regular silica gel (12 g) with EtOAc/CH_2Cl_2 (1:7)

as the mobile phase furnished 12 mg (yield 90%) of **12** as a colorless solid. HPLC: t_R = 11 min (A/B 50:50→0:100 in 25 min; HPLC area 98%, detection at 254 nm). TLC: R_f = 0.30 (regular silica gel plates; CH_2Cl_2/EtOAc 7:1; seen under UV-light). The cleavage of the amide to the free acid lactone **13a** with hydrochloric acid (ca. 25 wt.%) required 1 day heating and furnished some 60% of **13a** (isolated, as described for compound **11**). MS (ESI) *m*/*z* (positive mode,%) = 554 (100%) $[M+H]^+$. HRMS ($C_{30}H_{34}F_3N_3O_2Si^+$): 554.2442 (found), 554.2445 (calc.). ^{1}H-NMR (400 MHz, $CDCl_3$) δ = 0.49, 0.55 (s, 6 H, $SiCH_3$), 1.09 (s, 6 H, CH_3), 2.98 (s, 12 H, NCH_3), 3.60 (s, 2 H, CH_2), 6.11 (ddd, J = 8.3, 5.5, 1.5 Hz, 1H), 6.71 (d, J = 8.8 Hz, 2H), 6.83 (d, J = 2.9 Hz), 6.85 (m, 1H), 6.90 (d, J = 9.0 Hz, 2H) ppm. ^{13}C NMR (100.6 MHz, $CDCl_3$) δ = –1.4 (CH_3), 0.3 (CH_3), 14.3 (CH_3), 22.4 (C), 23.5 (CH_3), 31.9 (C), 63.3 (CH_2), 73.1 (C), 106.3 (CH), 110.2 (C), 129.1 (CH), 133.8 (C), 149.4 (C), 166.3 (C=O) ppm. ^{19}F NMR (376.4 MHz, $CDCl_3$): δ = –161.5 (s, 1 F), –139.2 (s, 1 F), –124.1 (s, 1 F), ppm.

The Si-rhodamine ester **13-**O-*i*Pr as a model ester with a fixed opened form (the isopropyl ester of Si-rhodamine dye **13a**).

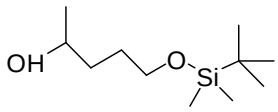


13-O-*i*Pr

Compound **13-**O-*i*Pr (the isopropyl ester of rhoadamine **13a**) was obtained and isolated in an 80% yield in exactly same fashion (via the acid chloride) and a large excess of isopropanol, as the alcohol substrate and a solvent. Purity and identity was confirmed by analytical methods. The stability towards bases (NaOH, Et_3N) was also explored (see the main text) by means of HPLC analyses. Its complete saponification can be also witnessed by the loss of the initial color (dark blue), as the colorless rhodamine lactone **13a** is formed. Analytical data for **13-**O-*i*Pr: t_R = 17 min (HPLC, *standard analytical program; see General Remarks*); TLC: R_f = 0.40 (regular silica gel plates, $CH_3CN/CH_2Cl_2/H_2O$ 10:1:1; seen by a naked eye as a bright blue spot). MS (ESI) *m*/*z* (positive mode,%) = 525 (100%) $[M^+]$. HRMS ($C_{29}H_{32}F_3N_2O_2Si^+$): 525.2184 (found), 525.2180 (calc.). ^{1}H-NMR (400 MHz, $CDCl_3$): δ = 0.53, 0.64 (s, 6 H, $SiCH_3$), 0.94 (dd, J = 6.3, 0.7 Hz, 6 H, CH_3, *i*-Pr) 3.44 (s, 12 H, NCH_3), 4.89 (hept., J = 6.4 Hz, 1H, *i*-Pr), 6.70 (dd, J = 9.6, 2.7 Hz, 2H), 6.88 – 6.98 (m, 1H), 7.01 (d, J = 9.6 Hz, 2H), 7.32 (m, 2H), ppm; ^{19}F NMR (376.4 MHz, $CDCl_3$): δ = –156.3 (td, J = 21.0, 6.6 Hz, 1 F), –130.8 (ddd, J = 20.9, 11.6, 2.2, 1 F), –124.1 (ddd, J =

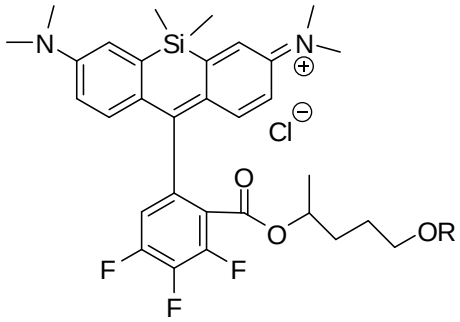
21.0, 11.6, 9.3 Hz, 1 F), ppm. UV/Vis spectral data for **13-**O-*i*Pr: λ_{max} abs. = 662 nm, λ_{max} fl.= 679 nm; Φ_{fl} = 60% (in H_2O, with Atto AZ 237 as a reference), $\varepsilon \times 10^{-5}$ = 1.3 $M^{-1}cm^{-1}$.

1-OTBDMS-pentanol-4 ($HOCH(CH_3)(CH_2)_3OTBDMS$, the spacer with a protected primary hydroxyl group).



TBDMS chloride (1.65 g, 1.05 mmol) was added dropwise to an ice-cold solution of 1,4-pentanediol (1.04 g, 1 mmol) and imidazole (1.36 g, 2 mmol) in DMF (6 mL) and stirred at r. t. for 3 days under an argon atmosphere. The solution was quenched with CH_2Cl_2 (20 mL), hexane (60 mL), and deionized water (80 mL). The organic layer was dried and evaporated. Column chromatography over silica gel (35 g) with EtOAc/hexane (1:5) afforded 1.76 g (yield 80%) of the target compound as colorless viscous oil. TLC: R_f = 0.50 (regular silica gel plates; hexane/EtOAc 5:1; with phosphomolybdic acid as a developer). Analytical data: MS (ESI) *m/z* (positive mode,%) = 219 (100%) $[M+H]^+$. HRMS ($C_{11}H_{26}O_2Si^+$): 219.1706 (found), 219.1702 (calc.). ^{1}H-NMR (400 MHz, $CDCl_3$) δ = 0.03 (s, 6 H, $SiCH_3$), 0.86 (s, 9 H, *t*-Bu), 1.35 – 1.69 (m, 4H, CH_2), 2.74 (br. s, 1H, OH), 3.78 (m, 2H, CH_2), 3.78 (m, 1H, CHOH) ppm. ^{13}C NMR (100.6 MHz, $CDCl_3$) δ = –5.4 (CH_3), 23.4 (CH_3), 25.9 (CH_3), 29.3 (CH_2), 36.6 (CH_2), 67.6 (CH). Si-rhodamine dye **1a-H**.

Si-rhodamine dye **1a**-H: synthesis of an ester from precursor **13a** (**13-**O) and a partially protected diol ($HOCH(CH_3)(CH_2)_3OTBDMS$, for the preparation see above), followed by the cleavage of the protective group.



1a-TBDMS (R = TBDMS)
1a-H (R = H)

In an argon-flushed Schlenck flask (25 mL) oxalyl chloride (0.30 mL, 3.5 mmol) was added through a septum to a solution continuing compound **13a** (30 mg, 0.062 mmol) and DMF (2 μL, catalyst) in 1,2-

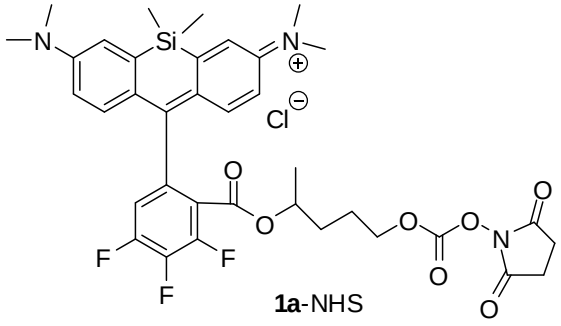
dichloroethane (3 mL). Some small internal pressure, which developed in the first 30 min of stirring, was being periodically relieved through a needle, and stirring continued overnight at RT A small distillation bridge with a condenser and a receiver flask was connected under an argon purge, the solution chilled in a dry ice bath for 1-2 min, and the solvent carefully evaporated *in vacuo* upon stirring (the receiver flask placed in a dry ice bath). The dry residue was kept under vacuum for 30 more min, the flask was filled with argon and sealed with a septum, as the distillation bridge was removed. A solution of 1-OTBDMS-pentanol-4 ($HOCH(CH_3)(CH_2)_3OTBDMS$, 300 mg, 1.38 mmol; see above for preparation and structure) in CH_2Cl_2 (3 mL) was added to the residue in one portion, and the resulting dark blue solution stirred for 1 h at RT under an argon atmosphere. The solution was diluted with a double volume of CH_3CN and purified by column chromatography first over 15 g of regular silica gel with a mixture of $CH_3CN/CH_2Cl_2/H_2O$ (30:3:1→10:1:1) to collect the colored fractions. The latter were combined, evaporated (t < 30°C) and purified by column chromatography one more time over regular silica gel (25 g) first with $CH_3CN/CH_2Cl_2/H_2O$ (30:3:1→20:1:1), then with CH_3CN/H_2O (5:1) containing 0.1 vol.% TFA. There fractions were collected: the very first contained compound **1a**-TBDMS (14 mg, yield 32%) as a dark blue amorphous solid. The compound is quite unstable and difficult to isolate in the pure form: the loss of the protective group was observed even in neutral solutions. Analytical data for **1a**-TBDMS: t_R = 26 min (HPLC, *standard analytical program; see General Remarks*); TLC: R_f = 0.60 (regular silica gel plates, $CH_3CN/CH_2Cl_2/H_2O$ 10:1:1). MS (ESI) *m/z* (positive mode,%) = 683 (100%) [M^+]. HRMS ($C_{37}H_{50}F_3N_2O_2Si^+$): 683.3310 (found), 683.3307 (calc.).

Other two fractions contained dye **1a**-H (the deprotected compound is divided in two fractions due to different counter-ions; their HPLC is identical). The latter were combined and concentrated to the volume of ca. 50 mL. The residue was mixed with CH_2Cl_2 (100 mL), deionized water (50 mL), brine (50 mL) and well-shaken. The organic layer was separated, dried (Na_2SO_4), filtered through syringe filters (0.45 μm), and carefully evaporated to furnish 21 mg (yield 53%, with chloride as counter-ion) of compound **1a**-H as a dark blue amorphous powder. Purity and identity was confirmed by analytical methods. Properties: well-soluble in chlorinated solvents (e.g., CH_2Cl_2), THF, CH_3CN, and alcohols. The compound is slightly soluble in deionized water and sufficiently stable towards towards saponification with weak bases (Et_3N, $NaHCO_3$), even more than the model ester **13-**O-*i*Pr (see description above). The fluorescence of its greenish-blue solutions is virtually not seen by a naked eye. The cleavage of the protective group was performed as follows: compound **1a-**TBDMS (14 mg, 0.02 mmol) was dissolved and left overnight in a plastic vial containing a mixture of CH_3CN (5 mL) and the commercial 50 wt. % HF (0.25 mL). The solution was quenched with CH_2Cl_2 (20 mL), deionized water (10 mL), brine (10 mL) and well-shaken. The organic layer was separated, dried (Na_2SO_4), filtered through syringe filters (0.45 μm), and carefully evaporated to furnish 11 mg (yield 92%) of a pure dye **1a**-H.

Analytical data for **1a**-H: t_R = 14 min (HPLC, *standard analytical program; see General Remarks*); TLC: R_f = 0.30 (regular silica gel plates, $CH_3CN/CH_2Cl_2/H_2O$ 10:1:1; seen by a naked eye as a bright blue

spot). MS (ESI) *m/z* (positive mode,%) = 569 (100%) [M^+]. HRMS ($C_{31}H_{36}F_3N_2O_3Si^+$): 569.2447 (found), 569.2442 (calc.). ^{1}H-NMR (400 MHz, $CDCl_3$) δ = 0.55, 0.62 (s, 6 H, $SiCH_3$), 1.04 (d, 6.1 Hz, 3 H, CH_3) 1.06 – 1.43 (m, 4 H, CH_2), 2.89 (br. s, 1H, OH), 3.44 (s, 12 H, NCH_3), 3.59 (m, 1H, CH), 3.98 (td, 6.6, 1.8 Hz, 2 H, CH_2), 6.71 (dd, 9.7 and 2.8 Hz, 2H), 6.91 – 6.95 (m, 1H), 6.98 (dd,9.7, 1.3 Hz, 2H), 7.20 (dd, 10.9 and 2.8 Hz, 2H) ppm; ^{13}C NMR (100.6 MHz, $CDCl_3$) δ = 0.8 (CH_3), 23.3 (CH_3), 24.8 (CH_2), 34.8 (CH_2), 66.4 (CH_3), 66.8 (CH), 114.1 (CH), 121.1 (CH), 127.4 (C), 140.8 (CH), 147.7 (C), 153.9 (C), 164.2 (C=O) ppm. ^{19}F NMR (376.4 MHz, $CDCl_3$): δ = –156.3 (m, 1 F), –130.3 (m, 1 F), –127.9 (m, 1 F), ppm.

The reactive marker **1a**-CONHS (the NHS carbonate).



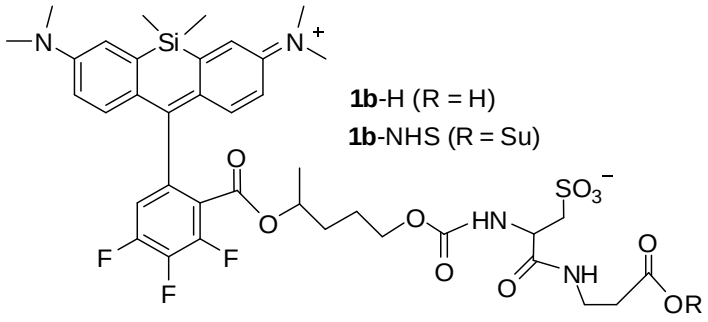
1a-NHS

In a typical experiment, the dye NHS carbonate (**1a**-CONHS) was prepared as follows: solid *N,N* '-disuccinimidyl carbonate (DSC reagent; 150 mg, 0.6 mmol) was added in one portion to a solution of compound **1a-**H (6 mg, 1 μmol) and DIPEA (70 μL, 4 μmol) in a mixture of CH_2Cl_2 (3 mL) and CH_3CN (0.1 mL) and stirred at RT for 4h. The solution was quenched with CH_2Cl_2 (15 mL) and deionized water (15 mL) and well-shaken. The organic layer was washed with brine (10 mL), dried, concentrated to the volume of ca. 2 mL (t < 20°C), and purified by column chromatography over regular silica gel (6 g) with $CH_3CN/CH_2Cl_2/H_2O$ (20:1:1) as the mobile phase. The homogeneous (blue) fractions were concentrated to the volume of ca. 20 mL and mixed up with equal volumes of brine, deionized water and CH_2Cl_2. The aqueous layer was extracted with CH_2Cl_2 (10 mL) and the combined extracts were dried, filtered and carefully evaporated *in vacuo*, as usually, to furnish **1a**-COONHS (6 mg, yield 80%) as a blue amorphous solid. Probably due to kinetic reasons, the reaction requires a very large excess of the DSC reagent. Attempts to reduce its amount, e. g., using DMAP as a catalyst, led to lower yields. On the other hand, the use of CH_2Cl_2 as a solvent and DIPEA as a base were found to be crucial.

Product properties: well-soluble in chlorinated solvents (e.g., CH_2Cl_2), THF, CH_3CN, and alcohols. The compound is slightly soluble in water, quite stable toward hydrolysis in neutral media but rapidly reacts with ammonia and amines. Analytical data for **1a**-CONHS: t_R = 16 min (HPLC, *standard analytical program; see General Remarks*); TLC: R_f = 0.40 (regular silica gel plates, $CH_3CN/CH_2Cl_2/H_2O$ 10:1:1).MS (ESI) *m/z* (positive mode,%) = 710 (100%) [M^+]. HRMS ($C_{36}H_{39}F_3N_3O_7Si^+$): 710.2501 (found), 710.2504

(calc.). ^{1}H-NMR (400 MHz, $CDCl_3$) δ = 0.59, 0.66 (s, 6 H, $SiCH_3$), 0.98 (d, J = 7.4 Hz, 3 H, CH_3) 1.03 – 1.82 (m, 4 H, CH_2), 2.88 (s, 4H, CH_2CO), 3.45 (s, 12 H, NCH_3), 4.03 (m, 2 H, CH_2), 4.71 (q, J = 6.5, 5.8 Hz, 1H), 6.71 (dd, J = 9.6, 2.8 Hz, 2H), 6.97 – 7.01 (m, 3H), 7.32 (m, 2H) ppm; ^{13}C NMR (100.6 MHz, $CDCl_3$) δ = 1.1 (CH_3), 20.2 (CH_3), 24.8 (CH_2), 41.3 (CH_3), 51.8 (CH_2), 65.0 (CH_2), 78.7 (CH), 114.5 (CH), 121.5 (CH), 126.8 (C), 140.6 (CH), 147.9 (C), 150.7 (C), 153.5 (C), 164.5 (C=O) ppm. ^{19}F NMR (376.4 MHz, $CDCl_3$): δ = –156.0 (m, 1 F), –129.5 (m, 1 F), –127.0 (m, 1 F), ppm.

The hydrophilic dye **1b-H**.

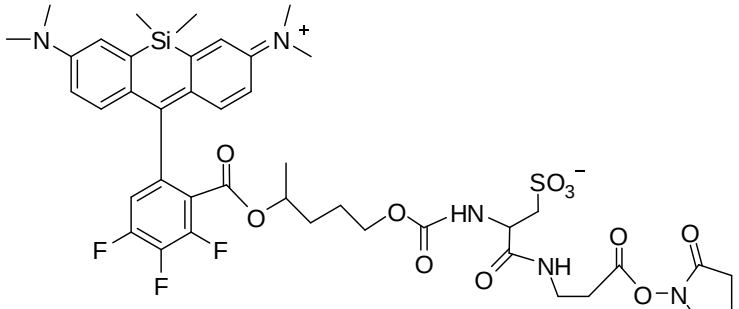


The active carbonate **1a**-CONHS was reacted with a solubilizing peptide-type spacer containing a cysteic acid moiety. It was prepared by conventional methods and utilized in our previous study as a solubilizing moiety or a “hydrophilizer” (see ref. [4]).

The reaction was typically performed as follows: compound (**1a**-CONHS (4 mg, 5.3 μmol), compound $H_3N^+CH(CH_2SO_3^-)CONH(CH_2)_2CO_2H$ (6.4 mg, 27 μmol) and DIPEA (12μL, 66 μmol) were stirred overnight in DMF (1 mL).The solution was diluted with a triple volume of CH_3CN and purified by column chromatography over 6 g of regular silica gel with a mixture of CH_3CN/H_2O (10:1→3:1) to collect the colored main fraction (HPLC analysis). The latter was filtered through syringe filters (0.45 μm), and evaporated *in vacuo* (t < 25°C) to the volume of ca. 10 mL, basified with Et_3N (30 μL) and extracted with CH_2Cl_2 (2 x 3 mL). The aqueous phase was freeze-dried, the residue re-dissolved in acetone (7 mL) containing Et_3N (3 μL), the solution filtered and carefully evaporated to furnish 5.2 mg (yield 70%) of **1b**-H as a Et_3N-salt. Properties: amorphous blue solid, well-soluble in acetone and MeOH, sparingly soluble in chlorinated hydrocarbons and pure water. The solubility and the distribution between the water and the organic phase strongly depend on the pH (see also the discussion in the main text). Compound **1b**-H (in the H-form) can be completely extracted with CH_2Cl_2 from aqueous solutions containing an excess of CF_3COOH (> 10 equiv.) On the other hand, bases ($NaHCO_3$, Et_3N) keep the dye predominantly in the aqueous phase. Analytical data for **1b-H**: t_R = 9 min (HPLC, *standard analytical program; see General Remarks*); TLC: R_f = 0.10 (regular silica gel plates, $CH_3CN/CH_2Cl_2/H_2O$ 10:1:1). MS (ESI) *m/z* (positive mode, %) = 835 (90%) $[M+H]^+$; HRMS ($C_{38}H_{45}F_3N_4O_{10}SSi^+$): 835.2649 (found), 835.2651 (calc.).^{1}H-NMR

(400 MHz, Acetonitrile-d_3) δ = 0.60 (br. s, 6 H, $SiCH_3$), 0.83 (m, 3 H, CH_3), 0.96 (m, 3H, CH_3, Et_3N), 1.05 – 1.25 (br. m, 4 H, CH_2), 1.30, 2.10, (br. m, 4 H, CH_2, β- Ala), 2.48 (m, 2H, CH_2, Et_3N), 3.44 (s, 12 H, NCH_3), 3.60 (m, 1H, CH), 3.70 (m, 2 H, CH_2SO_3), 3.96 (m, 2 H, CH_2), 6.80 (m, 2H), 7.05 – 7.16 (m, 1H), 6.98 (m, 2H), 7.20 (m, 2H) ppm; ^{19}F NMR (376.4 MHz, Acetonitrile-d_3: δ = –146.2 (m, 1 F), –131.5 (m, 1 F), –126.3 (m, 1 F), ppm.

The hydrophilic active ester **1b**-NHS.



1b-NHS

In a typical experiment, the active ester was prepared as follows: solid *N,N* '-disuccinimidyl carbonate (DSC reagent; 10 mg, 40 μmol) was added in one portion to a solution of acid **1b**-H (4 mg, 4.3 μmol; as a Et_3N salt) and DIPEA (14 μL, 77 μmol) in a mixture of CH_3CN (3 mL) and CH_2Cl_2 (2 mL) and stirred for 30 min at RT The solution was quenched with CH_2Cl_2 (20 mL), water (40 mL), and brine (20 mL). The organic layer separated, shaken with brine (20 mL), dried, filtered, and carefully evaporated *in vacuo* (t < 20 °C) to furnish 3.5 mg (yield 88%) of **1b-**NHS, whose purity (> 90%) and identity was confirmed by analytical methods. Properties: well-soluble in chlorinated solvents (e.g., CH_2Cl_2), THF, CH_3CN, and alcohols. The compound is slightly soluble in water, quite stable toward hydrolysis in neutral media but rapidly reacts with ammonia and amines.

Analytical data: HPLC: t_R = 9.6 min (A/B + 0.1% (v/v) TFA, 70:30→0:100 in 20 min; HPLC area 94%, detection at 254 nm; *see the analytical protocol below*); the standard analytical program *(which has slightly different gradient parameters; see General Remarks for details*) gave the t_R = 11 min TLC: R_f = 0.20 (regular silica gel plates, $CH_3CN/CH_2Cl_2/H_2O$ 10:1:1). MS (ESI) *m/z* (positive mode, %) = 954 (90%) $[M+H]^+$; HRMS ($C_{42}H_{48}F_3N_5O_{12}SSi^+$): 954.2631 (found), 954.2634 (calc.). The active ester was dissolved in DMF and aliquoted. The aliquots (each containing approx. 200 μg of the compound) were evaporated in the vials, flushed with argon, and stored at –20 °C. When necessary, they were re-dissolved in DMF (10 μL each) and used for antibody labelling experiments, according to the standard protocol described in ref. [1].

Operator: J. Bienert Timebase:HPLC Sequence:Trennung

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Max-Planck-Institut für biophysikalische Chemie - Göttingen
Facility für synthetische Chemie

Probe : **KK1444b**

Lösungsmittel : ? **Konzentration :** ? %
Aufgabeart : Handaufgabe **Aufgabemenge :** 1.0 µl

Säule : Kinetex 5µm C18 100
Länge : 25 cm **Innendurchmesser :** 4.6 mm

Fluß (ml / Min) : 1,20
Temperatur : 20 °C

Detektor : UltiMate 3000

Laufmittel : A = Acetonitril B = Wasser 0.1% TFA

Gradient : A% = 30 B% = 70 ----> A% = 100 B% = 0 T = 20 Min.

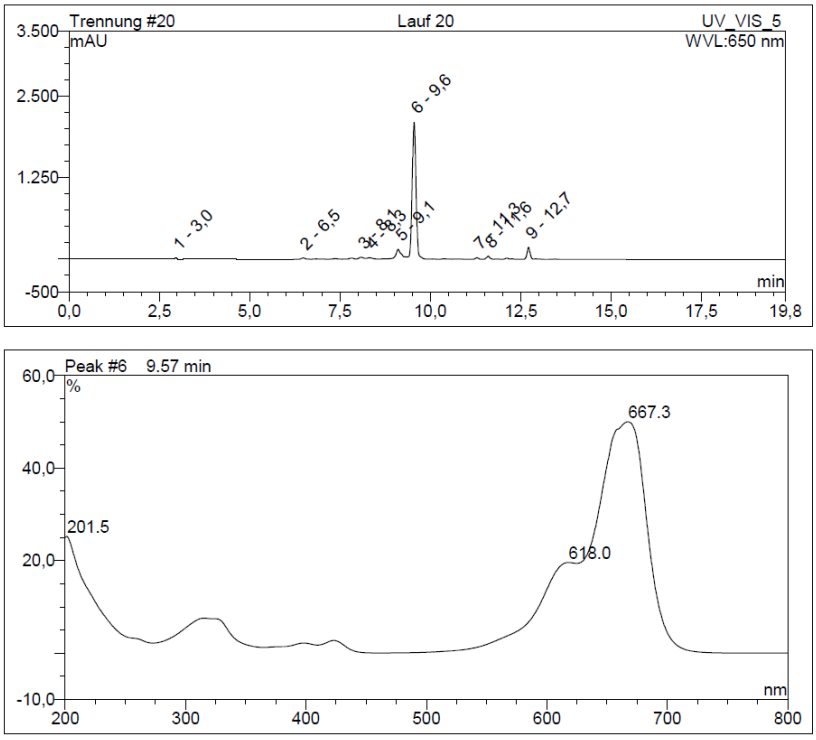
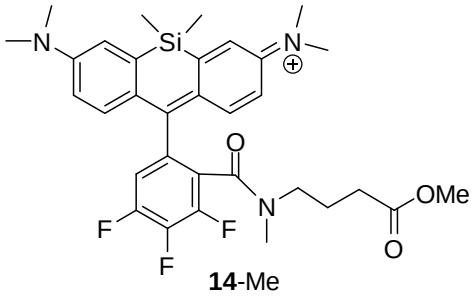


Fig. S03

HPLC analysis and UV/vis spectrum of the dye active ester **1b**-NHS.The analysis was performed at the Chemistry Facility (Max Planck Institute for Biophysical Chemisty, Göttingen) by means of Knauer Smartline semi-preparative high pressure gradient system with two pumps, mixing chamber, column thermostat 4000.and an UV detector UltiMate 3000. Other details are presented herein.

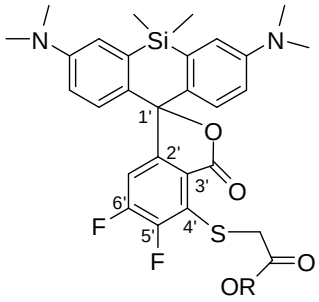
Amido ester **14-**Me and its saponification (an attempted attachment of an amide-type spacer to the silicon-rhodamine dye **13a**).



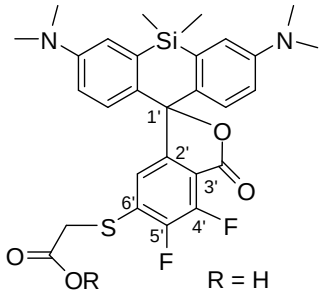
14-Me

Si-Rhodamine **13a** (10 mg, 0.020 mmol) was converted to the acid chloride exactly as described in the synthesis of compound **1**-H (see above) by the action of oxalyl chloride (0.30 mL, 3.5 mmol). The solvent was thoroughly evaporated and the residue re-dissolved in CH_2Cl_2 (4 mL) an argon atmosphere. A freshly prepared solution of the amide linker salt $HCl{*}CH_3NH(CH_2)_3COOCH_3$ (20 mg, 0.12 mmol; for preparation and properties see ref. [2]) in CH_3CN (1 mL) and then Et_3N (36 μL, 0.25 mmol) was added at 0 °C under argon. The solution was stirred for 20 min at this temperature, diluted with an equal volume of water, and well-shaken. The dark-blue organic solution was separated, washed one more time with deionized water, and dried (Na_2SO_4). The HPLC analysis showed the completion of the reaction: the starting material **13a** had completely vanished, while a new compound with the purity of some 96% was formed. The MS and HRMS spectra confirmed the structure **14**-Me. Analytical data: t_R = 13 (HPLC, *standard analytical program; see General Remarks*); TLC: R_f = 0.15 (regular silica gel plates, $CH_3CN/CH_2Cl_2/H_2O$10:1:1; seen by a naked eye as a bright blue spot). MS (ESI) *m/z* (positive mode,%) = 596 (90%) $[M]^+$; HRMS ($C_{32}H_{37}F_3N_3O_3Si^+$): 596.2564 (found), 596.2551 (calc.). However, even upon careful evaporation at RT, the product had undergone partial decomposition to form up to 20% of the starting Si-rhodamine **13a**, which was detected by HPLC and TLC (see above). The decomposition proceeded even upon storage at 5 °C. The crude product was subjected to careful saponification at 5 °C in a mixture of THF (3 mL) and deionized water (6 mL) containing NaOH (0.20 mL of a 1 M solution, 0.20 mmol). When compound **14**-Me had completely reacted (HPLC monitoring) only the starting material (Si-rhodamine **13a**) was detected, accompanied with some minor impurities. The amide complete cleavage (and the failure of the saponification reaction) was also witnessed by the absence of the initial intense blue color. Some 85% of the starting compound **13a** was recovered by column chromatography over regular silica gel (12 g) with $EtOAc/CH_2Cl_2$ (1:7) as the mobile phase.

Thioglycolic acid derivatives **15a**,**b**-H – analogs of the dye **SiR-**COOH (see ref [4] and the main text).



15a-H (4' -SCH_2CO_2H)



15b-H (6' -SCH_2CO_2H)

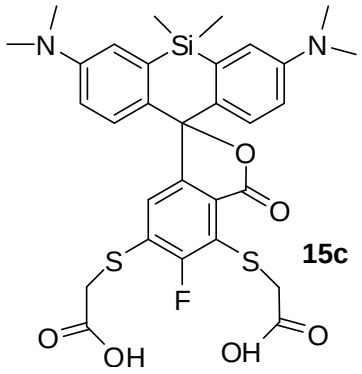
Title compounds were obtained by nucleophilic aromatic substitution of fluorine in **13a**.

Compound **13a** (15 mg, 0.03 mmol) was reacted with thioglycolic acid (11 mg, 0.12 mmol) in the presence of Et_3N (36 μL, 0.25 mmol) using CH_3CN (6mL) as a solvent. After a 3h-exposure at RT (with a HPLC monitoring) the reaction solution was neutralized with a slight excess of AcOH (20 μL, 0.35 mmol), diluted with a double volume of CH_3CN and purified by column chromatography over silica gel (20 g) with $CH_3CN/CH_2Cl_2/H_2O$ (20:1:1) as the mobile phase. The main colored fraction was collected, filtered, and evaporated to furnish 10 mg (yield 62%) of isomers **15a**-H and **15b**-H. The mixture of isomers was separated by means of preparative HPLC (A/B with 0.1% TFA, 70:30→0:100 in 25 min., preparative column, flow rate 3 mL/min) followed by freeze-drying of the fractions to furnish 7.5 mg of **15a-**H (4'-isomer, the major product) and 2.2 mg of **15b**-H (6'-isomer). The exact position of the substituent was established by comparing ^{1}H and ^{19}F-NMR spectra of the two isomers and the starting material (**13a**).

Analytical data for **15a-**H (4'-isomer): t_R = 11 min (HPLC, *standard analytical program; see General Remarks*); TLC: R_f = 0.30 (regular silica gel plates, MeOH/CH_2Cl_2 8:1). MS (ESI) *m*/*z* (positive mode,%) = 555 (100%) $[M+H]^+$. HRMS ($C_{28}H_{28}F_2N_2O_4SSi^+$): 555.1503 (found), 555.1507 (calc.). ^{1}H-NMR (400 MHz, Acetonitrile-d_3): δ = 0.56, 0.66 (s, 6 H, $SiCH_3$), 3.06 (s, 12 H, NCH_3), 3.98 (s, 2H, CH_2S), 6.94 (q, *J* = 1.7 Hz, 4H), 7.06 (dd, *J* = 8.7 Hz, *orto*-, 6.3 Hz, *meta*- , 1 H, H-7'), 7.37 (dt, *J* = 3.3, 1,6 Hz, 2H) ppm; ^{19}F NMR (376.4 MHz, Acetonitrile-d_3): δ = –132.6 (d, *J* = 19 Hz, 1 F), –126.1 (s, 1 F) ppm. UV/Vis spectral data: λ_{max} abs. = 657 nm, λ_{max} fl.= 676 nm; Φ_{fl} = 40% (in H_2O, with Atto AZ 237 as a reference), $\varepsilon \times 10^{-5}$ = 0.4 $M^{-1}cm^{-1}$.

Analytical data for **15b**-H (6'-isomer): t_R = 10.4 min (HPLC, *standard analytical program; see General Remarks*); TLC: R_f = 0.30 (regular silica gel plates, MeOH/CH_2Cl_2 8:1). MS (ESI) *m*/*z* (positive mode,%) = 555 (100%) $[M+H]^+$. HRMS ($C_{28}H_{28}F_2N_2O_4SSi^+$): 555.1501 (found), 555.1507 (calc.). ^{1}H-NMR (400 MHz, Acetonitrile-d_3): δ = 0.53, 0.65 (s, 6 H, $SiCH_3$), 3.08 (s, 12 H, NCH_3), 3.80 (s, 2H, CH_2S), 6.77 (dt, *J* = 9.1, 4.2, Hz, 2H), 6.92 (dd, *J* = 11.1, 6.6 Hz, 2 H), 7.01 (dd, *J* = 5.6 Hz, *meta*-, 1,4 Hz, *para*-, 1H, H-7'), 7.29 – 7.18 (m, 2H), ppm; ^{19}F NMR (376.4 MHz, Acetonitrile-d_3): δ = –141.1 (s, 1 F), –137.9 (s, 1 F) ppm.

Di-substituted thioglycolic acid derivative **15c**.

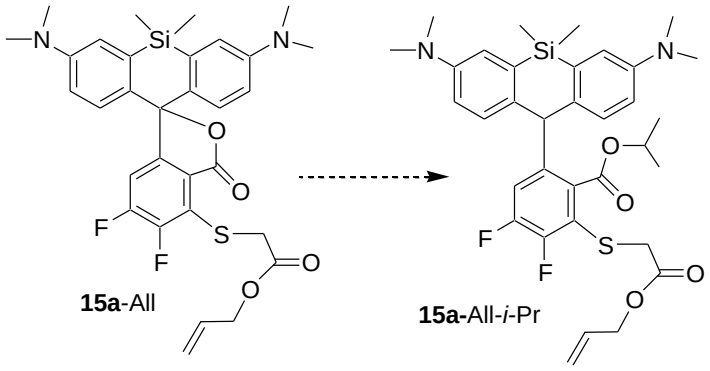


15c

The di-substituted derivative was obtained as an undesired reaction product when the substitution of a fluorine was performed exactly as described above for compounds **15a,b-**H, the reaction mixture quenched with a large excess of AcOH (5 equiv, relative to Et_3N), and evaporated to dryness at RT *in vacuo* as it was before chromatographic separation. Unexpectedly, further fluorine substitution proceeded in the residue, despite the acidic media. Compound **15c** was isolated in an 80% yield by means of preparative HPLC (A/B 70:30→0:100 in 25 min, preparative column, flow rate 3 mL/min, detection at 254 nm) followed by freeze-drying.

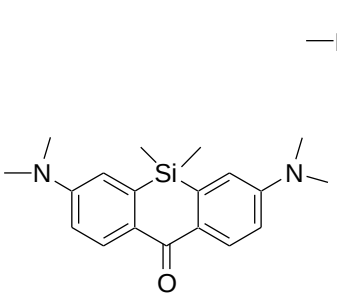
Analytical data for **15c**: t_R = 7 min (HPLC, *standard analytical program; see General Remarks*); TLC: R_f = 0.10 (regular silica gel plates, MeOH/CH_2Cl_2 5:1). MS (ESI) *m/z* (positive mode,%) = 627 (100%) $[M+H]^+$. HRMS ($C_{30}H_{31}FN_2O_6S_2Si^+$): 627.1371 (found), 627.1377 (calc.). ^{1}H-NMR (400 MHz, Acetonitrile-d_3): δ = 0.54, 0.64 (s, 6H, $SiCH_3$), 3.06 (s, 12H, NCH_3), 4.02 (s, 4H, $2CH_2S$), 6.94 (m, 4H), 7.09 (m 1 H), 7.30 (m, 2H) ppm; ^{19}F NMR (376.4 MHz, Acetonitrile-d_3): δ = –136.3 (s, 1 F) ppm.

Allyl ester **15a**-All and its attempted esterification.

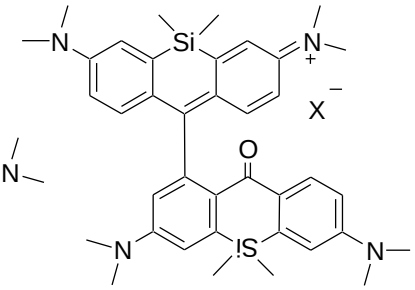


Compound **15a-**H was converted to the allyl ester **15a**-All by the conventional method, using an excess of allyl alcohol in the presence of DCC (*N,N'*-dicyclohexylcarbodiimide) as activating reagent and DMAP as a catalyst (see ref. [5]). The product was isolated by column chromatography over regular silica gel with EtOAc/CH_2Cl_2 (1:7) as the mobile phase with a 60% yield as a colorless solid (exists predominantly in the closed form, as expected). The purity and identity was confirmed by analytical methods: HPLC: t_R = 11 min (A/B 50:50→0:100 in 25 min; HPLC area 99%, detection at 254 nm). TLC: R_f = 0.80 (regular silica gel plates, $CH_3CN/CH_2Cl_2/H_2O$ 10:1:1). MS (ESI) *m/z* (positive mode,%) = 595 (100%) $[M+H]^+$. HRMS ($C_{31}H_{32}F_2N_2O_4SSi^+$): 595.1524 (found), 595.1820(calc.). Compound **15a**-All was treated consecutively with oxalyl chloride and isopropanol, exactly as described for **13-**O-*i*Pr (see above). However, despite the very large excess of $(COCl)_2$, the rhodamine chloride was not formed, which was witnessed by the absence of the blue color (the dye opened form). Also, no significant amount of the desired product was isolated, which was confirmed by analytical methods. The starting material was almost completely recovered.

Attempted lithiation of ketone **10a** using *n*-BuLi and the *bromine-free* oxazoline derivative **7** in the presence of Li-TMP reagent.



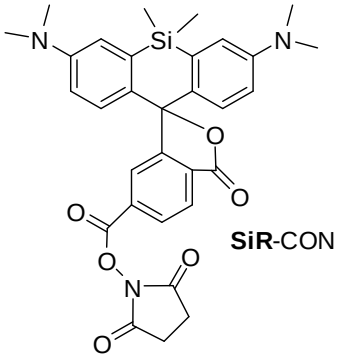
10a



10a-"dimer"

A flame-dried and argon-flushed Schlenck flask was loaded through a septum with a solution of 2,2,6,6-tetramethylpiperidine (TMP reagent, 60 μL, 0.41 mmol) in a freshly distilled THF (2mL), chilled to -78 °C (dry ice bath), and the commercial 1.6 M *n*-BuLi solution (250 μL, 0.40 mmol) was introduced within 1 min. upon stirring. About 30 min. later a solution of the oxazoline derivative **7** (60 mg, 0.28 mmol) in dry THF (2 mL) was added. The reaction solution was kept for 3 h at this temperature and the ketone substrate **10** (20 mg, 0.06 mmol) in the same solvent (2 mL) was added within 2 min upon vigorous stirring. The stirring was continued for 30 min at this temperature and for >10 h in an ice-water bath (0 °C) under an argon atmosphere. The solution was poured onto ice-cold deionized water acidified with a 1 N aqueous HCl solution (3 mL) and the mixture extracted with CH_2Cl_2 (2 X 30 mL). The extract was dried, evaporated, and purified by column chromatography over silica gel (20 g) with $CH_3CN/CH_2Cl_2/H_2O$ (20:1:1) as the mobile phase. Most of compound **7** was recovered as the less polar fraction. Its identity was confirmed by MS-spectrometry, HPLC, and TLC. The main dark purple fraction was collected, filtered, and evaporated to furnish 9 mg (yield 46%, X = OH) of the so-called **10a**-"dimer" as a black amorphous solid. The compound is soluble in most organic solvents and very slightly in water, to form dark brown solutions with a red fluorescence.

Analytical data: t_R = 13 min (HPLC, *standard analytical program; see General Remarks*); TLC: R_f = 0.10 (regular silica gel plates, $CH_3CN/CH_2Cl_2/H_2O$ 10:1:1). MS (ESI) *m/z* (positive mode, %) = 631 (100%) [M^+]. HRMS ($C_{38}H_{47}N_4OSi_2^+$): 631.3264 (found), 631.3283 (calc.). ^{1}H-NMR (400 MHz, Acetone-d_6): δ = 0.63, (s, 6 H, $SiCH_3$), 0.67, 0.71 (2s, 6 H, $SiCH_3$), 3.09 (s, 12 H, NCH_3), 3.23, 3.31 (2s, 6H, NCH_3); 11 H_{Ar}: 6.63 (dd, *J* = 9.1, 2.9 Hz, 2H), 6.77 (d, *J* = 2.3 Hz, 1 H), 6.88 – 6.92 (m, 1H), 6.96 (d, *J* = 9.1 Hz, 2 H), 7.25 (2 d, *J* = 7.24 and 7.26 Hz 3H), 7.59 (d, *J* = 2.4 Hz, 1 H), 7.86 (d, *J* = 9.2 Hz, 1 H) ppm.



SiR-CONHS

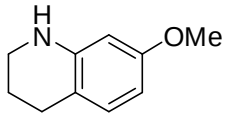
The NHS ester of 6-carboxy**-**TMR-**SiR** (**SiR**-CONHS).

The active ester for conjugation to antibodies was prepared on a small scale as follows: solid *N,N* '-disuccinimidyl carbonate (DSC reagent; 10 mg, 40 μmol) was added in one portion to a solution of 6-carboxy-TMR-SiR (**SiR**-CO_2H, 1 mg, 2 μmol; see ref [6] for preparation), DIPEA (14 μL, 77 μmol) and DMAP (0.2 mg, a catalyst) in a mixture of CH_3CN (0.3 mL) and CH_2Cl_2 (2 mL). The mixture was stirred overnight at RT to complete the reaction (TLC and HPLC monitoring). The solution was quenched with CH_2Cl_2 (5 mL) and well-shaken two times with deionized water (10 mL). The organic layer was separated dried, filtered, and carefully evaporated *in vacuo* (t < 20 °C) to furnish 0.9 – 1.0 mg (yield 80%) of **SiR**-CONHS, whose purity (> 85%) and identity was confirmed by analytical methods.

Being sufficiently pure, the NHS ester was used as a reference dye marker without further treatment in evaporated aliquots (DMF was used as a solvent) containing 200 μg of compound each. Analytical data for **SiR**-CONHS: t_R = 11 min (HPLC, *standard analytical program; see General Remarks*); TLC: R_f = 0.5 for the reaction product and 0.2 for the starting material – **SiR**-CO_2H (regular silica gel plates, CH_2Cl_2/MeOH 10:1). MS (ESI) *m/z* (positive mode,%) = 570 (100%) $[M+H]^+$. HRMS ($C_{31}H_{31}N_3O_6Si^+$): 570.2045 (found), 570.2055 (calc.).

Oxazine dyes and their precursors

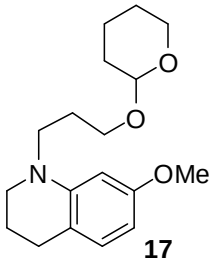
Compound **16**-Me (7-methoxy-1,2,3,4-tetrahydroquinoline by *O*-alkylation of amino phenol **16**; for an alternative preparation recipe see [7a]).



16-Me

Trimethylsilyl diazomethane (2 M in Et_2O, 2 mL, 4 mmol) was slowly added to stirred a solution of 7-hydroxy-1,2,3,4-tetrahydroquinoline (amino phenol **16**, 300 mg, 2 mmol; for the preparation see [7b]) and DIPEA (0.85 mL, 5 mmol) in a mixture of MeCN (9 mL) and MeOH (1 mL) at 0 °C. The mixture was slowly warmed up to RT, stirred overnight, and the solvents were evaporated *in vacuo*. The residue was dissolved in Et_2O (20 mL) and well-shaken with an aqueous 0.1 M NaOH solution. The aqueous phase was extracted 2x with an equal volume of Et_2O, the combined extracts dried and evaporated. The residue was purified by column chromatography over regular silica gel (20 g) with EtOAc/Hexane (1:10) as the mobile phase to afford compound **16-**Me (208 mg, yield 64%) as a colorless oil, whose purity and identity was confirmed by MS and NMRNMR spectroscopy. The analytical data were in a good agreement with the previously reported [7a]. MS (ESI) *m*/*z* (positive mode,%) = 164 (90%) $[M+H]^+$. HRMS ($C_{10}H_{13}NO^+$): 164.1069 (found), 164.1070 (calc.). ^{1}H-NMR (400 MHz, $CDCl_3$) δ = 1.91 – 1.97 (m, 2H, CH_2), 2.71 (t, 6 Hz, 2H, CH_2) 3.26 – 3.33 (m, 2H, CH_2), 3.75 (s, 3H, OCH_3), 6.05 6.21 (d, J = 2.5 Hz, 1H), 6.22 (dd, J = 8 and 2.5 Hz, 1H), 6.86 (d, J = 8.0 Hz, 1H) ppm; ^{13}C NMR (100.6 MHz, $CDCl_3$) δ = 22.5 (CH_2), 26.3 (C), 42.02 (CH_2), 55.2 (OCH_3), 99.5 (CH_2), 99.1 (CH), 102.9 (C), 114.3 (C), 130.2 (CH), 145.6 (C), 159.4 (C) ppm.

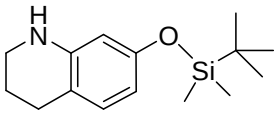
Compound **17** (*N*-alkylation of **16-**Me).



17

In a test tube sealed with a screw cup compound **16-**Me (163 mg, 1.0 mmol) was stirred for 48 h at 85 °C in MeCN (2 mL) containing the following reagents: 1-tetrahydropyranyloxy-3-bromopropane ($Br(CH_2)_3OTHP$, 268 mg, 1.2 mmol; for preparation see ref. [8]), finely powdered Na_2CO_3 (127 mg, 1.2 mmol), and KI (199 mg, 1.2 mmol). The mixture was quenched with deionized water (20 mL) and CH_2Cl_2 (20 mL). The organic layer was evaporated and the residue purified by column chromatography over a column with silica gel (20 g) and EtOAc/Hexane (1:15) as the mobile phase to afford compound 17 (282 mg, yield 92%) as a colorless oil. Its purity and identity was confirmed by MS and NMRNMR spectroscopy. MS (ESI) *m/z* (positive mode,%) = 306 (95%) $[M+H]^+$. HRMS ($C_{18}H_{27}NO_3^+$): 306.2062 found), 306.2064 (calc.). ^{1}H-NMR (400 MHz, $CDCl_3$) δ = 1.49 – 1.84 (m, 10H, $5CH_2$), 2.69 (t, ***J* =** 6 Hz, 2H, CH_2) 3.27 – 3.54 (m, 6H, $3CH_2$), 3.76 (s, 3H, OCH_3), 3.82 – 3.87 (m, 2H, CH_2), 4.58 (dd, ***J* =** 4 and 3 Hz, 1H, CHO), 6.13 (dd, ***J* =** 8 and 2 Hz, 1H), 6.21 (d, 2 Hz, 1H), 6.84 (d, 8.0 Hz, 1H) ppm; ^{13}C NMR (100.6 MHz, $CDCl_3$) δ = 19.8 (CH_2), 22.6 (CH_2), 25.9 (C), 26.7 (CH_2), 27.6 (CH_2), 30.1 (CH_2), 48.7 (CH_2), 49.5 (CH_2), 55.3 (OCH_3), 62.5 (CH_2), 65.3 (CH_2), 97.5 (CH_2), 99.1 (CH), 99.9 (CH), 115.3 (C), 129.5 (CH), 146.2 (C), 159.4 (C) ppm.

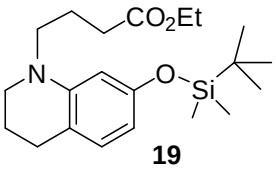
Compound **16-**TBDMS (the *O*-protected amino phenol).



16-TBDMS

Phenol **16** (7-hydroxy-1,2,3,4-tetrahydroquinoline) was reacted with TBDMS-Cl (1.1 equiv) in the presence of imidazole (2 equiv) within 2 days, following the conventional recipe (analogously to **1**-OTBDMS-pentanol-4; see above) The reaction mixture was quenched with CH_2Cl_2, washed with deionized water, and the product isolated by means of flash chromatography over regular silica gel with EtOAc/Hexane (1:25) as the mobile phase with a 96% yield as a colorless oil. Its purity and identity was confirmed by MS and NMR spectroscopy. MS (ESI) *m/z* (positive mode,%) = 264 (90%) $[M+H]^+$. HRMS ($C_{15}H_{25}NOSi^+$): 264.1786 (found), 264.1778 (calc.). ^{1}H-NMR (400 MHz, $CDCl_3$) δ = 0.18 (s, 6 H, 2Me), 0.98 (s, 9 H, *t*-Bu), 1.92 (m, 2H, CH_2), 2.99 (m, 2H, CH_2), 3.26 (m, 2H, CH_2N), 3.70 (br. s. 1H, NH) 5.99 (d, *J* = 2.4 Hz, 1H), 6.12 (dd, *J* = 8.1, 2.4 Hz, 1H), 6.77 (d, *J* = 8.1 Hz, 1H) ppm; ^{13}C NMR (100.6 MHz, $CDCl_3$) δ = 4.2 (CH_3), 18.3(CH_3), 22.6 (C), 25.9 (CH_2), 42.0 (CH_2), 105.7 (CH), 109.2 (CH), 114.8 (C), 130.0 (CH), 145.5 (C), 154.6 (C) ppm.

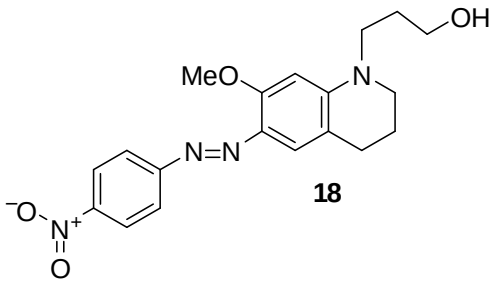
Synthone **19** (*N*-alkylation of the *O*-protected amino phenol **16-**TBDMS).



19

In a test tube sealed with a screw cup compound **16-**TBDMS (163 mg, 0.38 mmol) was stirred in MeCN (1 mL) containing ethyl 4-bromobutyrate (90 mg, 1.2 mmol), finely powdered Na_2CO_3 (50 mg, 0.46 mmol), and KI (76 mg, 1.2 mmol) for 60 h at 85 °C. The mixture was quenched with deionized water (20 mL) and CH_2Cl_2 (20 mL). The organic layer was evaporated and the residue purified by column chromatography over a column with regular silica gel (15 g) and EtOAc/Hexane (1:30) as the mobile phase to afford compound **19** (132 mg, yield 90%) as a colorless oil. The purity and identity was confirmed by MS and NMRNMR spectroscopy. MS (ESI) *m/z* (positive mode,%) = 378 (100%) $[M+H]^+$. HRMS ($C_{21}H_{35}NO_3Si^+$): 378.2458 (found), 378.2459 (calc.). ^{1}H-NMR (400 MHz, $CDCl_3$) δ = 0.19 (s, 6 H, 2Me), 0.98 (s, 9 H, *t*-Bu), 1.26 (t, *J* = 7 Hz, 3H, Et) 1.96 (m, 2H, CH_2), 2.35 (t, *J* = 7 Hz, 2H, CH_2), 2.67 (t, *J* = 6 Hz, 2H, CH_2), 3.23 (m, 2H, CH_2N), 4.14 (q, *J* = 7 Hz, 2H, Et), 3.70 (br. s. 1H, NH) 6.07 (dd, *J* = 8 and 2 Hz, 1H), 6. 14 (d, *J* = 2 Hz, 1H), 6.77 (d, *J* = 8 Hz, 1H) ppm; ^{13}C NMR (100.6 MHz, $CDCl_3$) δ = 4.3 (CH_3), 14.3(CH_2), 18.2(CH_3), 22.4 (C), 25.8 (CH_2), 31.8 CH_2), 42.0 (CH_2), 49.4 (NCH_2), 50.8 (NCH_2), 102.7 (CH), 107.2 (CH), 115.4 (C), 129.4 (CH), 145.8 (C), 154.9 (C), 173.2 (C=O) ppm.

The azo dye **18**.



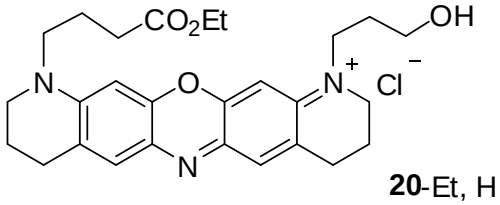
18

Attention! Being potentially explosive, dry diazonium salts require precautions while handling: Use plastic spatula! Do not use glassware with ground joints for storage! Do not heat the compound! Store at t < +5°C!

To a stirred suspension of *4-nitrobenzenediazonium tetrafluoroborate* (48 mg, 0.20 mmol) in an aqueous H_2SO_4 (10 wt.% solution, 0.5 mL) a solution of compound **17** (61 mg, 0.20 mmol) in MeOH (1.5 mL) was added within 2 min. After additional 2 h of stirring at RT, the dark red solution was carefully neutralized

with NaOH (1.26 mL of a 1 M solution, 1.26 mmol), quenched with CH_2Cl_2 (50 mL), saturated $NaHCO_3$ solution (10 mL), and well-shaken. The aqueous phase was extracted with CH_2Cl_2 (2 x 10 mL), the combine extracts washed with brine (10 mL), evaporated, and the residue purified by column chromatography over a column with silica gel (35 g) and MeOH/CH_2Cl_2 (1:60) as the mobile phase to afford compound **18** (63 mg, yield 85%) as dark brown crystalline solid with m.p. of 56 °C. TLC: R_f = 0.20 (regular silica gel plates, CH_2Cl_2/MeOH 30:1; seen by a naked eye as a dark orange spot). MS (ESI) *m/z* (positive mode,%) = 371 (95%) $[M+H]^+$. HRMS ($C_{19}H_{22}N_4O_4^+$): 371.1716 (found), 371.1714 (calc.). ^{1}H-NMR (400 MHz, DMSO-d_6) δ = 1.79 – 1.85 (m, 4H, $2CH_2$), 2.67 (t, J = 6 Hz, 2H, CH_2) 3.42 (m, 2H, CH_2), 3.48 – 3.56 (m, 4H, $2CH_2$), 3.93 (s, 3H, OCH_3), 4.68 (t, J = 5 Hz, 1H, OH), 6.35 (s, 1H), 7.47 (s, 1H), 7.80 (d, J = 9 Hz, 2H), 8.40 (d, J = 9 Hz, 2H) ppm; ^{13}C NMR (100.6 MHz, DMSO-d_6) δ = 21.5 (CH_2), 26.6 (CH_2), 29.3 (CH_2), 47.9(CH_2), 49.2(CH_2), 55.8 (OCH_3), 58.0 ($\underline{CH_2}OH$), 93.1 (CH), 115.9 (CH), 116.5 (C), 119.3 (CH), 121.9 (CH), 132.5 (CH), 157.2 (CH), 160.1 (CO) ppm.

Oxazine dye **20-**Et,H (the condensation of two synthones).



20-Et, H

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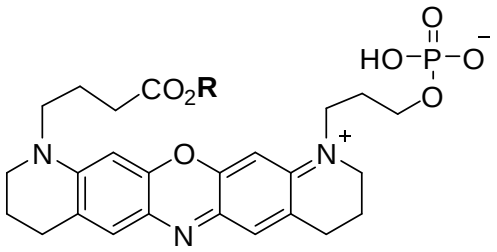
In a typical experiment, the condensation of two building blocks was performed as follows: azo dye **18** (7.4 mg, 20 μmol) and synthone **19** (7.5 mg, 20 μmol) were combined in abs. EtOH (0.5 mL), acidified with 4 M HCl (20μL), and heated in a sealed test tube for 1 h at 90°C. The solution was evaporated (t < 25°C) *in vacuo* and the residue purified by column chromatography over regular silica gel (20g) $CH_3CN/CH_2Cl_2/H_2O$ (10:1:1). The homogeneous fractions were pooled, filtered through syringe filters (0.45 μm), and evaporated. The residue was re-dissolved in CH_2Cl_2, filtered again, and the solvent was evaporated to furnish 6.8 mg (yield 67%) of compound **20-**Et,H as a dark blue solid. Properties: slightly soluble in water, well-soluble in most organic solvents. The fluorescence of its greenish-blue solutions is virtually not seen by a naked eye. Analytical data **20-**Et,H: t_R = 12 min (HPLC, *standard analytical program; see General Remarks*); TLC: R_f = 0.10 (regular silica gel plates $CH_3CN/CH_2Cl_2/H_2O$ 10:1:1; seen by a naked eye as a bright blue spot). MS (ESI) *m/z* (positive mode, %) = 464 (100%) $[M^+]$. HRMS ($C_{19}H_{22}N_4O_4^+$): 464.2546 (found), 464.2544 (calc.). ^{1}H-NMR (400 MHz, $CDCl_3$) δ = 1.26 (t, J = 7 Hz, 3H, CH_3) 2.02 – 2.10 (m, 8H, $4CH_2$), 2.90 (t, J = 6 Hz, 4H, $2CH_2$), 3.63 – 3,70 (m, 4H, $2CH_2$) 3.75 (t, J = 6 Hz, 2H, CH_2), 3.80 (t, J = 5 Hz, 2H, CH_2), 3.80 (t, J = 7 Hz, 2H, CH_2), 4.16 (q, J = 7 Hz, 2H, OCH_2), 6.90 (s, 1H), 7.13 (m, 1H), 7.35 (s, 1H), 7.38 (s, 1H), ppm; ^{13}C NMR (100.6 MHz, $CDCl_3$) δ = 14.4 (CH_3), 20.9 (CH_2), 21.0 (CH_2), 21.6 (CH_2), 30.0 (CH_2), 30.1 (CH_2), 50.9 (CH_2), 51.3 (CH_2), 52.5 (CH_2), 58.7 ($\underline{CH_2}OH$),

61.0 ($\underline{CH_2}CO$), 95.7 (CH), 96.5 (CH), 127.7 (CH), 129.3 (C), 130.9 (CH), 133.3 (C), 155.1 (C), 172.8 (C=O) ppm. UV/Vis spectral data for **20-**Et,H: λ_{max} abs. = 660 nm, λ_{max} fl.= 676 nm; Φ_{fl} = 50% (in H_2O, with Atto AZ 237 as a reference), $\varepsilon \times 10^{-5} = 1.2\ M^{-1}cm^{-1}$

The phosphorylated oxazine dye **2**-H.



20-Et,P(O)$(OH)_2$ (R = Et)
20-H,P(O)$(OH)_2$ (or **2**-H**,** R = H)
20-Su,P(O)$(OH)_2$ (or **2**-NHS, R = *N′*-succinimidyl)

Direct phosphorylation with $POCl_3$: compound **20**-Et,H (15 mg, 0.03 mmol, as a chloride salt) in 1,2-dichloroethane (1.5 mL) was added in 1 min to a precooled (0°C, under an argon atmosphere) mixture of $POCl_3$ (1.5 mL) and THF (1.5 mL) upon vigorous stirring. The reaction was continued for 30 min at this temperature and then for 2 h at RT. The solution was diluted with an equal volume of chlorobenzene and evaporated to dryness *in vacuo* (t < 25°C). Chlorobenzene (3 mL) was added again, the evaporation repeated, and the residue exposed for 1h to a high vacuum. The solid was dissolved in a mixture of CH_3CN (2 mL), water (6 mL), and Et_3N (0.3 mL). The solution was left overnight at 5 °C to hydrolyze the intermediate phosphoric dichloride. The solution was mixed with an equal volume of CH_2Cl_2 and well-shaken. The organic extract was washed with an equal volume of deionized water, the aqueous phases combined, carefully concentrated to the volume of ca. 3 - 4 mL, and freeze-dried. The residue was dissolved in a mixture of deionized water (10 mL) and CH_3CN (1 mL), the dark blue solution acidified with HOAc (0.3 mL) and loaded onto a column with reverse-phase silica gel (Polygoprep 60-50 C_{18}, Macherey-Nagel, 15 g). The column was eluted first with deionized water containing 0.1% (v/v) HCOOH and then with an CH_3CN/H_2O mixture (containing 0.2% (v/v) HCOOH), where the proportion of CH_3CN was gradually increased up to 60%, as the more polar impurities (of yellow and green color) had been washed off. The dark blue fraction was collected (HPLC control), filtered through a syringe filter (0.45 µm), and evaporated *in vacuo* at temperatures not exceeding 30 °C. That furnished ca. 16 mg of a dark blue solid, containing the ethyl ester **20**-Et,P(O)$(OH)_2$ and, probably, some inorganic or triethylammonium salts (not detected by HPLC).

The analytical data of the compound was consistent with the structure **20-**Et,P(O)(OH)$_2$: t_R = 10 min (HPLC, *standard analytical program; see General Remarks*); and 21 min (A/B 20:80→50:50 in 25 min); TLC: R_f = 0.10 (regular silica gel plates CH_3CN/H_2O 4:1). MS (ESI) *m/z* (positive mode,%) = 544 (100%) $[M+H]^+$. HRMS ($C_{27}H_{34}N_3O_7P^+$): 544.2179 (found), 544.2207 (calc.).

The ethyl ester saponification was performed as follows: the crude product was dissolved in an aqueous 0.1 N NaOH solution (10 mL, ca. 20 equiv.) and left for 2h at RT, until the reaction was complete (HPLC monitoring). The solution was acidified with HOAc (0.2 mL) and purified by column chromatography precisely as described above with the same amount of reverse-phase silica gel. First, a purple impurity was separated, and then the main (intense blue) fraction was collected, pooled, and concentrated *in vacuo* to the volume of 50 mL. Some CH_3CN (ca. 10 mL) was added to completely dissolve the precipitate. The solution was filtered through a syringe filter (0.45 µm), evaporated to 1/3 of its volume (t < 25 °C), and freeze-dried to furnish 11 mg (yield 72%, over two steps) of dye **2-**H (or **20**-H,P(O)(OH)$_2$, see also Scheme 5 in the main text).

Properties: dark heavy crystalline powder, almost insoluble in CH_3CN, CH_2Cl_2 and even DMSO. The dye is perfectly soluble in basic or alkaline aqueous solutions, slightly soluble in neutral water, better in MeOH and DMF. The fluorescence maximum of the dye solutions is shifted mostly to the near-IR region (see the main text, Table 1) and therefore practically not seen under the daylight or under an incandesced lamp (see Fig. S05). The HPLC analysis of compound **2**-H was performed using two distinct gradients and two distinct mobile phases (basic and acidic, respectively) : 1) t_R = 7 min (A = MeCN, B = H_2O + 0.1 M TEAB buffer with pH = 8.5), gradient 90:10→0:100 in 20 min; HPLC area 97%, detection at 254 nm *(see actual analysis protocol below)* and 2) t_R = 15 min (A = MeCN, B = H_2O + 0.1% (v/v) TFA, with pH = 2, gradient 20:80→50:50 in 25 min). TLC: R_f = 0.10 (regular silica gel plates CH_3CN/H_2O 4:1). MS (ESI) *m/z* (positive mode,%) = 516 (40%) $[M+H]^+$, 538 $[M+Na]^+$ (50%); HRMS ($C_{25}H_{30}N_3O_7P^+$): 516.1894 (found, M+H), 516.1898 (calc.); 538.1717 (found, M+Na), 538.1714 (calc.).

To obtain the NMR spectra, compound **2**-H was dissolved in dilute aqueous solution of Et_3N and freeze-dried. That provided a water-soluble salt. However, in the ^{1}H-NMR spectrum (500 MHz) in D_2O only broad unresolved signals were obtained due to the limited solubility and high viscosity: δ = 1.30 (m, 6H, 2CH_3, Et_3N), 1.80 – 2.10 (m, 8H, 4CH_2), 2.30 – 2.40 (m, 6H, 3CH_2), 2.60 (m, 4H, 2CH_2), 3.20 – 3.30 (m, 2H, CH_2; m, 4H, 2 CH_2, Et_3N), 3.45 –3.70 (m, 2H, CH_2), 4.05 (m, 2H, CH_2O), 6.70 (m, 2H), 6.80 (m, 1H), 7.04 – 7.10 (m, 3H) ppm. The best resolution for the proton spectrum of **2**-H (as a salt with 2 equiv. of Et_3N) was obtained in MeOH-d_4 as a solvent (400 MHz): δ = 1.30 (t, *J* = 7 Hz, 6H, Et_3N), 2.05 (m, 8H, 4CH_2), 2.38 (br. m., 6H, 3CH_2), 2.94 (m, 4H, 2CH_2), 3.18 (q, *J* = 7 Hz, 4H, Et_3N), 3.75 (t, *J* = 5.6 Hz, 2H, CH_2), 3.83 (m, 2H, CH_2O), 3.97 (m, 2H, CH_2O), 7.00 br. s (1H), 7.09 br. s (1H), 7.46 s (2H). DMSO-d_6 proved unsuitable in this case because of the unexpectedly low solubility of **2**-H in it (as mentioned above). ^{13}C NMR (125.7 MHz, D_2O) δ = 17.4 (CH_3, Et_3N), 17.5 (CH_2), 20.0 (CH_2), 24.2(CH_2), 24.8 (CH_2), 31.4 (CH_2), 47.5 (CH_2), 47.9 (CH_2), 48.2 (CH_2), 49.8 (CH_2), 59.1 (CH_2), 61.0 (CH_2), 92.6 (CH_2), 92.8 (CH_2),

126.2 (CH), 126.7 (CH), 129.6 (CH), 129.8 (CH), 145.4 (C), 145.6 (C), 151.0 (C), 151.2 (C), 178.8 (C=O) ppm. ^{31}P NMR (121.5 MHz, D_2O): δ = +3.12 (s, primary phosphate) ppm;

The active ester **2**-NHS (or **20**-Su,P(O)(OH)$_2$) was obtained as follows: a freshly prepared solution of TSTU (2 mg, 7 µmol) in DMF (0.2 mL) was added to a suspension of dye **2**-H (1 mg, 2 µmol) in DMF (0.3 mL) containing Et_3N (4 µL, 27 µmol) in an Eppendorf vial with a stirring bar. The vial was flushed with argon and the mixture stirred at r. t. for ca. 30 min, until the reaction is complete (as established by HPLC). The homogeneous solution was fast evaporated to dryness straight from the vial under high vacuum (< 0.2 mbar) and he residue sonificated with EtOAc (0.5 mL). The suspension was centrifuged, the supernatant liquid separated, the residue re-dissolved in DMF (250 µL) and divided into 5 aliquots (50 µL each). The aliquots were evaporated from the vials. The HPLC analysis showed only small amounts of the starting acid and the major peak with t_R =10 min (HPLC, A/B, A = MeCN, B = H_2O + 0.1 M TEAB) 90:10→0:100 in 20 min; HPLC area 97%, detection at 254 nm) or t_R = 11 min (HPLC, *standard analytical program; see General Remarks*). In this case, different to the previously described rhodamine dyes with two primary phosphate groups (*see ref.* [9]) or cyanine dyes decorated with phosphonate groups (*ref.* [10]), the NHS ester at the P-site (phosphate or phosphonate) was not observed to form. The active ester **2**-NHS, being relatively stable in neutral water or PBS, rapidly reacts with ammonia at RT in very dilute solutions (which is not the case for the P-NHS esters, as shown in *ref.* [9]). Apart from the different dye core (oxazine chromophore), the short reaction time and the special treatment of the crude NHS ester (washing with an inert solvent) are obviously important for the selective activation at the C-site (the carboxyl group).

MS (ESI) *m/z* (positive mode,%) = 516 (100%) $[M+H]^+$. HRMS ($C_{29}H_{33}N_4O_9P^+$): 613.1989 (found), 613.1985 (calc.). The solid material is well-soluble in water, PBS, and $NaHCO_3$ solutions. The aliquots (containing approx. 200 µg of the dye active ester each) were evaporated, flushed with argon, and stored at –20 °C. For labelling, the aliquots were re-dissolved in a small amount of DMF (10 µL) and used antibody labelling, according to the standard protocol described (see ref. [1a]) with no difficulties.

Operator: J. Bienert Timebase:HPLC Sequence:Trennung

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Max-Planck-Institut für biophysikalische Chemie - Göttingen
Facility für synthetische Chemie

Probe : **KK106T**

Lösungsmittel : H2O/MeCN **Konzentration :** ? %
Aufgabeart : Handaufgabe **Aufgabemenge :** 1.0 µl

Säule : Kinetex 5µm C18 100
Länge : 25 cm **Innendurchmesser :** 4.6 mm

Fluß (ml / Min) : 1,2
Temperatur : 20 °C

Detektor : UltiMate 3000

Laufmittel : A = Acetonitril B = Wasser 0.1M TEAB

Gradient : A% = 10 B% = 90 ----> A% = 100 B% = 0 T = 20 Min.

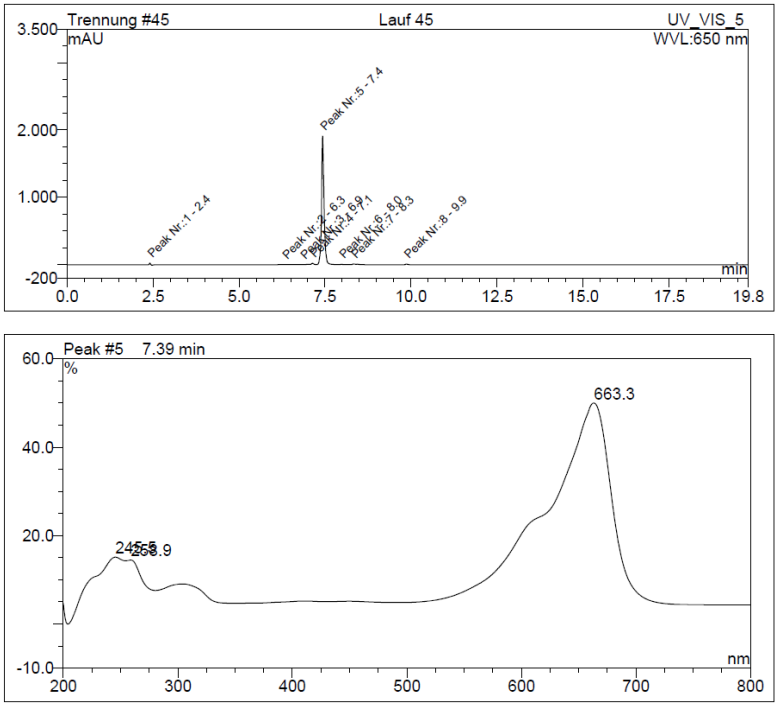

Fig. S04

HPLC analysis and UV/vis spectrum of the dye **2**-H. The analysis was performed at the Chemistry Facility (Max Planck Institute for Biophysical Chemisty, Göttingen) by means of Knauer Smartline semi-preparative high pressure gradient system with two pumps, mixing chamber, column thermostat 4000 and an UV detector UltiMate 3000. Other details are presented herein.

Fig. S05

The dye **KK114** (left) and the far-red-emitting dye **2-**H (right) as aqueous 0.05 mmol/L solutions upon illumination with an incandescent lamp from the bottom. The fluorescence maxima of the dye **2**-H is almost completely shifted to the IR region and therefore *not seen* by a naked eye (λ_{max} fl. at 680 nm). For spectral properties see Table 1 (main text) and the analytical protocol of **2**-H (above).

Fig. S06

The "amphiphilic dyes": **1-**H (λ_{max} abs. at 663 nm, left) and Abberior **STAR 635** (λ_{max} abs. at 635 nm, right; see ref. [4] and compound **6**-H therein for the structure) in aqueous 0.1 mmol/L solutions and after prolonged shaking with an equal volume of a lipophilic solvent (dichloromethane, the lower phase, see the vials in the middle). Note the sufficient solubility of **1-**H in water and the even distribution of both compounds between two phases. See also the subtle difference in the colors and the solvatochromic effect in the case of **1-**H (left).

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