Electronic Supplementary Material (ESI) for Photochemical & Photobiological Science

Electronic Supplementary Material

Masked red-emitting carbopyronine dyes with photosensitive 2-diazo-1-indanone caging group

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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 indicated additionally. Normal phase analytical TLC was performed on MERCK ready-to-use plates with silica gel 60 (F_{254}) with UV-detector. Preparative column chromatography was followed by stepwise filtration from SiO_2 through Rotilabo® syringe filters (45 µm and 22µm).

Syntheses

Caged dye 1a (carbopyronine-containing spiro-diazoketone as a model compound).

Compound **8a** (see Scheme 3) with two (2-methoxyethyl) groups was the key precursor for carbopyronine dyes described in our recent study. [1] Analogously to rhodamines, it was converted to the corresponding acid chloride and then reacted (one-pot) with a large excess of diazomethane to form the spiro-diazoketone. [2]

Compound **8a** (42 mg, 0.076 mmol) was dissolved in a mixture of CH_2Cl_2 (6 mL) and THF (2 mL) in an argon-flushed Schlenck flask, neat oxalyl chloride (0.20 mL, 2.4 mmol) was introduced through a septum, and the dark-blue solution was stirred for 2 h at r. t. under an argon atmosphere. 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 further 30 min, the flask was filled with argon, sealed with a septum, and the residue dissolved in CH_2Cl_2 (5 mL; introduced through a syringe). The flask was chilled down to –78°C (dry ice bath, a small pressure of argon for the first few min. was applied), a syringe filled with granulated $CaCl_2$ (for protection from moisture) connected, and an etheral solution of diazomethane (9 mL, containing ca. 0.15 mmol/mL CH_2N_2, 1.35 mmol in total; generated from Diazald®, Sigma-Aldrich) was added in one portion through another syringe. (ATTENTION: diazomethane is a highly toxic, light and temperature-sensitive gas; note that ground joints and porous glass might cause explosion; follow all

safety precautions, while handling CH_2N_2!). Soon afterwards, Et_3N (1.1 mL of 1% v/v solution in CH_2Cl_2, 0.076 mmol) was added, and the cooling bath was changed to a mixture of crushed ice and brine (equal volumes), which maintained the temperature in the range of –7…– 5°C. The solution was stirred for 4 h at this temperature, then overnight in an ice-water bath (protected from light), and evaporated at temperatures t < +10°C (rotary evaporator, cold water bath). The residue (temperature and light-sensitive!) was immediately subjected to column chromatography over 45 g of silica gel with CH_2Cl_2 /EtOAc (20:1) as a mobile phase to afford 57 mg (79%) of **1a** as pale yellow crystals. Special attention was paid to the thorough separation of the less polar brown impurity. That compound was isolated, and the analytical data suggested structure **9** (Scheme 3), as expected. The same compound was also isolated as the side product of the preparative photolysis (for details see below). Pure target compound **1a** is fairly stable at r. t. in the dark, yet rapidly decomposes at 60–70°C, R_f = 0.30 (silica, $CHCl_3$ /EtOAc, 20:1). HPLC: t_R = 16 min (A/B 50:50 – 0:100 in 25 min; detection at 254 nm).

All spiro-diazoketone derivatives described here are pale yellow compounds. Remarkably, they all are visualized on TLC plates as intense blue spots even after short exposure to UV or even daylight. ^{1}H NMR (300 MHz, $CDCl_3$) for **1a**: δ 7.81 (d, J = 9Hz, 1H), 7.40 (m, 2H), 6.83 (d, J = 9, 1H), 6.72 (br. s, 2H), 6.38 (s, 2H), 3.62 (m, 4H, CH_2O), 3.50 (m, 4H, CH_2N), 3.38 (s, 6H, OCH_3), 3.30 (m, 4H, CH_2), 2.48 (m, 4H, CH_2), 1.82 (m, 4H, CH_2), 1.80 (s, 3H, CH_3), 1.71 (s, 3H, CH_3); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 188.5 (C=O), 157.8, 144.4, 142.9, 135.1, 134.4, 128.5, 127.0, 125.0, 122.0, 119.8, 107.7, 77.4, 77.0, 76.6, 70.1, 59.1, 51.4, 50.2, 37.2, 36.2, 35.2, 31.8, 28.9, 27.5, 22.7, 22.1, 14.1; MS (ESI+): *m*/*z* (%) = 599 (100) [M+Na], 577 (80) [M+H], HRMS: calcd for $C_{36}H_{40}N_4O_3$ $[M+Na]^+$ 599.2993; found 599.2995. For spectral properties (UV/vis, emission spectrum of the uncaging product, etc.) see Table1.

Preparative photolysis of compound **1a**.

Diazoketone **1a** (11 mg, 0.018 mmol) in methanol (6 mL) was placed into three quartz cuvettes (3 x 2 mL) and irradiated upon stirring (magnetic bars) by a medium-pressure mercury lamp (250 W) equipped with a water jacket and a pyrex filter (λ > 330 nm). At the distance of 3 cm from the jacket of the lamp, the photolysis was complete in 10 – 12 min., as determined by HPLC and TLC. HPLC: A/B 50:50 – 0:100 in 25 min; t_R = 16 min., detection at 254 nm, for the starting material (**1a**), and 14 min for compound **3a**, the major photolysis product (see Scheme 3). TLC: R_f = 0.9 and 0.1, for **1a** and **3a**, respectively (silica, CH_3CN/H_2O, 10:1). The reaction solution was evaporated and the residue separated over a column with 7g of SiO_2 and CH_3CN/H_2O (10:1 → 5:1) as a mobile phase. Two fractions (probably, due to different counter ions in the dye, see Scheme 3), containing the dark blue compound were collected. The polar fraction (zone) required the addition of CF_3COOH (1/1000 v/v) to the eluent to get it washed off the column. HPLC and MS analyses confirmed the identity of the blue compound **3a** in both fractions, which were combined, evaporated to the volume of 5 mL, diluted with CH_2Cl_2 (20 mL), washed with sat. $NaHCO_3$ solution (4 mL), dried and evaporated again to furnish 7.5 mg (60%, for the CF_3COOH-salt with M = 695) of compound **3a**. The compound is as a dark blue solid slightly soluble in water and most organic solvents, with a very intense red fluorescence in solutions. ^{1}H NMR (300 MHz, $CDCl_3$) for **3a**: δ 7.40–7.48 (m, 3H), 7.20 (s, 2H), 7.12 (d, J = 9Hz, 1H), 6.61 (s, 2H), 3.88 (m, 4H, CH_2O), 3.72 (m, 4H, CH_2N), 3.60 (m, 4H, CH_2), 3.39 (s, 3H, CO_2CH_3), 3.36 (s, 6H, OCH_3), 3.34 (s, 2H, $PhCH_2$), 3.30 (m, 4H, CH_2), 2.53 (m, 4H, CH_2), 1.91 (m, 4H, CH_2), 1.78 (s, 3H, CH_3), 1.70 (s, 3H, CH_3); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 171.0, 160.5, 155.9, 153.7, 135.9, 134.2, 129.5, 129.4, 127.4, 123.5, 120.5, 111.1, 77.4, 77.2, 77.0, 76.6, 70.1, 59.2, 52.5, 51.8, 34.8, 33.1, 27.2, 21.0. MS (ESI+): *m*/*z* (%) = 581 (100) $[M]^+$, HRMS: calcd for $C_{37}H_{45}N_2O_4$ $[M]^+$ 581.3374; found 581.3382.

Fig 1.

Fluorescent dye **3a**, uncaged, (left) in a 10 μmol aqueous solution under daylight and under an incandescent lamp light (middle). Caged dye **1b** in the solid state (right); turns green and then blue upon exposure to daylight. See also Figs. 1, 2 and Table 1 in the *main text* for spectral properties of dyes **1a-c** and the uncaging products **3a-c**.

A brown compound of structure **9** (3.7 mg, 37%), the side product of the photolysis was separated as a far less polar chromatographic fraction (see Scheme 3 for structure). HPLC: t_R = 21 min (A/B 50:50 – 0:100 in 25 min; detection at 254 nm). ^{1}H NMR (300 MHz, $CDCl_3$) for compound **9**: δ 7.92 (d, J = 9Hz, 1H), 7.40–7.60 (m, 3H), 7.15 (d, J = 9Hz, 1H), 6.80 (br. s, 2H), 6.14 (s, 1H), 3.70 (m, 4H, CH_2O), 3.50 (m, 4H, CH_2N), 3.40 (s, 6H, OCH_3), 3.22–3.35 (m, 4H, CH_2), 3.15 (m, 4H, CH_2), 2.51 (m, 4H, CH_2), 1.95 (m, 4H, CH_2), 1.78 (s, 3H, CH_3), 1.70 (s, 3H, CH_3). MS (ESI+): *m/z,* % = 571 (100) [M+Na], 549 (80) [M+H]; HRMS (ESI+): calcd for $C_{36}H_{40}N_2O_3$ 571.2931 [M+Na], found 571.2929.
Later we established that the addition of acetic acid (up to 5% v/v) to methanolic solutions of the diazo ketone before the photolysis increases the selectivity virtually to 100% (as determined by HPLC). Meanwhile, we made sure that compound **1a** in acidified (HOAc) methanolic solutions remained unchanged for several days in the dark at ambient temperature.

Caged dye 1b (the caged dye with a linker for conjugation, see Schemes 1 and 3, Main Text).

Precursor **8b** (see Scheme 3) with a free 2-hydroxyethyl group was synthesized in the course of our previous research work on carbopyronine dyes. [1] Its acetylation (protection of the OH group) was carried out as follows: 60 mg (0.11 mmol) of **8b** was heated for 15 min in 4 mL of glacial acetic acid containing 0.40 mL (4.1 mmol) of acetyc anhydride and 0.05 mL of conc. hydrochloric acid at 40^{o}C. The full conversion of the starting material was detected by TLC. The overheating of the reaction mixture and/or longer reaction time leads to the replacement of the 2-methoxyethyl group with the acetyl residue in **8b**. After the reaction was complete, the HCl was neutralized with KOAc (70 mg, 0.71 mmol), and the reaction mixture evaporated to dryness *in vacuo* at temperatures not exceeding 20–25^{o}C. The residue was shaken with water (10 mL) and $CHCl_3$ (40 mL), the aqueous layer separated and extracted with $CHCl_3$ (2 × 20 mL). The combined organic extract was washed with sat. $NaHCO_3$ solution (20 mL), dried over Na_2SO_4, evaporated, and the residue subjected to column chromatography over 40g of SiO_2 with

CH_3CN/H_2O (20:1) as a mobile phase. The main fraction was evaporated, the residue dissolved in CH_2Cl_2, filtered from SiO_2, and evaporated again to afford 62 mg (96%) of a pale blue crystalline powder, slightly soluble in water, well soluble in most organic solvents; decomp. temp. 140–150°C. The purity and identity of compound **8c** was confirmed by MNR spectroscopy, mass-spectrometry, and TLC. R_f = 0.60 (silica, CH_3CN/H_2O,10:1). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.95 (d, *J* = 9Hz, 1H), 7.53 (m, 2H), 7.06 (d, *J* = 9Hz, 1H), 6.82 (s, 1H), 6.74 (s, 1H), 6.22 (d, *J* = 8Hz, 2H), 4.28 (m, 4H, CH_2OAc), 3.44–3.62 (m, 4H, CH_2N, m, 2H, CH_2OMe), 3.39 (s, 6H, OCH_3), 3.30 (m, 4H, CH_2), 2.50 (m, 4H, CH_2), 2.06 (s, 3H, $COCH_3$), 1.83 (m, 4H, CH_2), 1.80 (s, 3H, CH_3), 1.76 (s, 3H, CH_3); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 170.9, 170.8, 155.6, 145.5, 145.2, 144.8, 144.5, 134.2, 128.5, 128.1, 128.0, 127.0, 124.6, 123.8, 121.5, 121.4, 118.8, 118.2, 107.4, 69.9, 60.9, 59.1, 51.3, 50.3, 50.1, 49.9, 37.8, 35.1, 33.4, 27.6, 22.1, 21.0; HRMS (ESI+): calcd for $C_{36}H_{40}N_2O_5$ [M+H] 581.3010; found 581.3008.

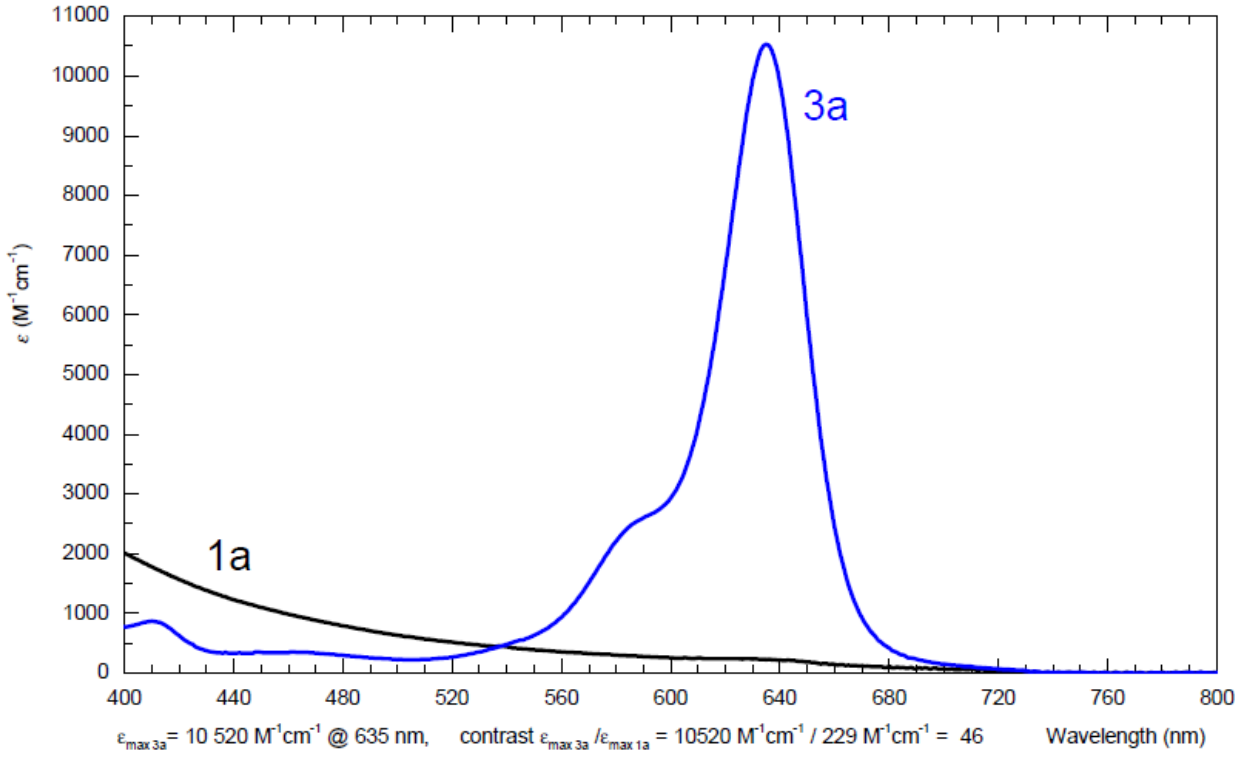

Fig 2.

Activation factor of caged dye **1a** in an aqueous solution (the visible region).

A 40 µmol solution of caged dye **1a** in aqueous PBS buffer (pH 7.4) was irradiated for 3 min with a medium-pressure mercury lamp (250 W) through a Pyrex glass (λ > 330 nm) from the distance of 5 cm; ca. 50% conversion of **1a** (HPLC). The absorbance spectrum of the solution (400 – 800 nm) was taken before and after irradiation (**3a**, blue curve). The conditional contrast factor at 635 nm (the absorbance maximum of dye **3a**) is estimated about 46. See also Fig 2 in the main text for the full spectra of compounds **1a** and **3a**.

Diazo ketone **8d** (carbopyronine-containing spiro-diazoketone).

The compound was best prepared as follows: to compound **8c** (23 mg, 0.040 mmol) in a mixture of CH_2Cl_2 (4 mL) and THF (1 mL) in an argon-flushed Schlenck flask, oxalyl chloride (0.1 mL, 1.2 mmol) was added through a septum, and the resulting solution was stirred for 2 h at r. t. under an argon atmosphere. A small distillation bridge with a condenser and a receiver flask were 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, sealed with a septum, and the

residue dissolved in CH_2Cl_2 (8–9 mL). The flask was chilled down to –78 ^oC (dry ice bath, a small pressure of argon for the first few min. was applied), a syringe filled with granulated $CaCl_2$ (protection from moisture) connected, and an ethereal solution of diazomethane (6 mL, containing approx. 0.15 mmol/mL CH_2N_2, 0.9 mmol in total) was added in one portion through an extra syringe. Triethylamine (0.040 mmol, 0.60 mL of 1% vol. solution in CH_2Cl_2) was added; the solution stirred 4 h at –5^oC and then overnight in an ice-water bath with protection from light and moisture (as described above for **1a**), and, finally, evaporated at temperatures not exceeding +10^oC (rotary evaporator, cold water bath). The residue was immediately subjected to column chromatography over 24 g of silica gel with CH_2Cl_2/EtOAc (20:1) as a mobile phase to afford 14 mg (58%) of **8d** as pale yellow crystals. This diazo compound, when pure, is fairly stable at +5°C (fridge) in the dark, yet slowly decomposes at room temp. and rapidly above > 60^oC.

Analytical data for **8d**: R_f = 0.15 (silica, $CHCl_3$/EtOAc, 20:1). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.81 (d, J = 9Hz, 1H), 7.40 (m, 2H), 6.88 (d, J = 9Hz, 1H), 6.78 (s, 1H), 6.71 (br.s, 1H), 6.37 (d, J = 9Hz, 2H), 4.23 (t, J = 8Hz, 2H, CH_2OAc), 3.61 (m, 2H, CH_2O), 3.50 (m, 4H, CH_2N), 3.39 (s, 6H, OCH_3), 3.30 (m, 4H, CH_2), 2.51 (m, 4H, CH_2), 2.03 (s, 3H, Ac), 1.82 (m, 4H, CH_2), 1.80 (s, 3H, CH_3), 1.63 (s, 3H, CH_3); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 188.8, 170.6, 157.2, 145.3, 144.4, 143.0, 142.8, 135.7, 134.3, 130.2, 127.6, 126.1, 123.3, 122.2, 122.0, 121.0, 120.8, 109.0, 107.9, 70.3, 60.6, 59.2, 54.3, 52.7, 51.5, 50.8, 50.2, 37.8, 36.6, 35.8, 28.1, 23.1, 22.8, 21,4; HRMS (ESI+): calcd for $C_{37}H_{40}N_4O_4$ [M+Na] 627.2942; found 627.2931.

Compound **8e** (carbopyronine-containing spiro-diazoketone with a free OH group).

The acetate protective group in compound **8d** was removed with almost a quantitative yield as follows: Acetyl derivative **8d** (27 mg, 0.045 mmol) in 30 mL of MeOH was stirred with finely powdered K_2CO_3 (42 mg, 0.30 mmol) for 1 h at r. t. The completion of the reaction was determined by TLC (silica plates, CH_2Cl_2/ EtOAc (5:1); R_f for **8d** and **8e** = 0.50 and 0.20, respectively). The solution was neutralized with an excess of HOAc (0.09 mL, 1.5 mmol) and evaporated to the volume of 5 mL at temperatures below 20°C. The residue was diluted with CH_2Cl_2 (30 mL) and heptane (10 mL), washed with sat. $NaHCO_3$ solution (5 mL), dried over Na_2SO_4, and evaporated *in vacuo* at ambient temperature to furnish 18 mg (96%) of compound **8e**, whose purity (ca. 98%) and identity was confirmed by HPLC, TLC, NMR and MS. HPLC: t_R = 14 min (A/B 50:50 – 0:100 in 25 min; detection at 254 nm). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.81 (d, J = 9Hz, 1H), 7.40 (m, 2H), 6.83 (d, J = 9Hz, 1H), 6.77 (s, 1H), 6.64 (s, 1H), 6.36 (d, J = 9Hz, 2H), 3.86 (t, J = 8Hz, CH_2OH), 3.61 (m, 2H, CH_2OMe), 3.50 (m, 4H, CH_2N), 3.39 (s, 6H, OCH_3), 3.30 (m, 4H, CH_2), 2.51 (m, 4H, CH_2), 1.82 (m, 4H, CH_2), 1.80 (s, 3H, CH_3), 1.63 (s, 3H, CH_3); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 188.3, 157.6, 144.9, 144.4, 143.0, 142.8, 135.0, 134.3, 128.7, 127.6, 125.4, 122.6, 122.2, 122.0, 120.5, 119.6, 108.3, 107.6, 70.1, 60.1, 59.2, 54.3, 52.7, 51.5, 50.4, 50.2, 37.3, 36.3, 35.3, 27.6, 22.7, 22.2; HRMS (ESI–): calcd for $C_{35}H_{38}N_4O_3$ [M–H] 561.2871; found 561.2857.

Compound **1b** (the caged dye with a linker for conjugation).

Diazoketone-containing precursor with a free OH group (**8e**) was reacted with an isocyanate reagent containing ethoxycarbonyl group (protected carboxyl moiety). Subsequent alkaline saponification furnishes the dye with a free carboxyl function, as illustrated in Scheme 3.

In a typical experiment, compound **8e** (10 mg, 18 μmol) in DMF (2 mL) was heated with ethyl 3-isocyanatopropionate (O=C=N$(CH_2)_2CO_2$Et, 32 mg, 0.22 mmol) and Et_3N (3 μL, 20 μmol) at 45–50°C for 5 – 7 h in a sealed screw-cap test tube (protected from light, Ar-flushed) upon stirring until a 90% conversion had been achieved, as determined by HPLC: t_R = 14 min for the starting material (**8e**) and 18 min for the reaction product (**8f**), respectively (A/B 50:50 – 0:100 in 25 min; detection at 254 nm). The solution was diluted with CH_2Cl_2 (20 mL), shaken with water (3 x 10 mL), the organic extract (light sensitive!) dried over Na_2SO_4, and evaporated *in vacuo* at ambient temperature. As soon as the evaporation was complete, the residue was dissolved in a mixture of THF (6 mL) and water (4 mL), and 1M aqueous NaOH added (0.4 mL, 0.40 mmol). The homogeneous solution was left overnight in a fridge at +5°C to complete the saponification (monitored by HPLC). To isolate the free acid, the solution was first neutralized with an

excess of HOAc (0.1 mL, 1.8 mmol), then carefully (t < 20°C, with protection from light) evaporated to the volume of 4–5 mL, diluted with an equal volume of brine, and extracted with CH_2Cl_2 (3 × 10 mL). The combined organic solution was dried over Na_2SO_4, evaporated carefully, as described before, and the residue separated over 15 g of SiO_2 with CH_3CN/H_2O (20:1) as a mobile phase. Pure fractions were filtered and carefully evaporated to dryness. The residue was dissolved in CH_2Cl_2, and filtration and evaporation were repeated to furnish 8.5 mg (70%) of a pale-yellow solid with a 96% purity (MNR, HPLC).

Analytical data for **1b**: t_R = 13 min (HPLC, A/B 50:50 – 0:100 in 25 min; detection at 254 nm) TLC: R_f = 0.3 (silica, CH_3CN/H_2O,10:1). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.82 (d, J = 9Hz, 1H), 7.40 (m, 2H), 6.90 (d, J = 9Hz, 1H), 6.72 (s, 1H), 6.68 (s, 1H), 6.36 (d, J = 8Hz, 2H), 5.30 (br. s, 1H, NH), 4.24 (m, 2H, CH_2OCO), 3.60 (m, m, 6H, CH_2N, CH_2O), 3.50 (m, 2H, CH_2), 3.40 (s, 6H, OCH_3), 3.30 (m, 4H, CH_2), 2.40 – 2.62 (m, m, 6H, CH_2), 1.84 (m, 4H, CH_2), 1.81 (s, 3H, CH_3), 1.69 (s, 3H, CH_3); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 188.5, 157.6, 156.2, 144.4, 144.2, 143.0, 142.9, 134.9, 134.4, 128.5, 128.4, 127.7, 125.4, 122.2, 122.1, 122.0, 119.9, 107.8, 78.8, 70.0, 62.1, 59.1, 52.7, 51.4, 50.7, 50.2, 50.1, 37.3, 36.4, 36.3, 35.3, 31.9, 29.1, 27.6, 27.5, 22.8, 22.2, 22,1, 14.2; HRMS (ESI–): calcd for $C_{39}H_{43}N_5O_6$ [M–H] 676.3141; found 676.3140. In earlier experiments, compound **8f** (the ethyl ester that formed in the reaction of **8e** with ethyl 3 – isocyanatopropionate) was isolated by column chromatography and identified it by its NMR and MS spectra. This ester was saponificated to the corresponding acid with almost a quantitative yield. Later we found that the isolation of ester **8f** is unnecessary and, therefore, we did the saponification in a one-pot fashion with a large excess of dilute alkali, as described above.

Compound **1b-NHS** (the active ester of caged fluorescent **1b**).

HATU reagent (8.4 mg, 22 μmol) in dry CH_3CN (1 mL) was added in one portion to a solution containing compound **1b** (8 mg, 12 μmol), *N*-hydroxysuccinimide (6 mg, 52 μmol l), and Et_3N (0.090 mL of a 10% v/v solution in CH_3CN, 60 μmol) in the same solvent (2 mL). The solution was stirred under an argon atmosphere at 0…+5°C until the reaction was complete, as determined by TLC: R_f = 0.7 and 0.1 for **1b**-NHS and **1b**, respectively (silica, CH_3CN/H_2O, 20:1). The reaction solution was diluted with CH_2Cl_2 (1 mL), loaded straight onto a column with SiO_2 (3g) and subjected to column chromatography with CH_2Cl_2/CH_3CN (3:1 → 1:1). Pure fractions were combined, carefully evaporated (r. t., protection from light), and the purification was repeated over 1.5 g of SiO_2 with CH_2Cl_2/EtOAc (1:1) to furnish 7.5 mg (80%) of the active ester (HPLC area 97%; t_R = 17 min; A/B 50:50 – 0:100 in 25 min; detection at 254 nm). The predominant peak in the MS spectrum agreed with the required structure (**1b-**NHS). HRMS (ESI–): calcd for $C_{43}H_{46}N_6O_8$ [M–H] 773.3304; found 773.3321. The active ester was stored at –20 °C, protected from light, and used immunolabeling experiments as a stock solution in DMF.

Synthesis of julolidine-containing caged fluorescent dyes (as illustrated in Scheme 4).

Carbinol **14**, containing a julolidine moiety, was synthesized according to a known procedure. [3]

6-Bromoindan-1-one (compound **11a**).

Following the known procedure,[4] 3-(4-bromophenyl)propionic acid (**10**, 9 g; 39 mmol) was added into a cooled (0°C) flask charged with 120 mL of chlorosulfonic acid. After 1 h stirring at this temperature, reaction mixture was poured onto 200 g of ice (ATTENTION: chlorosulfonic acid reacts with water extremely violently and causes heavy burns!) and extracted with CH_2Cl_2 (3×150 mL). The combined organic solutions were washed with sat. aq. $NaHCO_3$ and dried over Na_2SO_4. Evaporation of the solvent *in vacuo* furnished 7.1 g (88%) of crude product **11a**. The purity and identity of the compound was confirmed by its ^{1}H NMR spectrum, which agreed with previously reported.[5] The product was used without purification in the next step.

7-Bromo-1,2,3,4-tetrahydroquinoline (compound **12**).

Compound **11a** (7.6 g, 36 mmol) and hydroxylamine hydrochloride (10.8 g, 36 mmol) in 120 mL of MeOH was refluxed with stirring for 1 h. After cooling to r. t., the mixture was evaporated *in vacuo* to dryness; the residue was taken up with 140 mL of sat. aq. $NaHCO_3$, and extracted with $CHCl_3$ (3×200 mL). The combined organic extracts were dried over Na_2SO_4 and evaporated *in vacuo* to furnish 7.90 g (98%) of oxime **11b**. A Beckman Rearrangement on the crude oxime **11b** was performed according to the following recipe (see ref. [6] for other examples): 1M solution of DIBAL-H in hexanes (40 mL) was added dropwise under stirring at 0°C in 40 min into a 250 mL Schlenk Flask flushed with argon and charged with a suspension of oxime **11b** (1.4 g; 6.2 mmol) in dichloromethane (40 mL). After an overnight stirring at r. t., the reaction mixture was cooled (0 °C), NaF (4.5 g; 106 mmol) and water (1.5 mL) were added carefully. The resulted mixture was stirred for an additional 30 min and then filtered through Celite®. The filter cake was washed with $CHCl_3$ (500 mL), and the combined organic liquids were evaporated to afford a crude product. The followed column chromatography purification on 80 g of silica gel (hexane/EtOAc, 10:1 → 5:1) gave 880 mg (67%) of a colorless crystalline substance, whose ^{1}H NMR spectrum and R_f were identical to the previously reported. [1] Note that this compound was first obtained by direct bromination of 1,2,3,4-tetrahydroquinoline with a moderate yield. The preparation of alkene **13** from bromide **12** is also described in our previous publication.[1]

Carbopyronine-containing precursors 15a-c, and **16**.

The condensation of alkene **13** and carbinol **14** was carried out as follows.

A 100 mL Schlenk Flask was flushed with argon and charged with a CH_2Cl_2 solution (40 mLl) of alkene **13** (526 mg; 2 mmol) and carbinol **14** (406 mg; 2.00 mmol). Then a solution of BCl_3 (1 M in CH_2Cl_2, 2.3 mL; 2.3 mmol) was injected at 0°C upon stirring, and the resulted mixture was left overnight at r. t. Polyphosphoric acid (VWR International, 17 g) was mixed with the conc. (85% wt.) phosphoric acid (8 mL) and heated to 80–100 °C with manual stirring; the melt was cooled to r. t. and poured into the reaction flask (containing the initial condensation product of **13** with **14**). Through a thick cannula as an outlet, the CH_2Cl_2 was slowly evaporated in an argon purge with stirring and a gentle heating. The temperature was gradually raised to 110 °C and the mixture was maintained at this temperature for 2 h with stirring under a slow argon stream. The homogeneous reaction mixture was allowed to cool and then poured onto a mixture of ice (100 g), CH_2Cl_2 (100 mL) and KOH (23 g). The dark blue organic phase was separated and the aqueous layer was extracted with CH_2Cl_2 (2×100 mL). The combined organic extracts were dried and evaporated to afford the dark-blue solid **15a** (partly oxidized with the formation of a blue dye). The compound was used without further purification. MS (ESI+): *m/z* = 381 [M+Na]. HRMS: calcd. for $C_{25}H_{30}N_2Na$ [M+Na] 381.2301; found 381.2287.

The removal of the benzyl protecting group in **15a** was carried out as follows: In an argon atmosphere, with stirring, the crude compound was dissolved in a mixture of MeOH (60 mL) and Et_2O (20 mL) in a flask equipped with a gas inlet, a reflux condenser, and a bubble counter. Pd/C catalyst (10% Pd, oxidized form, VWR International; 650 mg) and ammonium formate (880 mg, 14 mmol) were added, the flask was flushed with argon, and the reaction mixture was stirred for 50 min at 50 °C and at r. t. for 1 h. The mixture was diluted with toluene (80 mL), filtered through Celite®, and the solvents were evaporated (t< 25°C) to give crude intermediate **15b** (an oxidizable compound, stored under Ar-atmosphere) which was used in the next step without purification.

The introduction of 2-methoxyethyl group and further oxidation to salt **16** were carried out as follows: Crude compound **15b** in DMF (10 mL) was placed in a screw-cap test tube containing 2-methoxyethyl bromide (2.0 g; 14.4 mmol) and finely powdered K_2CO_3 (1.0 g; 7.3 mmol). The test tube was flushed with argon and sealed, and the mixture was stirred at 100°C for 3 h. The reaction mixture was diluted with H_2O (10 mL) and extracted with toluene (4×20 mL). The combined organic extracts were dried over Na_2SO_4 and evaporated *in vacuo*. The residue was taken up in EtOH (85 mL) and oxidized with Bu_4NIO_4 (115 mg, 0.26 mmol) in presence of $HClO_4$ (70 %, 1.1 mL) for 1h at r. t., with stirring. The dark blue solution was diluted with aq. 0.2 M $NaClO_4$ (200 mL) and extracted with CH_2Cl_2 (4×100 mL). The combined organic extract was dried and evaporated. The residue was subjected to a column

chromatography (SiO_2,150 g, CH_2Cl_2/MeOH, 20:1) to furnish 738 mg of salt **16** (perchlorate, 72% over 4 steps from compound **13**). Properties: amorphous dark blue solid, unstable upon storage at r. t., well-soluble in CH_2Cl_2, alcohols, DMF, HMPTA, slightly soluble in acetone, CH_3CN, THF, insoluble in water. R_f = 0.20 (silica, CH_2Cl_2/MeOH, 20:1). HPLC: t_R = 13 min (A/B 50:50 – 0:100 in 25 min; detection at 636 nm). ^{1}H NMR (300 MHz, CD_3OD): δ 7.77 (s, 1H), 7.26 (s, 1H), 7.23 (s, 1H), 7.06 (s, 1H), 3.83 (t, J = 5 Hz, 2H, OCH_2), 3.70 (t, J = 5 Hz, 2H, NCH_2), 3.62–3.67 (m, 2H, NCH_2), 3.54–3.61 (m, 4H, NCH_2), 3.36 (s, 3H, OCH_3), 2.74–2.82 (m, 4H, CH_2), 1.93–2.07 (m, 6H, CH_2), 1.76 (s, 6 H, CH_3), 1.61–1.70 (m, 2H, CH_2); ^{13}C NMR (75.5 MHz, CD_3CN): δ 159.4, 158.6, 153.2, 137.3, 135.0, 133.2, 124.3, 124.2, 120.0, 119.7, 111.7, 70.7, 52.8, 52.4, 52.1, 52.0, 51.9, 30.1, 27.7, 27.5, 27.4, 21.6, 21.0. MS (ESI+): m/z = 415 $[M]^+$. HRMS: calcd. for $C_{28}H_{35}N_2O$ $[M]^+$ 415.2744; found 415.2744. Note that the use of chloroform ($CHCl_3$) as solvent in the syntheses of **15a-c**, as well as of $CDCl_3$ for taking NMR spectra, should be avoided. We found that this solvent reacts with carbinol **14** and some other julolidine-containing dye precursors, especially **15a**,**b**.

Compound 18 (the 2-oxazoline-protected acid).

2-(2-Bromophenyl)-4,4-dimethyl-2-oxazoline was synthesized according to the recipe by *J. Sedelmeier and T. Hammerer*. [7] The lithium reagent of structure **17** was prepared and used *in situ* as follows: in an argon-flushed Schlenk Flask (250 mL) with septum 2-(2-bromophenyl)-4,4-dimethyl-2-oxazoline (13 g; 5.12 mmol) in anidrous THF (12 mL) was lithiated with *t*-BuLi (1.5 M in pentane; 3.8 mL; 5.6 mmol in total) at –78 °C (dry ice bath), and the mixture was kept stirring at this temperature for 1 h. To this solution, salt **16** (168 mg, 0.32 mmol) in anhydrous THF (80 mL, with TMEDA, 0.8 mL, 5.4 mmol; the salt was dissolved upon sonication for 10 min at room temp.) was added through a syringe over a period of 40 min upon vigorous stirring. The stirring was continued for additional 2 hours at –78 °C, then overnight at 0°C (ice-bath). The reaction mixture was poured onto a stirred ice-cold aq. NH_4Cl solution (20%, 80 mL). The organic layer was separated, the aqueous phase extracted with dichloromethane (2×20 mL). The combined organic extracts were washed with brine, dried, and evaporated. The residue was dissolved in EtOH (60 mL), then Bu_4NIO_4 (43 mg; 0.10 mmol) and $HClO_4$ (70 %; 0.6 mL) were added. The deeply colored solution was stirred for 2h at r. t., then poured onto an aq. solution of $NaClO_4$ (0.2 M; 220 mL) and extracted with dichloromethane (2×150 mL). The combined organic extracts were washed with brine, dried and evaporated. The title compound (**18**) was isolated in a 65% yield (169 mg, HPLC-area ca. 96%) by means of column chromatography (silica gel, 130 g; CH_2Cl_2/MeOH, 25:1). Properties: amorphous very dark blue solid, unstable upon storage at r. t., well-soluble in CH_2Cl_2, alcohols, acetone, CH_3CN, DMF, slightly soluble in THF, insoluble in water. R_f = 0.25 (silica, CH_2Cl_2/MeOH, 20:1). For monitoring the reaction between **16** and **17** by HPLC, the probes of the reaction mixture were oxidized with a small amount of Bu_4NIO_4 in presence of HOAc HPLC: t_R = 8 min (A/B 50:50 – 0:100 in 25 min detection at 636 nm). ^{1}H NMR (300 MHz, CD_3COCD_3): δ = 7.99–8.03 (m, 1H), 7.66–7.76 (m, 2H), 7.34–7.38 (m, 1H), 7.23 (s, 1H), 6.74 (s, 1H), 6.61 (s, 1H), 3.93 (d, J = 5 Hz, 1H, OCH_2), 3.89 (d, J = 5 Hz, 1H, OCH_2), 3.74 (m, J = 3 Hz, 2H, NCH_2), 3.62–3.69 (m, 4 H, NCH_2), 3.60 (t, J = 6 Hz, 2H, OCH_2), 3.33 (s, 3H, OCH_3), 0.91 (s, 6H, CH_3), 3.23 (t, J = 6 Hz, 2H, NCH_2), 2.05–2.11 (m, 2H, CH_2), 2.53–2.60 (m, 4H, CH_2), 1.95 (s, 3H, CH_3), 1.91 (m, 4H, CH_2), 1.87 (s, 3H, CH_3), 1.79–1.84 (m, 2H, CH_2); ^{13}C NMR (75.5 MHz, CD_3COCD_3): δ = 164.3, 160.8, 159.1, 153.9, 153.8, 151.0, 137.6, 134.9, 133.4, 131.3, 131.2, 130.3, 130.0, 129.8, 123.8, 123.6, 121.7, 120.1, 111.7, 79.4, 70.9, 68.7, 59.1, 52.7, 52.4, 52.0, 41.5, 30.9, 30.4, 28.2, 28.0, 27.9, 21.9, 21.4, 21.3. MS (ESI+): m/z = 588 $[M]^+$. HRMS: calcd. for $C_{39}H_{46}N_3O_2$ $[M]^+$ 688.3585; found 688.3579.

Acid 19a (deprotection of the carboxyl group).

In a typical experiment, compound **18** (224 mg, 0.32 mmol) was dissolved in a mixture of conc. HCl (22 mL; 0.26 mol) and H_2O (11 mL) and heated with stirring in a flask with a reflux condenser for 14 h at 80 °C. The mixture was diluted with an equal volume of H_2O, neutralized with solid $NaHCO_3$ (24 g; 0.29 mol) and extracted with CH_2Cl_2 (7×70 mL), until the aqueous layer becomes colorless. The combined organic extracts were dried, evaporated *in vacuo*, the

residue was subjected to a column chromatography (150 g SiO_2; CH_2Cl_2/MeOH 5:1→1:2) to afford 84 mg of **19a** (49%) and **19b** (12%) as dark blue crystalline solids, slightly soluble in water and well-soluble in most organic solvents (except alkanes). The demethylated product **19b** (for properties see below) was added to the bulk amount of this compound, which was later obtained in the course of the demethylation, performed as a separate step (see Scheme 4). Analytical data for the free acid **19a**: HPLC: t_R = 10 min (A/B 50:50 – 0:100 in 25 min; detection at 636 nm). TLC: R_f = 0.4 (silica, MeOH/CH_2Cl_2, 1:4). ^{1}H NMR (300 MHz, CD_3OD) for **19a**: δ 8.00–8.05 (m, 1H), 7.50–7.60 (m, 2H), 7.07–7.11 (m, 1H), 7.03 (s, 1H), 6.74 (s, 1H), 6.61 (s, 1H), 3.78 (m, J = 5 Hz, 2H, OCH_2), 3.69 (m, J = 5 Hz, 2H, NCH_2), 3.50–3.59 (m, 4H, NCH_2), 3.43–3.50 (m, 2H, NCH_2), 3.36 (s, 3H, OCH_3), 3.14–3.22 (m, 2H, CH_2), 2.45–2.61 (m, 4H, CH_2), 1.98–2.07 (m, 2H, CH_2), 1.88–1.93 (m, 4H, CH_2, overlapped), 1.86 (s, 3H, CH_3), 1.87 (s, 3H, CH_3), 1.81–1.85 (m, 2H, CH_2); ^{13}C NMR (75.5 MHz, CD_3OD): δ 165.3, 159.1, 153.7, 153.5, 151.1, 139.2, 135.5, 134.1, 130.4, 130.3, 129.6, 123.9, 123.7, 123.4, 122.1, 120.7, 111.4, 71.3, 59.4, 52.9, 52.6, 52.2, 41.7, 31.2, 31.1, 28.7, 28.3, 22.5, 22.0, 21.9; MS (ESI+): m/z = 535 [M+H]. HRMS: calcd. for $C_{35}H_{38}N_2O_3$ [M+H] 535.2955; found 535.2948.

Demethylation of 19a to 19b and acylation to 19c.

The commercial 1M solution of BBr_3 in CH_2Cl_2 (4 mL, 4 mmol) was added to a cold (0°C) solution of compound **19a** (53 mg, 0.10 mmol) in chlorobenzene (150 mL) upon stirring under an argon atmosphere. The solution was allowed to warm up to r. t. and stirred for 1.5 h, until the starting material had completely reacted (TLC on silica, MeOH/CH_2Cl_2 1:4, R_f for **19a** and **19b** were 0.4 and 0.15, respectively). The reaction mixture was quenched with sat. $NaHCO_3$ solution (100 mL) and CH_2Cl_2 (50 mL) and well stirred. The organic layer was separated, dried, evaporated, and the residue subjected to column chromatography over a column with SiO_2 (50 g) with MeOH/CH_2Cl_2 1:4 → 1:3 as a mobile phase. That afforded 34 mg (64%) of the demethylation product **19b** with an HPLC-area of ca. 94%. The compound is a dark blue crystalline solid, rather unstable at r. t., slightly soluble in water and well-soluble in most organic solvents. **19b**: MS (ESI+): m/z = 521 $[M+H]^+$; HRMS: calcd. for $C_{34}H_{36}N_2O_3$ $[M+H]^+$ 521.2799; found 521.2801; HPLC: A/B 50:50 – 0:100 in 25 min., t_R = 6 min). Compound **19b** was acylated (in the next step) without additional purification, as described above for **8b** (Scheme 3) the solvents were thoroughly evaporated (KOAc was not added), and the crude acetate **19c** was used directly for the preparation of diazoketone **20a**. Analytical data for **19c**: HPLC: t_R = 9 min (A/B 50:50 – 0:100 in 25 min; detection at 636 nm); TLC: R_f = 0.6 (silica, MeOH/CH_2Cl_2,1:4). MS (ESI+): m/z = 563 [M+H]. HRMS: calcd. for $C_{36}H_{38}N_2O_4$ [M+H] 563.2904; found 563.2905.

Diazoketone-containing intermediates 20a,b,c and caged dyes 1c and 1c-NHS.

Following the procedure described for diazoketones **1a** and **8c** (see above for details; see Schemes1 and 3 for structures) crude compound **19c** (28 mg, 0.05 mmol) in 1,2-dichloroethane (4 mL) was reacted with a large excess of oxalyl chloride (0.25 mL) upon stirring under argon for 3 h at r. t.; a small amount of DMF (0.070 mL of 1% vol. soln. in CH_2Cl_2) was added as a catalyst beforehand. The solvent was thoroughly evaporated, the residue was dissolved in CH_2Cl_2 (7 mL) and reacted with diazomethane (5 mL of solution containing approx. 0.15 mmol/mL CH_2N_2, 0.8 mmol) in presence of Et_3N (0.77 mL of 1% vol. solution in Et_2O, 0.053 mmol) at –5°C (2 h) and then at 0°C overnight. Chlorobenzene (3 mL) was added, the reaction solution was carefully evaporated (t < 20°C, protection from light) to the volume of 3–5 mL and subjected to column chromatography over 20 g of Al_2O_3 (neutral) with EtOAc/hexane/CH_2Cl_2 (1:5:20) as a mobile phase. That afforded 16 mg (50%) of diazoketone **20a** as an amorphous solid. HPLC area 93%; t_R = 12 min (A/B 50:50 – 0:100 in 25 min; detection at 254 nm). The acetyl protective group in **20a** was removed with a quantative yield (as determined by HPLC) by stirring with finely powdered K_2CO_3 (40 mg, 0.37 mmol) in MeOH (50 mL). The reaction mixture was acidified with HOAc (0.2 mL, 3.5 mmol), 1 mL of chlorobenzene (prevents evaporation to dryness, which causes decomposition of diazo ketones, especially in the presence of inorganic salts) was added, and the solution evaporated to the volume of 2–3 mL. The residue was diluted with CH_2Cl_2 (40 mL), shaken with sat. $NaHCO_3$ solution (10 mL), dried and evaporated to the volume of 0.5–1

mL. Analytical data for **20b**: MS (ESI+): m/z = 545 [M+H]; HRMS: calcd. for $C_{35}H_{36}N_4O_2$ [M+H] 521.2911; found 545.2897; HPLC: t_R = 9 min (A/B 50:50 – 0:100 in 25 min; detection at 254 nm).

In the next step of the synthesis, crude compound **20b**, containing chlorobenzene, was mixed up with CH_2Cl_2 (2 mL) and DMF (5 mL). After that, ethyl 3-isocyanatopropionate ($O=C=N(CH_2)_2CO_2Et$, 220 mg, 1.5 mmol) and DMAP (catalyst, 1.0 mg) were added, the solution was transferred into a screw-cap tube and heated at 60°C for 26 h, until the reaction was almost (>90%, HPLC) completed. The reaction mixture was diluted with CH_2Cl_2 (30 mL), heptane (15 mL), and shaken with water (3×15 mL). The organic layer was dried, evaporated to the volume of 5–6 mL and subjected to column chromatography over 30 g of Al_2O_3 with EtOAc/ hexane/CH_2Cl_2 (1:5:20) as an eluent. The colored (yellow) fraction was collected, while the DMF and the isocyanate were separated. Further, chromatography was repeated over 13 of SiO_2 with the same eluent, and the pure fractions were combined and evaporated volume of 0.5–1mL. Chlorobenzene (1 mL) was added to stabilize the compound (**20c**) before the evaporation. Analytical data for **20c**: MS (ESI+): m/z = 710 [M+Na]; HRMS: calcd. for $C_{41}H_{45}N_5O_5$ [M+Na] 710.3313; found 710.3308; HPLC: t_R = 13 min (A/B 50:50 – 0:100 in 25 min). Crude intermediate **20c** (containing chlorobenzene) was hydrolyzed with aq. NaOH (1 mL of 1N solution, 1 mmol) in of a mixture of H_2O and THF (10+10 mL) in a fridge at 5°C overnight. The solution was acidified with HOAc (0.12 mL, 2.0 mmol), evaporated to the half of its initial volume and the residue, containing a yellow-green oil, extracted with CH_2Cl_2 (2 × 20 mL). The extract was dried, evaporated and separated over a column with SiO_2 (12g) and EtOH/CH_2Cl_2 (10:1) as a mobile phase to afford 15 mg of free acid **1c** in a pure state (35%, over 5 steps from compound **19b**). Caged dye **1c** is a greenish-yellow solid, almost insoluble in water (precipitates at concentrations higher than 20μmol/L), slightly soluble in MeOH and CH_3CN, well-soluble in chlorinated solvents and acetone. The compound is more stable than its precursors with the diazoketone moiety. Dye **1c** is fairly stable at –20°C, but undergoes a noticeable decomposition already at +5°C to form some polar red and brown compounds, as determined by TLC.

Analytical data for **1c**: ^{1}H NMR (300 MHz, CD_3COCD_3): δ 7.71–7.76 (m, 1H), 7.40–7.53 (m, 2H), 6.86–6.90 (m, 1H), 6.86 (s, 1H), 6.26 (s, 2H), 4.22 (t, J = 6 Hz, 2H, OCH_2), 3.39 (t, J = 7 Hz, 2H, NCH_2), 3.32 (t, J = 6 Hz, 2H, NCH_2), 3.11–3.21 (m, 4H, NCH_2), 2.98–3.05 (m, 2H, CH_2), 2.53 (t, J = 7 Hz, 2H, CH_2), 2.31–2.50 (m, 4H, CH_2), 1.99 (s, 3H, CH_3), 1.73–1.82 (m, 4H, CH_2), 1.90–1.96 (m, 2H, CH_2), 1.86 (s, 3H, CH_3); ^{13}C NMR (125.7 MHz, CD_3COCD_3): δ 187.8, 173.0, 159.1, 157.0, 148.3, 145.2, 144.5, 140.4, 135.5, 135.2, 128.5, 128.0, 127.4, 126.2, 122.7, 122.4, 121.8, 120.7, 118.7, 109.0, 77.8, 61.5, 54.5, 51.2, 51.0, 50.7, 50.4, 38.1, 37.5, 34.5, 33.4, 28.7, 28.2, 23.3, 23.2, 23.0, 22.5. HRMS (ESI–): calcd for $C_{39}H_{41}N_5O_5$ [M–H] 658.3035; found 658.3032; HPLC area 97%; t_R = 8 min (A/B 50:50 – 0:100 in 25 min; detection at 254 nm).

Active ester **1c-NHS** was obtained from dye **1c** with an 80% yield precisely as described for dye **1b** using HATU reagent in CH_3CN. HRMS (ESI+): calcd for $C_{43}H_{44}N_6O_7$ [M+Na] 779.3164; found 779.3161; HPLC area 95%; t_R = 12 min (A/B 50:50 – 0:100 in 25 min; detection at 254 nm). The compound was stored at –20 °C, protected from light, and used in immunolabeling experiments as a stock solution in DMF.

Dye 1d and its active ester 1d-NHS. (Modification with a hydrophilic spacer).

In a typical experiment, compound **1c** taken as a 2 mmol solution in dry CH_3CN, was reacted with amino ester $H_2NCO(CH_2)_2O(CH_2)_2O(CH_2)_2OCH_2CO_2Me$ (1.5 equiv) in presence of HATU (2 equiv) and Et_3N (3 equiv) at 0°C (see Scheme 4). The amino ester was prepared by the conventional esterification of the commercially available amino acid, according to *G. Clavé* and co-workers.[7] After the reaction was complete (0.5 – 1 h), the reaction solution was charged straight onto a column with silica gel (1 g of SiO_2 per 1mg of the starting compound **1c**) and eluted with CH_3CN/H_2O (50:1) as a mobile phase. Fractions containing the pure reaction product (**1d-**Me) were combined, evaporated, and the residue saponificated with a large excess of dilute alkali, exactly as described for compounds **8f** and **20a** (see above).

1d-Me: HRMS: calcd. for $C_{46}H_{54}N_6O_8$ [M+Na] 841.54521; found 841.54520; HPLC: t_R = 15 min (A/B 50:50 – 0:100 in 25 min). Column chromatography of the saponification product over silica gel with CH_3CN/H_2O (10:1) as mobile phase furnished dye **1d** as a free acid in a 69% yield. HPLC area 98%, t_R = 11 min (A/B 50:50 – 0:100 in 25 min; detection at 254 nm). The compound is a pale-yellow photo-sensitive amorphous solid, sparingly soluble in water (> 0.2 mmol/L) and well soluble in most organic solvents. The thermal stability of the modified dye is similar to that of **1c**. ^{1}H NMR for **1d**: (500 MHz, CD_3COCD_3): δ 7.76–7.82 (br.s, 1H), 7.44–7.60 (m, 2H), 6.95 (s, 1H), 6.89–6.92 (m, 1H), 6.32 (s, 2H), 4.27 (t, J = 6 Hz, 2H, OCH_2), 4.12 (s, 2H, OCH_2), 3.68–3.76 (m, 2H, OCH_2), 3.62–3.68 (m, 2H, OCH_2), 3.53–3.60 (m, 4H, CH_2), 3.34–3.47 (m, 6H, NCH_2), 2.98–3.05 (m, 2H, CH_2), 3.17–3.26 (m, 4H, CH_2), 2.49–2.66 (m, 2H, CH_2), 2.40–2.48 (m, 4H, CH_2), 2.04 (s, 3H, CH_3), 1.96–2.02 (m, 2H, CH_2), 1.92 (s, 3H, CH_3), 1.78 (m, 4H, CH_2); ^{13}C NMR (125.7 MHz, CD_3COCD_3): 187.8, 171.5, 159.1, 157.0, 148.3, 145.2, 144.5, 140.4, 135.6, 135.2, 128.0, 128.6, 127.4, 126.2, 122.7, 122.4, 121.8, 120.7, 118.7, 109.0, 77.8, 71.2, 70.7, 70.5, 61.4, 54.5, 51.2, 51.0, 50.7, 50.4, 39.6, 38.1, 36.4, 34.5, 33.8, 28.7, 28.2, 22.5, 23.3, 23.2, 23.0; HRMS (ESI–): calcd for $C_{45}H_{52}N_6O_7$ [M–H] 803.3774; found 803.3771.

Active ester **1d**-NHS was obtained with ca. 70% yield as described for dyes **1b** and **1c** using HATU reagent in dry CH_3CN. HRMS (ESI+): calcd for $C_{49}H_{55}N_7O_{10}$ [M+Na] 924.3903; found 924.3909; HPLC: t_R = 13 min (A/B 50:50 – 0:100 in 25 min; detection at 254 nm). The compound was stored at –20 ^{o}C, protected from light, and used in immunolabeling experiments as a stock solution in DMF.

Feasibility study on caged rhodamine dye 2b (see Scheme 2).

Sulfonated rhodamine **5-H** was prepared by direct sulfonation of the corresponding fluorine-substituted rhodamine (a known compound) with sulfuric acid, precisely as described in our previous publication on rhodamine fluorescent dyes.[9] The purity and identity of **5-H** was confirmed by NMR and MS spectrometry.

Allyl ester 5-All.

Sulfonated rhodamine **5-H** was esterified by heating in allyl alcohol as follows:

Compound **5-H** (50 mg, 0.06 mmol) in allyl alcohol (8 mL), containing 10 mg of Tos-OH (catalyst), was heated upon stirring in a screw-cup test tube placed in an oil bath at 120–130°C until the reaction was complete (6 – 10 h), as indicated by TLC: R_f = 0.2 for **5**-All and 0.1 for **5**-H, respectively (silica, MeOH/CH_2Cl_2, 1:4) and HPLC: t_R = 21 min (A/B 80:20 – 50:50 in 25 min; detection at 636 nm). The reaction solution was evaporated to dryness *in vacuo* and subjected to column chromatography over 20 g of silica gel with CH_3CN /CH_2Cl_2/H_2O (10:1:1) as mobile phase to furnish 38 mg (72%) of **5-**All as a dark blue solid, well-soluble in water and alcohols, slightly soluble in CH_3CN, insoluble in chloroform. ^{1}H NMR (300 MHz, CD_3OD) for **5-**All: δ 7.27 (br. s, 2 H, $H^{ar.}$), 5.86 (br. s, 2 H, C<u>H</u>=), 5.49 (ddd, 1 H, J 6.1, 12 and 16 Hz, C<u>H</u>= in allyl), 4.99–5.10 (m, 2 H, CH_2=), 4.44 (d, 2 H, J 6.1 Hz, CH_2O), AB-system (δ_A 3.68, δ_B 3.79, J_{AB} 14 Hz, CH_2S), 3.65 (m, 4 H, CH_2), 3.02 (m, 4 H, CH_2), 2.06 (m, 4 H, CH_2), 1.54/1.56 (s×2, 12 H, CH_3) ppm; ^{19}F NMR (282.4 MHz, CD_3OD): δ = –136.6 (m, 1 F), –137.9 (m, 1 F), –150.0 (m, 1 F), –153.1 (dt, J 6 and 20.6 Hz, 1 F) ppm; ^{13}C NMR (75 MHz, CD_3OD, due to very low intensities, signals of the fluorinated carbon atoms were not detected): δ 154.5, 152.0, 138.4 , 132.0, 124.6, 123.2, 122.6, 119.9, 114.6, 107.3, 67.9, 61.3, 55.0, 44.6, 28.7, 28.3, 21.7, 21.1. HRMS (ESI–): calcd for $C_{41}H_{38}N_2O_9F_4S_2$ [M–H] 841.1882; found 841.1883.

Thioether 6-All,H.

Compound **5**-All (200 mg, 0.24 mmol) was dissolved in CH_3CN (70 mL) upon sonication at r. t., and the solution cooled to –7°...—5°C in a bath with a mixture of saturated brine and crushed ice (approx.1:1). Ethyl thioglycolate (115 mg, 0.96 mmol) in CH_3CN (10 mL) and Et_3N (0.14 mL, 0.96 mmol) was added upon stirring. The stirring was

continued for 3 h at this temperature until the starting material almost completely reacted, as indicated by TLC: R_f = 0.25 (silica, MeOH/CH_2Cl_2 1:4, for **6-**All,H), and HPLC: t_R = 19 min (A/B 80:20 – 50:50 in 25 min; detection at 636 nm). The solution was acidified with an excess of HOAc (0.2 mL, 3.5 mmol; to stop the exchange of the second fluorine atom), evaporated to dryness *in vacuo* at t < 25°C, and separated over 320 g of silica gel with MeOH/CH_2Cl_2 (1:4) as a mobile phase to furnish 116 mg (51%) of **5-**All as a dark blue solid, well-soluble in water and alcohols, slightly soluble in CH_3CN, almost insoluble in chloroform. ^{1}H NMR (300 MHz, CD_3OD for **6-**All,H: δ = 7.21 (br. s, 2 H, H), 5.88 (br. s, 2 H, C<u>H</u>=), 5.48 (ddt, 1 H, *J* 6.1, 10 and 16 Hz, C<u>H</u>= in allyl), 5.03 (m, 2 H, CH_2=), 4.43 (dm, 2 H, *J* 6 Hz, CH_2O in allyl), 4.17 (q, 2 H, *J* 7.1 Hz, $CH_3C\underline{H}_2O$), AB-system (δ_A 3.73, δ_B 3.77, J_{AB} 14 Hz, CH_2S), 3.65 (m, 4 H, CH_2), 3.02 (t, 4 H, *J* 6.5 Hz, CH_2), 2.06 (m, 4 H, CH_2), 1.54/1.55 (s×2, 12 H, CH_3), 1.21 (t, 2 H, *J* 7.1 Hz, $C\underline{H}_3CH_2O$) ppm; ^{19}F NMR (282.4 MHz, CD_3OD): δ = –107.7 (dd, *J* 3.6 and 14.1 Hz, 1 F), –124.1 (dd, *J* 4 and 23 Hz, 1 F), –140.2 (dd, *J* 14 and 23 Hz, 1 F) ppm; ^{13}C NMR (75 MHz, CD_3OD, due to very low intensities, signals of the fluorinated carbon atoms w not detected): δ = 170.6, 162.6, 154.7, 152.2, 146.5, 138.3, 132.2, 124.7, 123.2, 122.6, 119.9, 114.9, 107.3, 67.9, 62.9, 61.2, 54.4, 44.6, 36.7, 28.6, 28.4, 21.7, 21.1, 14.4. HRMS (ESI–): calcd for $C_{45}H_{45}N_2O_{11}F_3S_3$ [M–H] 941.2065; found 941.2077.

Thioethers 6-All,R^2.

The esterification of the sulfonic acid groups was performed as follows:

Compound **6-**All,H (80 mg. 0.085 mmol) was suspended (by sonication) in dry CH_3CN (30 mL) under an argon atmosphere, cooled to 0°C, and trimethyloxonium tetrafluroborate (**R^2** = Me; 100 mg, 0.68 mmol) in CH_3CN (5 mL) was introduced through the septum. The mixture was sonicated for 1-2 min. until the solid had completely dissolved, and diethylisopropylamine (0.12 mL, 0.68 mmol), was introduced at 0°C upon stirring. To complete the reaction, the solution was warmed to r. t., the same amount of trimethyloxonium tetrafluroborate (100 mg, 0.68 mmol) and 0.06 mL (0.34 mmol) of diethylisopropylamine were added, and the stirring was continued for 15 min. The course of the reaction was monitored by TLC: R_f = 0.50 (silica, EtOH/CH_2Cl_2,1:6) and HPLC for **6**-All,Me: t_R = 24 min (A/B 80:20 – 50:50 in 25 min); t_R = 20 min (A/B 70:30 – 100 in 25 min; detection at 636 nm). The solution was evaporated to dryness *in vacuo* at t < 25°C, and subjected to column chromatography over 60 g of silica gel with MeOH/CH_2Cl_2 (1:10) as a mobile phase to furnish 95 mg (97%, for **6-**All,Me x 2HBF_4, M = 1146) of the methyl sulfonyl ester as a dark blue solid, insoluble in water, well soluble in acetone and CH_3CN, slightly soluble in chloroform. Ethyl ester **6-**All,Et was obtained precisely as described above, using the commercial triethyloxonium tetrafluroborate. ^{1}H NMR (300 MHz, CD_3COCD_3) for **6-All,Me**: δ 7.14 (br. s, 2 H, $H^{ar.}$), 6.13 (br. s, 2 H, C<u>H</u>=), 5.70 (ddt, 1 H, *J* 5.8, 10.4 and 17.2 Hz, C<u>H</u>= in allyl), 5.22 (ddd, 1 H, *J* 1.5, 3.1 and 17.2 Hz, C<u>H</u>H= in allyl), 5.12 (ddd, 1 H, *J* 1.2, 2.7 and 10.4 Hz, CH<u>H</u>= in allyl), 4.54 (dt, 2 H, *J* 1.3 and 5.8 Hz, CH_2O in allyl), AB-system (δ_A 4.30, δ_B 4.40, J_{AB} 14.7 Hz, CH_2S), 4.13 (q, 2 H, *J* 7.1 Hz, $CH_3C\underline{H}_2O$), 3.76 (m, 4 H, CH_2), 3.09 (t, 4 H, *J* 6.5 Hz, CH_2), 2.13 (m, 4 H, CH_2), 1.62/1.61 (s×2, 12 H, CH_3), 1.19 (t, 2 H, *J* 7.1 Hz, $C\underline{H}_3CH_2O$) ppm; ^{19}F NMR (282.4 MHz, CD_3COCD_3): δ = –108.2 (dd, *J* 3.5 and 13.9 Hz, 1 F), –125.5 (dd, *J* 3.6 and 23 Hz, 1 F), –137.5 (dd, *J* 13.9 and 23 Hz, 1 F) ppm; ^{13}C NMR (75 MHz, CD_3COCD_3, due to very low intensities, signals of the fluorinated carbon atoms were not detected): δ 169.2, 161.3, 154.1, 151.4, 147.1, 140.6, 132.0, 122.6, 120.6, 119.2, 114.2, 107.4, 67.3, 62.4, 61.4, 57.6, 52.0, 44.3, 35.8, 28.3, 28.4, 21.2, 20.4, 14.3. HRMS (ESI+): calcd for $C_{47}H_{50}N_2O_{11}F_3S_3$ [M+H] 971.2523; found 971.2534.

Compounds 6-H,R^2. Deprotection of the carboxylic acid group.

In a typical experiment, the allyl ester protective group was removed as follows: to 1 equiv. of compound **6**-All,Me, taken as a 2 mg/mL (1.7 μmol) solution in dry THF, upon stirring under an argon atmosphere, was added Et_3N*HCOOH (0.5 M in THF, 5 equiv.) and $Pd(PPh_3)_4$ (20% mol., as freshly prepared soln. in THF). The reaction was complete in 1 h at r. t., as established by HPLC (t_R = 17 min. for **6**-H,Me; A/B 30:70 – 100 in 25 min; detection at 636

nm) and TLC: R_f = 0.30 (silica, EtOH/CH_2Cl_2, 1:6). The reaction solution was loaded straight onto a column with approx. 0.5 g SiO_2 per 1 mg of the starting compound and eluted with MeOH/CH_2Cl_2 (1:10 → 1:4). To the main pure fraction, containing compound **6-**H,R^2, a stabilizer – chlorobenzene was added in amount of 20 mg per 1 mg of the substrate. The solution was filtered from SiO_2 and carefully (t < 20°C) evaporated *in vacuo,* so that the residue still contained chlorobenzene (some ten-fold amount, relative to the expected yield of the product). That stabilized the free acid from disproportion, and the purity maintained at the level of 80 – 90% (HPLC, see above). MS (ESI+) m/z, %: 953 (90) [M+Na]; HRMS (ESI+): calcd for $C_{44}H_{45}N_2O_{11}F_3S_3$ [M+H] 931.2210; found 931.2207. A higher purity of compound **6-**H,Me was impossible to achieve, because it undergoes disproportion upon concentrating, being unstable even at low temperatures (– 20°C) in a neat state.

The preparation and isolation of the ethyl analog (**6-H,Et**) was performed in the exactly same fashion. The compound with ethyl group proved more stable: we could isolate it neat in an 80%, yield with ca 90 – 95% purity (the overall total yield was thus about 70%). However, we failed to obtain clean NMR spectra due to the disproportion. The purity soon dropped to 70% in concentrated solutions, especially in polar solvents, and at ambient temperature. Analytical data for **6**-H,Et: HPLC: t_R = 18min (A/B 70:30 – 100 in 25 min; detection at 636 nm); MS (ESI+) m/z, %: 981(100) [M+Na]; HRMS (ESI+): calcd for $C_{46}H_{49}N_2O_{11}F_3S_3$ [M+H] 959.2523; found 959.2522.

All attempts to convert compounds **6-**H,R^2 to the corresponding diazoketones **7**-R^2 (see Scheme 2), following the recipes for **1a** and **8c** resulted in a very low yields of the target compounds: 10-15% for **7**-Et and even lower (few %) for **7**-Me. Under these conditions, carbopyronines and other, less sophisticated rhodamines gave good yields of the diazo ketones. Unfortunately, in both cases (**6-**H,R^2, R^2 = Me, Et), large amounts of non-photosensitive (as established by TLC) side-products were formed, which proved impossible to separate. MS-analysis of the yellow non-polar products (considered to be diazo ketones) agreed with the required structures for **7**-R^2. Meanwhile, the main reaction products were polar coloured compounds (that do not represent any interest in the context of caged dyes), and were difficult to identify. Among them, in the reaction mixtures, the “normal” Wolff Rearrangement products of **7-R^2** were isolated and identified by MS-spectroscopy. The hydrolysis of the diazketone-containing alkyl sulfonates **7**-R^2 (in the last stage of this synthesis, Scheme 2) proved impossible to accomplish. While carboxyethyl group in the thioglycol acid moiety of **7-R^2** reacted very easily, the saponification of the sulfonate was far from being smooth, despite our expectations. With ethyl ester **7-Et** the reaction conditions were prohibitingly severe. The hydrolysis involved prolonged heating (several hours) at 50–60°C in methanolic solutions of NaOH, which resulted in complicated product mixtures. In the course of the preparation of methyl esters **6-**H,Me (see Scheme 2), the methyl group more easily drifts to other reaction sites, particularly in the course of the acid chloride preparation and further, while preparing the diazoketone (**7**-Me). The reactions with alkali and *N*-methyl imidazole of **7**-Me, isolated in a very poor yield itself, anyway proved to be insufficiently slow and did not proceed cleanly (see discussion in the Main Text of the paper).

Immunofluorescence labeling and mounting of the samples

For the preparation of cell samples, PtK2 cells were grown on cover slips. Cells were fixed with ahhydrous 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 antiserum directed against the alpha-tubulin (Sigma-Aldrich, St. Louis, MO, USA). The primary antibodies were detected with secondary antibodies (sheep anti-mouse; Jackson ImmunoResearch Laboratories, West Grove; PA; USA) custom labelled with the fluorescent dyes. After several washing steps with PBS the samples were mounted in Mowiol.

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