CHEMISTRY
A EUROPEAN JOURNAL

Supporting Information

Polar Red-Emitting Rhodamine Dyes with Reactive Groups: Synthesis, Photophysical Properties, and Two-Color STED Nanoscopy Applications**

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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. Reactions were carried out upon magnetic stirring in Schlenk flasks equipped with septa or reflux condensers with bubble-counters under argon using a standard manifold with vacuum and argon lines. 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; analytical column: Eurospher-100 C18, 5 µm, 250×4 mm, 1.1 mL/min; solvent A: water + 0.1 % v/v trifluoroacetic acid (TFA); solvent B: CH_3CN + 0.1 % v/v TFA; detection at 636 or 254 nm, as specified. Analytical TLC was performed on MERCK ready-to-use plates with regular silica gel 60 (F_{254}) and UV-detector (unless specified otherwise). Preparative column chromatography performed on regular silica gel with the particle size 40--63 µm, unless otherwise stated.

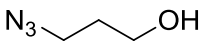
Preparation of mammalian cell samples for confocal and STED microscopy. Staining and sample preparation were carried out according to the standard protocols, described by *C. A. Wurm* and co-workers. [1,2] Primary human dermal fibroblasts and HeLa cells were seeded on cover slips one day before the experiment. After fixation with formaldehyde (4%/ RT/ 5 min) or cold methanol (-20 °C/ 5 min), extraction in 0.5 % Triton X 100 in PBS and blocking in 5% bovine serum albumin in PBS, the cells were incubated with a mouse monoclonal antibody targeting Nup153, Nup214 and Nup62 (Abcam, Cambridge, UK), rabbit polyclonal antibodies targeting Giantin (Abcam), a mouse monoclonal antibody targeting GM130 (BD Biosciences, San Jose, Ca, USA), a mouse monoclonal antibody targeting Vimentin (Sigma-Aldrich St. Louis, MO, USA) or a mouse monoclonal antibody targeting Tubulin (Sigma-Aldrich). The detection of these primary antibodies was performed using secondary antibodies (Dianova, Hamburg, Germany) custom labelled with the dyes Atto590, Atto594, Alexa594, Atto647N (Atto-Tec GmbH, Siegen, Germany), Abberior Star 580 and Star 635P (Abberior GmbH, Göttingen, Germany), KK114 (reference) and the novel dyes described here. Finally, the samples were mounted in Mowiol containing DABCO.

Light microscopy. Single and dual color light microscopy was performed as described. [2,3] In brief, for single color confocal and STED microscopy a home build STED microscope, equipped with a pulsed excitation laser at 640 nm and a Titanium-Sapphire STED laser emitting 760 nm pulses 76 MHz (chirped up to a pulse duration of ~200 ps) was used. Dual color confocal and STED microscopy was performed at a custom build STED microscope as well. The excitation of fluorophores in this microscope was performed using two pulsed excitation lasers at 595 nm and 640 nm. Light from a

frequency-doubled fiber laser emitting 1.2 ns-pulses at 775 nm and 20 MHz repetition rate was used for inhibiting fluorescence (STED) in both color channels.

Red-emitting dyes and their precursors

3-Azidopropan-1-ol (8-OH,N_3)

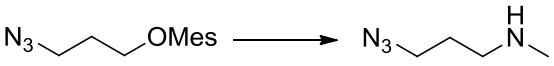


8-OH,N_3

3-Chloropropan-1-ol (**8**-OH,Cl, 3.2 g, 34 mmol) was refluxed upon stirring for 22 h in a solution containing NaN_3 (6.5 g, 100 mmol) and KI (0.1 g) in 20 mL of water. The mixture was cooled to room temperature and extracted with Et_2O (3x50 mL). The combined organic layers were washed with brine and dried with Na_2SO_4. The solvent was carefully removed *in vacuo* (at r.t.) at ca. 100 mbar by means of rotary evaporator to afford 3.42 g of a colourless liquid (94%; purity > 90%, as established by NMR). ESI-MS+: *m*/*z* (rel. int., %) = 101 (100) $[M+H]^+$. ^{1}H-NMR (400 MHz, $CDCl_3$) δ = 1.79–1.85 (m, 2 H, $CH_2C\underline{H}_2CH_2$), 2.52 (s, 1 H, OH), 2.63–2.64 (t, *J* = 4 Hz, $C\underline{H}_2OH$), 3.39–3.42 (t, *J* = 4 Hz, CH_2N_3).

***N*-Methyl-3-azidopropylamine (8-NHMe,N_3)**

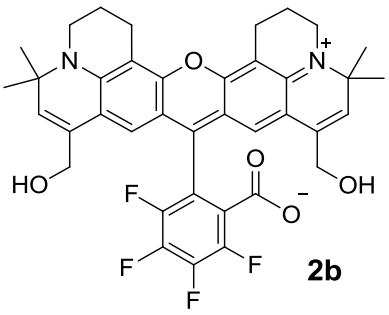
A one-pot synthesis (see also Scheme 4 in the main text)



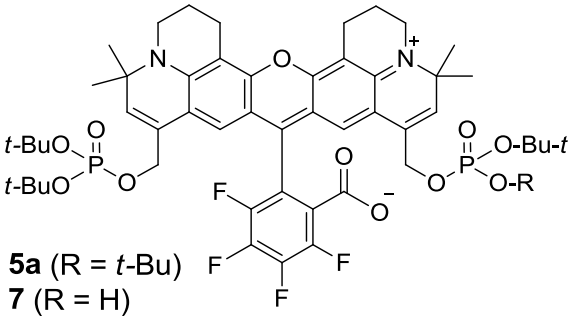
8-Mes,N_3 **8**-NHMe,N_3

The compound was first obtained by *Lebreton et al.* [4] from 1-iodo-3-azidopropane. Their paper also describes a method for conversion of alcohols to azides (via mesylates) which we applied to the preparation of **8**-NHMe,N_3. Our synthesis was performed in a one-pot-fashion as follows: mesyl chloride (0.93 g, 8 mmol) was slowly added to a solution of compound **8**-OH,N_3 (0.52 g, 5.0 mmol) and Et_3N (1.9 mL, 13 mmol) in CH_2Cl_2 (20 mL) upon stirring at 0°C. The reaction mixture was warmed up to r.t. (within ca. 20 min) and evaporated to dryness in rotary evaporator at 10 mbar. The residue was refluxed for 20 h in the mixture of THF (40 mL), Et_3N (0.2 mL, 1.4 mmol), and aqueous (40 wt. %) solution of methylamine (10 mL, 116 mmol) in an oil bath at 65°C. The solution was then evaporated at 90°C (bath temperature) using a distillation bridge *at ambient pressure* to furnish 1.40 g of a colourless liquid that contained 35-40 wt. % of the target compound (full conversion, yield > 90%), as calculated from a ^{1}H-NMR spectrum. The crude compound contains THF and Et_3N. None of these, however, reacts in the further step – amidation of rhodamine-containing building block with an excess of amine (**8**-NHMe,N_3). ESI-MS+: *m*/*z* % = 115 (100%) $[M+H]^+$. ^{1}H-NMR (400 MHz, $CDCl_3$): δ = 1.72–1.79 (m, 2 H, $CH_2C\underline{H}_2CH_2$), 2.43 (s, 3 H, NCH_3), 2.63–2.64 (t, *J* = 4 Hz, $C\underline{H}_2NCH_3$), 3.30–3.38 (t, *J* = 4 Hz, CH_2N_3).

Phosphorylated building block 5a



2b

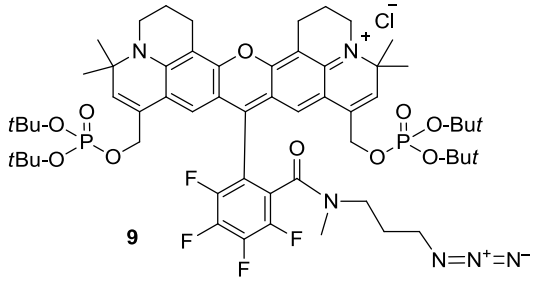


5a (R = *t*-Bu)
7 (R = H)

The title compound was prepared by phosphorylation of compound **2b** [5] with di-*tert*-butyl *N,N*-diisopropyl phosphoramidite, followed by oxidation. In a typical experiment, compound **2b** (90 mg, 0.133 mmol) in CH_2Cl_2 (20 mL) was placed into an argon-flushed dry Schlenk flask equipped with a reflux condenser, magnetic stirring bar, septum, and a bubble counter. 1*H*-Tetrazole solution (0.45 M) in acetonitrile (ALDRICH, 1.05 mL, 0.48 mmol) and di-*tert*-butyl *N,N*-diisopropyl phosphoramidite (180 mg, 0.63 mmol) in CH_2Cl_2 (10 mL) were consecutively added, and the mixture was stirred at reflux for 3 h under argon. The starting compound had completely reacted, as established by HPLC (for **2b** t_R = 10 min, A/B 50:50→0:100 in 25 min, detection at 636 nm). After cooling to r.t., the reflux condenser was disconnected and the solution was chilled with a salt-ice mixture to ca. –10 °C (an argon purge was maintained to prevent condensation of the atmospheric moisture). 3-Chloroperoxybenzoic acid (MCPBA, 0.7 g, 4 mmol) in 20 mL of CH_2Cl_2 was added in one portion, and the stirring continued for 5 min. at –10 °C. The cooling mixture was changed to ice-cold water, while a solution of anhydrous Na_2SO_3 (1.5 g) in water (30 mL) and a saturated solution of $NaHCO_3$ (10 mL) were added. The mixture was stirred slowly for 15–20 min and warmed up to r.t. The organic layer was separated, washed with an equal volume of water, stabilized with Et_3N (50 μL), dried (Na_2SO_4) and evaporated to the volume of ca. 5 mL *in vacuo* at $t \leq 30$°C. The chromatographic isolation was performed over a column with 100 g of SiO_2 using a mixture of $CH_3CN/CH_2Cl_2/H_2O$ (10:1:1) containing 0.2 vol. % Et_3N. The evaporation residue of the worked-up reaction mixture containing the crude compound (see above) was diluted with 20 mL of the liquid phase and loaded onto the column. The homogeneous fractions (HPLC and TLC control; for details, see below) were combined and evaporated ($t < 30$°C) to the volume of ca. 20 mL. The residue was mixed with CH_2Cl_2 (100 mL), water (100 mL), brine (100 mL) and well-shaken. The organic layer separated, dried (Na_2SO_4), filtered through syringe filters (0.45 μm), and evaporated to furnish 94 mg (67%) of compound **5a** as a dark blue amorphous powder. Purity and identity was confirmed by analytical methods. Properties: well-soluble in chlorinated solvents, THF, CH_3CN, and alcohols. Insoluble in water, sufficiently stable towards bases and alkali (unchanged for 3 h); HPLC in a 0.1 M NaOH solution in water reveals no products which can be attributed to the aromatic S_N reactions of the fluorine atoms in a tetrafluoro substituted phenyl ring), yet rapidly decomposes (with the cleavage of *t*-Bu groups) in acidic media, and also on silica gel (stabilization with Et_3N is therefore required). Mass-spectrometry of the decomposition product

suggested structure **7**, where one of the *t*-Bu groups is cleaved. Analytical data on compound **7**: t_R = 16 min (HPLC, A/B 50:50→0:100 in 25 min). MS (ESI) *m*/*z* (negative mode, %) = 1001 (100) $[M - H]^-$, HRMS ($C_{50}H_{60}F_4N_2O_{11}P_2$): 1001.3622 (found for M–H), 1001.3608 (calc.). Analytical data on title compound **5a**: t_R = 22 min (HPLC, A/B 50:50→0:100 in 25 min, HPLC area above 98%, detection at 254 or 636 nm). TLC: R_f = 0.15 (regular silica gel plates, $CH_3CN/CH_2Cl_2/H_2O$ 10:1:1 + 0.2% Et_3N). MS (ESI) *m*/*z* (positive mode, %) = 1081 (80) $[M+Na]^+$, 1059 (15) $[M+H]^+$. HRMS ($C_{54}H_{68}F_4N_2O_{11}P_2Na$): 1081.4130 (found M+Na), 1081.4127 (calc.). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.35/1.39 (s×2, 36 H), 1.41/1.44 (s×2, 12 H), 1.91–2.02 (m, CH_2, 4 H), 2.89 (t, J = 6.5 Hz, 4 H), 3.40–3.48 (m, 4 H), 4.50–4.70 (br. m, 4 H), 5.69 (s, 2 H), 6.89 (s, 2 H) ppm; ^{13}C NMR (100.6 MHz, $CDCl_3$): δ = 20.1/20.7 (CH_3×2), 28.1 (CH_2), 29.6 (CH_2), 31.6 (CH_3), 42.9 (CH_3), 59.0 (CH), 64.9 (C), 82.6 (C), 105.8 (C), 112.2 (C), 119.0 (C), 121.0 (CH_2), 131.8 (CH_2), 149.0 (CH), 152.8 (C), 163.1 (C=O) ppm; ^{19}F NMR (376.4 MHz, $CDCl_3$): δ = –156.52 (t, J = 20.7 Hz, 1 F), –152.90 (dd, J = 23.2, 19.4 Hz, 1 F), –139.78 (dd, J = 21.8, 13.7 Hz, 1 F), –138.30 (m, 1 F) ppm; ^{31}P NMR (161.9 MHz, $CDCl_3$): δ = –9.8 (s, tertiary phosphate) ppm.

Precursor 9

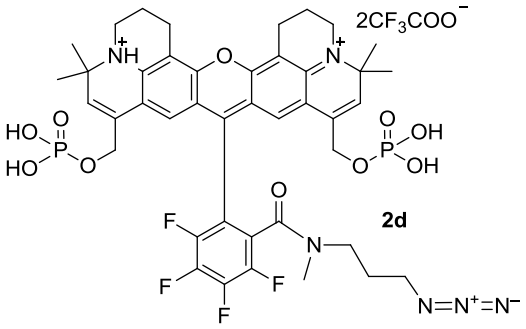


9

Amidation of the rhodamine-containing building block was performed as follows: compound **5a** (30 mg, 0.028 mmol), Et_3N (10 μL, 0.070 mmol), HATU (13 mg, 0.034 mmol), and **8-**NHMe,N_3 (0.1 mmol as 33 mg of a 35-40 wt.% solution in THF; for preparation and structure, see above) were combined in dry CH_3CN (17 mL) under an argon atmosphere at 0°C. After 1 h stirring at this temperature, the reaction was complete, as established by TLC (regular silica gel plates; $CH_3CN/CH_2Cl_2/H_2O$ (10:1:1) + 0.2 vol. % Et_3N; R_f-values for **5a** and **9** are 0.20 and 0.35, respectively) and HPLC (see below). Note that compound **9** is very acid-sensitive (cleavage of the *t*-Bu groups) and decomposes on silica gel or drying agents, so it was crucial to maintain its solutions basic (Et_3N) throughout all the manipulations. The reaction was quenched with CH_2Cl_2 (40 mL), ice-cold water (50 mL), and well-shaken. The organic phase was separated, washed with brine (20 mL), stabilized with Et_3N (10 μL), and dried over Na_2SO_4. The solution was evaporated *in vacuo* to the volume of ca. 10-15 mL at temperatures not exceeding 25°C, and the residue was loaded (as it was, with precipitated colorless salts) onto a column with 35 g of SiO_2 and a mixture of $CH_3CN/CH_2Cl_2/H_2O$ (10:1:1) containing 0.2 vol. % Et_3N. Pure fractions were collected, evaporated to the volume of ca. 30–50 mL *in vacuo* at r.t., mixed up with water (100 mL), brine (150 mL), and CH_2Cl_2 (100 mL). After shaking in a separatory funnel, the organic phase was

separated, dried (Na_2SO_4), filtered through syringe filters (0.45 μm), and evaporated to furnish 26 mg (78%) of compound **9** (presumably with chloride as a counter ion and M = 1191); t_R = 17 min (HPLC, A/B 50:50→0:100 in 25 min; HPLC area above 98%, detection at 636 nm). MS (ESI) *m/z* (positive mode, %) = 1155 (90) $[M]^+$; HRMS ($C_{58}H_{77}F_4N_6O_{10}P_2$): 1155.5124 (found M^+), 1155.5107 (calc.). ^{1}H NMR (400 MHz, $CDCl_3$, *mixture of amide rotamers*): δ = 1.34/1.38 (s×2, 36 H, CH_3), 1.40/1.43 (s×2, 12 H, CH_3), 1.52–1.57 (m, 2 H, CH_2) 2.06 (m, 4 H, CH_2), 2.93 (s, 3 H, NCH_3), 3.02 (m, 4 H, CH_2), 3.27–3.37 (m, 2 H, CH_2), 3.60–3.66 (m, 4 H, CH_2), 4.32–4.51 (br. m, 4 H, 2×CH_2O, 2 H, CH_2N_3), 5.78/5.81 (s×2, 2 H), 6.89/7.07 (s×2, 2 H) ppm; ^{13}C NMR (100.6 MHz, $CDCl_3$): δ = 19.5/19.7 (CH_3×2), 25.8 (CH_3), 28.6 (CH_2), 29.7 (CH_2), 36.6 (CH_2), 43.8 (CH_3), 48.4 (CH_3), 60.1 (CH), 64.9 (C), 65.9 (C), 82.5 (C), 106.1 (C), 107.0 (C), 112.3 (C), 114.4 (C), 119.7 (C), 125.8 (CH_2), 133.6 (CH), 136.7 (CH), 143.0 (C), 151.0 (C), 155.4 (C), 162.2 (CO) ppm; ^{19}F NMR (376.4 MHz, $CDCl_3$): δ = –152.8 (d, *J* = 15.0 Hz, 2 F), –141.4 (m, 1 F), 138.7 (m, 1 F) ppm; ^{31}P NMR (161.9 MHz MHz, $CDCl_3$): δ = –9.3 (s, tertiary phosphate) ppm.

Azide-substituted water-soluble rhodamine dye 2d



The cleavage of the *t*-Bu protecting groups was typically performed as follows: compound **9** (26 mg, 0.022 mmol) was sonicated for 5 min in 0.3 mL of CF_3COOH (in an ultra-sound bath) and left for 2 h at r.t. The completion of the reaction was checked by TLC and HPLC (see below). The solution was then diluted with water (20 mL) and freeze-dried to afford 24.5 mg of a heavy dark-blue crystalline powder (quantative yield of a trifluoroacetate salt, as established by its ^{19}F NMR; M = 1158). Properties of an an "amphiphilic" compound: soluble in water, PBS buffer (pH = 7.4) and $NaHCO_3$ solutions, alcohols, DMF, CH_3CN; insoluble in chlorinated solvents (e.g., CH_2Cl_2, $CHCl_3$) and very slightly soluble in THF. However, compound **2d** can be completely extracted with CH_2Cl_2 from the acidic aqueous solutions containing 5–10 equiv. of CF_3COOH. On the other hand, bases ($NaHCO_3$, Et_3N) keep the dye in the aqueous phase. HPLC: t_R = 9.5 min (A/B 70:30→0:100 in 25 min; HPLC area 99%, detection at 254 nm). TLC: R_f = 0.10 (regular silica gel plates; CH_3CN/H_2O 5:1). MS (ESI) *m/z* (negative mode, %) = 929 (100) $[M–H]^–$. HRMS ($C_{42}H_{44}F_4N_6O_{10}P_2$): 929.2464 (found M–H), 929.2457 (calc.). ^{1}H NMR (400 MHz, CD_3OD *mixture of amide rotamers*): δ = 1.38–1.42 (m, 2 H, CH_2), 1.54 (s, 12 H, CH_3), 2.05 (m, 4 H, CH_2), 2.67–2.78 (m, 2 H, CH_2), 2.84 (s, 3 H, NCH_3), 3.01 (m, 4 H, CH_2), 3.66 (m, 4 H, CH_2), 4.58 (m, 2 H, CH_2N_3), 4.72–4.89 (br. m, 4 H, 2×CH_2O), 5.97 (s, 2 H),

6.96/7.07 (s×2, 2 H), ppm; ^{13}C NMR (100.6 MHz, CD_3OD): δ = 18.8/19.5 (CH_3×2), 28.6 (CH_2), 25.2 (CH_3), 27.2 (CH_2), 34.9 (CH_2), 44.0 (CH_3), 60.1 (CH), 64.9/65.8 (CH), 105.7 (C), 106.7 (C), 113.1 (C), 114.4 (C), 119.6 (C), 121.8 (CH_2), 119.6 (C), 125.5 (CH), 133.7 (C), 136.2 (C), 141.5 (C), 149.9 (C), 152.1 (C), 158.2 (C), 162.5 (C=O) ppm; ^{19}F NMR (376.4 MHz, CD_3OD): δ = –152.7 (m, 2 F), –141.4 (m, 1 F), –138.7 (m, 1 F) , –77.2 (s, 3 F, CF3COO) ppm; ^{31}P NMR (161.9 MHz, CD_3OD): δ = –0.36 (s, primary phosphate) ppm.

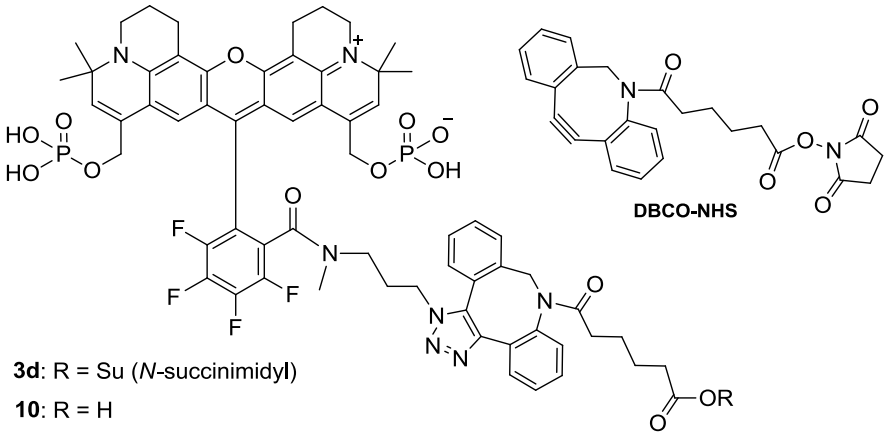
Preparation of dyes 3d and 10 (a click reaction on rhodamine substrate 2d)

In DMF solution, the reaction was performed as follows: azide substrate **2d** (9 mg, 7.8 µmol) and DBCO-NHS reagent (7 mg, 16 µmol, 2 equiv.; supplied by *Jena Bioscience*) were combined and left at r.t. overnight in 0.4 mL of the anhydrous solvent under argon. The reaction mixture was quenched with water (4 mL) and CH_2Cl_2 (40 mL), organic phase (deeply colored) separated and filtered through a small piece of cotton wool rinsed with CH_2Cl_2 beforehand. The reaction product was extracted with a solution of $NaHCO_3$ (3 mg, 35 µmol, 4.5 equiv.) in 15 mL of water, the aqueous phase separated and immediately acidified with an excess of CF_3COOH (10 µL, 130 µmol). The dye was again extracted with CH_2Cl_2 (60 mL), the extract was washed with equal volume of water containing CF_3COOH (10 µL), dried (Na_2SO_4) and carefully evaporated to dryness *in vacuo* to afford 11 mg (94%) of dye **3d** (as a trifluoroacetate; M = 1474), containing ca. 10% of the corresponding acid, as established by HPLC. Analytical data on **3d**: t_R = 11–11.5 min, broad peak (HPLC, A/B 70:30→0:100 in 25 min; HPLC area 90%, detection at 254 nm). TLC: R_f = 0.15 (regular silica gel plates; CH_3CN/H_2O 5:1). MS (ESI) *m/z* (negative mode, %) = 1359 (100) $[M–H]^–$, HRMS ($C_{67}H_{66}F_4N_8O_{15}P_2$): 1359.4087 (found M–H), 1359.4059 (calc.). ^{1}H NMR (400 MHz, CD_3OD, *mixture of amide rotamers*): δ = 1.12–1.59 (m, 4 H, CH_2), 1.57/1.58 (s×2, 12 H, CH_3), 1.63–1.88 (m, 2 H, CH_2), 2.02 (m, 4 H, CH_2), 2.62 (s, 4 H, CH_2CO), 2.73–3.10 (m, 2 H, CH_2), 2.83/2.86/2.89 (s×3, 3 H, NCH_3), 3.01 (m, 4 H, CH_2), 3.41–3.80 (m, 2 H, CH_2), 3.96–4.25 (m, 2 H, CH_2), 3.62 (s, 2 H, CH_2N), 3.66 (m, 4 H, CH_2), 4.55 (m, 2 H, CH_2N=N), 4.72–4.83 (br. m, 4 H, 2×CH_2O), 5.95/6.02 (s×2, 2 H), 6.64 (m, 1 H), 6.86 (m, 1 H), 7.03/7.04 (s×2, 2 H) 7.06–7.65 (m, 6 H) ppm; ^{19}F NMR (376.4 MHz, $CDCl_3$): δ = –152.7 (m, 2 F), –141.5 (m, 1 F), –138.8 (m, 1 F), –77.3 (s, 3 F, CF_3COO) ppm; ^{31}P NMR (161.9 MHz, CD_3OD): δ = –0.46 (s, primary phosphate) ppm.

Free acid (**10**) was obtained as follows: the NHS ester **3d** (10.5 mg, 7 µmol) was dissolved in 4 mL of water containing Na_2CO_3 (11 mg, 0.10 mmol). After standing for 2 h at r.t., the solution was acidified with CF_3COOH (50 µL, 660 µmol), extracted with CH_2Cl_2 (60 mL), the extract dried (Na_2SO_4) and evaporated to afford 9 mg (94%) of dye **10**. Analytical data: t_R = 10.5–11 min, broad peak (HPLC, A/B 70:30→0:100 in 25 min; HPLC area 96%, detection at 254 nm). TLC: R_f = 0.12 (regular silica gel plates; CH_3CN/H_2O 5:1). MS (ESI) *m/z* (positive mode, %) = 1286 (100) $[M + Na]^+$, 1264 (20) $[M + H]^+$. HRMS ($C_{63}H_{63}F_4N_7O_{13}P_2$): 1264.3976 (found M+H), 1264.3968 (calc.). ^{1}H NMR (400 MHz, CD_3OD, *mixture of amide rotamers*): δ = 1.10–1.62 (m, 4 H, CH_2), 1.57 (s, 12 H, CH_3), 1.63–1.88 (m, 2 H, CH_2), 2.02 (m, 4 H, CH_2), 2.73–3.10 (m, 2 H, CH_2), 2.83/2.86/2.89 (s×3, 3 H, NCH_3), 3.01 (m, 4

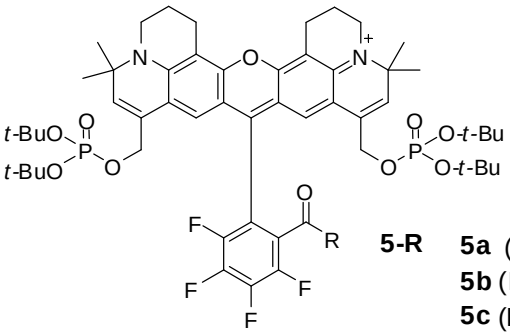
H, CH_2), 3.41–3.80 (m, 2 H, CH_2), 3.96–4.25 (m, 2 H, CH_2), 3. 62 (s, 2 H, CH_2N), 3.66 (m, 4 H, CH_2), 4.54 (m, 2 H, $CH_2N{=}N$), 4.72–4.83 (br. m, 4 H, 2×CH_2O), 5.97/6.03 (s×2, 2 H), 6.64 (m, 1 H), 6.85 (m, 1 H), 7.03/7.04 (s×2, 2 H) 7.06–7.70 (m, 6 H) ppm; ^{19}F NMR (376.4 MHz, $CDCl_3$): δ = –152.9 (m, 2 F), –141.6 (m, 1 F), –138.4 (m, 1 F), –77.3 (s, 3 F, CF_3COO) ppm; ^{31}P NMR (161.9 MHz, CD_3OD): δ = –0.43 (s, primary phosphate) ppm.

Both compounds **10** and **3d** are "amphiphilic": perfectly soluble in water, PBS buffer (pH=7.4) and $NaHCO_3$ solutions, alcohols, DMF, CH_3CN. The solubility and the distribution between aqueous and organic phases strongly depend on pH (data in the main text). These dyes are only slightly soluble in chlorinated solvents (e.g., CH_2Cl_2, $CHCl_3$) and THF. Both compounds can be completely extracted with CH_2Cl_2 from the acidic aqueous solutions containing 5–10 equiv. of CF_3COOH. On the other hand, bases ($NaHCO_3$, Et_3N) and even PBS buffer keep the dye in the aqueous phase. As seen in the photo in the main text, in presence of the acetate buffer or acetic acid, comparable amounts of this dye are distributed between water and CH_2Cl_2.



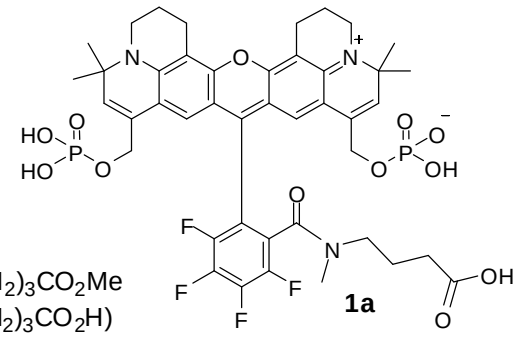
Another protocol for this click reaction utilized aqueous THF as a solvent: azide substrate **2d** (1 mg, 0.9 μmol) and DBCO-NHS reagent (0.9 mg, 2 μmol, 2 equiv.) were dissolved in a mixture of water (50 μL) and THF (100 μL) and left until the reaction was complete (3 h, as established by HPLC). In *aqueous* THF, the reaction is faster than in DMF (usually 8 h were required). The solution was mixed up with water (1 mL), CH_2Cl_2 (1.5 mL), and heptane (0.5 mL). The organic phase (and the excess of the alkyne reagent) was separated, and the aqueous phase extracted with CH_2Cl_2 (2 x 4 mL). The organic extract was thoroughly shaken with water (6 mL), and the dye (**3d**) was completely returned to the aqueous phase, as witnessed by the color. The solution was freeze-dried to afford 1.2 mg of **3d** (90%; as a trifluoroacetate), which contained ca. 15% of the corresponding acid (**10**), due to the hydrolysis. Remarkably, under the conditions of the click reaction (substrate was used as a trifluoroacetate in THF–H_2O, as described above), the hydrolysis was not detected.

An improved synthesis of the water-soluble phosphorylated dye 1a (Star 635P)



5-R

5a (R=OH)
5b (R= $NH(CH_3)(CH_2)_3CO_2Me$
5c (R= $NH(CH_3)(CH_2)_3CO_2H$)



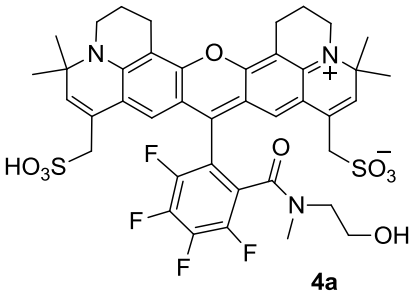
1a

Compound **5a** was amidated with an aminoester $CH_3NH(CH_2)_3CO_2CH_3$ (hydrochloride). The reaction product was subsequently treated with an alkali and trifluoroacetic acid to furnish a red-emitting water-soluble dye **1a**.

To a stirred solution of compound **5a** (30 mg, 0.028 mmol) in DMF (3 mL) under an argon flush at 0°C the following reagents were added one by one in one portion: HATU (60 mg, 0.16 mmol), solid methyl *N*-methyl-ω-aminobutyrate hydrochloride (30 mg, 0.18 mmol), and Et_3N (0.08 mL, 0.55 mmol). The solution was stirred overnight at this temperature, and the completion of the reaction checked by HPLC: for the starting compound (**5a**), t_R = 10 min, and for the reaction product (**5b**), t_R = 13 min (A/B 20:80→0:100 in 25 min; detection at 636 nm). Then CH_2Cl_2 (60 mL), water (60 mL), and saturated $NaHCO_3$ solution (20 mL) were added to the reaction mixture, and it was well-shaken. The organic layer was separated, diluted with hexane (20 mL), washed two times with an equal volume of water, and evaporated to dryness *in vacuo* at temperatures not exceeding 30°C. ESI-MS of crude **5b**: (*m*/*z*, ESI, positive mode, %) = 1173 (100) $[M]^+$ $(C_{60}H_{80}F_4N_3O_{12}P_2)^+$. The residue was dissolved in a mixture of THF (6 mL) and water (12 mL), 0.6 ml of 1 M aq. NaOH (0.60 mmol) was added, and the solution left at r.t. under Ar. The saponification was complete in 3 hours, as established by HPLC. Reaction product **5c**: t_R = 10 min (A/B 20:80→0:100 in 25 min; detection at 636 nm. TLC: $R_f \leq 0.10$ for **5c** and 0.3–0.4 for **5b** (regular silica gel plates; CH_3CN/H_2O 10:1). The solution was neutralized with HOAc (40 mg, 0.66 mmol), mixed with CH_2Cl_2 (80 mL), water (20 mL), brine (40 mL) and well-shaken. The organic layer was separated, and the solvents removed *in vacuo*. MS of crude **5c**: (*m*/*z*, ESI, positive mode, %) = 1159 (100%) $[M+H]^+$, as calc. for $M=C_{59}H_{77}F_4N_3O_{12}P_2$. The product was purified by column chromatography with regular silica gel (20 g) and CH_3CN/H_2O (5:1) containing 0.2 vol. % Et_3N, as a mobile phase. The pure fractions were pooled and evaporated to the volume of ca. 20 mL. The residue was again mixed with CH_2Cl_2 (80 mL), water (20 mL), brine (40 mL), the organic layer separated, dried (Na_2SO_4), filtered of inorganic materials through syringe filters (0.45 µm), and evaporated. The solid residue was dissolved in a mixture of CH_3CN (0.4 mL), water (0.5 mL), and CF_3COOH (0.8 mL), sonicated for 3 min. and left for 2 h at r.t. The completion of the reaction was checked by HPLC; for the reaction product (**1a**) t_R = 6.5 min (A/B 70:30→0:100 in 25 min; detection at

254 nm). The solution was diluted with water (20 mL) and freeze-dried to furnish 21 mg (73%) of **1a** as a trifluoroacetate salt (as established by ^{19}F MNR) with M=1047. The analytical data were identical with these of the previously obtained samples. [5a] However, the extinction quotient (see Table 1, main text) proved to be ca. 20-25 % higher than previously reported, which indicates a higher purity of the new sample.

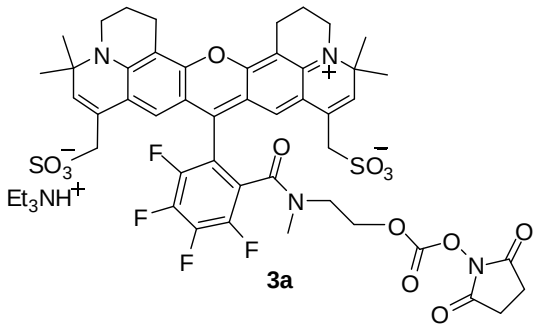
Sulfonated rhodamine precursor 4a



4a

Compound **4a** was prepared by amidation of building block **2c** [5b] by a conventional method, using HATU as a coupling agent. The reaction was best performed as follows: 2-methylamino ethanol (38 mg, 0.50 mmol; VWR International) in 2 mL DMF was added in one portion to a freshly prepared solution of **2c** (60 mg, 0.075 mmol) in DMF (6 mL), also containing Et_3N (0.11 mL, 0.75 mmol) and HATU reagent (150 mg, 0.40 mmol), upon stirring and cooling in an ice bath. The flask was flushed with argon and the stirring continued overnight at 0°. The solution was diluted with water (25 mL), acidified with CF_3COOH (0.4 mL) and loaded straight onto a column with reverse-phase silica gel (Polygoprep 60-50 C_{18}, 30 g, Macherey-Nagel). The column was eluted with pure water (containing 0.1% v/v CF_3COOH), to which CH_3CN was gradually added, until the ratio CH_3CN/H_2O reached 1:1. The colored fraction was collected and evaporated *in vacuo* at temperatures not exceeding 40°C. To obtain an extra pure sample (with 98% HPLC area and clean ^{1}H-NMR spectrum), the chromatographic purification was repeated over 20 g reverse-phase silica gel. The pure fractions (HPLC with detection at 254 nm) were pooled, filtered from inorganic materials through syringe filters (0.45 µm), concentrated and freeze-dried to afford 49 mg of **4a** (67%, as trifluoroacetate with M = 973). HPLC: t_R = 6.5 min (A/B 70:30→0:100 in 25 min; detection at 254 nm; for the starting material **2c** – 8 min). TLC: R_f = 0.2–0.3 (regular silica gel plates; CH_3OH/CH_2Cl_2 1:3). MS (ESI) *m/z* (negative mode, %) = 858 (100) $[M–H]^–$, HRMS ($C_{41}H_{41}F_4N_3O_9S_2$): 858.2163 (found M–H), 858.2148 (calc.). ^{1}H NMR (400 MHz, CD_3OD, *mixture of amide rotamers*) δ = 1.52/1.54 (s, 12 H, 4×CH_3), 2.03 (m, 4 H, 2×CH_2), 2.66/2.76 (s, 3 H, NCH_3), 2.99 (m, 4 H, 2×CH_2N), 3.05–3.56 (m, 2 H, NCH_2), 3.76 (m, 4 H, CH_2SO_3), 4.12–4.31 (m, 2 H, CH_2OH), 5.84 (s, 2 H, 2×CH=), 7.31/7.35/7.40 (s, 2 H) ppm; ^{13}C NMR (100.6 MHz, CD_3OD) δ = 19.5/20.2 (CH_3), 26.5 (CH_2), 31.9 (CH_2), 38.2 (CH_2), 43.1 (CH_3), 58.0 (CH_2), 59.4 (CH_2), 105.5/106.2 (C), 113.2 (C), 121.1 (CH), 121.3 (CH), 123.2 (C), 123.8 (CH), 125.0 (C), 136.5 (C), 150.2 (C), 152.6 (C) ppm; ^{19}F NMR (376.4 MHz, CD_3OD): δ = –154.6 (dt, *J* = 234.8, 19.3 Hz, 1 F), –153.2/ –152.5 (m, 1 F), –142.0/ –139.3 (m, 1 F), –137.3 (d, *J* = 148.3 Hz, 1 F) ppm.

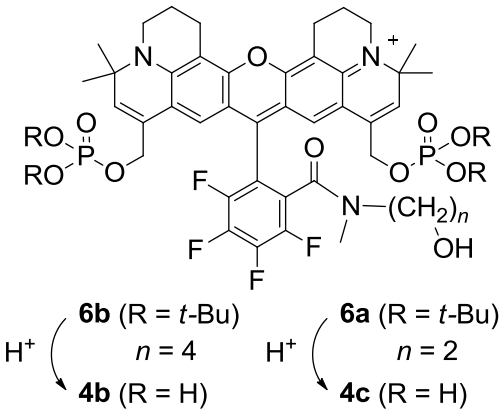
Amino-reactive sulfonated rhodamine marker 3a



3a

Solid *O,O'*-di(*N*-succinimidyl) carbonate (DSC; 40 mg, 0.16 mmol) was added to a solution of compound **4a** (10 mg, 0.01 mmol) and Et_3N (30 µL, 0.21 mmol) in dry CH_3CN (10 mL) in a small flask. The flask was sealed with a septum, flushed with argon, and sonicated for few minutes until the solid had dissolved. The solution was stirred overnight at r.t. and heated for 1 h at 45°C to complete the reaction (HPLC control). The solution was loaded straight onto a column with silica gel (25 g) and the elution was performed with $CH_3CN/CH_2Cl_2/H_2O$ (10:1:1→7:1:1) as the mobile phase. Pure fractions were pooled, filtered through syringe filters (0.45 µm), and evaporated *in vacuo* at temperatures not exceeding 20°C. The solid residue was dissolved in dry CH_3CN (15 mL) and centrifuged to completely separate the silica gel. The solution was evaporated, dissolved in water containing Et_3N (10 µL), and freeze-dried to afford 9.6 mg (87%, a triethyl ammonium salt, as established by ^1H NMR). Properties: a dark-blue heavy crystalline powder, well-soluble in water, alcohols, sparingly soluble in CH_3CN, insoluble in CH_2Cl_2. Stable in aqueous solutions, even in the presence of bases, yet rapidly and completely reacts with NH_3 (as established by HPLC analysis). **3a**: t_R = 6 min (HPLC, A/B 70:30→0:100 in 25 min; HPLC area 99%); TLC: R_f = 0.15 (regular silica gel plates; $CH_3CN/CH_2Cl_2/H_2O$ 10:1:1 + 0.2% Et_3N); MS (ESI) *m/z* (positive mode, %) = 1023 (100) $[M+Na]^+$; HRMS ($C_{46}H_{44}F_4N_4O_{13}S_2$): 1023.2178 (found M+Na), 1023.2175 (calc.). ^1H NMR (400 MHz, CD_3OD, *mixture of amide rotamers*) δ = 1.25 (t, J = 7.3 Hz, 9 H, $C\underline{H}_3CH_2$) 1.53 (s, 12 H, CH_3), 2.03 (m, 4 H, 2×CH_2), 2.61 (s, 4 H, CH_2CO), 2.69/2.75 (s×2, 3 H, NCH_3), 2.98 (m, 4 H, 2×CH_2N), 3.13 (q, J = 7.3 Hz, 6 H, $CH_3C\underline{H}_2$), 3.21–3.55 (m, 2 H, $NC\underline{H}_2CH_2$), 3.65–3.80 (m, 4 H CH_2SO_3), 4.27 (m, 2 H, CH_2O), 5.84 (s, 2 H), 7.33–7.41 (s, 2 H) ppm; ^{13}C NMR (100.6 MHz, CD_3OD) δ = 19.4/19.8 (CH_3), 24.6 (CH_3), 26.1 (CH_2), 31.7 (CH_2), 38.1 (CH_2), 43.0 (CH_3), 58.3 (CH_3), 59.4 (CH_3), 105.1/106.1 (C), 113.2 (C), 120.1 (CH), 121.0 (CH), 123.6 (C), 125.0 (C), 136.1 (C), 137.1 (C), 151.0 (C), 153.5 (C) ppm; ^{19}F NMR (376.4 MHz, CD_3OD): δ = –154.5/–154.0 (m, 1 F), –153.2/ –152.2 (m, 1 F), –142.0/ –141.5/–139.4/–136.5/–137.2 (m, 2 F) ppm.

Alcohols 4b and 4c



The title compounds were obtained via amidation of precursor **5a** followed by cleavage of the protective *tert*-butyl groups. For compounds **6b** and **4b** (see structure above) the synthesis was performed as follows: compound **5a** (35 mg, 0.033 mmol) was dissolved in dry DMF (6 mL) containing Et_3N (70 μL, 0.50 mmol) in an argon-flushed flask sealed with a septum. Upon stirring and cooling in an ice bath, solid HATU reagent (76 mg, 0.20 mmol) was added in one portion, the flask flushed again with argon, and, 10 min later, a solution of 4-(methylamino)butan-1-ol (26 mg, 0.25 mmol; the preparation is described below) in 1 mL of CH_2Cl_2 was promptly introduced through a syringe. The stirring continued at r.t. for 3 h or longer. The completion of the reaction was established by HPLC: for the starting compound (**5a**), t_R = 10 min, and for the reaction product (**6b**) - t_R = 12 min (A/B 20:80→0:100 in 25 min; detection at 636 nm). TLC (regular silica gel plates; MeOH/CH_2Cl_2 20:1): R_f-values for **5a** and **6b** are 0.10 and 0.20, respectively; a "blue shift" in the reaction product's color is also observed. The reaction mixture was quenched with CH_2Cl_2 (150 mL), water (80 mL), saturated $NaHCO_3$ solution (20 mL) and well-shaken. The organic layer was separated and washed first with an equal volume of water, then with a solution containing citric acid (0.5 g) in a mixture of water (150 mL) and brine (50 mL), then once again with water (50mL), stabilized with Et_3N (50 μL), and, finally, dried over Na_2SO_4. The solution was evaporated to the volume of ca. 3--5 mL *in vacuo* at temperatures not exceeding 30°C. The residue was diluted with CH_3CN/H_2O (10:1, 15 mL), loaded onto a column containing regular silica gel (25 g), and eluted with CH_3CN/H_2O (10:1→3:1) + 0.2 vol. % Et_3N, as a mobile phase. The homogeneous fractions (analyzed by HPLC) were pooled and concentrated to the volume of ca. 50 mL *in vacuo* at temperatures not exceeding 35°C. The residue was mixed up with brine 100 mL, water, 100 mL, CH_2Cl_2 (150 mL), and well-shaken. The organic layer was separated, and the aqueous layer extracted again with CH_2Cl_2 (100 mL). The combined extract was dried (Na_2SO_4), filtered through a syringe filter (0.45 μm), and evaporated to furnish 31 mg (80%) of **6b** as an amorphous dark blue solid. Analytical data on **6b**: MS spectrum; (*m*/*z*, ESI, positive mode, %) = 1144 (100) $[M]^+$, HRMS ($C_{59}H_{80}F_4N_3O_{11}P_2$): 144.5187 (found for M^+), 144.5199 (calc. for $C_{59}H_{80}F_4N_3O_{11}P_2$); TLC: R_f = 0.6 (regular silica gel plates; CH_3OH/CH_2Cl_2 1:5 + 0.2% Et_3N). Similarly to compound **5a** (see above), **6b** is unstable on silica gel and temperature-sensitive. As a cationic

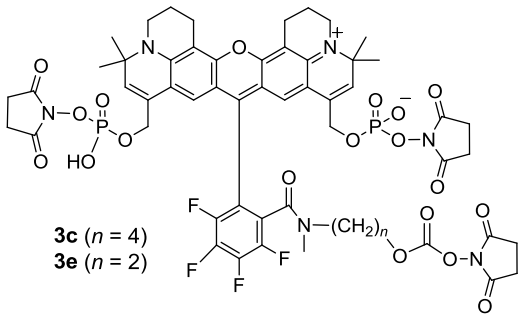
dye, compound **6b** may form two or even more zones, while performing analytical or preparative chromatography due the different counter ions that occur in the reaction mixture. On the other hand, the deprotected compound (**4b**) exists as a zwitter-ion, which is stable and relatively easy to purify and analyse.

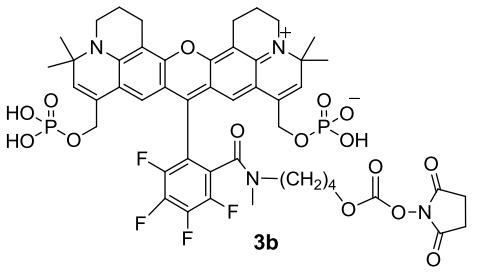
The deprotection (cleavage of the *t*-Bu groups) was done as follows: compound **6b** (31 mg) was dissolved in a mixture of CH_3CN (0.4 mL), water (0.8 mL), and CF_3COOH (0.6 mL), sonicated for 3 min., and left at r.t. overnight. The solution was diluted with water (40 mL) and freeze-dried. The deprotection reaction was monitored by HPLC and TLC (see below). The HPLC analysis of the reaction mixture (A/B 70:30→0:100 in 25 min; detection at 254 nm) reveals two peaks: at 6.5 and 11 min, respectively (in the ratio 3:1), while the starting material **6b** (t_R = 24--25 min) had completely vanished. The less polar peak at 11 min might be attributed to the product of a reversible cyclization at the OH and the $OP(OH)_2$ sites (see structure above), which is cleaved in a basic media. The solid residue was re-dissolved in a mixture of CH_3CN (10 mL), water (2mL), and % Et_3N (0.3 mL), left for 1 h, and evaporated to dryness *in vacuo*. The HPLC analysis of the dry residue revealed only one peak at 7 min (see above for conditions). The product was purified by column chromatography with regular silica gel (18 g) and CH_3CN/H_2O (7:1→3:1) as a mobile phase. The homogeneous fractions were pooled and evaporated *in vacuo* at temperatures not exceeding 35°C. The solid was re-dissolved in CH_3CN/H_2O (5:1, 20 mL), centrifuged, separated from the precipitate (silica gel) and additionally filtered through a syringe filter (0.22 µm). The evaporation afforded 21 mg (69%, over two steps) of **4b** as a dark blue crystalline solid, very slightly soluble in CH_3CN, better in DMF and alcohols, well-soluble in water.

Analytical data on **4b**: t_R = 6.5 min (HPLC, A/B 70:30→0:100 in 25 min; detection at 254 nm), TLC: R_f = 0.10 (regular silica gel plates; CH_3CN/H_2O 4:1); MS (ESI) *m/z* (negative mode, %) = 918 (100) $[M–H]^-$, HRMS ($C_{43}H_{47}F_4N_3O_{11}P_2$): 918.2542 (found M–H), 918.2549 (calc.). ^{1}H NMR (400 MHz, CD_3OD, *mixture of amide rotamers*) δ = 1.11–1.39 (m, 2 H, CH_2), 1.53 (s, 12 H, CH_3), 2.04 (m, 4 H, CH_2), 2.82–2.91 (m, 2 H, CH_2), 2.86 (s, 3 H, NCH_3), 3.01 (m, 4 H, CH_2), 3.23–3.28 (m, 2 H, CH_2), 3.42–3.53 (m, 2 H, CH_2), 4.43 (m, 2 H, CH_2O), 4.57 (m, 4 H, 2×CH_2OH), 5.96/6.01, (2×s, 2 H), 6.82/6.91 (2×s, 2 H) ppm; ^{13}C NMR (100.6 MHz, CD_3OD) δ = 19.5/20.1 (CH_3×2), 22.4 (CH_3), 27.3 (CH_2), 27.2 (CH_2), 28.7 (CH_3), 36.7 (CH_2), 43.4 (CH_3), 59.5 (CH_2), 60.6 (CH_2), 62.3/63.8 (CH_2), 105.7 (C), 106.3 (C), 109.8 (CH), 114.2 (CH), 119.7 (C), 120.5 (C), 119.6 (C), 126.3 (C), 131.4 (C), 133.8 (C), 150.2 (C), 150.6 (C), 152.9 (C), 162.8 (C=O) ppm; ^{31}P NMR (161.9 MHz, CD_3OD): δ = –0.83 (s, primary phosphate) ppm; ^{19}F NMR (376.4 MHz, CD_3OD): δ = 153.4 (m, 1 F), 152.5 (m, 1 F), 140.8 (m, 1 F), 139.0 (m, 1 F) ppm. Compound **4c** (see structure above and the main text for discussion) was synthesized in exactly same fashion, using commercially available 2-methylamino ethanol for amidation. Purity and identity of the compound was confirmed by proper analytical methods.

Reactive markers 3c and 3e

Alcohol **4b** was treated with and *O,O'*-di(*N*-succinimidyl) carbonate (DSC) to form mixed carbonate **3c** as the major product. The photo below shows the red fluorescence of dye **3c** (10 μM) in aqueous solution illuminated with an incandescent lamp. Dyes **1a**,**b**, **2d, 3a**,**c**,**d** and Abberior Star635 (Scheme 6 in the main text) are spectrally identical and behave in exactly the same way (see Table 1 in the main text and ref. [5a] for spectral properties).





In a typical experiment, *O,O'*-di(*N*-succinimidyl) carbonate (DSC reagent, 70 mg, 0.28 mmol) was added in one portion to a solution containing compound **4b** (7 mg, 7.6 μmol) and Et_3N (30 μL, 20 mmol) in dry DMF (3 mL). The flask was flushed with argon through a septum, sonicated for 10 min until all solids had completely dissolved, and left with stirring for 1 or 2 days at r.t, until the substrate had completely reacted to give compound **3c** predominantly, as established by HPLC and TLC (see below for details). The solution was diluted with two volumes of CH_3CN and loaded straight onto a column with regular silica gel (15 g). The elution was performed with CH_3CN/H_2O (10:1→6:1) as a mobile phase (no Et_3N was added). Pure fractions were pooled, filtered through syringe filters (0.45 μm), and evaporated *in vacuo* at temperatures not exceeding 25°C. The solid residue was dissolved in dry CH_3CN (10 mL) and centrifuged to completely separate the silica gel. The supernatant solution was separated, and the solvent was removed to afford 7.7 mg (80%) of **3c** (as a free secondary phosphate, as established by ^1H and ^{31}P NMR spectroscopy). Properties: amorphous dark blue solid, perfectly soluble in water, alcohols, CH_3CN, slightly soluble in acetone, almost insoluble in CH_2Cl_2. Analytical data on **3c**: t_R = 7 min (HPLC, A/B 70:30→0:100 in 25 min; detection at 254 nm, HPLC area above 97%), TLC: R_f = 0.60 (regular silica gel plates; CH_3CN/H_2O 5:1); MS (ESI) *m/z* (negative mode, %) = 1253 (100) $[M–H]^-$; HRMS ($C_{56}H_{56}F_4N_6O_{19}P_2$): 1253.2927 (found for M–H), 1253.2939 (calc.). ^1H NMR (400 MHz, CD_3OD, *mixture of amide rotamers*): δ = 1.20 (t, J = 7.4 Hz, 9 H, Et) 1.24–1.33 (m, 2 H, CH_2), 1.55 (s, 12 H, CH_3), 2.06 (m, 4 H, CH_2), 2.53 (s, 12 H, CH_2CO), 2.82–2.91 (m, 2 H, CH_2), 2.77 (s,

3 H, NCH_3), 2.98 (m, 4 H, CH_2), 3,10 (q, *J* = 7.4 Hz, 6 H, Et), 3.23–3.28 (m, 2 H, CH_2), 3.98–4.11 (m, 2 H, CH_2), 4.54–4.79 (m, 4 H, CH_2O), 5.93, (s, 2 H, CH=), 6.85/7.02 (2×s, 2 H) ppm; ^{31}P NMR (161.9 MHz, CD_3CN): δ = –2.93 (s) ppm; ^{19}F NMR (376.4 MHz, CD_3CN): δ = 154.1 (m, 1 F), 153.8 (m, 1 F), 141.5 (s, 1 F), 138.6 (d, *J* = 20.4 Hz, 1 F). Data on the hydrolytic stability of **3c**: in a 50 µmol aqueous solution, some 30–40% of the substrate is hydrolyzed after being left for one day at r. t. in pure water, and some 70–80% in aqueous PBS buffer (pH = 7.4), respectively.

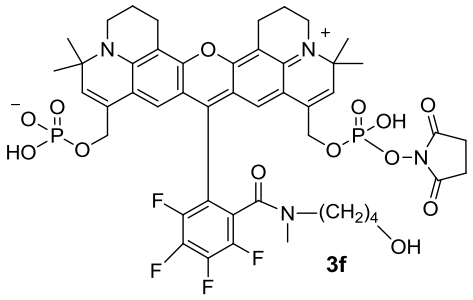
In the reaction with *O,O'*-di(*N*-succinimidyl) carbonate, the substrate with a shorter linker (**4c**, see structure above) proved to be far less active than **4b**. Under the conditions and scale described above, the reaction was not complete even after 3 days at r.t., and heating for 2 h at 50°C was required. Under these conditions, the mono-substituted NHS ester (analog of **3b** with a shorter linker) was not detected among the reaction products (as established by the mass-spectroscopic analysis of the fractions obtained in the course of column chromatography). Compound **3e** (tris-NHS derivative) was the major product, whose constitution was confirmed by ESI-MS: *m*/*z* (negative mode, %) = 1225 (100) $[M–H]^-$, HRMS ($C_{54}H_{52}F_4N_6O_{19}P_2$): 1225.2661 (found for M–H), 1225.2626 (calc.). However, the reaction was not always reproducible and the yield of **3e** always remained modest. Therefore, this dye was not used for bioconjugation and imaging.

4-(Methylamino)butan-1-ol (the linker) was obtained by reduction of the commercially available 4-(methylamino)butyric acid as follows. Into an argon-flushed Schlenk flask (0.5 L) containing dry diethyl ether (100 mL), was carefully added upon stirring $LiAlH_4$ (1.5 g, 40 mmol) and then finely ground 4-(methylamino)butyric acid hydrochloride (2.0 g, 13 mmol) through a connector with two cones, one being attached to a reflux condenser (CAUTION: hydrogen gas is evolved!) with a bubble counter mounted on the top. After the reagents were added, the cone was sealed, and the mixture was kept under reflux overnight with a slow argon flow. The content of the flask was carefully poured into a 0.5 L Erlenmeyer flask containing an ice-cold 30 wt. % solution of NaOH (100 mL). The solution was extracted with Et_2O (2×50 mL), the extract dried (NaOH), evaporated to the volume of 20–30 mL, filtered through a cotton wool, and further concentrated to 3–5 mL. In a 25-mL flask, the residual solvent (and other volatile compounds) were completely removed *in vacuo* at ca. 10 mbar, and 4-(methylamino)butan-1-ol was distilled “bulb-to-bulb”, using a small Schlenk flask as a receiver (the distillation flask was heated by a heat gun to 130–140°C, and the receiver cooled with ice). The distillation at ca. 10 mbar afforded 330 mg (50%) of the title compound as a colorless liquid. MS (ESI) *m*/*z* (positive mode, %) = 104 (100) $[M+H]^+$. ^1H-NMR (400 MHz, $CDCl_3$): δ = 1.53 (m, 4 H, CH_2), 2.32 (s, 3 H, NCH_3), 2.52 (t, *J* = 5 Hz, 2 H, CH_2N), 3.48 (m, 2 H, CH_2OH), 3.70 (br. s, 1 H, OH) ppm; ^{13}C NMR (75.5 MHz, $CDCl_3$) δ = 27.85 (CH_2), 31.97 (CH_2), 35.67 (CH_3), 51.57 (CH_2), 62.19 (CH_2) ppm.

Mono-substituted NHS ester 3b

The title compound was isolated in a poor yield as an unstable intermediate in the reaction of alcohol **4b** and *O,O'*-di(*N*-succinimidyl) carbonate. The reaction was stopped when the starting material had completely reacted. Otherwise, it would have been extremely difficult to chromatographically separate

3b from **4b,** due to the high polarity of two primary phosphate groups and only very subtle difference in the dye linkers. In a typical experiment, *O,O'*-di(*N*-succinimidyl) carbonate (15 mg, 60 μmmol) was added in one portion to the solution of **4b** (3 mg, 3.3 μmmol) and Et_3N (9 μL, 62 mmol) in 0.5 mL of DMF. The solution was sonicated for few minutes in a sealed vial and stirred for 2–3 h, until the starting material disappeared (HPLC monitoring: A/B 70:30→0:100 in 25 min, detection at 636 nm; t_R = 6.5 min for the starting material (**4b**), 8 min for **3b**, and 7 min for **3c** (major product; see above). The reaction mixture was immediately loaded onto a column with silica gel (5 g) and the elution was performed with CH_3CN/H_2O (10:1→5:1) as the mobile phase (with no Et_3N was added). The first fractions contained compound **3c**, the following (more polar) fractions were analyzed, collected, filtered as described above, acidified with CF_3COOH (1 μL), centrifuged, concentrated at temperatures not exceeding 20°C, and freeze-dried to afford 0.8–1 mg of **3b** (yield ca. 30%, with purity ~70%, as established by HPLC). MS (ESI) *m/z* (negative mode, %) = 1059 (80) $[M–H]^–$; HRMS ($C_{48}H_{50}F_4N_4O_{15}P_2$): 1059.2623 (found for M–H), 1059.2606 (calc.). This material was used for labeling of the secondary antibodies as it is. Properties: amorphous dark blue solid, perfectly soluble in water, alcohols, CH_3CN, insoluble in acetone and CH_2Cl_2. In aqueous solutions, especially under basic conditions (Et_3N or $NaHCO_3$), the NHS moiety in compound **3b** migrates (with decarboxylation) to the phosphate site to form a "P-NHS ester" of structure **3f** (see the structure below). Particularly, if an experiment is performed as described above on the same scale, but triethylamine (Et_3N, 3 μL) is added instead of CF_3COOH, the product is completely converted to **3f** after freeze-drying overnight. Analytical data on **3f**: t_R = 6.5 min (HPLC, A/B 70:30→0:100 in 25 min; detection at 254 nm, HPLC area above 90%), TLC: R_f = 0.10 (regular silica gel plates; CH_3CN/H_2O 5:1); MS (ESI) *m/z* (negative mode, %) = 1015 (80) $[M–H]^–$; HRMS ($C_{47}H_{50}F_4N_4O_{13}P_2$): 1015.2737 (found for M–H), 1015.2713 (calc.).

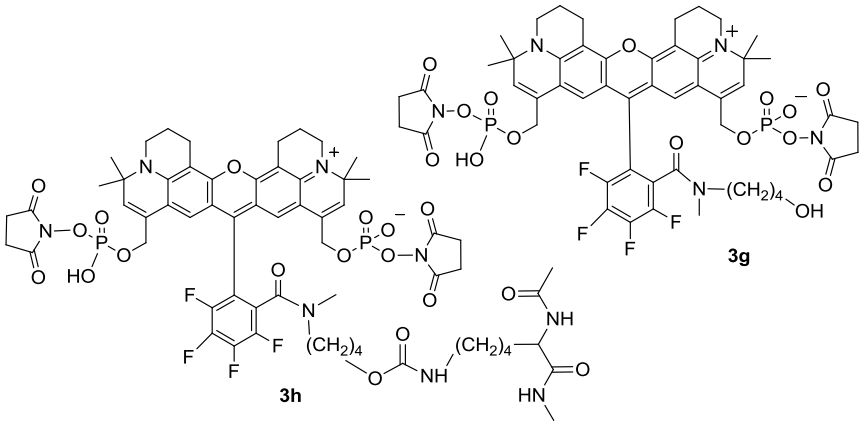


3f

Test reaction of 3c with a model amino acid derivative

The reaction was performed as follows: 12 equiv. of Ac-Lys-NHMe (Bachem) was added to a 1 equiv. of the substrate (**3c**) as a 0.5 mmol solution in: a) aqueous (70% v/v) CH_3CN, b) 0.1 M aq. $NaHCO_3$, and c) 0.1 M aq. $Et_3N*H_2CO_3$ buffer. The reaction in CH_3CN led in 20 min to a single product – **3h**, where P-NHS sites remained unreacted (see the structure below). In aqueous carbonate buffers, which imitate the standard conditions of the antibody labeling, a significant amounts of the hydrolysis product **3g** (40–60%) were detected. The same compound was isolated as the single reaction product in a

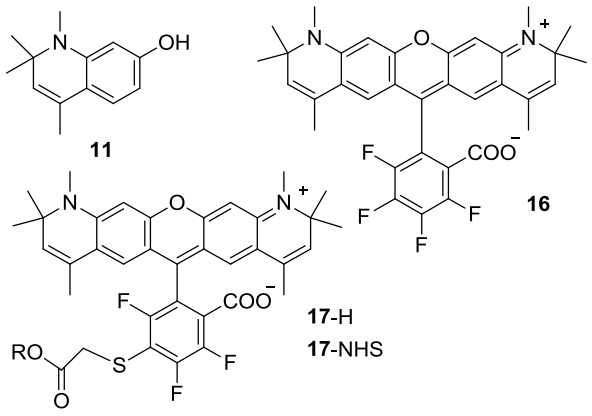
control experiment in pure water (1 day exposure) or in $NaHCO_3$ solution (3 h at r.t.) with no amine added.



Analytical data for **3h**: t_R = 6.2 min (HPLC, A/B 70:30→0:100 in 25 min; detection at 254 nm, HPLC area 98%); MS (ESI) *m/z* (positive mode, %) = 1363 (80) $[M+Na]^+$, HRMS ($C_{61}H_{70}F_4N_8O_{18}P_2$): 1363.4100 (found for M+Na), 1363.4112 (calc.). Analytical data for **3g**: t_R = 5.8 min (HPLC, A/B 70:30→0:100 in 25 min; detection at 254 nm, HPLC area 96%); MS (ESI) *m/z* (negative mode, %) = 1112 (100) HRMS ($C_{51}H_{53}F_4N_5O_{18}P_2$): 1112.2864 (found for M–H), 1112.2877 (calc.).

Fluorine-substituted rhodamine dye 16

Tetrafluoro phtalic anhydride (1.05 g, 4.80 mmol) and phenol **11** [6] (1.30 g, 6.40 mmol) were dissolved and refluxed for 15 h in propionic acid (8 mL) containing *p*-toluene sulfonic acid (0.10 g) under an argon atmosphere. The reaction mixture was evaporated to dryness, the residue chromatographed over 160 g of silica gel with acetone/MeOH (4:1) as the mobile phase, and the fractions containing a blue fluorescent dye combined and evaporated. An additional purification over 300 g of silica gel with CH_2Cl_2/MeOH (10:1) as the mobile phase afforded 390 mg (14%) of **16** as a dark blue heavy crystalline powder. Its purity and identity was confirmed by analytical methods. [6]



Rhodamine 17-H with thiogycolic acid residue and three fluorine atoms

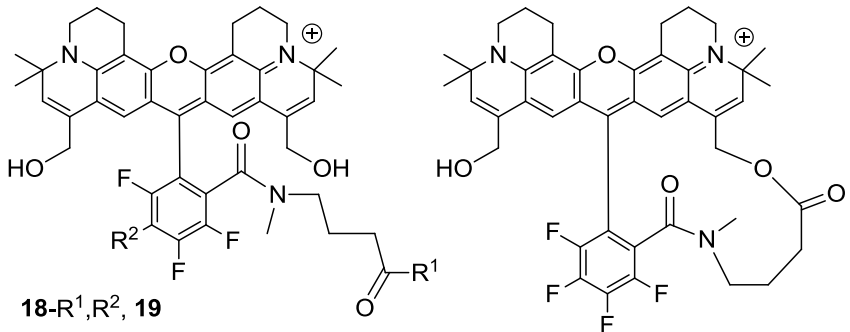
The substitution of one fluorine atom in **16** (see the structure above) was performed as follows: thioglycolic acid (11 mg, 0.12 mmol) in CH_3CN (1 mL) was added in one portion to an ice-cold stirred solution of **16** (18 mg, 0.03 mmol) and Et_3N (0.05 mL, 0.35 mmol) in 6 mL of acetonitrile. The stirring was continued for 3 h at 0±5°C until the reaction was complete (TLC control on regular silica gel plates with Me/CH_2Cl_2 (1:5); for **17**-H**,** R_f = 0.20, which is lower than R_f of **16**). The reaction mixture was diluted with equal volume of CH_2Cl_2 and loaded onto a column with silica gel (35 g). The elution was performed first with MeOH/CH_2Cl_2 (1:6) then with MeOH/CH_2Cl_2/H_2O (20:7:1) containing 0.2% v/v Et_3N. Pure fractions were pooled, filtered of silica gel, and evaporated to afford 19 mg of **17**-H (83%, as a Et_3N salt (according to NMR spectra) with MW = 763). Properties: a dark blue powder, slightly soluble in water, perfectly soluble in basic aqueous solutions, well soluble in alcohols and CH_2Cl_2, sparingly soluble in CH_3CN. Analytical data for **17**-H: t_R = 10 min (HPLC, A/B 50:50→0:100 in 25 min; detection at 254 nm, HPLC area 98%). MS (ESI) *m*/*z* (positive mode, %) = 663 (100) $[M+H]^+$; HRMS: ($C_{36}H_{33}F_3N_2O_5S$) 662.2141 (found for M+H) 1023.2135 (calc.). ^{1}H NMR (400 MHz, DMSO-d_6): δ = 1.05 (t, J = 7.2 Hz, 9 H, Et) 1.37 (s, 12 H, CH_3), 1.81 (m, 6 H, CH_3), 2.66 (q, J = 7.2 Hz, 6 H, Et), 2.97 (s, 6 H, NCH_3), 3.50 (s, 2 H, CH_2S), 5.49 (q, J = 1.5 Hz, 2 H), 6.51/6.63 (2×s, 4 H) ppm; ^{13}C NMR (100.6 MHz, DMSO-d_6) δ = 18.4 (CH_3), 28.8 (CH_3), 32.4 (CH_3), 45.1 (CH_2), 58.3 (CH_2), 96.1 (C), 96.3 (C), 109.4 (C), 109.7 (C), 117.3 (CH), 121.5 (C), 121.9 (CH), 125.2 (C), 131.5 (CH), 151.0 (C), 155.6 (C), 169.8 (C=O) ppm; ^{19}F NMR (376.4 MHz, DMSO-d_6): δ = 143.4 (dd, J = 25.8, 17.3 Hz, 1 F), 125.8 (dd, J = 25.5, 3.6 Hz, 1 F), 112.4 (d, J = 17.1 Hz, 1 F) ppm.

***N*-hydroxysuccinimidyl ester 17-NHS**

HATU reagent (3.8 mg, 10 µmol) was added to a stirred solution of **17**-H (2 mg, 3 µmol), *N*-hydroxysuccinimide (3.6 mg, 18 µmol), and Et_3N (3 µL, 20 µmol) in a mixture of dry CH_3CN (3 mL) and CH_2Cl_2 (1 mL) at 0+5°C. The solution was stirred under an argon atmosphere for 1 h at 0+5°C, until the reaction was complete (TLC, HPLC) and loaded straight onto a column with silica gel (3 g), and the elution was performed with CH_3CN/CH_2Cl_2/H_2O (10:1:1) as the mobile phase (no Et_3N was added). Pure fractions were pooled, filtered through syringe filters (0.45 µm), and evaporated *in vacuo* at temperatures not exceeding 20°C. The solid residue was dissolved in dry acetone (10 mL) and centrifuged to completely separate the silica gel. The solvent was removed to afford 1.7 mg (80%) of **17**-NHS. Properties: a dark blue powder, slightly soluble in water, well-soluble in most organic solvents, except alkanes. Analytical data: t_R = 16 min (HPLC, A/B 50:50→0:100 in 25 min; detection at 254 nm, HPLC area 98%). MS (ESI) *m*/*z* (positive mode, %) = 760 (100) $[M+H]^+$; HRMS: ($C_{40}H_{36}F_3N_2O_7S$) 760.2221 (found for M+H) 760.2226 (calc.).

Derivatives of dye 18: cyclization product 20-F and conjugate 19

The cyclization product (lactone) was formed from dye **18**-OH,F with free OH groups and a linker containing CO_2H function (see main text for discussion and details).



18-R^1,R^2, **19**
18-OH,F
18-NHS,F
19 (R^1 = $HN(CH_2)_2O(CH_2)_2O(CH_2)_6Cl$, R^2 =F)

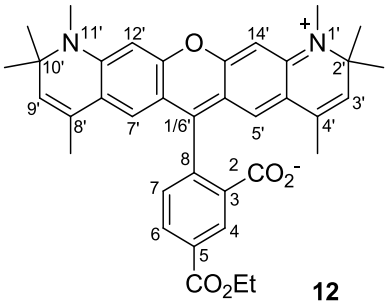
20-F

Compound **18**-NHS,F was first obtained in our previous study. [5a] Later, we found that attempts to additionally purify it by means of column chromatography or to let react it with amines under basic conditions (Et_3N) lead to one major product (>80%), whose analytical data suggested structure **20**-F. Unexpectedly, the same compound proved to be the major product in the reaction of **18**-NHS,F with primary amines under basic conditions (see the main text for details). Properties: a dark blue crystalline powder, very slightly soluble in water, well-soluble in most organic solvents, except alkanes. Analytical data for compound **20**-F: TLC: R_f = 0.30 (regular silica gel plates; CH_3CN/H_2O 10:1); t_R = 14 min (HPLC, A/B 50:50→0:100 in 25 min, detection at 254 nm, HPLC area 98%). MS (ESI) *m/z* (positive mode, %) = 756 (100) $[M]^+$; HRMS: ($C_{43}H_{42}F_4N_3O_5$) 756.3052 (found M^+), 756.3055 (calc.). ^{1}H NMR (400 MHz, $CDCl_3$, *mixture of two amide rotamers*): δ = 1.31 (m, 4 H, $2CH_2$), 1.49/1.51/1.60/1.66 (s, 12 H, CH_3), 2.06–2.22 (m, 4 H, $2CH_2$), 2.69/2.88 (s×2, 3 H, NCH_3), 2.85 (m, 2 H, CH_2), 3.05 (br. m, 4 H, CH_2), 3.54–3.77 (m, 6 H, 3×NCH_2), 3.98 (m, 1 H, CH_2O), 4.03 (d, *J* = 12 Hz, 1 H, CH_2O), 4.13 (d, *J* = 13 Hz, 1 H, CH_2O), 4.55 (d, *J* = 13 Hz, 1 H, CH_2O), 5.79 (s, 1 H), 5.83 (s, 1 H), 6.69/9.97 (s×2, 2 H, Ar) ppm; ^{19}F NMR (376.4 MHz, $CDCl_3$): δ = –151.3 (m, 1 F), –148.2 (m, 1 F), –136.3 (m, 1 F), –134.4 (m, 1 F), 75,3 (s, 3 F, CF_3COO^-) ppm.

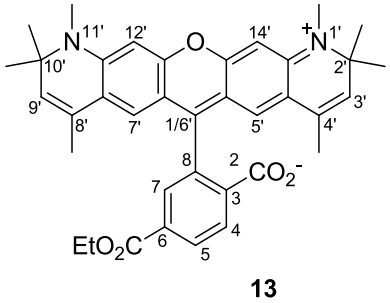
Satisfactory yields of the conjugate with HaloTag ® amine (structure **19**) and with other amines, as well, were obtained from acid **18**-OH,F only when the reagents were combined in the following order: HATU reagent (3.8 mg, 14 μmol) in 0.8 mL of dry CH_3CN was slowly (10 min) added upon stirring to the solution containing the amine substrate (5.2 mg of HaloTag ® amine, 20 μmol), Et_3N (2 μL, 14 μmol), and **18**-OH,F (1.5 mg, 2 μmol) in 1.5 ml of the same solvent at 0+5°C and left for 2 h at this temperature under an argon atmosphere. The solution was transferred onto a column with silica gel (8

g) and the elution was performed with CH_3CN/H_2O (10:1→3:1) as the mobile phase, containing 0.2% v/v Et_3N. The main colored fraction was collected, concentrated *in vacuo* to the volume of 5 mL, diluted with equal volume of brine, and extracted with CH_2Cl_2. The extract was filtered and evaporated to afford 1.1 mg (52%) of compound **19** (dye derivative with a positive net charge; presumably as a hydrochloride). Properties: a dark blue amorphous material, slightly soluble in water, well-soluble in most organic solvents, except alkanes. Analytical data: R_f = 0.20 (regular silica gel plates; CH_3CN/H_2O 10:1); t_R = 15.5 min (HPLC, A/B 50:50→0:100 in 25 min, detection at 254 nm, HPLC area 98%). MS (ESI) *m/z* (positive mode, %) = 979 (100) $[M]^+$; HRMS: ($C_{53}H_{64}ClF_4N_7O_7$) 979.4392 (found M^+) 979.4395 (calc.).

Isomeric rhodamines 12 and 13



12



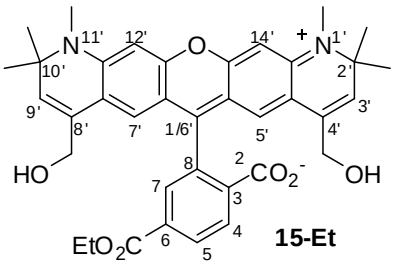
13

Phenol **11** (1.00 g, 4.92 mmol, 2.00 eq.) was dissolved in 1,2-dichlorobenzene (2.8 mL) and stirred under an argon atmosphere. Trimellitic anhydride ethyl ester (4-carboxyethyl phthalic anhydride, 90% purity, 601 mg, 2.46 mmol, 1.00 eq.) was added, and the mixture was heated to reflux for 8 h. The reaction mixture was cooled to r.t. and diluted with a mixture of CH_2Cl_2 (20 mL) and MeOH (2 mL). The mixture was loaded directly onto a silica gel column. The product was isolated by column chromatography on regular silica (142 g SiO_2, CH_2Cl_2/MeOH 10:1 → 6:1) as a mixture of the two regioisomers **12** and **13** in the ratio 4:3. The separation of the regioisomers was accomplished by silica column chromatography (MeCN/CH_2Cl_2/H_2O 10:2:1, three subsequent columns) and afforded 584 mg (989 µmol, 40%) of **12** and 435 mg (736 µmol, 30%) of **13**. Both isomers are dark violet crystalline solids, very slightly soluble in water, soluble in most organic solvents, except alkanes. The solutions are purple-pink with intense red florescence. Analytical data on compound **12** (the less polar isomer; *eluted first*, while performing preparative LC on regular silica gel): t_R = 16 min (HPLC, A/B 70:30→0:100 in 25 min; detection at 595 nm, HPLC area above 96%); TLC R_f = 0.25 (regular silica gel plates, MeCN/CH_2Cl_2/H_2O 10:2:1). Analytical data on compound **13** (the more polar isomer): t_R = 15 min (HPLC, A/B 70:30→0:100 in 25 min; detection at 595 nm, HPLC area above 98%); TLC R_f = 0.20 (regular silica gel plates, MeCN/CH_2Cl_2/H_2O = 10:2:1). MS (ESI) *m/z* (positive mode, %) = 591 (100) $[M+H]^+$. Purity and structure of the isomers are confirmed by NMR spectroscopy; the assignment of all signals is given below (note the atom numbering in the figure above). ^{1}H NMR (CD_3OD, 400.1 MHz) for **12** *(5-isomer)*: δ = 1.46 (t, $^3J_{H,H}$ = 7.1, 3 H, $CH_2C\underline{H_3}$), 1.48 (s, 6 H, 2'-CH_3,

10'-CH_3), 1.49 (s, 6 H, 2'-CH_3, 10'-CH_3), 1.77 (d, $^4J_{H,H}$ = 1.1, 6 H, 4'-CH_3, 8'-CH_3), 3.16 (s, 6 H, 1'-CH_3, 11'-CH_3), 4.46 (q, $^3J_{H,H}$ = 7.1, 2 H, C$\underline{H_2}$CH$_3$), 5.62 (d, $^4J_{H,H}$ = 1.1, 2 H, 3'-H, 9'-H), 6.77 (s, 2 H, 12'-H, 14'-H), 6.84 (s, 2 H, 5'-H, 7'-H), 7.41 (d, $^3J_{H,H}$ = 7.9, 1 H, 7-H), 8.26 (dd, $^3J_{H,H}$ = 7.9, $^4J_{H,H}$ = 1.7, 1 H, 6-H), 8.77 (d, $^3J_{H,H}$ = 1.7, 1 H, 4-H) ppm. ^{13}C NMR (CD_3OD, 100.6 MHz): δ = 14.7 (CH$_2$C$\underline{H_3}$), 18.3 (4'-C$\underline{H_3}$, 8'-C$\underline{H_3}$), 29.2 (2'-C$\underline{H_3}$, 10'-C$\underline{H_3}$), 29.2 (2'-C$\underline{H_3}$, 10'-C$\underline{H_3}$), 33.4 (1'-C$\underline{H_3}$, 11'-C$\underline{H_3}$), 61.0, 62.7, 96.2, 115.1, 123.2, 124.5, 126.9, 131.2, 131.5, 132.2, 133.2, 133.7, 138.1, 141.4, 154.7, 159.6, 167.1 ($\underline{C}O_2$Et), 171.7 (C-2) ppm.

^{1}H NMR (CD_3OD, 400.1 MHz) for **13** *(6-isomer)*: δ = 1.38 (t, $^3J_{H,H}$ = 7.1, 3 H, CH$_2$C$\underline{H_3}$), 1.47 (s, 6 H, 2'-CH_3, 10'-CH_3), 1.49 (s, 6 H, 2'-CH_3, 10'-CH_3), 1.77 (d, $^4J_{H,H}$ = 1.1, 6 H, 4'-CH_3, 8'-CH_3), 3.15 (s, 6 H, 1'-CH_3, 11'-CH_3), 4.39 (q, $^3J_{H,H}$ = 7.1, 2 H, C$\underline{H_2}$CH$_3$), 5.62 (d, $^4J_{H,H}$ = 1.1, 2 H, 3'-H, 9'-H), 6.77 (s, 2 H, 12'-H, 14'-H) , 6.87 (s, 2 H, 5'-H, 7'-H), 7.92 (d, $^3J_{H,H}$ = 1.7, 1 H, 7-H), 8.17 (d, $^3J_{H,H}$ = 8.0, 1 H, 4-H), 8.27 (dd, $^3J_{H,H}$ = 8.0, $^4J_{H,H}$ = 1.7, 1 H, 5-H) ppm. ^{13}C NMR (CD_3OD, 100.6 MHz): δ = 14.6 (CH$_2$C$\underline{H_3}$), 18.3 (4'-C$\underline{H_3}$, 8'-C$\underline{H_3}$), 29.2 (2'-C$\underline{H_3}$, 10'-C$\underline{H_3}$), 29.2 (2'-C$\underline{H_3}$, 10'-C$\underline{H_3}$), 33.4 (1'-C$\underline{H_3}$, 11'-C$\underline{H_3}$), 61.0, 62.7, 96.2, 115.4, 123.3, 124.4, 126.9, 131.3, 131.6, 131.8, 132.6, 133.6, 133.7, 145.6, 154.7, 159.2, 159.7, 166.9 ($\underline{C}O_2$Et), 172.1 (C-2) ppm.

Dihydroxylated rhodamine 15-Et *(6-isomer)*



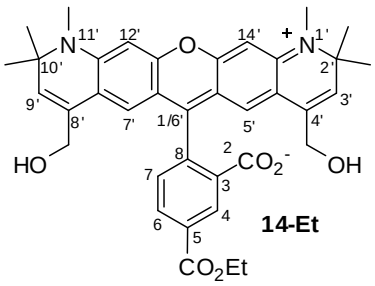
15-Et

The title compound was prepared by direct oxidation of **13** as follows: selenium dioxide (2.00 g, 18.0 mmol, 42.6 eq.) was dissolved in hot water (2.0 mL) and added to a stirred suspension of compound **13** (250 mg, 423 µmol, 1.00 eq.) in 1,4-dioxane (20 mL). The reaction mixture was heated under reflux for 23 h (oil bath temperature 117 °C). The reaction mixture was poured into a stirred mixture of water (90 mL), brine (30 mL) and CH_2Cl_2 (210 mL). Silica (1.5 g) was added; the mixture was stirred for 5 min and then filtered through a glass filter (Nr. 4). The residue was washed with MeOH (3×10 mL), and the filtrate was collected. Phases of the filtrate were separated, and the aqueous phase was re-extracted with CH_2Cl_2 (3×10 mL). All organic phases including the methanolic filtrate were combined, dried over Na_2SO_4 and concentrated *in vacuo* to give 637 mg of a blue solid. The blue solid was dissolved in absolute EtOH (90 mL) to give an intensely purple solution. It was cooled to 0 °C, and sodium borohydride (450 mg, 11.8 mmol, 27.9 eq.) was added in small portions over a period of 15 min. The mixture was stirred for 1 h at 0 °C, while the purple color became less

intense. The mixture was diluted with absolute EtOH (30 mL), and additional sodium borohydride (300 mg, 7.86 mmol, 18.6 eq.) was added in portions over a period of 2.5 h at 0 °C. The purple color of the reaction mixture was almost vanished, and the mixture was poured into a stirred mixture of ice-cold water (300 mL), CH_2Cl_2 (300 mL) and brine (150 mL). The aqueous phase was separated and extracted with CH_2Cl_2 (2×75 mL). The combined organic phases were dried over Na_2SO_4 and concentrated *in vacuo* to give 240 mg of a blue solid. The blue solid was dissolved in anhydrous EtOH (30 mL). Glacial HOAc (0.9 mL) and tetrabutylammonium periodate (210 mg, 485 µmol, 1.15 eq.) were added and the reaction mixture was stirred for 2 h at room temperature. It was concentrated *in vacuo* to one third of its initial volume and diluted with CH_2Cl_2 (120 mL). The solution was washed with a mixture of water (90 mL) and brine (90 mL). The aqueous phase was re-extracted with CH_2Cl_2 (50 mL). The combined organic phases were washed with a mixture of sat. aq. $NaHCO_3$ (45 mL) and water (45 mL). The aqueous phase was re-extracted with CH_2Cl_2 (50 mL). The organic phases were combined, dried over Na_2SO_4 and concentrated *in vacuo* to give blue oil. The crude product was purified by flash column chromatography on regular silica gel (300 g SiO_2, CH_2Cl_2/MeOH = 8:1→ 5:1). Pure fractions were combined, concentrated *in vacuo*, dissolved in MeOH (1 mL), diluted with water (9 mL) and brine (9 mL), and extracted with CH_2Cl_2 (4 × 10 mL). The combined organic extracts were dried over Na_2SO_4 and concentrated *in vacuo*. The pure product was obtained as dark violet solid, sparingly soluble in water and well-soluble in most organic solvents except alkanes. The solutions have intense red florescence. Yield 116 mg (188 µmol, 44 %). Analytical data for **15-Et**: t_R = 11 min (HPLC, A/B 70:30→0:100 in 25 min; detection at 595 nm, HPLC area above 95%). TLC: R_f = 0.30 (regular silica gel plates, CH_2Cl_2/MeOH 5:1). ^{1}H NMR (CD_3OD, 300.5 MHz): δ = 1.35 (t, $^3J_{H,H}$ = 7.1, 3 H, $CH_2C\underline{H}_3$), 1.50 (s, 6 H, 2'-CH_3, 10'-CH_3), 1.52 (s, 6 H, 2'-CH_3, 10'-CH_3), 3.15 (s, 6 H, 1'-CH_3, 11'-CH_3), 4.08 – 4.20 (m, 4 H, $C\underline{H}_2OH$), 4.34 (q, $^3J_{H,H}$ = 7.1, 2 H, $C\underline{H}_2CH_3$), 5.84 (s, 2 H, 3'-H, 9'-H), 6.79 (s, 2 H, 12'-H, 14'-H), 6.96 (s, 2 H, 5'-CH_3, 7'-CH_3), 7.88 (d, $^4J_{H,H}$ = 1.7, 1 H, 7-H), 8.10 (d, $^3J_{H,H}$ = 8.1, 1 H, 4-H), 8.23 (dd, $^3J_{H,H}$ = 8.1, $^4J_{H,H}$ = 1.7, 1 H, 5-H) ppm. ^{13}C NMR (CD_3OD, 125.7 MHz): δ = 14.6 ($CH_2\underline{C}H_3$), 29.1 (2'-$\underline{C}H_3$, 10'-$\underline{C}H_3$), 29.1 (2'-$\underline{C}H_3$, 10'-$\underline{C}H_3$), 33.3 (1'-$\underline{C}H_3$, 11'-$\underline{C}H_3$), 61.0, 61.6, 62.6, 96.4, 115.3, 122.1, 123.1, 130.2, 130.9, 131.7, 131.8, 132.2, 132.6, 133.1, 146.5, 154.8, 159.5, 159.6, 166.9 ($\underline{C}O_2Et$), 172.8 (C-2) ppm. MS (ESI) *m/z* (positive mode, %) = 623 (100) $[M+H]^+$, HRMS: ($C_{37}H_{38}N_2O_7$) 623.2745 (found M+H) 623.2752 (calc.).

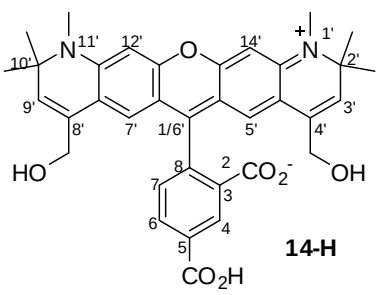
Hydroxylated rhodamine 14-Et *(5-isomer)*

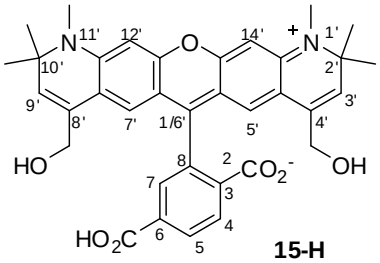
Following the recipe described above, rhodamine **12** was oxidized to **14-Et** with the yields of 35-40%.



Analytical data for **14-Et**: t_R = 11.5 min (HPLC, A/B 70:30→0:100 in 25 min; detection at 595 nm, HPLC area above 95%). TLC: R_f = 0.33 (regular silica gel plates, CH_2Cl_2/MeOH 5:1) and 0.35 (MeCN/H_2O 5:1 + 0.1 % TFA). ^{1}H NMR (CD_3OD, 400.1 MHz): δ = 1.41 (t, $^3J_{H,H}$ = 7.1, 3 H, $CH_2C\underline{H}_3$), 1.47 (s, 6 H, 2'-CH_3, 10'-CH_3), 1.49 (s, 6 H, 2'-CH_3, 10'-CH_3), 3.13 (s, 6 H, 1'-CH_3, 11'-CH_3), 4.09–4.13 (m, 4 H, $C\underline{H}_2OH$), 4.42 (q, $^3J_{H,H}$ = 7.1, 2 H, $C\underline{H}_2CH_3$), 5.81 (s, 2 H, 3'-H, 9'-H), 6.76 (s, 2 H, 12'-H, 14'-H), 6.93 (s, 2 H, 5'-CH_3, 7'-CH_3), 7.35 (d, $^4J_{H,H}$ = 8.0, 1 H, 7-H), 8.19 (dd, $^3J_{H,H}$ = 8.0, $^4J_{H,H}$ = 1.7, 1 H, 6-H), 8.70 (d, $^3J_{H,H}$ = 1.7, 1 H, 4-H) ppm. ^{13}C NMR (CD_3OD, 100.6 MHz): δ = 14.7 ($CH_2\underline{C}H_3$), 29.1 (2'-$\underline{C}H_3$, 10'-$\underline{C}H_3$), 29.1 (2'-$\underline{C}H_3$, 10'-$\underline{C}H_3$), 33.4 (1'-$\underline{C}H_3$, 11'-$\underline{C}H_3$), 49.9, 61.1, 61.8, 62.6, 96.4, 111.5, 115.2, 122.2, 123.2, 130.2, 131.2, 131.3, 132.0, 132.9, 133.1, 137.8, 154.8, 159.5, 160.3, 167.2 ($\underline{C}O_2Et$) ppm. MS (ESI) *m/z* (positive mode, %) = 623 (100) $[M+H]^+$, HRMS ($C_{37}H_{38}N_2O_7$): 623.2741 (found M+H); 623.2752 (calc.).

Free isomeric acids 14-H and 15-H with two carboxyl groups





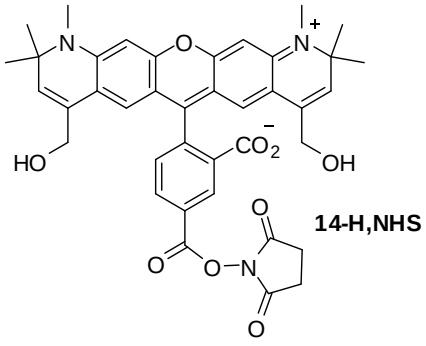
The saponification was performed as follows: compound **14-Et** (5.0 mg, 8.0 µmol, 1.00 eq.) was treated with a mixture of aq. NaOH (1 M, 360 µL, 0.36 mmol, 45 eq.), water (9.6 mL) and THF (4.0 mL) at room temperature. The resulting dark violet solution was sonicated in an ultrasonic bath for 5 min. The solution was stirred for 17 h at r.t. and the reaction was quenched by addition TFA (91 mg, 62 µL, 0.80 mmol, 100 eq.). The THF was removed *in vacuo* and the resulting aqueous solution was loaded directly on a reversed phase column (15 g, RP C_{18}, MeCN/H_2O = 2:1 → 5:1 + 0.1 % TFA). Pure fractions were combined and concentrated *in vacuo* followed by lyophilization. The product was obtained in a yield of 3.3 mg (5.5 µmol, 69 %). Dark violet crystalline solid, soluble in water and most organic solvents (except alkanes) producing pink solutions with intense red fluorescence. Analytical data on **14-H** *(5-isomer)*: t_R = 8.9 min (HPLC, A/B 70:30→0:100 in 25 min; detection at 595 nm, HPLC area above 95%). TLC R_f = 0.10 (regular silica gel plates,

(MeCN/H_2O 6:1). ^{1}H NMR (CD_3OD, 400.1 MHz): δ = 1.48 (s, 6 H, 2'-CH_3, 10'-CH_3), 1.49 (s, 6 H, 2'-CH_3, 10'-CH_3), 3.14 (s, 6 H, 1'-CH_3, 11'-CH_3), 4.07 (d, $^2J_{H,H}$ = 13.7, 2 H, 4'-C$\underline{H_a}H_b$, 8'-C$\underline{H_a}H_b$), 4.12 (d, $^2J_{H,H}$ = 13.7, 2 H, 4'-C$H_a\underline{H_b}$, 8'-C$H_a\underline{H_b}$), 5.80 (s, 2 H, 3'-H, 9'-H), 6.76 (s, 2 H, 12'-H, 15'-H), 6.95 (s, 2 H, 5'-H, 7'-H), 7.23 (d, $^3J_{H,H}$ = 7.7, 1 H, 7-H), 8.14 (d, $^3J_{H,H}$ = 7.7, 1 H, 6-H), 8.71 (br. s, 1 H, 4-H) ppm. MS (ESI): *m/z* (positive mode, %) = 595 (100) $[M+H]^+$, HRMS: ($C_{35}H_{34}N_2O_7$) 595.2438 (found M+H) 595.2439 (calc.).

Analogously was prepared acid **15-H** *(6-isomer)* with the yield of 74%.

Analytical data: t_R = 8.6 min (HPLC, A/B 70:30→0:100 in 25 min; detection at 595 nm, HPLC area above 95%); TLC R_f = 0.12 (regular silica gel plates, (MeCN/H_2O 6:1). ^{1}H NMR (CD_3CN, 300.1 MHz): δ = 1.48 (s, 12 H, 2 × 2'-CH_3, 2 × 10'-CH_3), 3.13 (s, 6 H, 1'-CH_3, 11'-CH_3), 4.03 (s_{br}, 4 H, C$\underline{H_2}$OH), 5.79 (s, 2 H, 3'-H, 9'-H), 6.71 (s, 2 H, 12'-H, 14'-H), 6.74 (s, 2 H, 5'-H, 7'-H), 7.91 (s_{br}, 1 H, 7-H), 8.31 – 8.33 (m, 2 H, 4-H, 5-H) ppm. MS (ESI): *m/z* (positive mode, %) = 595 (100) $[M+H]^+$, HRMS ($C_{35}H_{34}N_2O_7$): 617.2258 (found M+Na); 617.2245 (calc.).

_N_-hydroxysuccinimidyl ester 14-NHS *(5-isomer)*



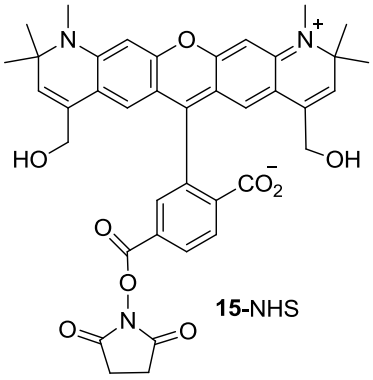
14-H,NHS

Rhodamine **14**-H (3.7 mg, 6.2 µmol, 1.0 eq.) and *N*-hydroxysuccinimide (7.2 mg, 62 µmol, 10 eq.) were dissolved in dry acetonitrile (8.0 mL), and the solution was stirred under a nitrogen atmosphere. A solution of HATU (2.4 mg, 6.2 µmol, 1.0 eq.) in dry acetonitrile (240 µL) was added at 0 °C. The mixture was allowed to warm up to r.t. and stirred for additional 10 min. A solution of NEt_3 (4.3 µL, 3.5 mg, 31 µmol, 5.0 eq.) in dry acetonitrile (1.0 mL) was added dropwise at room temperature. Additional HATU (4.8 mg, 12.4 µmol, 2.0 eq.) was added as solution in dry acetonitrile (480 µL) in three portions after 1.5 h, 2.5 h and 3.5 h. After stirring for additional 60 min, the reaction mixture was quenched by addition of TFA (2.4 µL, 3.5 mg, 31 µmol, 5 eq.) in dry acetonitrile (240 µL). After stirring for 10 min, the reaction mixture was loaded directly onto a silica column (9 g SiO_2, MeCN/CH_2Cl_2/H_2O 10:1:1→ 10:1:2). Pure fractions were combined and concentrated *in vacuo*. The product was obtained as a dark blue solid, purple-pink in solutions, with a red fluorescence. Yield 3.5 mg (5.1 µmol, 82 %).

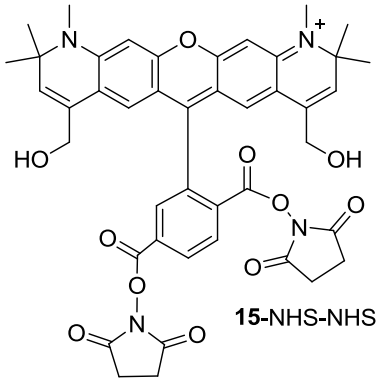
Analytical data: t_R = 10 min (HPLC, A/B 70:30→0:100 in 25 min; detection at 595 nm, HPLC area above 95%); TLC R_f = 0.40 (regular silica gel plates, (MeCN/H_2O 5:1). MS (ESI): *m/z* (positive mode, %) = 692 (100) $[M+H]^+$, 714 (20) $[M+Na]^+$, HRMS: ($C_{35}H_{34}N_2O_7$) 692.2602 (found M+H) 692.2603 (calc.)

***N*-hydroxysuccinimidyl ester 15-NHS *(6-isomer)* and the corresponding bis-*N*-hydroxisuccinimidyl ester 15-NHS-NHS (*3,6-isomer*)**

The procedure described above, when applied to isomer **15**-H, led to the formation of the double NHS ester (3,6-isomer) as the major product (>50 %, as established by HPLC and witnessed by the dark blue color of the product, which was not detected for 5-isomer). It was isolated by preparative column chromatography (the first, less polar fraction) as a dark blue solid; also blue in solutions (with a red fluorescence). Its constitution was confirmed by mass-spectrometry: MS (ESI): *m/z* (positive mode, %) = 789 (100) $[M]^+$, HRMS ($C_{35}H_{34}N_2O_7^+$): 789.2763 (found for M^+), 789.2766 (calc.). Pure chromatographic fractions containing compound **15**-NHS were collected, and the identity was confirmed by MS-spectrometry: MS (ESI): *m/z* (positive mode, %) = 692 (100) $[M+H]^+$, HRMS ($C_{35}H_{34}N_2O_7$): 692.2601 (found M+H), 692.2603 (calc.). Upon concentrating, even at 16–18°C, the product disproportioned into bis-*N*-hydroxy succinimidyl ester (*3,6-isomer*) and the starting acid **15**-H (as established by HPLC and MS). Attempts to improve the yield of **15**-NHS by using TSTU reagent and/or changing solvent to DMF, failed. In view of those difficulties, compound **15**-NHS did not find any practical applications.



15-NHS



15-NHS-NHS

Supplementary Figures

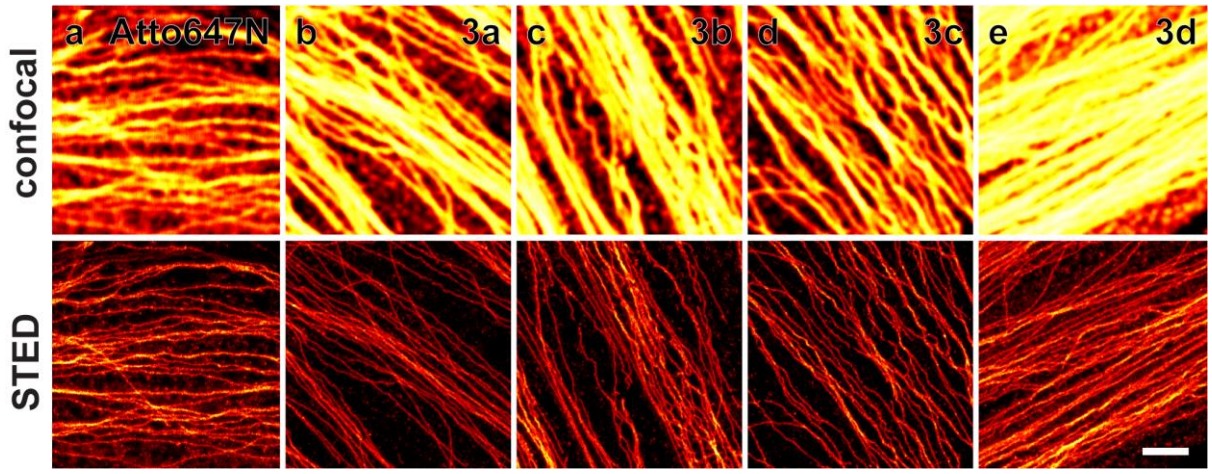


Figure S1. *Single color STED microscopy in the far red spectral region. The performance of the different compounds for imaging of biological specimen was tested by indirect immunolabeling of tubulin cytoskeleton in fixed mammalian cells, followed by confocal and STED microscopy. Scale bar: 1 µm.*

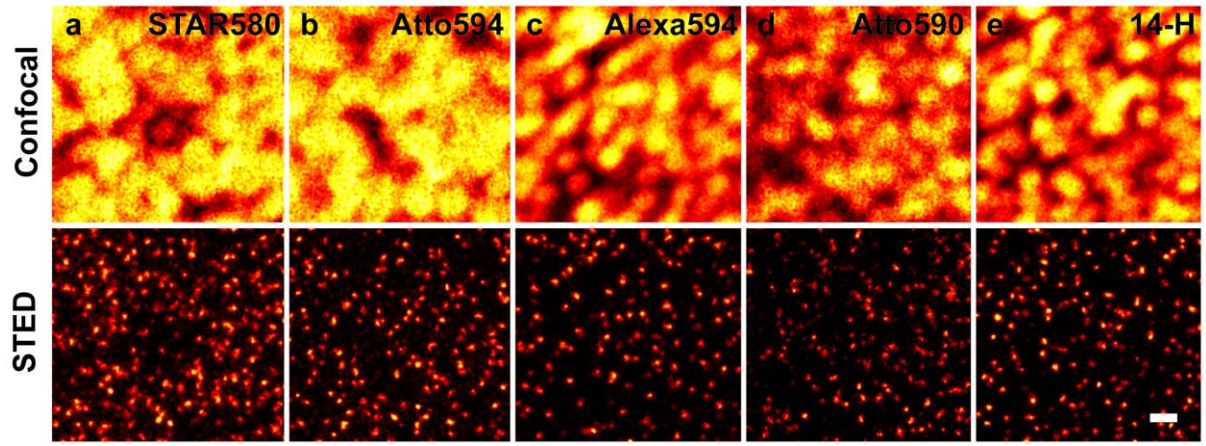


Figure S2. *Single color STED nanoscopy in the red spectral region. The performance of different compounds for imaging of biological specimen was testest by indirect immunolabeling of nuclear pore complex (NPC) subunits in the central channel in fixed mammalian cells, followed by confocal microscopy and STED nanoscopy. Shown is smoothed raw data. Scale bar: 500 nm*

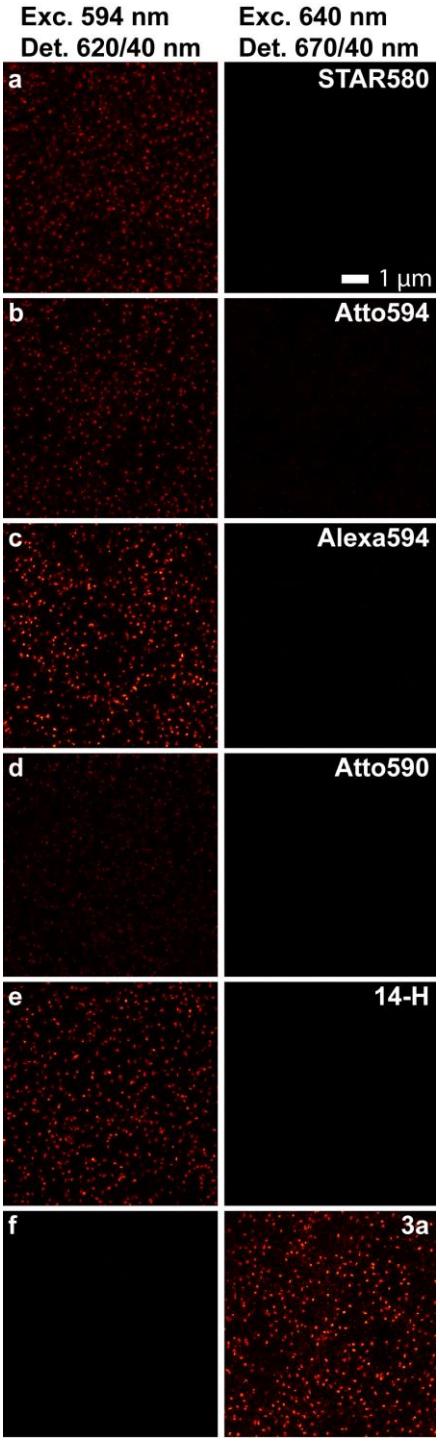


Figure S3. *Spectral crosstalk observed with the different dyes. For two-color imaging, the crosstalk of different dyes in biological specimen was testest by indirect immunolabeling of NPC subunits in the central channel in fixed mammalian cells, followed by STED nanoscopy of both channels. Shown is smoothed raw data. Scale bar: 1 µm*

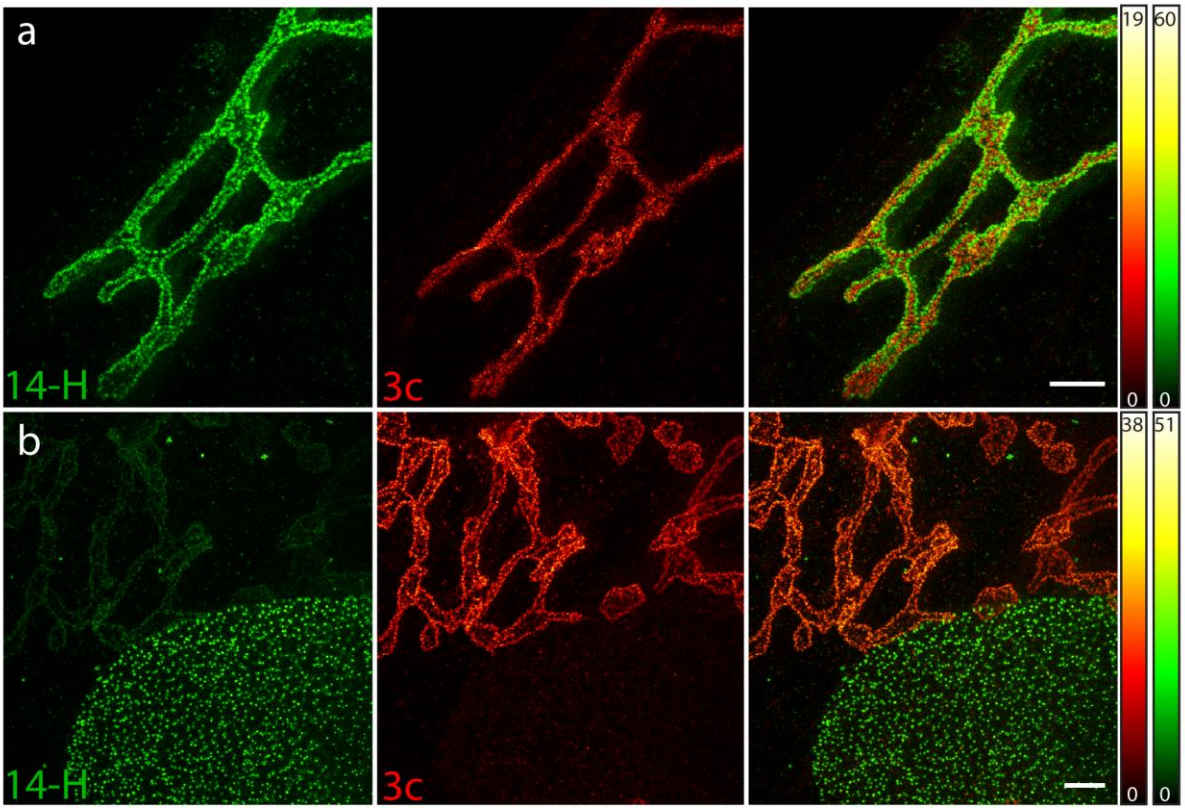


Figure S4. *Two color STED nanoscopy with dyes **14**-H and **3c**. Single color channels for the images are shown in Figure 5 in the main text. (a) Cellular nanoscopy of Giantin (**14**-H) and GM130 (**3c**). (b) Cellular nanoscopy of Giantin (**3c**) and the NPC (**14**-H). Scale bars: 2 µm.*

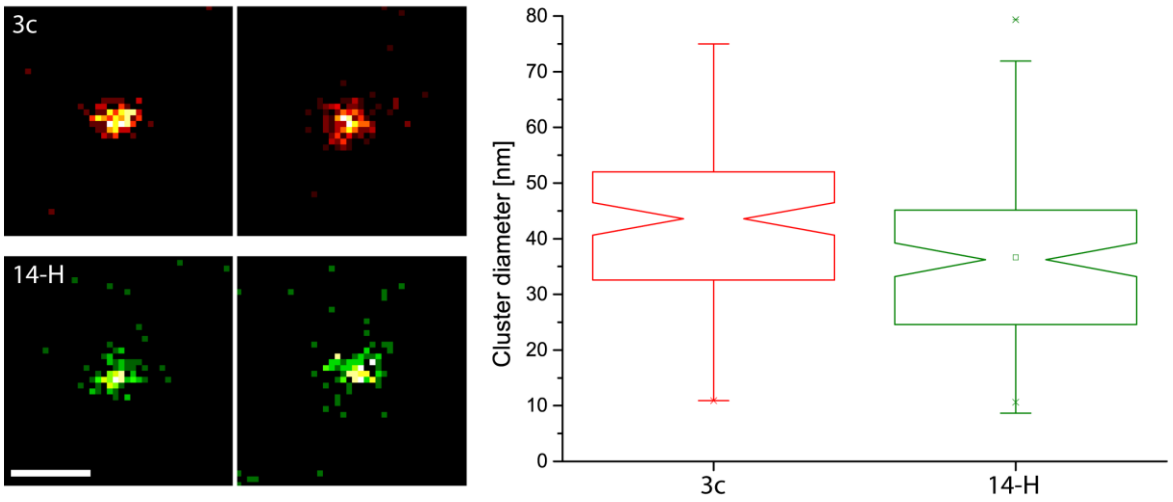


Figure S5. *Evaluation of the optical resolution using new dyes **3c** and **14**-H. The resolution was analyzed by determining the dimensions of more than 100 background clusters in immunofluorescence samples (i.e. antibody aggregates) (left). The result of these measurements are shown as notched boxplots (right). In this representation, the boxed intervals contain the data points from the first to third quartiles of the distribution. With a size of 1.5 times the interquartile range, the*

whiskers encompass ~99% of all data. The notches describe a confidence interval around the median, which equals the 95% confidence interval.
*As a result of this statistics, a median resolution of 44 nm was determined for compound **3c** and 36 nm for compound **14**-H. Since small clusters often tend to aggregate and form larger ones, these numbers are likely to underestimate the actual optical resolution provided by a dye in STED nanoscopy. Scale bar: 100 nm.*

References:

[1] C. A. Wurm, D. Neumann, R. Schmidt, A. Egner and S. Jakobs in *Methods in Molecular Biology* **2010**, *591*, Part 2, 185–199, DOI: 10.1007/978-1-60761-404-3_11 (Sample Preparation for STED Microscopy).

[2] C. A. Wurm, D. Neumann, M. A. Lauterbach, B. Harke, A. Egner, S. W. Hell, S. Jakobs, *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 13546–13551.

[3] F. Göttfert, C. A. Wurm, V. Müller, S. Berning, V. C. Cordes, A. Honigmann, S. W. Hell, *Biophys. J.* **2013**, *105*, L01 – L03.

[4] L. Lebreton, E. Jost, B. Carboni, J. Annat, M. Vaultier, P. Dutartre, P. Renaut, *J. Med. Chem.* **1999**, *42*, 4749–4763.

[5] a) K. Kolmakov, C. A. Wurm, R. Hennig, E. Rapp, S. Jakobs, V. N. Belov, S. W. Hell. *Chem.–Eur. J.* **2012,** *18***,** 12986–12998**;** b) K. Kolmakov, V. N. Belov, J. Bierwagen, C. Ringemann, V. Müller, C. Eggeling, S. W. Hell, *Chem.–Eur. J.* **2010**, *16*, 158−166.

[6] V. N. Belov, M. L. Bossi, J. Fölling, V. P. Boyarskiy, S. W. Hell, *Chem.–Eur. J.* **2009**, *15*, 10762−10776.