Chemistry—Methods

Supporting Information

A Bright Surprise: Live-Cell Labeling with Negatively Charged Fluorescent Probes based on Disulfonated Rhodamines and HaloTag

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Abbreviations

aq. (aqueous), calcd (calculated), Cys (cysteine), DCM (dichloromethane), DIPEA (*N*,*N*-diisopropyl ethyl amine), DMF (*N*,*N*-dimethylformamide), equiv. (equivalent(s)), ESI (electrospray ionization), EtOH (ethanol), h (hour), HATU ([*O*-(7-Azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorphosphate), HPLC (high performance liquid chromatography), HRMS (high resolution mass-spectrometry), MeCN (acetonitrile), MeOH (methanol), min (minute), NaOH (sodium hydroxide), $Na_2HPO_4*2H_2O$ (sodium phosphate dibasic dihydrate), NMR (nuclear magnetic resonance), RP (reversed phase), r.t. (room temperature), SiO_2 (regular silica gel), TAT (transactivator of transcription), TCEP (tris(2-carboxyethyl)phosphine hydrochloride), TFA (trifluoroacetic acid), THF (tetrahydrofuran), TLC (thin layer chromatography), Tris (tris(hydroxymethyl)aminomethane), UV (ultraviolet), v/v (volume ratio of two solvents).

General remarks for synthesis: Unless stated otherwise, chemicals and solvents were purchased from reputable suppliers and used as received. All glassware and magnetic stirrer bars were cleaned and then dried in an oven before use. The reactions were carried out with magnetic stirring, and evaporations in vacuum were performed in a rotary evaporator with bath temperature not exceeding 60 °C. Analytical RP-HPLC were carried out with a Knauer Azura (Ultimate 3000) systems equipped with a 250 × 4 mm column (Eurospher II, 100-5 C18); flow rate 1.2 mL/min. Solvent A: H_2O + 0.1% v/v TFA, solvent B: MeCN + 0.1% v/v TFA. Preparative RP-HPLC separations were performed on an Interchim puriFlash 4250 device with a 250 × 16 mm column (Eurospher II, 100-5 C18); flow rate 20 mL/min, gradient of acetonitrile in 0.1% aq. TFA is given in the synthesis description.

Nuclear magnetic resonance (NMR): NMR Spectra (^{1}H) were recorded on an Agilent 400MR DD2 (400 MHz). All ^{1}H-NMR spectra are referenced to the signals of the residual protons in CD_3OD (δ = 3.31), or $CDCl_3$ (δ = 7.26), or DMSO-d_6 (δ = 2.50). The FIDs were processed with the software MestReNova. Multiplicities of the signals are described as follows: s = singlet, d = doublet, t = triplet, m = multiplet or overlap. Coupling constants (J) are given in Hz.

High resolution mass spectrometry (HR-MS): Mass spectra with electro-spray ionization (ESI-MS) were recorded on a Varian 500MS spectrometer (Agilent). ESI-HRMS spectra were acquired using a MICROTOF spectrometer (Bruker) equipped with an Apollo ion source and a direct injector with an LC-autosampler Agilent RR 1200.

Absorption spectra, emission spectra, fluorescence lifetime, and fluorescence quantum yield: UV-Vis absorption spectra were recorded in a Cary 5000 UV-Vis-NIR spectrometer (Agilent Technologies), and emission spectra in a Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies), in 4 mL quartz cuvettes (model 119F-10-40, Hellma Analytics). Fluorescence lifetimes were measured with a Quantaurus-Tau fluorescence lifetime spectrometer (model C11367-32, Hamamatsu). Fluorescence quantum yields were obtained with a Quantaurus-QY absolute PL quantum yield spectrometer (model C11347-12, Hamamatsu). Extinction coefficient measurement was repeated three times as three independent experiments. All the measurements were performed at room temperature.

Ligation buffer preparation: Guanidine hydrochloride (57.3 g, 0.60 mol) and $Na_2HPO_4 \cdot 2H_2O$ (3.56 g, 20 mmol) were dissolved in 90 mL of distilled water. To this solution, 1 M aq. NaOH was added, until pH was changed to 8.5. Distilled water was added to make the total volume of 100 mL. Thus, the concentrations of guanidine hydrochloride and Na_2HPO_4 are 6.0 M and 200 mM, respectively.

Degree of labeling (DOL) of secondary antibodies: the DOL values were calculated according to the following equations (S1 and S2). For *N*-hydroxysuccinimidyl esters (**Rho590^{Me}**-NHS and **Rho565^{Me}**-NHS), CF_{280} was assumed to be the same as for the corresponding carboxylic acid (**Rho590**-COOH (compound 2) and **Rho565**-COOH (compound 8)) (see Figure S3).

$$DOL = \frac{A_{max}/\varepsilon_{max}}{A_{prot}/\varepsilon_{prot}} = \frac{A_{max} \times \varepsilon_{prot}}{(A_{280} - A_{max} \times CF_{280}) \times \varepsilon_{max}} \quad \text{(Equation S1)},$$

$$CF_{280} = \frac{\varepsilon_{280}}{\varepsilon_{max}} \quad \text{(Equation S2)},$$

where A_{max} and A_{prot} are the measured absorbance at the absorption maximum of the dye and the protein (280 nm), respectively; ε_{max} and ε_{prot} is the extinction coefficient at the absorption maximum of the dye and the protein, respectively. ε_{prot} = 210 000 $M^{-1}cm^{-1}$ for IgG at 280 nm. A_{280} and ε_{280} are the measured absorbance at 280 nm of the dye-protein conjugate and the measured extinction coefficient at 280 nm of the dye, respectively.

Secondary antibody conjugation: For coupling of proteins with NHS-esters of the dyes, 400 µL of the solution containing ~1 mg of goat-anti-rabbit secondary antibody (Dianova, Hamburg, DE; Cat no. 111-005-003), 40 µL of 1 M aq. $NaHCO_3$ and 100 µg of a dye NHS-ester dissolved in 20 µL DMSO were mixed with stirring and stirred for one hour. The conjugate was isolated using a PD10 size exclusion chromatography column (GE Healthcare) with phosphate buffered saline as eluent.

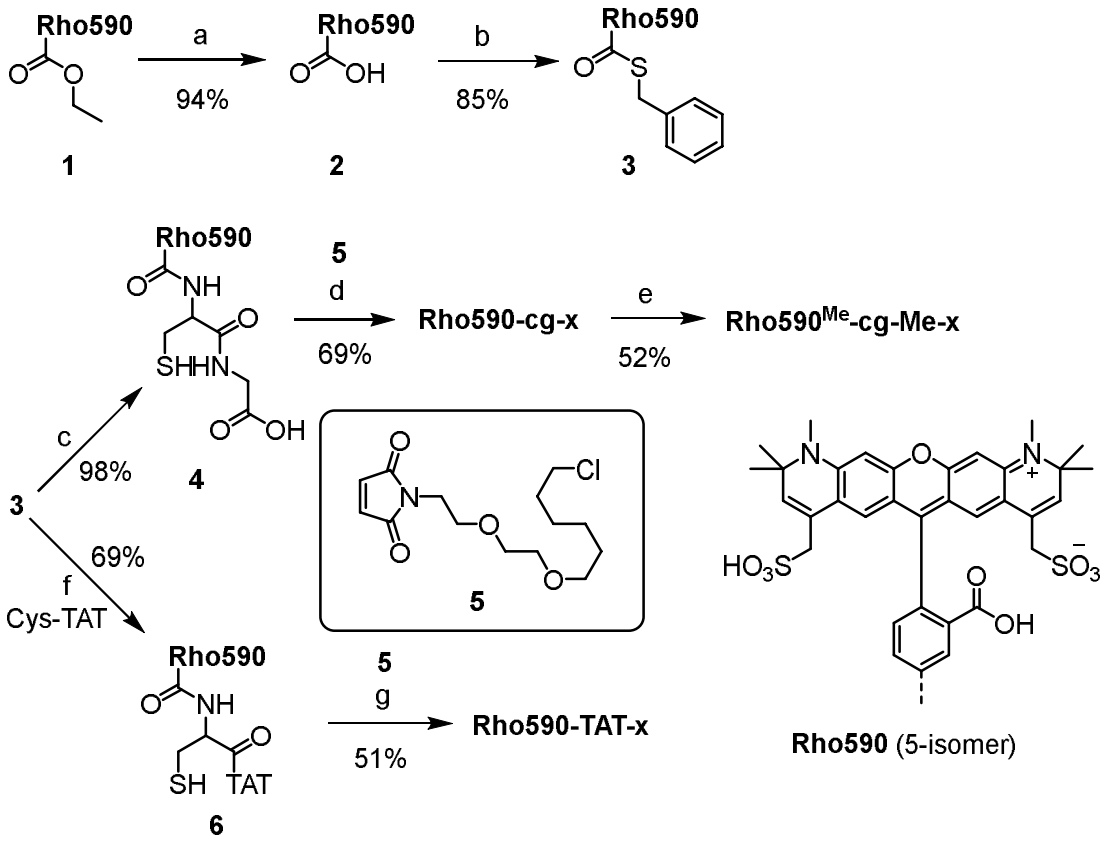
Cell culture of U-2 OS and monoclonal *vimentin-Halo* expressing U-2 OS cells: Human Osteosarcoma cells (ECACC, Porton Down, Salisbury, UK; Cat no. 92022711, Lot. 17E015) and monoclonal *vimentin-Halo* expressing U-2 OS cells[S1] were cultivated in McCoy´s medium (Thermo Fisher Scientific, Waltham, MA, USA), supplemented with 10% (v/v) fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA) and 1% (v/v) sodium pyruvate (Sigma Aldrich, St. Louis, MO, USA), in a humidified 5% CO_2 incubator at 37 °C.

Staining of living U2-OS cells expressing *vimentin-Halo*: The day before labeling, 30000 U2-OS cells per chamber of a chamber slide (ibidi GmbH, Gräfelfing, Germany; µ-Slide 8 Well Glass Bottom) were seeded in McCoy´s medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% (v/v) fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA) and 1% (v/v) sodium pyruvate (Sigma Aldrich, St. Louis, MO, USA). Staining, washing and imaging was performed in Opti-MEM™ (Thermo Fisher Scientific, Waltham, MA, USA). For labeling, the cells were incubated with 2 µM solution of a probe for 1 h at 37 °C in a humidified 5% CO_2 incubator. Then the cells were washed with Opti-MEM three times, for a total period of 30 min and afterwards imaged directly.

Immunofluorescence labeling of U2-OS cells: The day prior to fixation, $1{,}5 \times 10^6$ U2-OS cells were seeded on cover slips in a Cell Culture Petri Dish (diameter: 100 mm) and cultured in McCoy´s medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% (v/v) fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA) and 1% (v/v) sodium pyruvate (Sigma Aldrich, St. Louis, MO, USA). The initial fixation step was done via the addition of pre-warmed formaldehyde dissolved in phosphate buffered saline (PBS, 137 mM NaCl, 2.68 mM KCl and 10 mM Na_2HPO_4 , pH 7.4) to the growth medium, resulting in a formaldehyde concentration of 2% (v/v), for 5 min at 37 °C. Subsequently the fixation was facilitated via incubation of the cells in 8% (v/v) pre-warmed formaldehyde in PBS for additional 5 min at room temperature. Afterwards the cells were extracted with 0.5% (v/v) Triton X-100 in PBS and blocked with 5% (w/v) bovine serum albumin (BSA) in PBS/Glycine (0.1 M) for 15 min. Tom22 and ATP beta were labeled using specific primary antibodies (Anti-Tom22, Rabbit, Merck Millipore/Sigma-Aldrich, HPA003037, Lot: 000003317; Anti-ATPB, Mouse, abcam, [4.3E8.D10], ab5432, Lot: GR3177231-21). Incubation with appropriate antibodies was performed in 5% BSA (w/v) in PBS/Glycine (0.1 M) for 1 h at room temperature. After six times washing with PBS, the primary antibodies were detected by the secondary antibodies coupled to the fluorescent dyes **Rho565^{Me}-COOH** (goat-anti-rabbit) or **Abberior STAR RED** (Abberior, Göttingen, Germany) (goat-anti-mouse), respectively. Again, the incubation was done in 5% BSA (w/v) in PBS/glycine (0.1 M) for 1 h at room temperature. After six additional washing steps with PBS, the cells were mounted in Mowiol mounting medium containing 0.1% (w/v) 1,4-diazabicyclo[2.2.2]octan (DABCO) (Merck Millipore Sigma, Burlington, MA, USA).

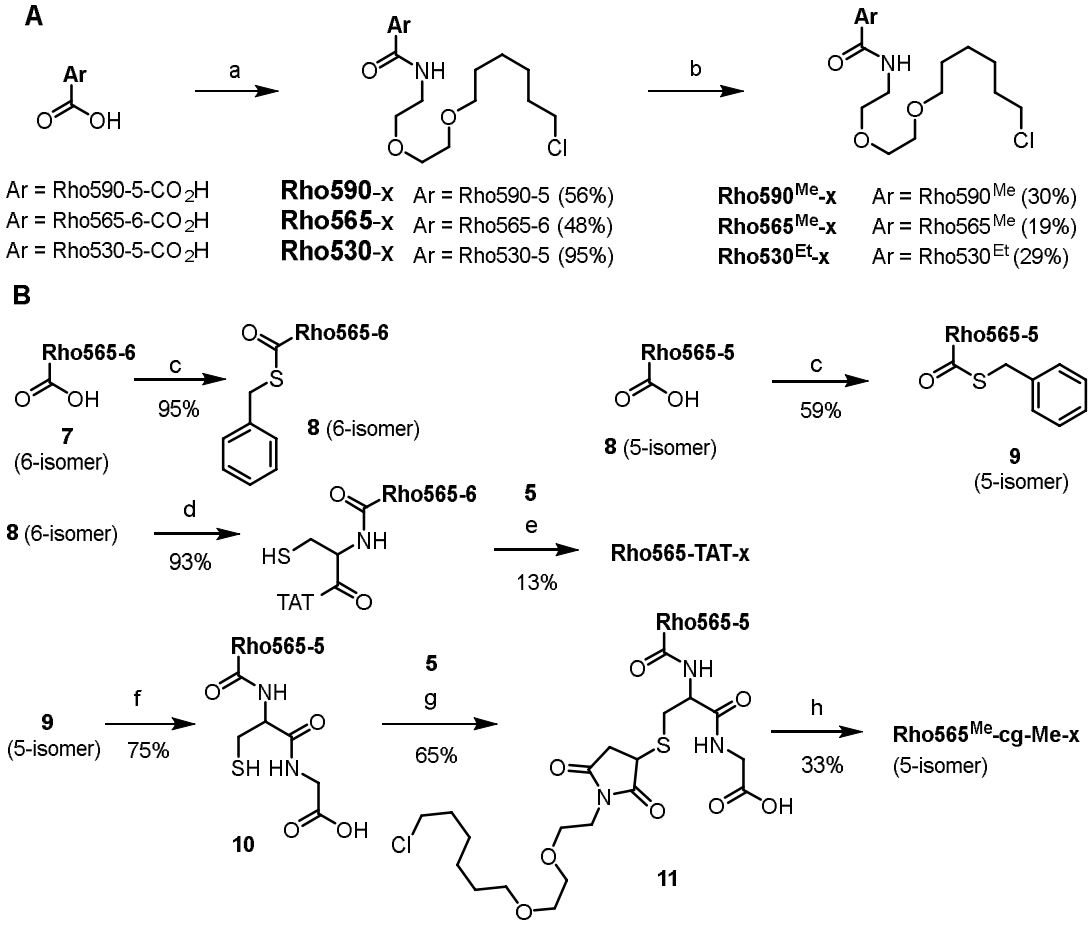
Confocal and STED microscopy of living and fixed U-2 OS cells: Confocal light microscopy was performed using a Leica TCS SP8 laser-scanning microscope (Leica Microsystems, Wetzlar, Germany).

STED microscopy was performed using a quad scanning STED microscope (Abberior Instruments, Göttingen, Germany) equipped with a UPlanSApo 100x/1,40 Oil objective (Olympus, Tokyo, Japan). For dye excitation, lasers with emission wavelengths of 485 nm (**Rho530**, **Rho530^{Et}**), 561 nm (**Rho530^{Et}, Rho565**, **Rho565^{Me}, Rho590 = Alexa Fluor 594, Rho590^{Me}**) and 640 nm (**KK114 = STAR RED**) were used. To achieve super-resolution imaging, STED lasers with emission wavelengths of 595 nm or 775 nm and a repetition rate of 40 MHz were applied. Filter sets designed for 3 detection ranges (500 – 550 nm, 580 – 630 nm and 650 – 720 nm) were used. With the exception of contrast stretching, no further image processing was applied.



Scheme S1. Synthesis of the HaloTag™ probes **Rho590-cg-x**, **Rho590^{Me}-cg-Me-x**, and **Rho590-TAT-x** (for full structures, see Figure 1 in the main text: a) NaOH, H_2O/EtOH (4:1, v/v), 0 °C, 2 h, then aq. HCl; b) HATU, DIPEA, $C_6H_5CH_2SH$, DMF, r.t., 20 min; c) H-Cys-Gly-OH, thiophenol (0.4%, v/v), 0.1 M TCEP, ligation buffer (pH 7.2), r.t., 8 h; d) **5**, tris buffer (pH 7.2), r.t., 1 h; e) diazomethane, MeOH, 0 °C,

1 h; f) Cys-TAT, thiophenol (0.4%, v/v), 0.1 M TCEP, ligation buffer (pH 7.2), r.t., 8h; g) **5**, tris buffer (pH 7.2), r.t., 3 h.



Scheme S2. For full structures, see Scheme A and Figure 1 in the main text. **A**. Synthesis of the HaloTag^{TM} probes Rho590-x, Rho565-x and Rho530-x, as well esters Rho590^{Me}-x, Rho565^{Me}-x and Rho530^{Et}-x: a) HATU, DIPEA, HaloTag® Amine (O2), DMF, r.t., 20 min; b) diazomethane, MeOH (diazoethane, ethanol/DMF for Rho530^{Et}-x), 0 ℃, 50 min. **B**. Synthesis of the HaloTag probes Rho565-TAT-x and Rho565^{Me}-cg-Me-x : c) HATU, DIPEA, benzyl mercaptan, DMF, r.t., 30 min; d) H-Cys-Gly-OH, thiophenol (0.4%, v/v), 0.1 M TCEP, ligation buffer (pH 7.2), r.t., 4 h; e) **5**, Tris buffer (pH 7.2), r.t., 30 min; f) Cys-TAT, thiophenol (0.4%, v/v), 0.1 M TCEP, ligation buffer (pH 7.2), r.t., 4 h; g) **5**, tris buffer (pH 7.2), 1 mM TCEP, r.t., 1 h h) CH_2N_2, ether / MeOH, 0 ℃, 30 min.

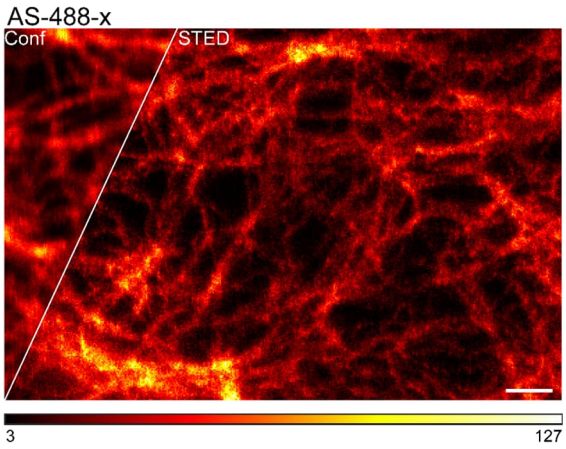


Figure S1. Live-cell STED microscopy with compound AS488-x. Confocal and STED image of a U2OS cell expressing *Vimentin-Halo* from the endogenous locus. Cells were labelled with 2 µM solution of the Halo Tag amine attached to Abberior STAR 488 (**AS488-x**). For nanoscopy an excitation laser with an emission wavelength of 485 nm and a STED laser with an emission wavelength of 595 nm were used. Scale bar: 1 µm.

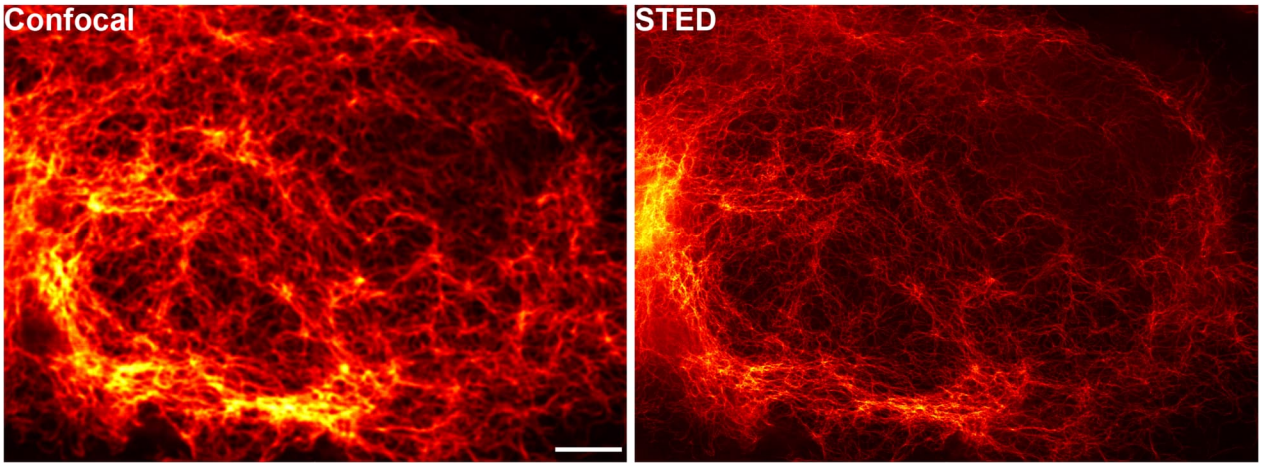


Figure S2. Live-cell STED microscopy with compound STAR RED-x. Confocal and STED image of a U2OS cell expressing *Vimentin-Halo* from the endogenous locus. Cells were labelled with 2 µM solution of the Halo Tag amine attached to Abberior STAR RED (**STAR RED-x**) containing 6% DMSO. For nanoscopy an excitation laser with an emission wavelength of 640 nm and a STED laser with an emission wavelength of 775 nm was used. Scale bar: 5 µm.

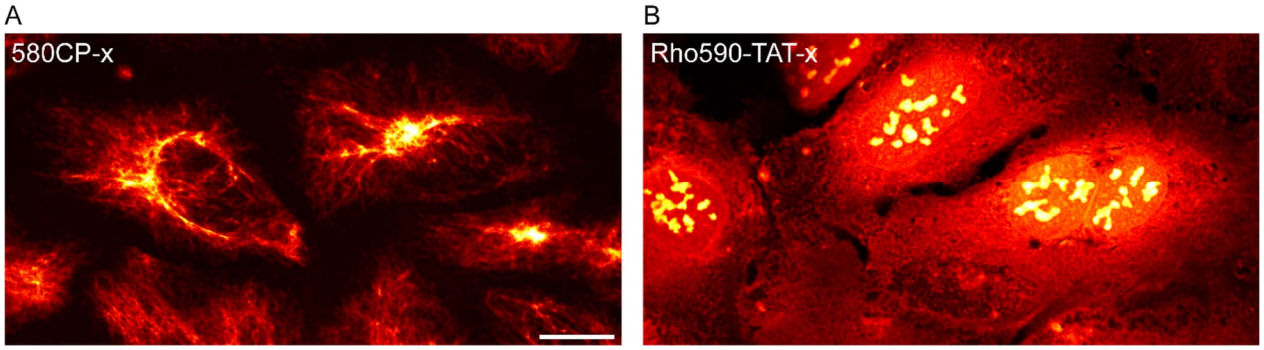


Figure S3. Compounds *580CP-x* (reference) and Rho590-TAT-x as Halo-Tag probes provide specific and non-specific staining of Vimentin-Halo fusion proteins. (A, B) Confocal images of U2OS cells expressing *Vimentin-Halo* from the endogenous locus. (A) Cells were labeled with 2 µM solutions of *580CP*-x or (B) **Rho590-TAT-x**. Scale bar: 20 µm.

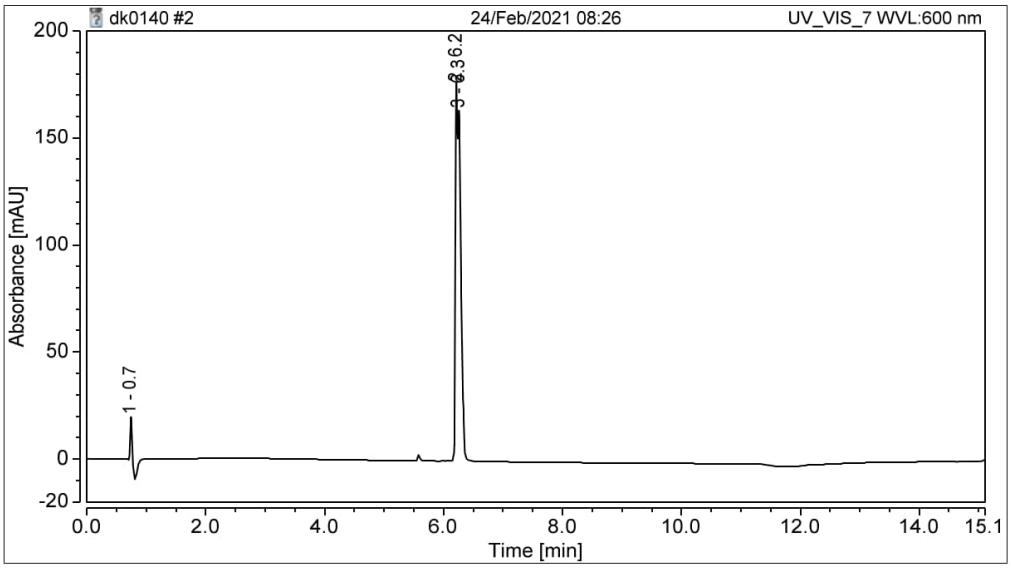
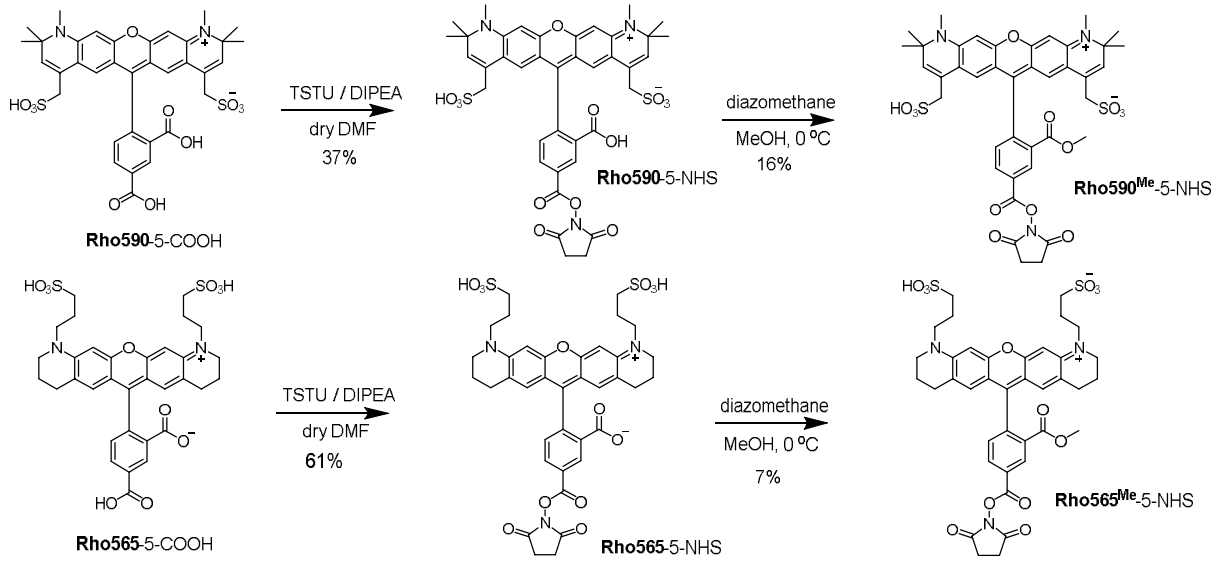


Figure S4. High performance liquid chromatography (HPLC) trace of **Rho590^{Me}-cg-Me-x**; detection at 600 nm.



Scheme S3. Synthesis of *N*-hydroxysuccinimidyl esters **Rho590^{Me}-5-NHS** and **Rho565^{Me}-5-NHS** from 5-carboxy isomers of Rho590 and Rho565.

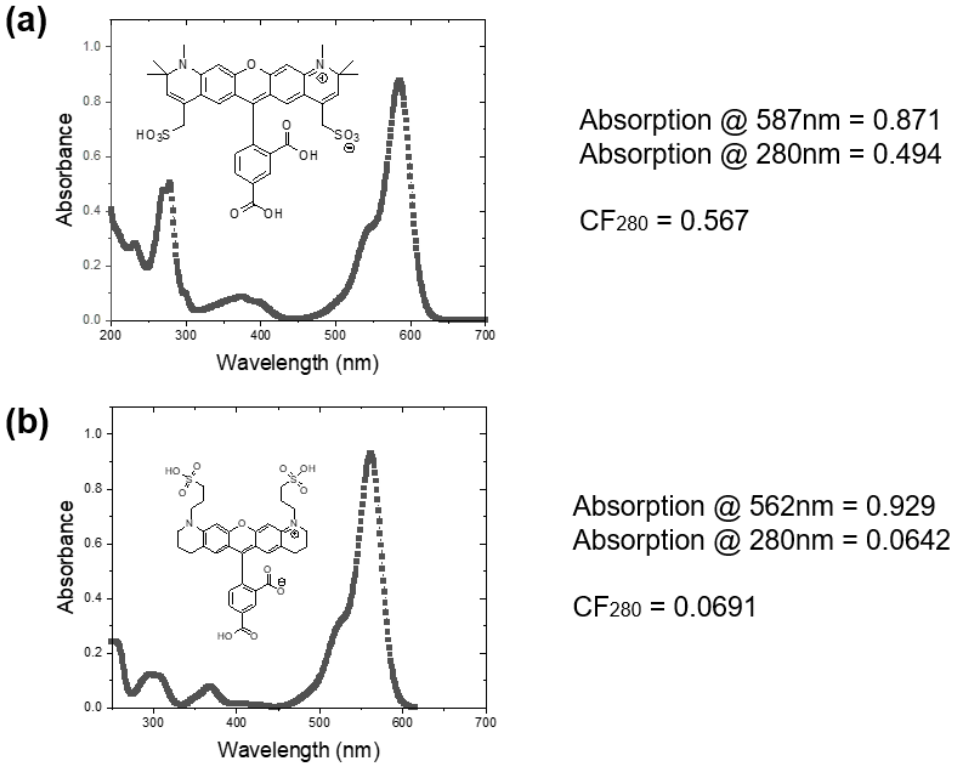


Figure S5. Calculation of CF_{280} values for compounds (a) **Rho590-COOH** and (b) **Rho565-COOH**.

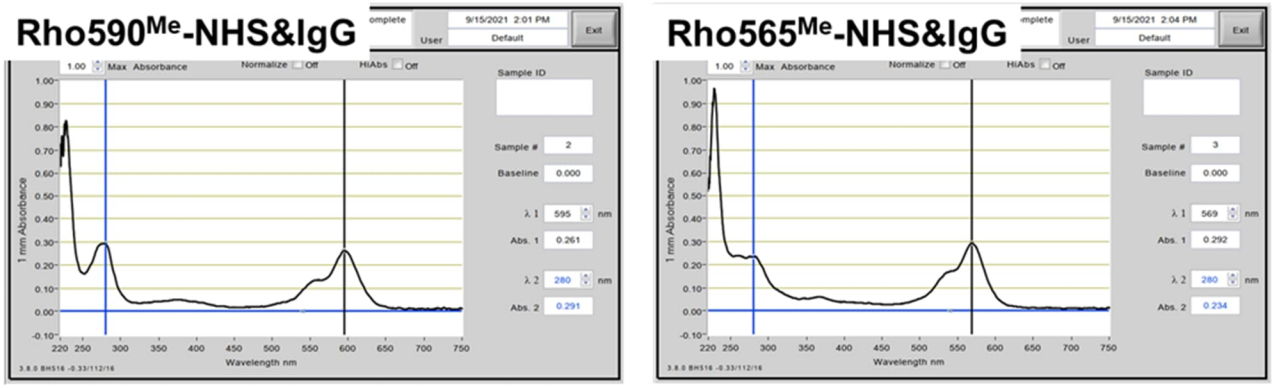


Figure S6. The degrees of labeling (DOL) obtained for the secondary antibodies conjugated with amino-reactive dyes **Rho590^{Me}**-NHS and **Rho565^{Me}**-NHS (the structures are given in Scheme S1) were calculated to be 4.2 and 3.1, respectively. The positions of the IgG (280 nm) and the dyes' absorption maxima are shown.

Figure S7. Photos of aqueous solutions of **Rho590-COOH**, **Rho565-COOH**, **Rho530-COOH**, and **AS488-COOH** under 365 nm illumination; for structures, see Figure 1 in the main text.

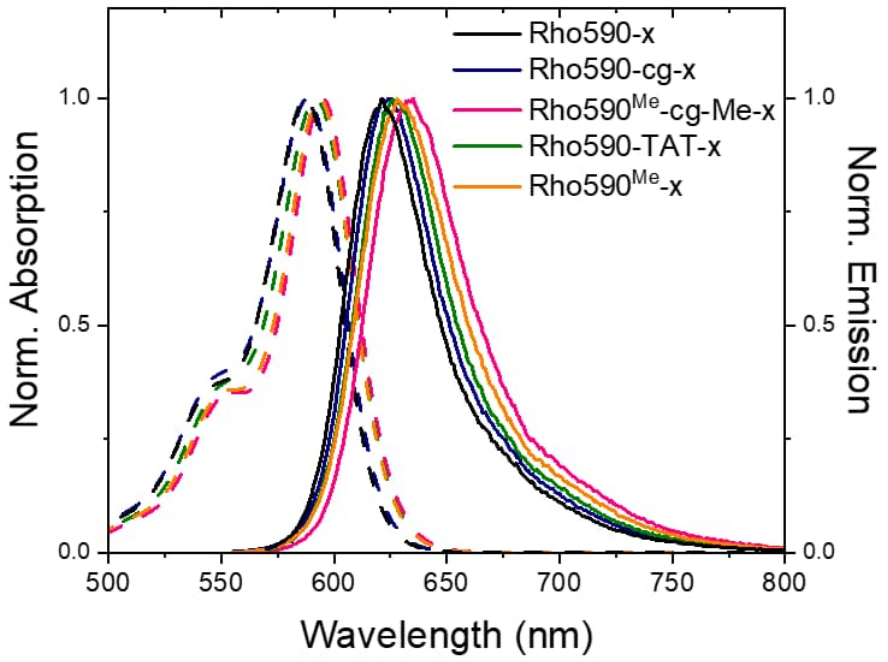


Figure S8. Normalized absorption and photoluminescence (PL) spectra of **Rho590-x**, **Rho590-cg-x**, **Rho590^{Me}-cg-Me-x**, **Rho590-TAT-x** and **Rho590^{Me}-x** (concentration: 10 μM in aqueous PBS buffer, pH 7.4).

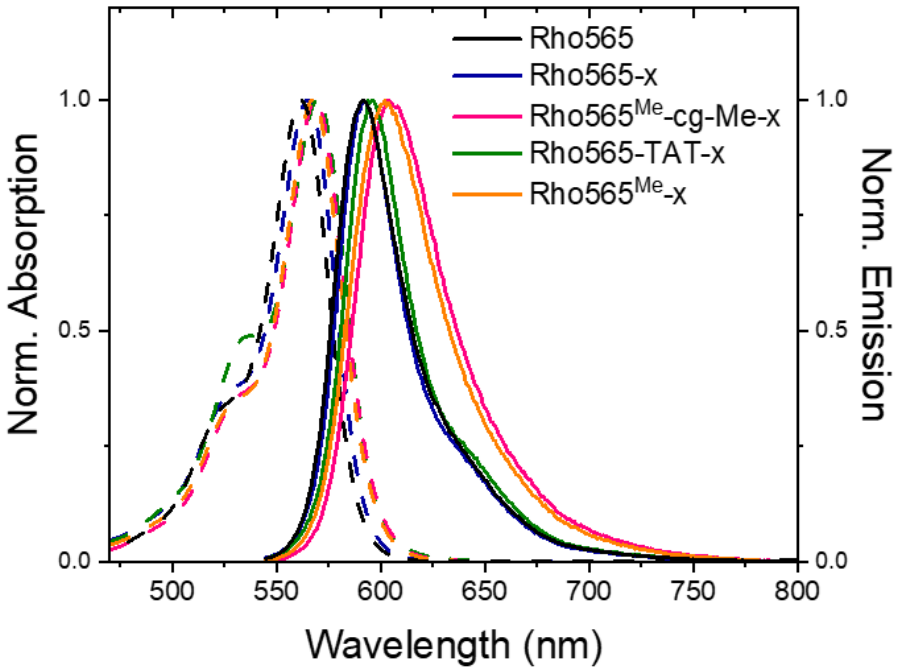


Figure S9. Normalized absorption and PL spectra of **Rho565**, **Rho565-x**, **Rho565^{Me}-cg-Me-x**, **Rho565-TAT-x** and **Rho565^{Me}-x** (concentration: 10 μM in aqueous PBS buffer, pH 7.4).

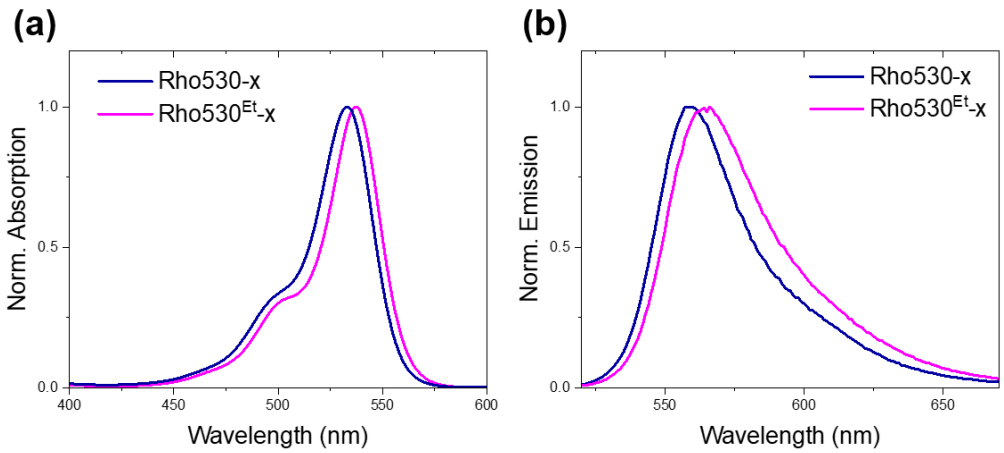


Figure S10. (a) Normalized absorption and (b) PL spectra of **Rho530-x** and **Rho530^{Et}-x** (concentration: 10 μM in aqueous PBS buffer, pH 7.4).

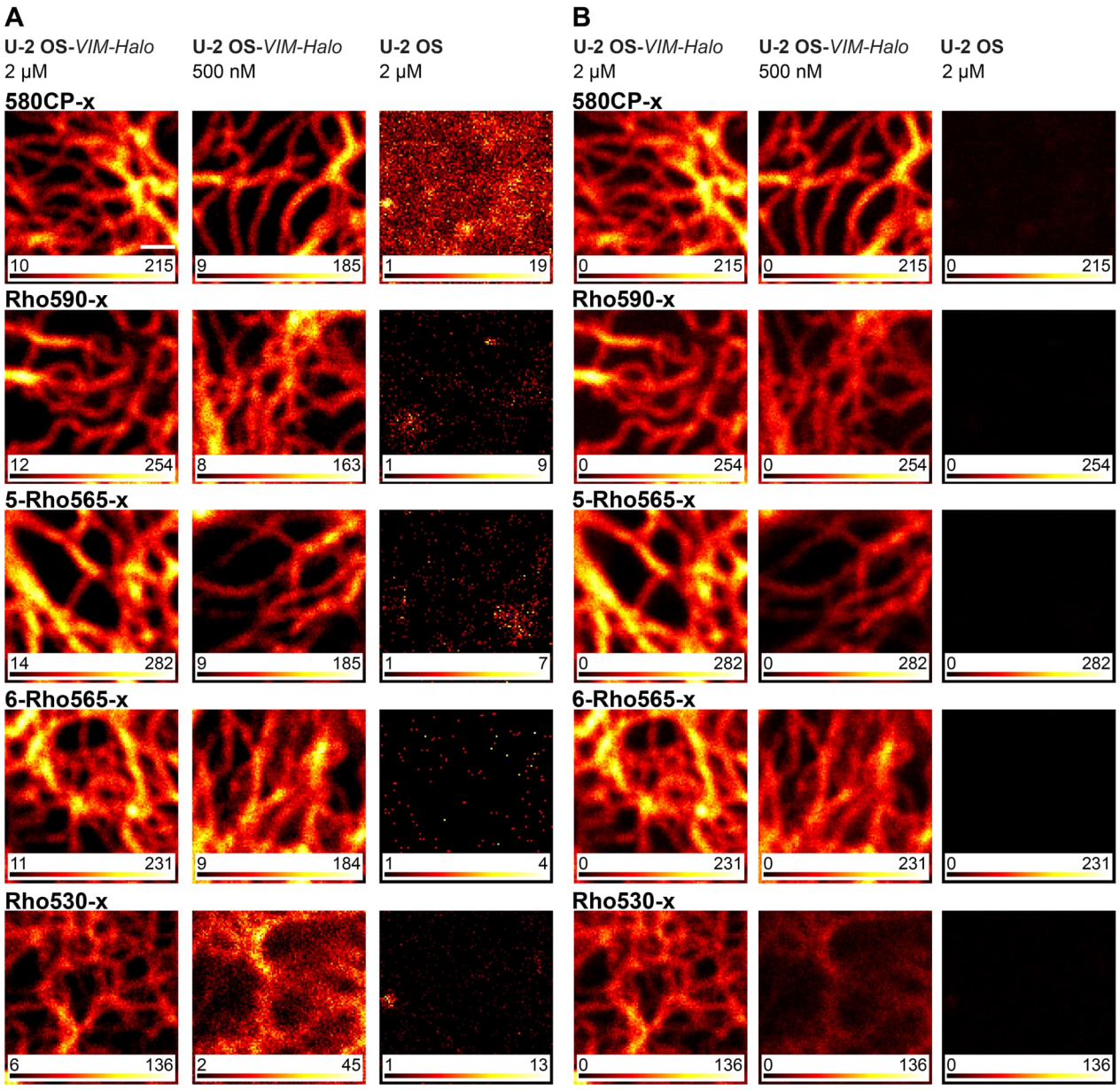


Figure S11. Labeling efficiency of Rhodamine Halo Ligands for live cell light microscopy is concentration dependent. (A-B) Confocal images of U-2 OS cells expressing *Vimentin-Halo* from the endogenous locus. Genetically unmodified U-2 OS cells were used for comparison to verify label specificity. Cells were labelled with 500 nM or 2 µM of the Halo Tag Ligands (x) of *580CP*, **Rho530**, **Rho565** and **Rho590**, respectively. For dye excitation, lasers with emission wavelengths of 485 nm (**Rho530**) and 561 nm (*580CP*, **Rho565**, **Rho590**) were used. Optimized settings were used for the image acquisition of each dye, but the same settings were used for different concentrations of the same dye. (A-B) Fluorescence images with (A) optimized and (B) identical settings for contrast stretching. Scale bar: 1 µm.

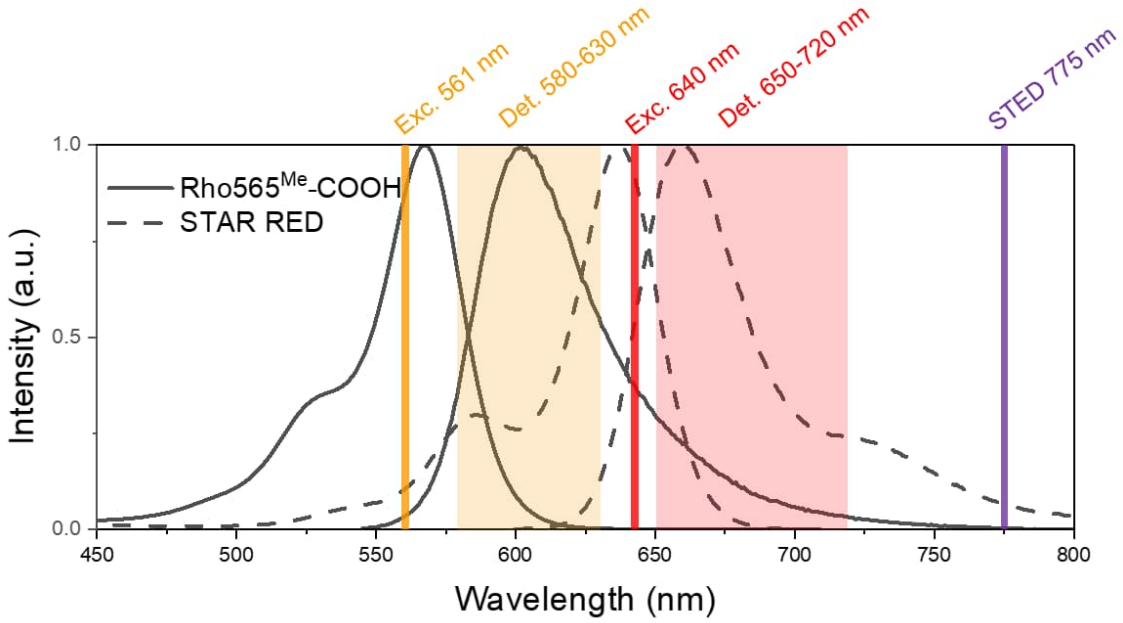


Figure S12. Two-color STED imaging scheme for **Rho565^{Me}-COOH** (for structure, see Figure 1 in the main text) and Abberior STAR RED (KK114). For a STED image, see Figure 4 in the main text. Excitation wavelengths and detection windows in accordance with this figure provide minimal cross-talk and excellent color separation (see Figure S15).

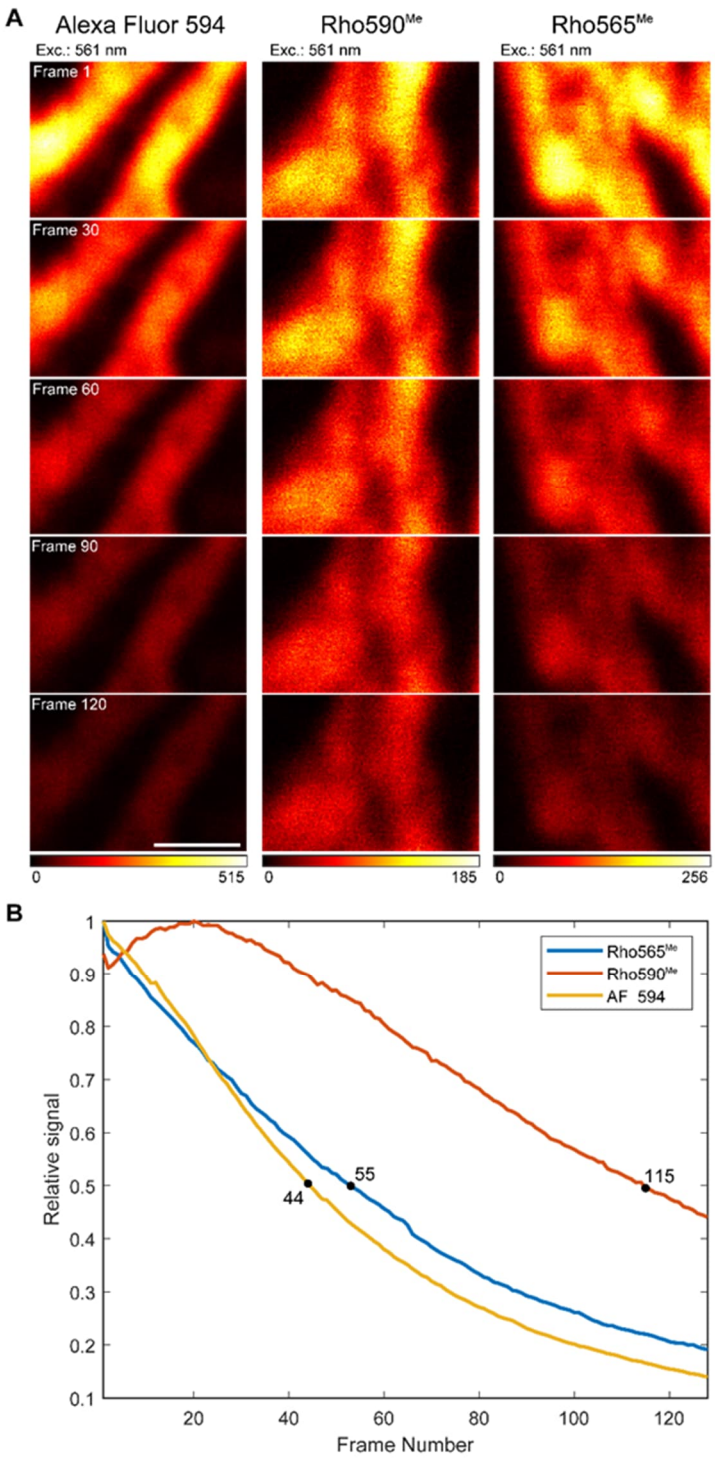


Figure S13. Comparison of the bleaching behavior of Alexa Fluor 594, Rho590^{Me} and Rho565^{Me} fluorophores in a confocal mode. (A) Confocal time series images (120 frames; emission signal) of fixed U2OS cells decorated with a Tom22 specific serum. The primary antibodies were detected by using secondary antibodies coupled with Alexa Fluor 594 (**Rho590**-COOH), **Rho590^{Me}**-COOH and **Rho565^{Me}**-COOH respectively. (B) Scale bar: 1 µm.

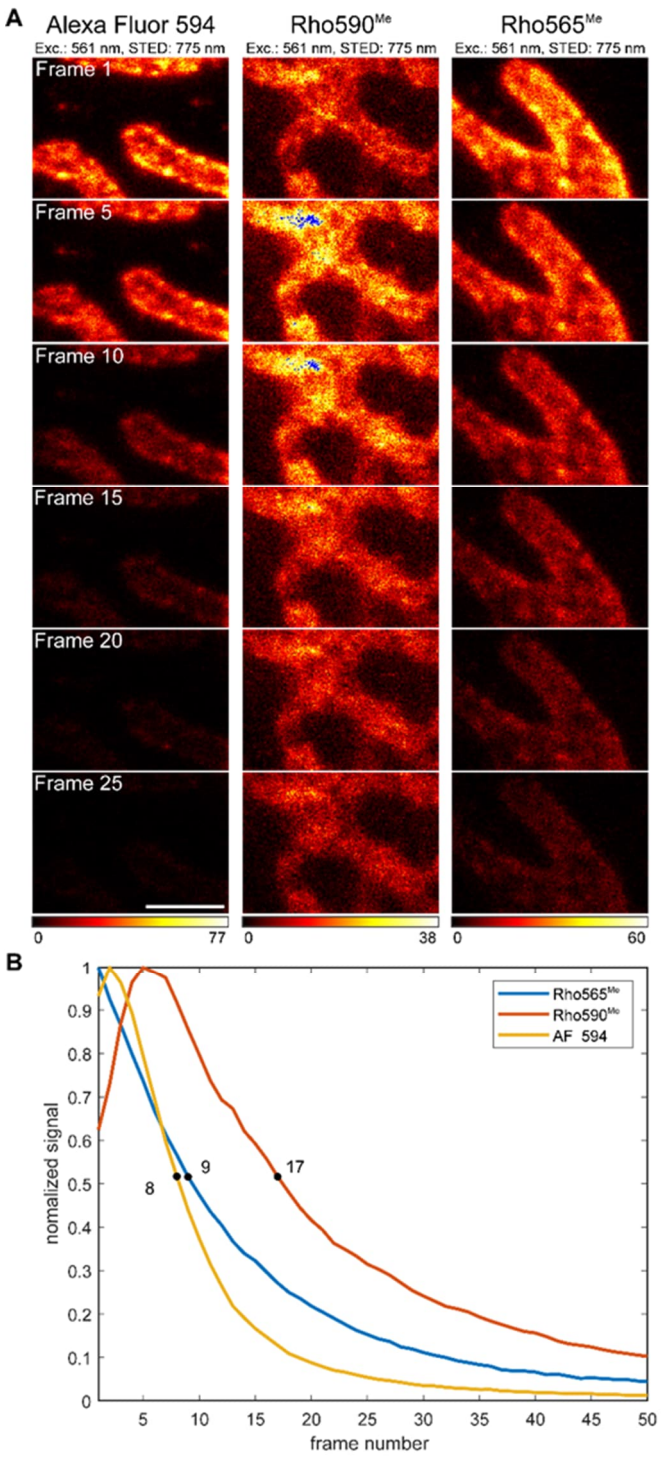


Figure S14. Comparison of bleaching behavior of Alexa Fluor 594, Rho590^{Me} and Rho565^{Me} fluorophores under STED conditions. (A) STED microscopy time series images (50 frames, emission signal) of fixed U2OS cells decorated with a Tom22 specific serum. The primary antibodies were detected by using secondary antibodies coupled with Alexa Fluor 594 (**Rho590**-COOH), **Rho590^{Me}**-COOH and **Rho565^{Me}**-COOH, respectively. (B) Scale bar: 1 µm.

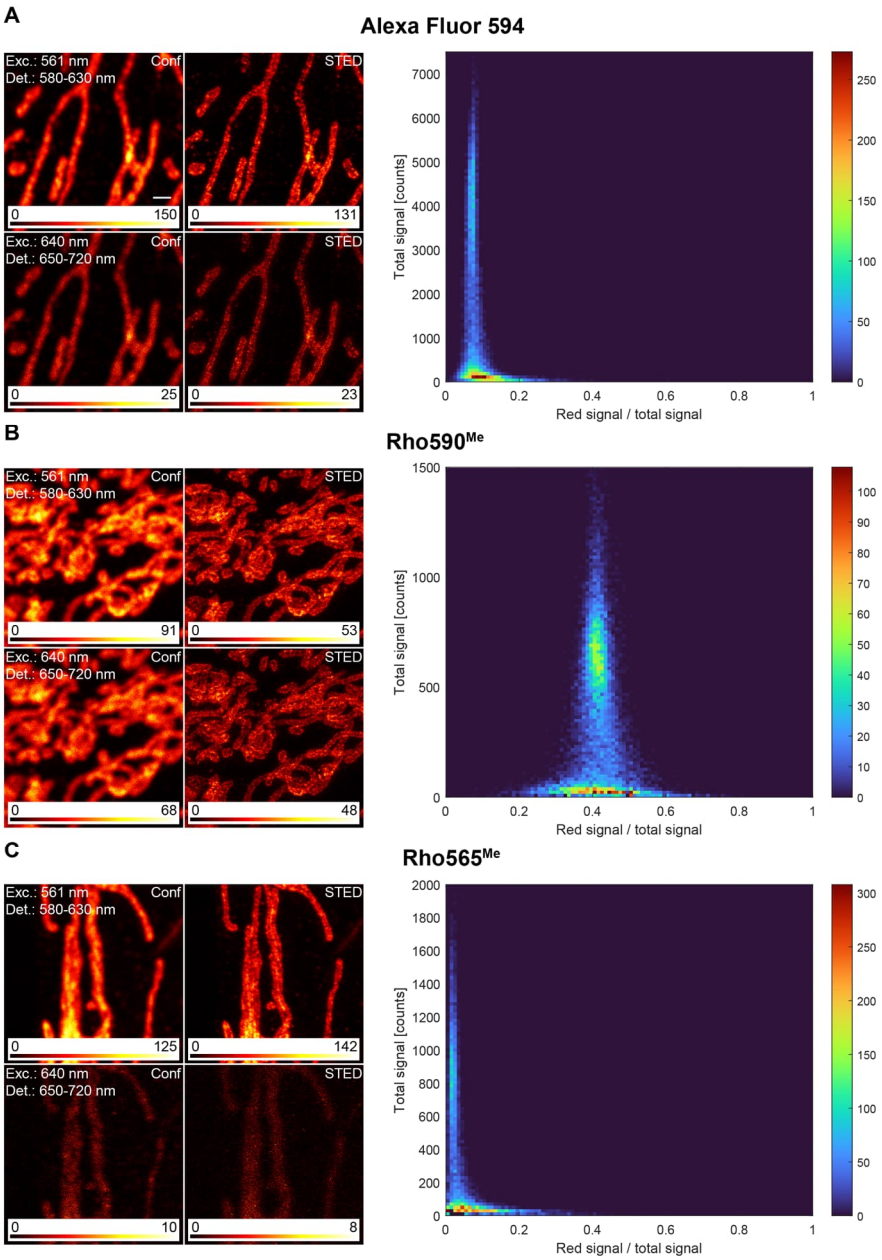
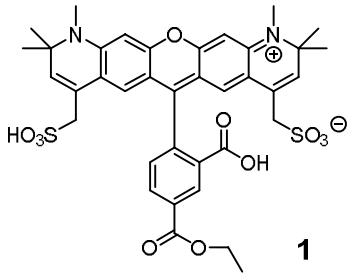


Figure S15. Signal separation between two detection channels measured for Alexa Fluor 594, Rho590^{Me} and Rho565^{Me} fluorophores. (A-C, left side) Confocal and STED images of fixed U2OS cells decorated with a Tom22 specific serum. The primary antibodies were detected by using secondary antibodies coupled with (A) Alexa Fluor 594 (**Rho590**-COOH), (B) **Rho590^{Me}**-COOH or (C) **Rho565^{Me}**-COOH, respectively. For the first channel, a 561 nm excitation laser and a detection range between 580 nm and 630 nm was used; for the second channel, a 640 nm excitation laser and a detection window between 650 nm and 720 nm was used. For super-resolution images, a 775 nm STED laser was used. Right column: distribution of the ratio "red signal" (detection window 650 – 720 nm) / total signal (both detection windows) *vs.* intensity of the total signal. Scale bar: 1 µm.

Table S1. STED efficiency of fluorophores with 775 nm STED laser wavelength. Photophysical properties of dye derivatives; measured in aqueous PBS buffer at pH 7.4

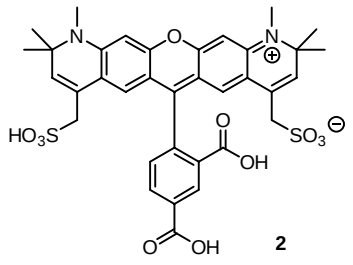
	Absorption		Emission			
	λ_{max} [nm]	ε [$Mol^{-1}cm^{-1}$]	λ_{max} [nm]	Emission at 775 nm	Relative ε at 775 nm [$Mol^{-1}cm^{-1}$]	Relative STED power
KK114-x	638	120,000	664	0.0363	4,356	1
Rho590-x	588	92,000	621	0.0131	1,205	3.6
580CP-x	587	90,000	613	0.0084	756	5.8
Rho565-x	565	93,000	592	0.0011	102	42.7
Rho530-x	533	74,000	558	0.0005	37	117.7
Atto532-x	532	120,000	553	0.0003	36	121

Synthesis



1

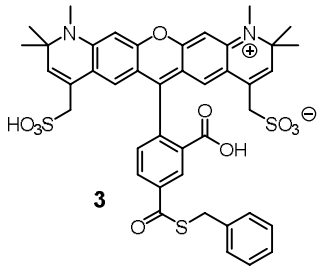
Compound **1:** Compound **1** was synthesized according to the reported procedure.[S2] ^{1}H-NMR (400 MHz, CD_3OD): δ (ppm) = 8.87 (ddd, *J* = 3.1, 1.7, 0.5 Hz, 1H), 8.44 (ddd, *J* = 7.9, 2.2, 1.7 Hz, 1H), 7.53 (dt, *J* = 7.9, 0.6 Hz, 1H), 7.16 (d, *J* = 0.6 Hz, 2H), 6.81 (d, *J* = 0.6 Hz, 2H), 5.89 (d, *J* = 2.9 Hz, 2H), 4.47 (q, *J* = 7.2 Hz, 2H), 3.71 – 3.53 (m, 4H), 3.18 (s, 6H), 1.54 (d, *J* = 4.6 Hz, 12H), 1.47 (t, *J* = 7.1 Hz, 3H). MS (ESI–, *m/z*): $[M-H]^-$ calcd for $C_{37}H_{37}N_2O_{11}S_2^-$, 749.2; found, 749.1.



2

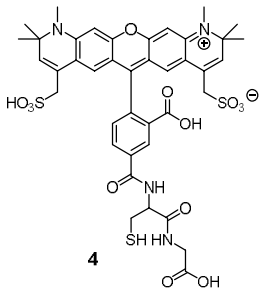
Compound **2:** Compound **1** (40 mg, 53 µmol, 1 equiv.) was dissolved in 1 mL of the solvent mixture H_2O/EtOH (4/1, v/v), which was stirred at 0 °C for 5 min. 266 µL of 1 M aq. NaOH (27 µmol, 5 equiv.) were added, and the reaction mixture was stirred for 2 h at 0 °C. The reaction was quenched by injection of 266 µL of 1 M HCl, and the solvent was evaporated. The product was isolated by preparative HPLC

(20 – 80% MeCN in H_2O + 0.1% TFA over 15 min; for details of prep. HPLC, see the general remarks for the synthesis). Lyophilization yielded 36 mg (94%) of **2** as a blue powder. ^{1}H-NMR (400 MHz, CD_3OD): δ (ppm) = 8.88 (d, *J* = 1.7 Hz, 1H), 8.44 (dd, *J* = 7.9, 1.7 Hz, 1H), 7.53 (d, *J* = 8.0 Hz, 1H), 7.16 (s, 2H), 6.81 (s, 2H), 5.89 (s, 2H), 3.73 – 3.52 (m, 4H), 3.18 (s, 6H), 1.54 (d, *J* = 5.2 Hz, 12H). MS (ESI+, *m/z*): $[M+H]^+$ calcd for $C_{35}H_{35}N_2O_{11}S_2^+$, 723.2; found, 723.2.



3

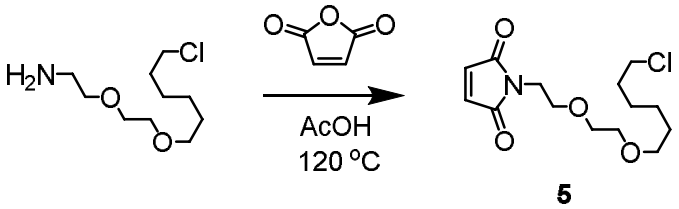
Compound **3:** To a solution of **2** (8.5 mg, 12 μmol, 1 equiv.) in anhydrous DMF (150 μL), HATU (6.7 mg, 18 μmol, 1.5 equiv.) and DIPEA (9.1 mg, 71 μmol, 6 equiv.) were added. The reaction mixture was stirred at r.t. for 10 min. Benzyl mercaptan (2.2 mg, 18 μmol, 1.5 equiv.) was added the reaction mixture, and was stirred for 20 min at r.t. DMF was evaporated in vacuum, and the product was isolated by preparative HPLC (10 – 80% MeCN in H_2O + 0.1% TFA over 20 min). Lyophilization yielded 8.3 mg (85%) of **3** as a blue powder. ^{1}H-NMR (400 MHz, CD_3OD): δ (ppm) = 8.82 (d, *J* = 1.8 Hz, 1H), 8.40 (dd, *J* = 8.0, 1.9 Hz, 1H), 7.55 (d, *J* = 7.9 Hz, 1H), 7.48 – 7.40 (m, 2H), 7.38 – 7.30 (m, 2H), 7.29 – 7.22 (m, 1H), 7.14 (s, 2H), 6.81 (s, 2H), 5.88 (s, 2H), 4.42 (s, 2H), 3.70 – 3.53 (m, 4H), 3.18 (s, 6H), 1.54 (d, *J* = 5.0 Hz, 12H). HRMS (ESI+, *m/z*): $[M+H]^+$ calcd for $C_{42}H_{41}N_2O_{10}S_3^+$, 829.1918; found, 829.1919.



4

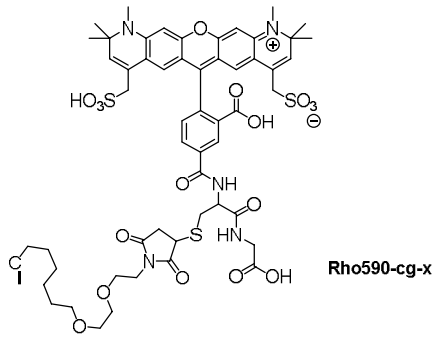
Compound **4:** To a solution of **3** (2.0 mg, 2.4 μmol, 1 equiv.) and H-Cys-Gly-OH (*Bachem*, Switzerland; 0.56 mg, 3.1 μmol, 1.3 equiv.) in the ligation buffer (500 μL), 2 μL of thiophenol and 125 μL of 0.5 M aq. TCEP (Sigma Aldrich GmbH) were added. In the resulting ligation buffer, the concentrations of thiophenol and TCEP were 0.4% v/v and 0.1 M, respectively. The pH of the reaction solution was

adjusted to pH 7.2 by adding 1 M aq. HCl. The reaction mixture was stirred at r.t. for 8 h. The product was isolated by preparative HPLC (10 – 80% MeCN in H_2O + 0.1% TFA over 20 min). Lyophilization yielded 2.1 mg (98%) of **4** as a blue powder. ^{1}H-NMR (400 MHz, CD_3OD): δ (ppm) = 8.79 (d, *J* = 1.8 Hz, 1H), 8.31 (dd, *J* = 8.0, 1.8 Hz, 1H), 7.52 (d, *J* = 7.9 Hz, 1H), 7.21 (d, *J* = 3.9 Hz, 2H), 6.81 (s, 2H), 5.87 (s, 2H), 4.83 – 4.79 (m, 1H), 3.97 (d, *J* = 2.6 Hz, 2H), 3.73 – 3.54 (m, 4H), 3.18 (s, 6H), 3.15 – 3.01 (m, 2H), 1.59 – 1.49 (m, 12H). HRMS (ESI–, *m/z*): $[M-H]^-$ calcd for $C_{40}H_{41}N_4O_{13}S_3^-$, 881.1838; found, 881.1858.



5

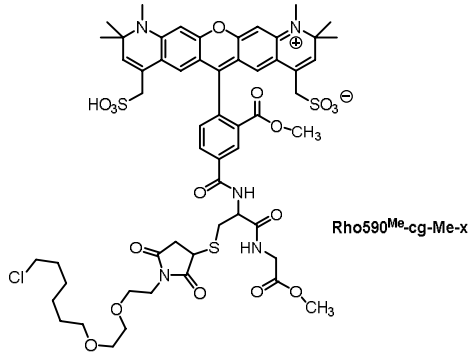
Compound **5:** HaloTag® Amine (O2) (*Promega GmbH*, USA) (1.9 mg, 8.5 μmol, 1 equiv.) and maleic anhydride (1.1 mg, 11 μmol, 1.3 equiv.) were dissolved in acetic acid (400 μL). The reaction mixture was then refluxed at heating in an oil bath (120 °C) for 2 h. After completion of the reaction, the reaction mixture was transferred into DCM (50 mL), organic layer separated and solvents removed in vacuum. The residue was subjected to chromatography on regular silica gel with gradient elution (MeOH/DCM: 90/10 → 10/90) to yield 2.0 mg (78%) of **5** as a pale yellow oil. ^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) = 6.70 (s, 2H), 3.76 – 3.69 (m, 2H), 3.67 – 3.63 (m, 2H), 3.62 – 3.58 (m, 2H), 3.56 – 3.51 (m, 4H), 3.43 (t, *J* = 6.6 Hz, 2H), 1.78 (dt, *J* = 14.4, 6.7 Hz, 2H), 1.61 – 1.52 (m, 2H), 1.48 – 1.34 (m, 4H). MS (ESI+, *m/z*): $[M+H]^+$ calcd for $C_{14}H_{22}ClNNaO_4^+$, 326.1; found, 326.2.



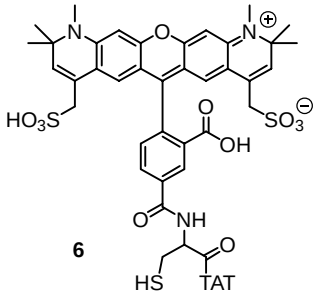
Rho590-cg-x

Rho590-cg-x: Compound **4** (1.3 mg, 1.5 μmol, 1 equiv.) and compound **5** (2.2 mg, 7.4 μmol, 5 equiv.) were dissolved in Tris buffer (pH 7.2, 500 μL). The reaction mixture was stirred for 1 h at r.t. The product was isolated by preparative HPLC (10 – 65% MeCN in H_2O + 0.1% TFA over 20 min). Lyophilization yielded 1.2 mg (69%) of **Rho590-cg-x** as a blue powder. ^{1}H-NMR (400 MHz, CD_3OD): δ (ppm) = 8.80 (t, *J* = 1.5 Hz, 1H), 8.33 (dd, *J* = 8.0, 1.8 Hz, 1H), 7.53 (dd, *J* = 7.9, 2.8 Hz, 1H), 7.20 (t, *J* = 2.4 Hz, 2H), 6.81 (d, *J* = 0.9 Hz, 2H), 5.92 – 5.83 (m, 2H), 5.06 – 4.98 (m, 1H), 4.11 (dd, *J* = 9.1, 3.9 Hz, 1H), 3.98 (s, 2H), 3.75 – 3.41 (m, 17H), 3.29 – 3.11 (m, 1H), 3.18 (s, 6H), 2.54 (ddd, *J* = 18.5, 5.1, 3.9 Hz, 1H), 1.81 –

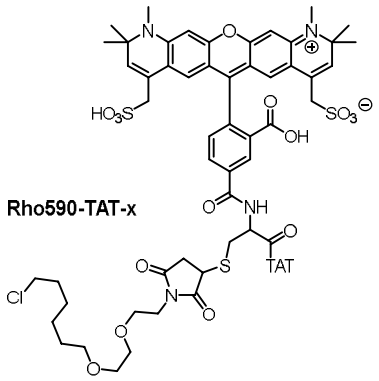
1.68 (m, 2H), 1.54 (d, J = 4.3 Hz, 14H), 1.50 – 1.32 (m, 4H). HRMS (ESI+, *m/z*): $[M+H]^+$ calcd for $C_{54}H_{65}ClN_5O_{17}S_3^+$, 1186.3221; found, 1186.3196.



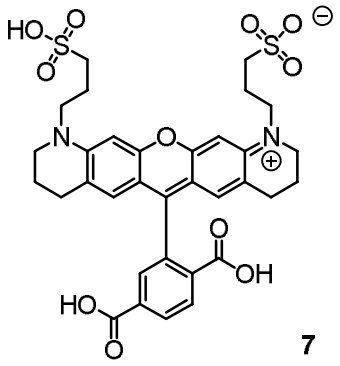
Rho590^{Me}-cg-Me-x: Rho590-cg-x (1.5 mg, 1.3 μmol) was dissolved in MeOH (1 mL), and the solution was stirred at 0 °C for 5 min. 500 μL of diazomethane solution (ca. 100 mM in ether) were added dropwise to the reaction mixture, which was stirred for 1 h at 0 °C. The solvents were evaporated in vacuo, and the product isolated by preparative HPLC (10 – 50% MeCN in H_2O + 0.1% TFA over 25 min). Lyophilization yielded 0.79 mg (51%) of **Rho590-cg-Me-x** as a blue powder. HRMS (ESI+, *m/z*): $[M+2Na]^{2+}$ calcd for $C_{56}H_{68}ClN_5O_{17}S_3Na_2^{2+}$, 629.6623; found, 629.6605.



Compound **6:** To a solution of **3** (1.0 mg, 1.2 μmol, 1 equiv.) and TAT peptide containing N-terminal cysteine (H-Cys-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Pro-Gln-NH_2) (*Genecust*, France) (2.6 mg, 1.5 μmol, 1.25 equiv.) in the ligation buffer (500 μL), 2 μL of thiophenol and 125 μL of 0.5 M aq. TCEP (Sigma Aldrich GmbH) were added. In the resulting ligation buffer, the concentrations of thiophenol and TCEP were 0.4% v/v and 0.1 M, respectively. The pH of the reaction solution was adjusted to pH 7.2 by adding 1 M aq. HCl solution. The reaction mixture was stirred at r.t. for 8 h. The product was isolated by preparative HPLC (10 – 60% MeCN in H_2O + 0.1% TFA over 20 min). Lyophilization yielded 2.9 mg (69%) of **6** as a blue powder. HRMS (ESI+, *m/z*): $[M+3H]^{3+}$ calcd for $C_{103}H_{165}N_{38}O_{25}S_3^{3+}$, 810.0651; found, 810.0639.



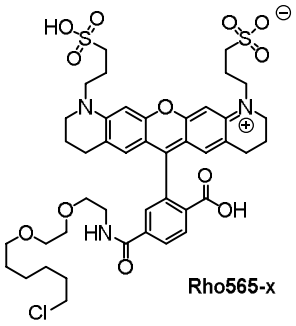
Rho590-TAT-x: Compound **6** (4.5 mg, 1.9 µmol, 1 equiv.) and compound **5** (1.7 mg, 5.6 µmol, 3 equiv.) were dissolved in 800 µL Tris buffer (0.1 M, pH 7.2). The reaction mixture was stirred for 3 h at r.t. The product was isolated by preparative HPLC (10 – 65% MeCN in H_2O + 0.1% TFA over 20 min). Lyophilization yielded 1.8 mg (51%) of **Rho590-TAT-x** as a blue powder. HRMS (ESI+, *m/z*): $[M+5H]^{5+}$ calcd for $C_{117}H_{189}ClN_{39}O_{29}S_3^{5+}$, 547.0667; found, 547.0665.



7

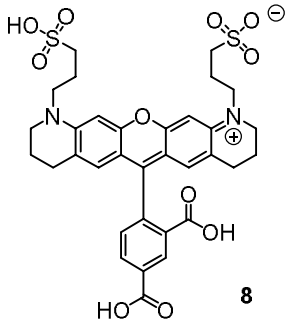
Compound **7:** Compound **7** was synthesized according to the literature method.[S3]

^{1}H-NMR (400 MHz, CD_3OD): δ (ppm) = 8.40 – 8.34 (m, 2H), 7.93 (dd, *J* = 1.5, 0.7 Hz, 1H), 7.08 (s, 2H), 6.74 (s, 2H), 3.87 – 3.74 (t, *J* = 7.5 Hz, 4H), 3.67 – 3.56 (t, *J* = 5.7 Hz, 4H), 3.02 – 2.90 (t, *J* = 6.9 Hz, 4H), 2.75 – 2.66 (t, *J* = 6.0 Hz, 4H), 2.28 – 2.16 (m, 4H), 2.00 – 1.88 (m, 4H). HRMS (ESI–, *m/z*): $[M-2H]^{2-}$ calcd for $C_{33}H_{32}N_2O_{11}S_2^{2-}$, 348.0729; found, 348.0749.



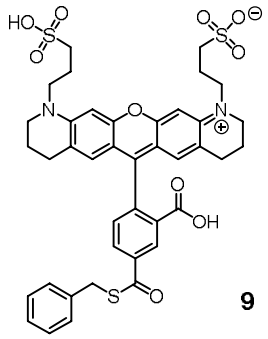
Rho565-x

Rho565-x: To a solution of **7** (1.0 mg, 1.4 µmol, 1 equiv.) in anhydrous DMF (80 µL), stock solutions (in DMF) of HATU (0.60 mg, 1.6 µmol, 1.1 equiv.) and DIPEA (0.92 mg, 7.2 µmol, 5 equiv.) were added. The reaction mixture was stirred at r.t. for 10 min. A DMF stock solution of HaloTag® Amine (O2) (*Promega GmbH*, USA) (0.45 mg, 2.0 µmol, 1.4 equiv.) was added to the reaction mixture which was then stirred for 20 min at r.t. DMF was evaporated in vacuum, and the product was isolated by preparative HPLC (10 – 70% MeCN in H_2O + 0.1% TFA over 20 min). Lyophilization yielded 1.2 mg (93%) of **Rho565-x** as a purple powder. ^{1}H-NMR (400 MHz, CD_3OD): δ (ppm) = 8.37 (d, *J* = 8.3 Hz, 1H), 8.19 (dd, *J* = 8.2, 1.8 Hz, 1H), 7.78 (d, *J* = 1.7 Hz, 1H), 7.08 (s, 2H), 6.76 (s, 2H), 3.85 – 3.76 (m, 4H), 3.68 – 3.54 (m, 14H), 3.52 (t, *J* = 6.7 Hz, 2H), 3.42 (t, *J* = 6.5 Hz, 2H), 2.95 (t, *J* = 7.3 Hz, 4H), 2.70 (t, *J* = 5.9 Hz, 4H), 2.26 – 2.18 (m, 4H), 1.99 – 1.89 (m, 4H), 1.78 – 1.65 (m, 2H), 1.49 (dt, *J* = 14.0, 6.6 Hz, 2H), 1.42 – 1.36 (m, 3H), 1.35 – 1.31 (m, 3H). HRMS (ESI–, *m/z*): $[M-H]^-$ calcd for $C_{43}H_{53}ClN_3O_{12}S_2^-$, 902.2765; found, 902.2765.



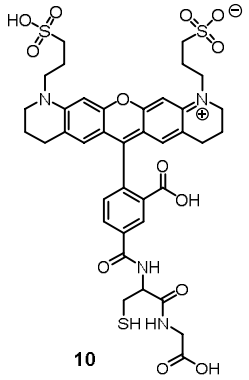
8

Compound **8:** Compound **8** was synthesized according to the literature method.[S3] ^{1}H-NMR (400 MHz, CD_3OD): δ (ppm) = 8.89 (dd, *J* = 1.8, 0.5 Hz, 1H), 8.42 (dd, *J* = 7.9, 1.7 Hz, 1H), 7.51 (dd, *J* = 7.9, 0.5 Hz, 1H), 7.07 (s, 2H), 6.73 (s, 2H), 3.79 (t, *J* = 7.6 Hz, 4H), 3.61 (t, *J* = 5.6 Hz, 4H), 2.96 (t, *J* = 6.5 Hz, 4H), 2.70 (t, *J* = 6.0 Hz, 4H), 2.27 – 2.16 (m, 4H), 1.98 – 1.88 (m, 4H). HRMS (ESI–, *m/z*): $[M-2H]^{2-}$ calcd for $C_{33}H_{32}N_2O_{11}S_2^{2-}$, 348.0729; found, 348.0730.



9

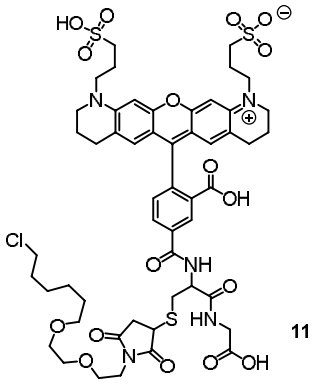
Compound **9:** To a solution of **8** (5.0 mg, 7.2 μmol, 1 equiv.) in anhydrous DMF (200 μL), stock solutions (in DMF) of HATU (3.0 mg, 3.1 μmol, 7.9 equiv.) and DIPEA (4.6 mg, 36 μmol, 5 equiv.) were added. The reaction mixture was stirred at r.t. for 10 min. A DMF stock solution of benzyl mercaptan (1.1 mg, 8.6 μmol, 1.2 equiv.) was added to the reaction mixture which was then stirred for 30 min at r.t. DMF was evaporated in vacuum, and the product was isolated by preparative HPLC (10 – 70% MeCN in H_2O + 0.1% TFA over 20 min). Lyophilization yielded 3.4 mg (59%) of **9** as a purple powder. ^{1}H-NMR (400 MHz, CD_3OD): δ (ppm) = 8.81 (dd, *J* = 1.9, 0.5 Hz, 1H), 8.36 (dd, *J* = 8.0, 1.9 Hz, 1H), 7.53 (dd, *J* = 8.0, 0.5 Hz, 1H), 7.47 – 7.41 (m, 2H), 7.38 – 7.31 (m, 2H), 7.31 – 7.24 (m, 1H), 7.07 (s, 2H), 6.73 (s, 2H), 4.44 (s, 2H), 3.79 (t, *J* = 7.6 Hz, 4H), 3.61 (t, *J* = 5.7 Hz, 4H), 2.94 (t, *J* = 7.1 Hz, 4H), 2.69 (t, *J* = 6.2 Hz, 4H), 2.26 – 2.15 (m, 4H), 1.99 – 1.86 (m, 4H). HRMS (ESI–, *m/z*): $[M-H]^-$ calcd for $C_{40}H_{39}N_2O_{10}S_3^-$, 803.1772; found, 803.1782.



10

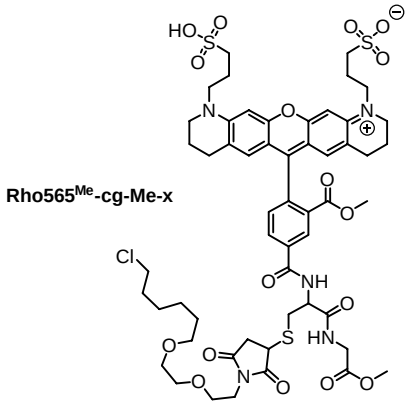
Compound **10:** To a solution of **9** (5.0 mg, 6.2 μmol, 1 equiv.) and H-Cys-Gly-OH (*Bachem*, Switzerland) (1.4 mg, 8.1 μmol, 1.3 equiv.) in the ligation buffer (500 μL), 2 μL of thiophenol and 125 μL of 0.5 M aq. TCEP (Sigma Aldrich GmbH) were added. In the resulting ligation buffer, the concentrations of thiophenol and TCEP were 0.4% v/v and 0.1 M, respectively. The pH of the reaction solution was adjusted to pH 7.2 by adding 1 M aq. HCl. The reaction mixture was stirred at r.t. for 4 h. The product was isolated by preparative HPLC (10 – 70% MeCN in H_2O + 0.1% TFA over 20 min). Lyophilization yielded 4.0 mg (75%) of **10** as a purple powder. ^{1}H-NMR (400 MHz, CD_3OD): δ (ppm) = 8.81 (d, *J* = 1.9 Hz,

1H), 8.31 (dd, *J* = 7.9, 1.9 Hz, 1H), 7.52 (d, *J* = 7.9 Hz, 1H), 7.07 (s, 2H), 6.75 (s, 2H), 4.84 – 4.81 (m, 1H), 4.05 – 3.93 (m, 2H), 3.79 (t, *J* = 7.6 Hz, 4H), 3.61 (t, *J* = 5.6 Hz, 4H), 3.17 – 3.10 (m, 1H), 3.04 – 2.98 (m, 1H), 2.95 (t, *J* = 7.0 Hz, 4H), 2.69 (t, *J* = 6.0 Hz, 4H), 2.27 – 2.16 (m, 4H), 1.99 – 1.88 (m, 4H). HRMS (ESI–, *m/z*): $[M-2H]^{2-}$ calcd for $C_{38}H_{40}N_4O_{13}S_3^{2-}$, 428.0882; found, 428.0898.



11

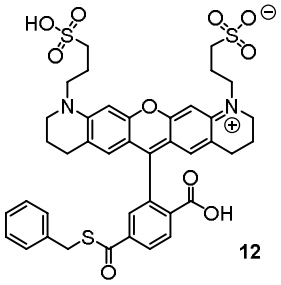
Compound **11:** Compound **10** (5.0 mg, 5.8 μmol, 1 equiv.) and compound **5** (6.8 mg, 22 μmol, 3.8 equiv.) were dissolved in Tris buffer (pH 7.2, 4 mL). The reaction mixture was stirred for 30 min at r.t. The product was isolated by preparative HPLC (10 – 60% MeCN in H_2O + 0.1% TFA over 20 min). Lyophilization yielded 4.4 mg (65%) of **11** as a purple powder. ^{1}H-NMR (400 MHz, CD_3OD): δ (ppm) = 8.85 – 8.79 (m, 1H), 8.32 (dt, *J* = 7.9, 2.1 Hz, 1H), 7.52 (dd, *J* = 8.0, 2.4 Hz, 1H), 7.07 (s, 2H), 6.78 – 6.72 (m, 2H), 5.08 – 4.98 (m, 1H), 4.10 (ddd, *J* = 9.1, 4.0, 2.0 Hz, 1H), 4.00 (d, *J* = 1.8 Hz, 2H), 3.85 – 3.75 (m, 4H), 3.75 – 3.69 (m, 2H), 3.69 – 3.64 (m, 2H), 3.64 – 3.11 (m, 14H), 2.95 (t, *J* = 7.0 Hz, 4H), 2.69 (t, *J* = 6.1 Hz, 4H), 2.56 (ddd, *J* = 25.4, 18.6, 4.0 Hz, 1H), 2.21 (p, *J* = 7.0 Hz, 4H), 2.00 – 1.88 (m, 4H), 1.74 (dq, *J* = 13.3, 6.6 Hz, 2H), 1.61 – 1.35 (m, 6H). HRMS (ESI–, *m/z*): $[M-2H]^{2-}$ calcd for $C_{52}H_{62}ClN_5O_{17}S_3^{2-}$, 579.6501; found, 579.6527.



Rho565^{Me}-cg-Me-x

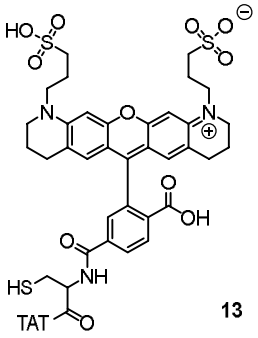
Rho565^{Me}-cg-Me-x: Compound **11** (3.0 mg, 2.6 μmol) was dissolved in methanol (1 mL), and the solution was stirred at 0 °C for 5 min. Diazomethane (200 μL of 100 mM solution in ether) was added dropwise to the reaction mixture which was then stirred for 30 min at 0 °C. The product was isolated by

preparative HPLC (10 – 60% MeCN in H_2O + 0.1% TFA over 20 min). Lyophilization yielded 0.2 mg (18%) of **Rho565^{Me}-cg-Me-x** as a purple powder. ^{1}H-NMR (400 MHz, CD_3OD): δ (ppm) = 8.84 – 8.78 (m, 1H), 8.34 (dt, *J* = 8.0, 2.1 Hz, 1H), 7.55 (dd, *J* = 8.0, 2.2 Hz, 1H), 7.09 (s, 2H), 6.72 (s, 2H), 5.07 – 4.99 (m, 1H), 4.13 – 4.07 (m, 1H), 4.05 – 4.02 (m, 2H), 3.85 – 3.76 (m, 4H), 3.76 – 3.69 (m, 5H), 3.69 – 3.49 (m, 15H), 3.48 – 3.11 (m, 4H), 2.95 (t, *J* = 7.1 Hz, 4H), 2.72 – 2.64 (m, 4H), 2.56 (ddd, *J* = 27.0, 18.6, 4.1 Hz, 1H), 2.22 (p, *J* = 7.4 Hz, 4H), 1.99 – 1.88 (m, 4H), 1.74 (dq, *J* = 13.2, 6.6 Hz, 2H), 1.56 (dt, *J* = 14.2, 7.1 Hz, 2H), 1.49 – 1.32 (m, 4H). HRMS (ESI+, *m/z*): $[M+H]^+$ calcd for $C_{54}H_{69}ClN_5O_{17}S_3^+$, 1190.3534; found, 1190.3527.



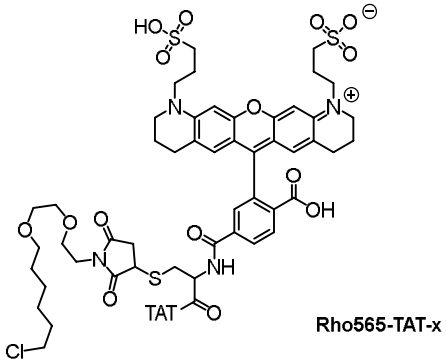
12

Compound **12:** To a solution of **7** (2.0 mg, 2.8 μmol, 1 equiv.) in anhydrous DMF (110 μL), stock solutions (in DMF) of HATU (1.2 mg, 3.1 μmol, 1.1 equiv.) and DIPEA (1.9 mg, 14 μmol, 5 equiv.) were added. The reaction mixture was stirred at r.t. for 10 min. A DMF stock solution of benzyl mercaptan (0.43 mg, 3.4 μmol, 1.2 equiv.) was added to the reaction mixture which was then stirred for 30 min at r.t. DMF was evaporated in vacuum, and the product was isolated by preparative HPLC (10 – 70% MeCN in H_2O + 0.1% TFA over 20 min). Lyophilization yielded 1.1 mg (96%) of **12** as a purple powder. ^{1}H-NMR (400 MHz, CD_3OD): δ (ppm) = 8.38 (d, *J* = 8.3 Hz, 1H), 8.30 (dd, *J* = 8.3, 1.9 Hz, 1H), 7.83 (d, *J* = 1.9 Hz, 1H), 7.40 – 7.35 (m, 2H), 7.33 – 7.27 (m, 2H), 7.27 – 7.21 (m, 1H), 7.08 (s, 2H), 6.72 (s, 2H), 4.36 (s, 2H), 3.79 (t, *J* = 7.8 Hz, 4H), 3.59 (t, *J* = 5.5 Hz, 4H), 2.97 (t, *J* = 7.2 Hz, 4H), 2.67 (t, *J* = 6.3 Hz, 4H), 2.28 – 2.16 (m, 4H), 1.96 – 1.85 (m, 4H). HRMS (ESI–, *m/z*): $[M-2H]^{2-}$ calcd for $C_{40}H_{38}N_2O_{10}S_3^{2-}$, 401.0850; found, 401.0874.



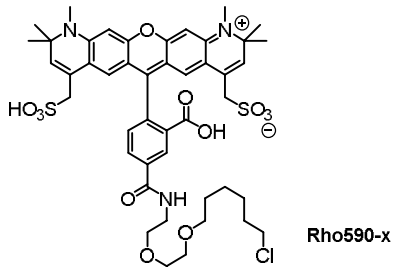
13

Compound **13:** To a solution of **12** (1.0 mg, 1.2 μmol, 1 equiv.) and TAT peptide containing N-terminal cysteine (H-Cys-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Pro-Gln-NH_2) (*Genecust*, France; 2.5 mg, 1.4 μmol, 1.2 equiv.) in the ligation buffer (500 μL), 2 μL of thiophenol and 125 μL of 0.5 M aq. TCEP (Sigma Aldrich GmbH) were added. In the resulting ligation buffer, the concentrations of thiophenol and TCEP were 0.4% v/v and 0.1 M, respectively. The pH of the reaction solution was adjusted to pH 7.2 by adding 1 M aq. HCl solution. The reaction mixture was stirred at r.t. for 4 h. The product was isolated by preparative HPLC (10 – 70% MeCN in H_2O + 0.1% TFA over 20 min). Lyophilization yielded 2.7 mg (93%) of **13** as a purple powder. HRMS (ESI+, *m/z*): $[M+5H]^{5+}$ calcd for $C_{101}H_{167}N_{38}O_{25}S_3^{5+}$, 481.6420; found, 481.6418.



Rho565-TAT-x

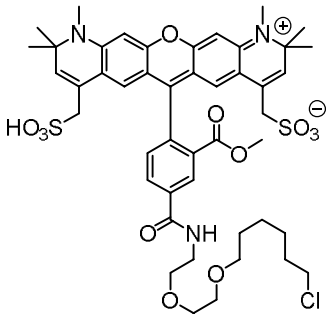
Rho565-TAT-x: Compound **13** (7.4 mg, 3.1 μmol, 1 equiv.) and compound **5** (2.2 mg, 7.1 μmol, 2.3 equiv.) were dissolved in Tris buffer (pH 7.2, 5 mL). TCEP (100 μL of 0.5 M aq. solution) was added to the reaction mixture which was then stirred for 1 h at r.t. The product was isolated by preparative HPLC (10 – 55% MeCN in H_2O + 0.1% TFA over 20 min). Lyophilization yielded 1.1 mg (13%) of **Rho565-TAT-x** as a purple powder. HRMS (ESI+, *m/z*): $[M+5H]^{5+}$ calcd for $C_{115}H_{189}ClN_{39}O_{29}S_3^{5+}$, 542.2667; found, 542.2672.



Rho590-x

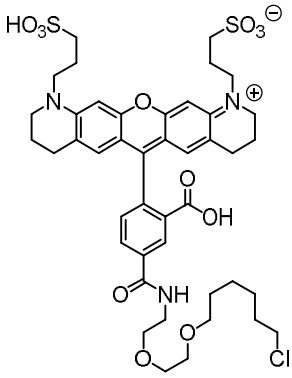
Rho590-x: To a solution of **2** (5.0 mg, 6.9 μmol, 1 equiv.) in anhydrous DMF (370 μL), stock solutions (in DMF) of HATU (2.9 mg, 7.6 μmol, 1.1 equiv.) and DIPEA (4.5 mg, 35 μmol, 5 equiv.) were added. The reaction mixture was stirred at r.t. for 10 min. A DMF stock solution of HaloTag® Amine (O2) (*Promega GmbH*, USA) (2.2 mg, 9.7 μmol, 1.4 equiv.) was added to the reaction mixture, which was then stirred for 20 min at r.t. DMF was evaporated in vacuum, and the product was isolated by preparative

HPLC (10 – 60% MeCN in H_2O + 0.1% TFA over 30 min). Lyophilization yielded 3.6 mg (56%) of **Rho590-x** as a blue powder. ^{1}H-NMR (400 MHz, CD_3OD): δ (ppm) = 8.71 (d, *J* = 1.6 Hz, 1H), 8.23 (dd, *J* = 8.0, 1.7 Hz, 1H), 7.51 (d, *J* = 7.9 Hz, 1H), 7.18 (s, 2H), 6.81 (s, 2H), 5.88 (s, 2H), 3.74 – 3.50 (m, 16H), 3.18 (s, 6H), 1.79 – 1.70 (m, 2H), 1.64 – 1.56 (m, 2H), 1.54 (d, *J* = 5.4 Hz, 12H), 1.50 – 1.35 (m, 4H). HRMS (ESI+, *m/z*): $[M+2H]^{2+}$ calcd for $C_{45}H_{56}ClN_3O_{12}S_2^{2+}$, 464.6491; found, 464.6480.



Rho590^{Me}-x

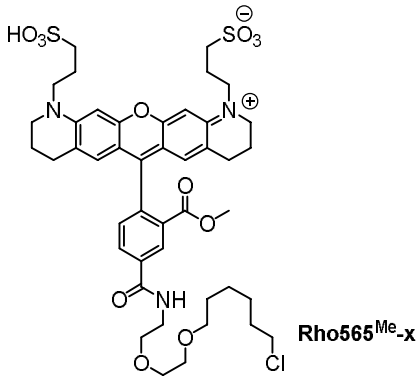
Rho590^{Me}-x: Compound **Rho590-x** (3.6 mg, 3.9 μmol) was dissolved in methanol (800 μL), and the solution was stirred at 0 °C for 5 min. Diazomethane (326 μL, *c* ~ 20 mg/mL in ether, 40 equiv.) was added dropwise to the reaction mixture, which was then stirred for 50 min at 0 °C. The product was isolated by preparative HPLC (10 – 50% MeCN in H_2O + 0.1% TFA over 30 min). Lyophilization yielded 1.1 mg (30%) of **Rho590^{Me}-x** as a blue powder. ^{1}H-NMR (400 MHz, CD_3OD): δ (ppm) = 8.72 (d, *J* = 1.8 Hz, 1H), 8.24 (dd, *J* = 8.0, 1.8 Hz, 1H), 7.51 (d, *J* = 8.0 Hz, 1H), 7.13 (s, 2H), 6.82 (s, 2H), 5.87 (s, 2H), 3.75 – 3.49 (m, 19H), 3.19 (s, 6H), 1.79 – 1.70 (m, 2H), 1.64 – 1.57 (m, 2H), 1.54 (s, 12H), 1.49 – 1.36 (m, 4H). HRMS (ESI–, *m/z*): $[M-H]^-$ calcd for $C_{46}H_{55}ClN_3O_{12}S_2^-$, 940.2921; found, 940.2888.



Rho565-x

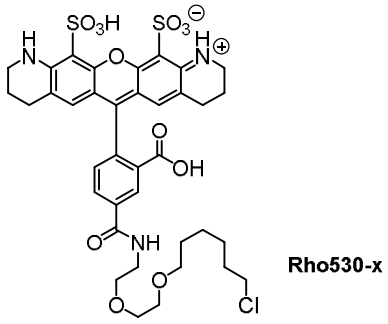
Rho565-x: To a solution of **8** (5.0 mg, 7.2 μmol, 1 equiv.) in anhydrous DMF (370 μL), stock solutions (in DMF) of HATU (3.0 mg, 7.9 μmol, 1.1 equiv.) and DIPEA (4.6 mg, 36 μmol, 5 equiv.) were added. The reaction mixture was stirred at r.t. for 10 min. A DMF stock solution of HaloTag® Amine (O2) (*Promega GmbH*, USA) (2.2 mg, 10 μmol, 1.4 equiv.) was added to the reaction mixture which was then stirred for 20 min at r.t. DMF was evaporated in vacuum, and the product was isolated by preparative HPLC (10 – 50% MeCN in H_2O + 0.1% TFA over 30 min). Lyophilization yielded 3.1 mg (48%) of

Rho565-x as a purple powder. ^{1}H-NMR (400 MHz, CD_3OD): δ (ppm) = 8.75 (dd, *J* = 1.8, 0.4 Hz, 1H), 8.25 (dd, *J* = 7.9, 1.9 Hz, 1H), 7.52 – 7.48 (m, 1H), 7.07 (s, 2H), 6.74 (s, 2H), 3.83 – 3.76 (m, 4H), 3.75 – 3.58 (m, 14H), 3.51 (t, *J* = 6.6 Hz, 4H), 2.95 (t, *J* = 7.2 Hz, 4H), 2.69 (t, *J* = 5.9 Hz, 4H), 2.21 (p, *J* = 7.5 Hz, 4H), 1.99 – 1.90 (m, 4H), 1.78 – 1.68 (m, 2H), 1.59 (p, *J* = 6.7 Hz, 2H), 1.47 – 1.35 (m, 4H). HRMS (ESI+, *m/z*): $[M+2Na]^{2+}$ calcd for $C_{43}H_{54}ClN_3Na_2O_{12}S_2^{2+}$, 474.6311; found, 474.6293.



Rho565^{Me}-x

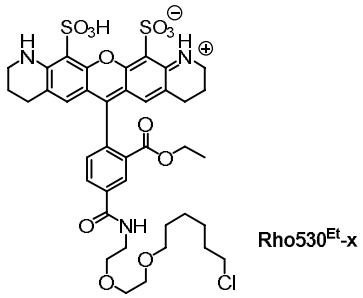
Rho565^{Me}-x: Compound **Rho565-x** (3.1 mg, 3.4 μmol) was dissolved in methanol (650 μL), and the solution was stirred at 0 °C for 5 min. Diazomethane (288 μL, *c* ~ 20 mg/mL in ether, 40 equiv.) was added dropwise to the reaction mixture which was then stirred for 50 min at 0 °C. The product was isolated by preparative HPLC (10 – 50% MeCN in H_2O + 0.1% TFA over 30 min). Lyophilization yielded 0.6 mg (19%) of **Rho565^{Me}-x** as a purple powder. HRMS (ESI–, *m/z*): $[M-H]^-$ calcd for $C_{44}H_{55}ClN_3O_{12}S_2^-$, 916.2921; found, 916.2931.



Rho530-x

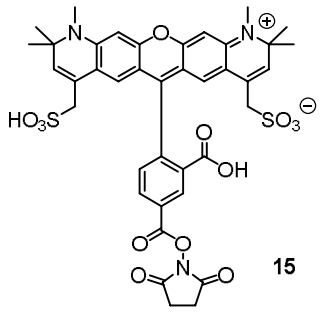
Rho530-x: To a solution of compound **14**[S4] (2.0 mg, 3.3 μmol) in anhydrous DMF (200 μL), stock solutions (in DMF) of HATU (1.4 mg, 3.6 μmol, 1.1 equiv.) and DIPEA (2.1 mg, 16 μmol, 5 equiv.) were added. The reaction mixture was stirred at r.t. for 10 min. A DMF stock solution of HaloTag® Amine (O2) (*Promega GmbH*, USA) (1.0 mg, 4.6 μmol, 1.4 equiv.) was added to the reaction mixture which was then stirred for 20 min at r.t. DMF was evaporated in vacuum, and the product was isolated by preparative HPLC (10 – 50% MeCN in H_2O + 0.1% TFA over 30 min). Lyophilization yielded 2.5 mg (95%) of **Rho530-x** as a magenta powder. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) = 10.24 (s, 2H), 8.94 (t, *J* = 5.7

Hz, 1H), 8.70 (d, *J* = 1.7 Hz, 1H), 8.27 (dd, *J* = 8.0, 1.9 Hz, 1H), 7.47 (d, *J* = 7.9 Hz, 1H), 6.64 (s, 2H), 3.62 – 3.49 (m, 16H), 2.69 – 2.64 (m, 4H), 1.79 – 1.72 (m, 4H), 1.71 – 1.65 (m, 2H), 1.51 – 1.45 (m, 2H), 1.39 – 1.24 (m, 4H). HRMS (ESI–, *m/z*): $[M-H]^-$ calcd for $C_{37}H_{41}ClN_3O_{12}S_2^-$, 818.1826; found, 818.1833.



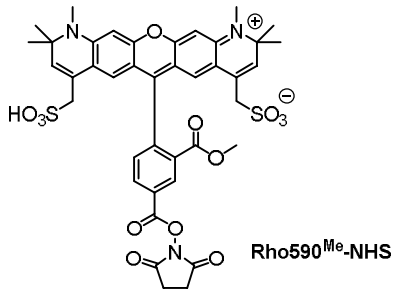
Rho530^{Et}-x

Rho530^{Et}-x: Rho530-x (1.0 mg, 1.2 μmol) was dissolved in the mixture of ethanol (400 μL) and DMF (200 μL), and the solution was stirred at 0 °C for 5 min. Diazoethane (48.8 μL of 1 M solution in ether, 48.8 μmol, 40 equiv.; prepared from N-nitroso-N-ethylurea [Sigma-Aldrich] in ether and 40% aq. KOH) was added dropwise to the reaction mixture, which was then stirred for 50 min at 0 °C. The product was isolated by preparative HPLC (10 – 50% MeCN in H_2O + 0.1% TFA over 30 min). Lyophilization yielded 0.3 mg (29%) of **Rho530^{Et}-x** as a magenta powder. HRMS (ESI–, *m/z*): $[M-H]^-$ calcd for $C_{39}H_{45}ClN_3O_{12}S_2^-$, 846.2139; found, 846.2134.

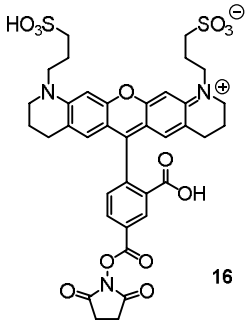


15

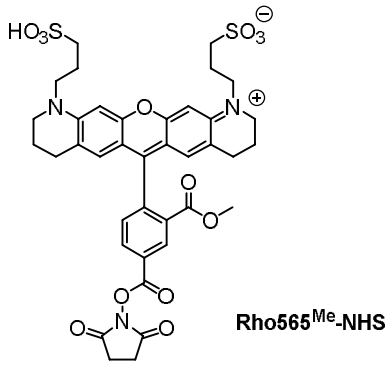
Compound **15:** To a solution of compound **2** (6.0 mg, 8.3 μmol) in anhydrous DMF (200 μL), stock solutions (in DMF) of TSTU (3.5 mg, 12 μmol, 1.4 equiv.) and DIPEA (4.3 mg, 33 μmol, 4 equiv.) were added. The reaction mixture was stirred at r.t. for 20 min. DMF was evaporated in vacuum, and the product was isolated by preparative HPLC (5 – 45% MeCN in H_2O + 0.1% TFA over 30 min). Lyophilization yielded 5.9 mg (87%) of **15** as a blue powder. ^{1}H NMR (400 MHz, CD_3OD): δ (ppm) = 8.97 (d, *J* = 1.7 Hz, 1H), 8.55 (dd, *J* = 8.0, 1.8 Hz, 1H), 7.66 (d, *J* = 8.0 Hz, 1H), 7.14 (s, 2H), 6.83 (s, 2H), 5.90 (s, 2H), 3.71 (d, *J* = 14.1 Hz, 2H), 3.59 (d, *J* = 14.2 Hz, 2H), 3.19 (s, 6H), 2.94 (s, 4H), 1.54 (d, *J* = 6.2 Hz, 12H). HRMS (ESI+, *m/z*): $[M+H]^+$ calcd for $C_{39}H_{38}N_3O_{13}S_2^+$, 820.1841; found, 820.1821.



Rho590^{Me}-NHS: Compound **15** (4.0 mg, 4.9 μmol) was dissolved in methanol (600 μL), and the solution was stirred at 0 °C for 5 min. Diazomethane (410 μL, *c* ~ 20 mg/mL dissolved in ether, 40 equiv.) was added dropwise to the reaction mixture, which was then stirred for 40 min at 0 °C. The product was isolated by preparative HPLC (5 – 45% MeCN in H_2O + 0.1% TFA over 30 min). Lyophilization yielded 1.6 mg (39%) of **Rho590^{Me}-NHS** as a blue powder. HRMS (ESI–, *m/z*): $[M-H]^-$ calcd for $C_{40}H_{38}N_3O_{13}S_2^-$, 832.1852; found, 832.1814.



Compound **16:** To a solution of compound **8** (4.0 mg, 5.7 μmol) in dry DMF (200 μL), stock solutions (in DMF) of TSTU (2.2 mg, 7.4 μmol, 1.3 equiv.) and DIPEA (2.2 mg, 17 μmol, 3 equiv.) were added. The reaction mixture was stirred at r.t. for 20 min. DMF was evaporated in vacuum, and the product was isolated by preparative HPLC (5 – 50% MeCN in H_2O + 0.1% TFA over 30 min). Lyophilization yielded 4.0 mg (88%) of **16** as a purple powder. ^{1}H NMR (400 MHz, CD_3OD): δ (ppm) = 8.99 (dd, *J* = 1.8, 0.5 Hz, 1H), 8.53 (dd, *J* = 8.0, 1.8 Hz, 1H), 7.65 (dd, *J* = 8.0, 0.4 Hz, 1H), 7.09 (s, 2H), 6.76 (s, 2H), 3.81 (t, *J* = 7.6 Hz, 4H), 3.62 (t, *J* = 5.7 Hz, 4H), 2.98 – 2.92 (m, 8H), 2.71 (t, *J* = 6.2 Hz, 4H), 2.22 (p, *J* = 7.4 Hz, 4H), 2.00 – 1.91 (m, 4H). HRMS (ESI+, *m/z*): $[M+Na]^+$ calcd for $C_{37}H_{37}N_3NaO_{13}S_2^+$, 818.1660; found, 818.1631.



Rho565^{Me}-NHS

Rho565^{Me}-NHS: Compound **16** (3.5 mg, 4.4 μmol) was dissolved in methanol (900 μL), and the solution was stirred at 0 °C for 5 min. Diazomethane (370 μL, *c* ~ 20 mg/mL in ether, 40 equiv.) was added dropwise to the reaction mixture which was then stirred for 40 min at 0 °C. The product was isolated by preparative HPLC (5 – 45% MeCN in H_2O + 0.1% TFA over 30 min). Lyophilization yielded 1.0 mg (28%) of **Rho565^{Me}-NHS** as a purple powder. HRMS (ESI–, *m/z*): $[M-H]^-$ calcd for $C_{38}H_{38}N_3O_{13}S_2^-$, 808.1852; found, 808.1882.

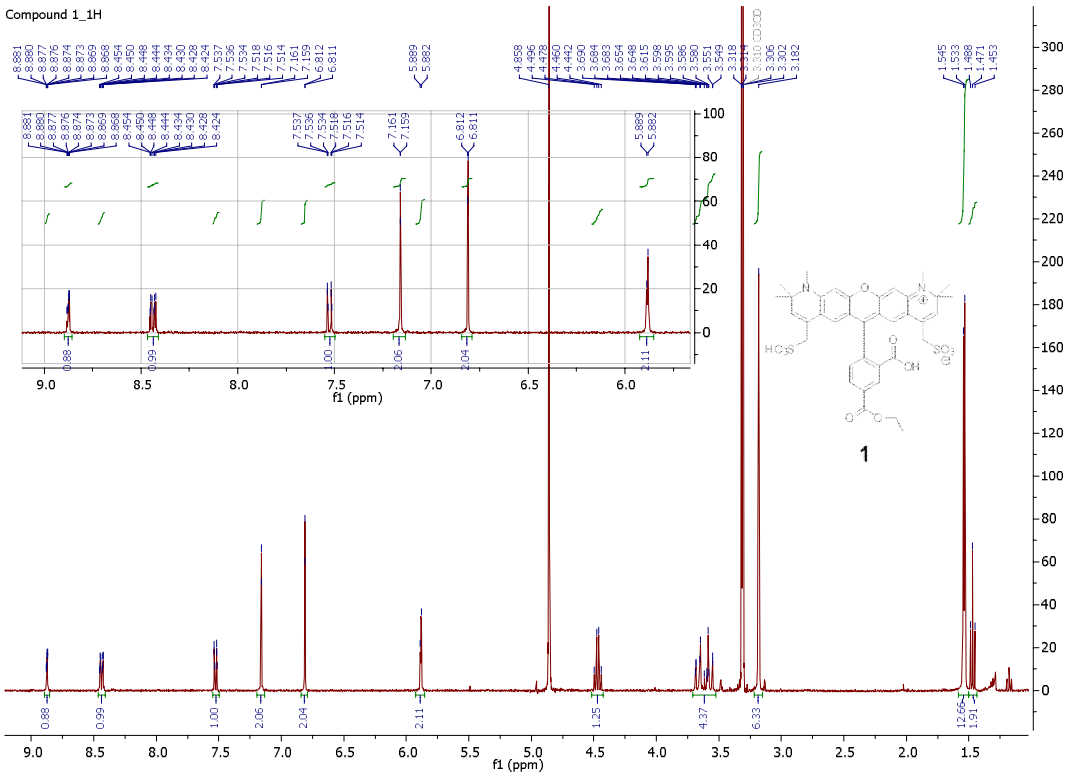


Figure S15. ^{1}H NMR spectrum (400 MHz, CD_3OD) of compound **1**.

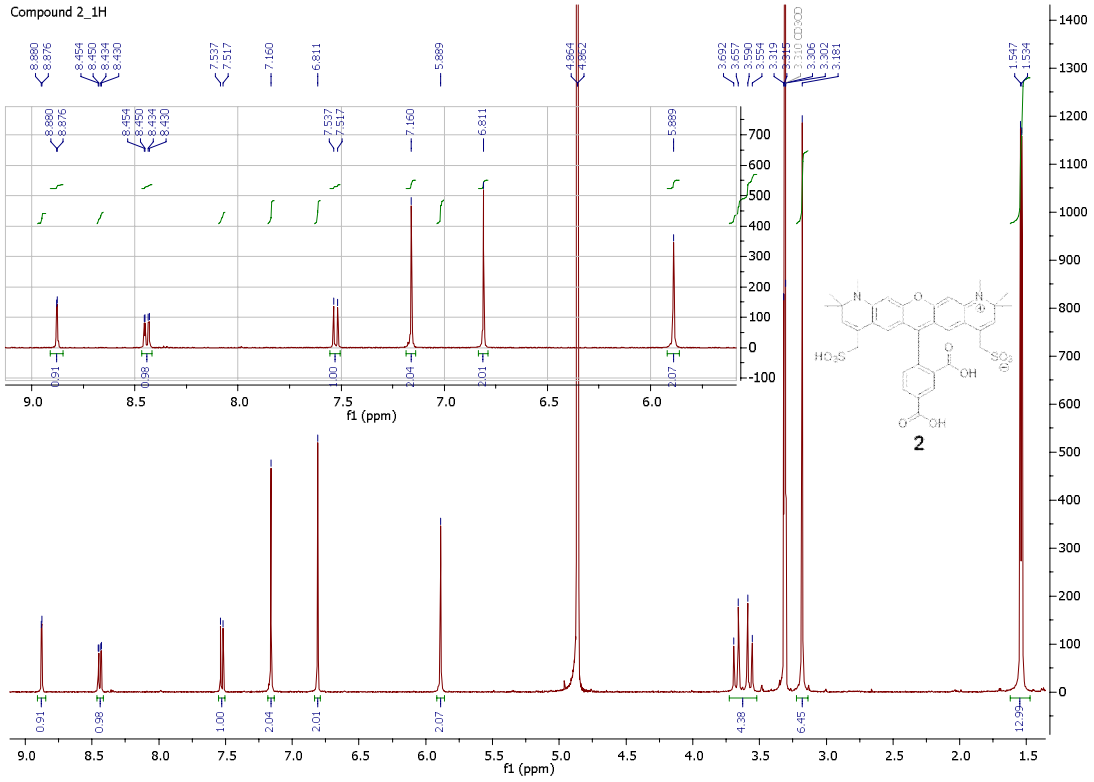


Figure S16. ^{1}H NMR spectrum (400 MHz, CD_3OD) of compound **2**.

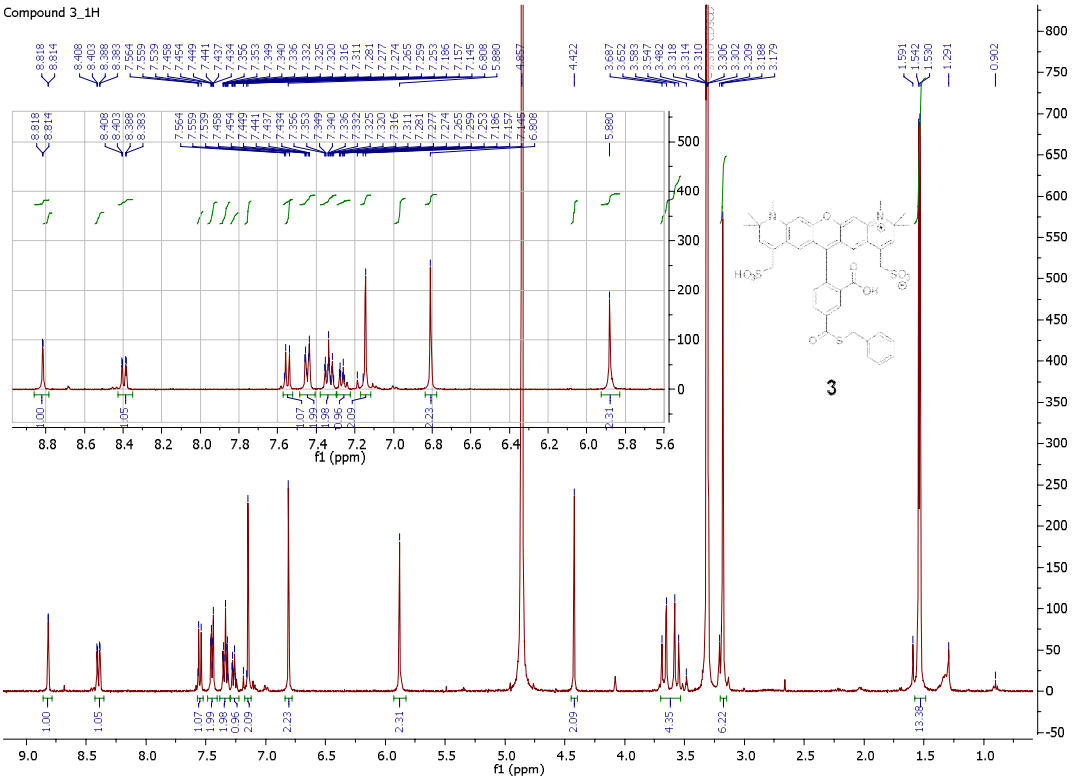


Figure S17. ^{1}H NMR spectrum (400 MHz, CD_3OD) of compound **3**.

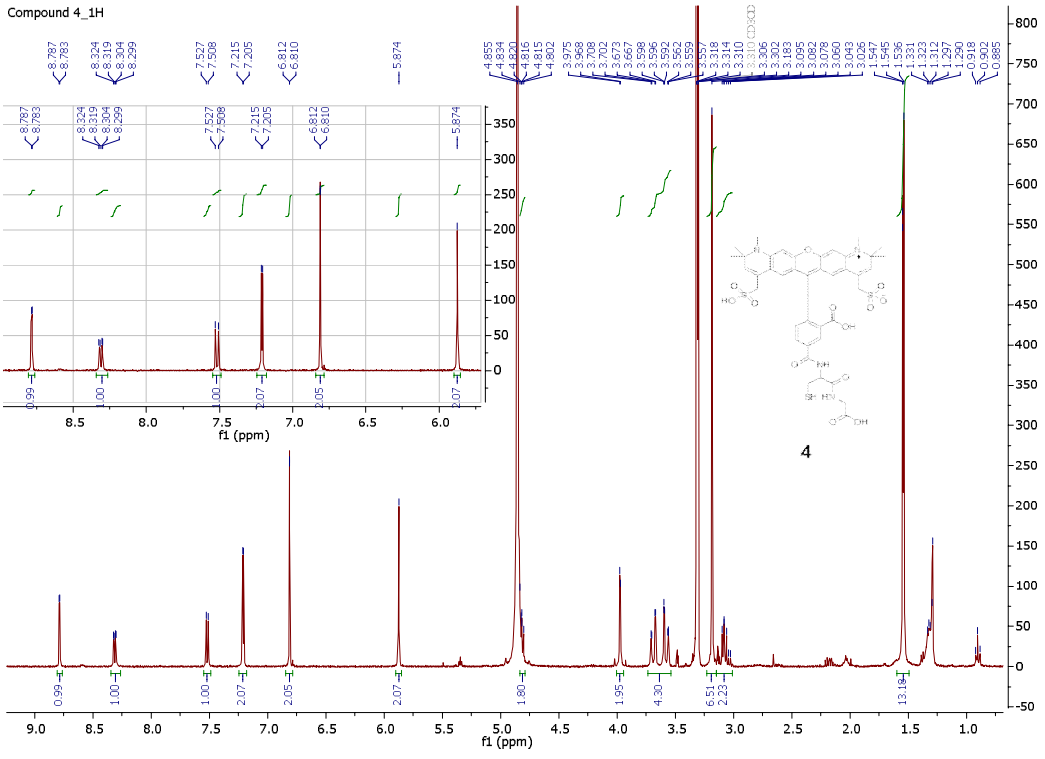


Figure S18. ^{1}H NMR spectrum (400 MHz, CD_3OD) of compound **4**.

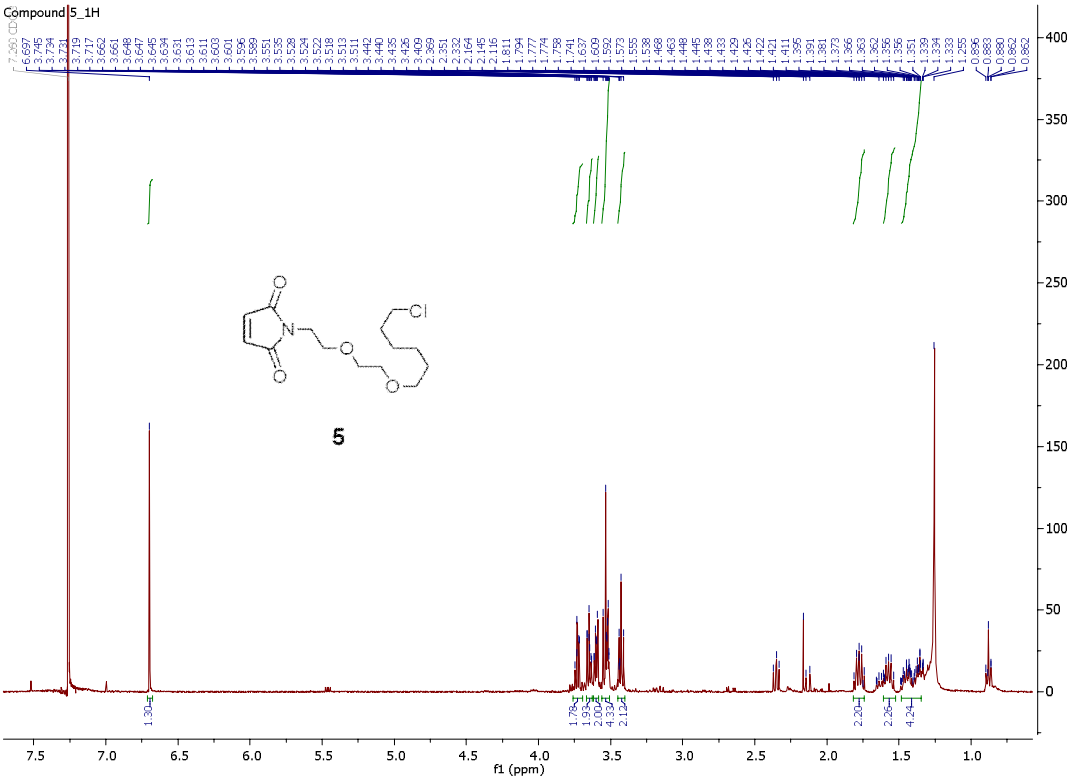


Figure S19. ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of compound **5**.

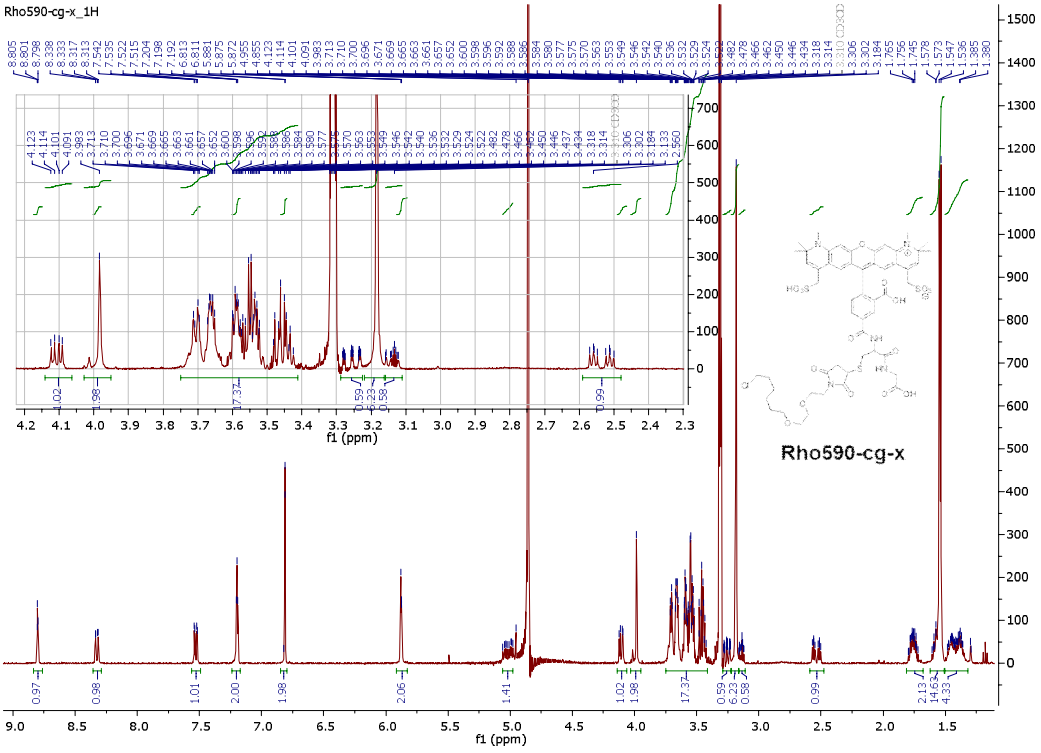


Figure S20. ^{1}H NMR spectrum (400 MHz, CD_3OD) of compound **Rho590-cg-x**.

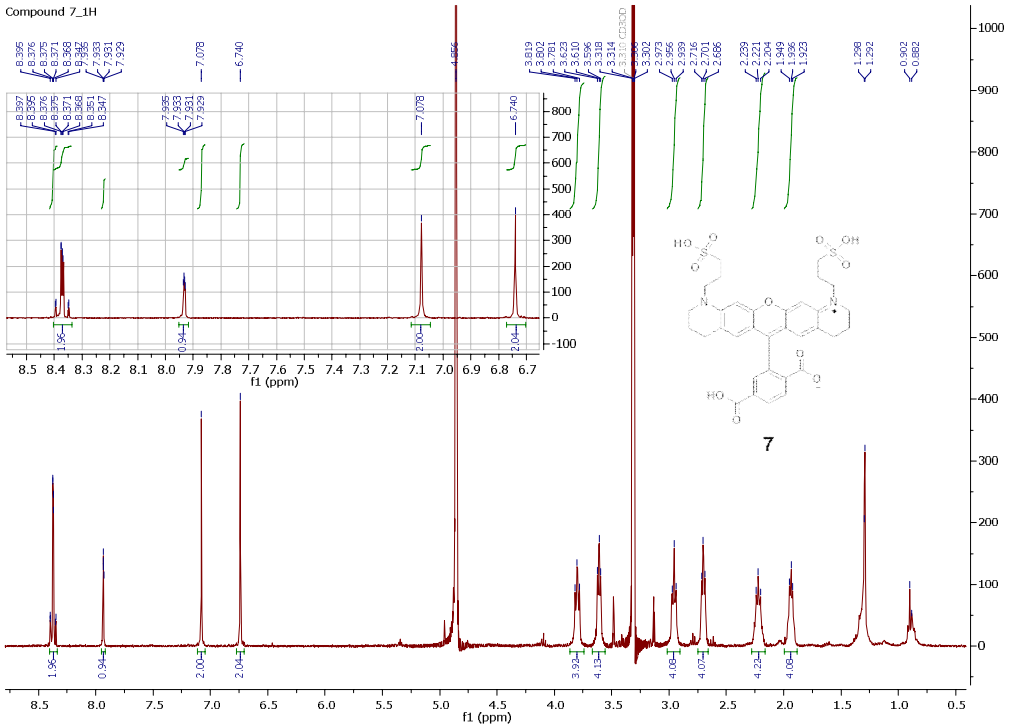


Figure S21. ^{1}H NMR spectrum (400 MHz, CD_3OD) of compound **7**.

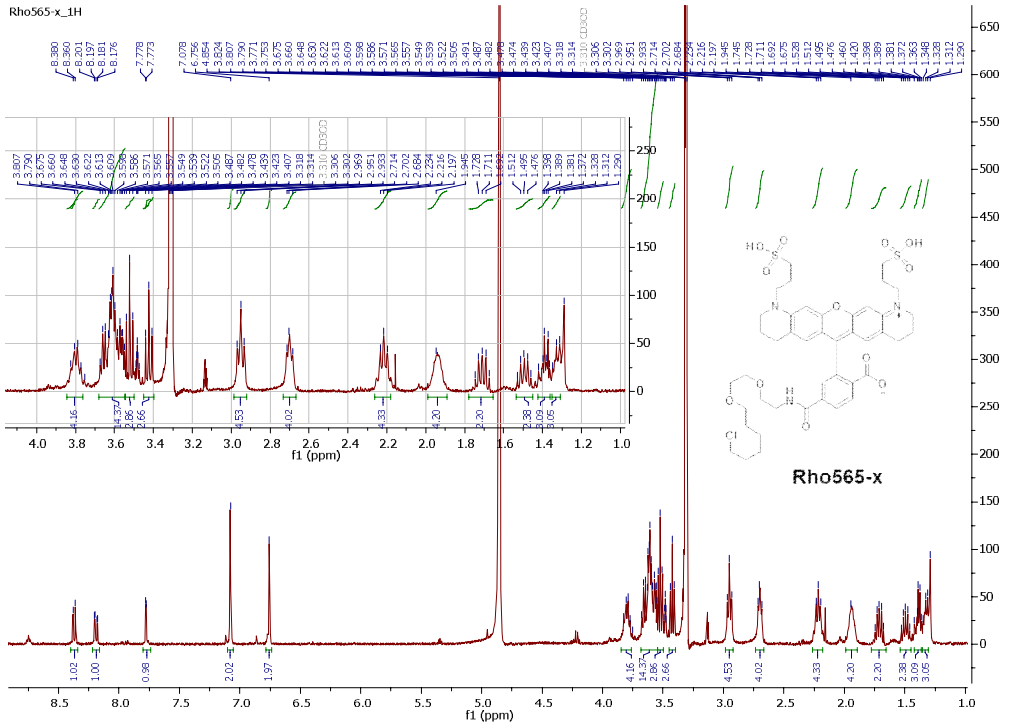


Figure S22. ^{1}H NMR spectrum (400 MHz, CD_3OD) of compound **Rho565-x**.

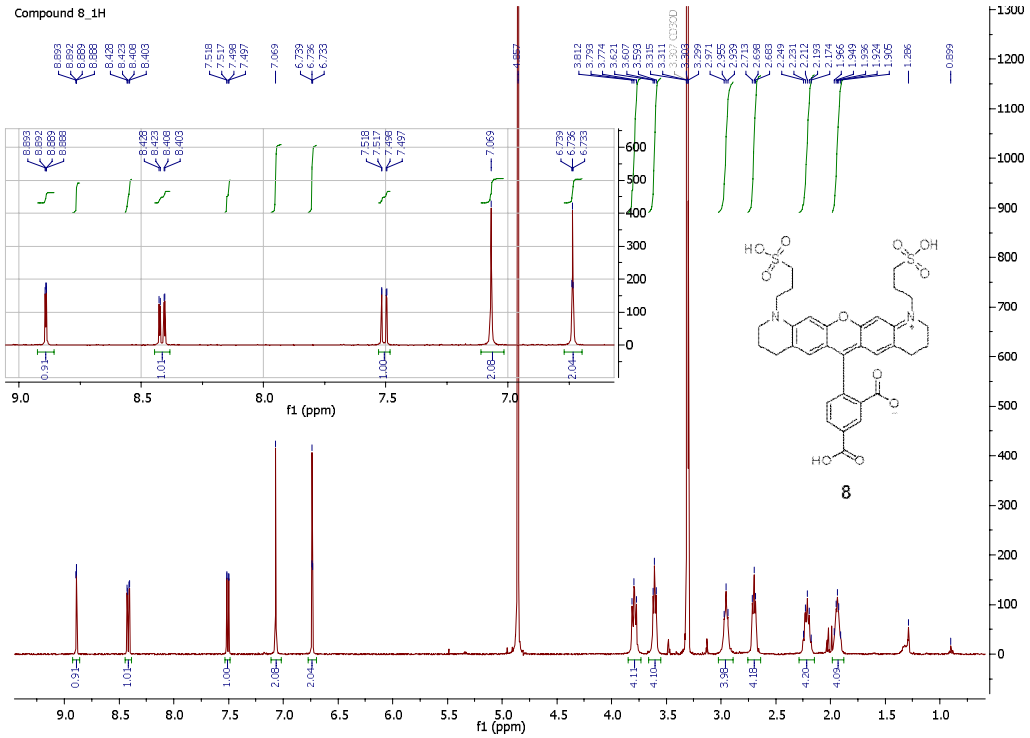


Figure S23. ^{1}H NMR spectrum (400 MHz, CD_3OD) of compound **8**.

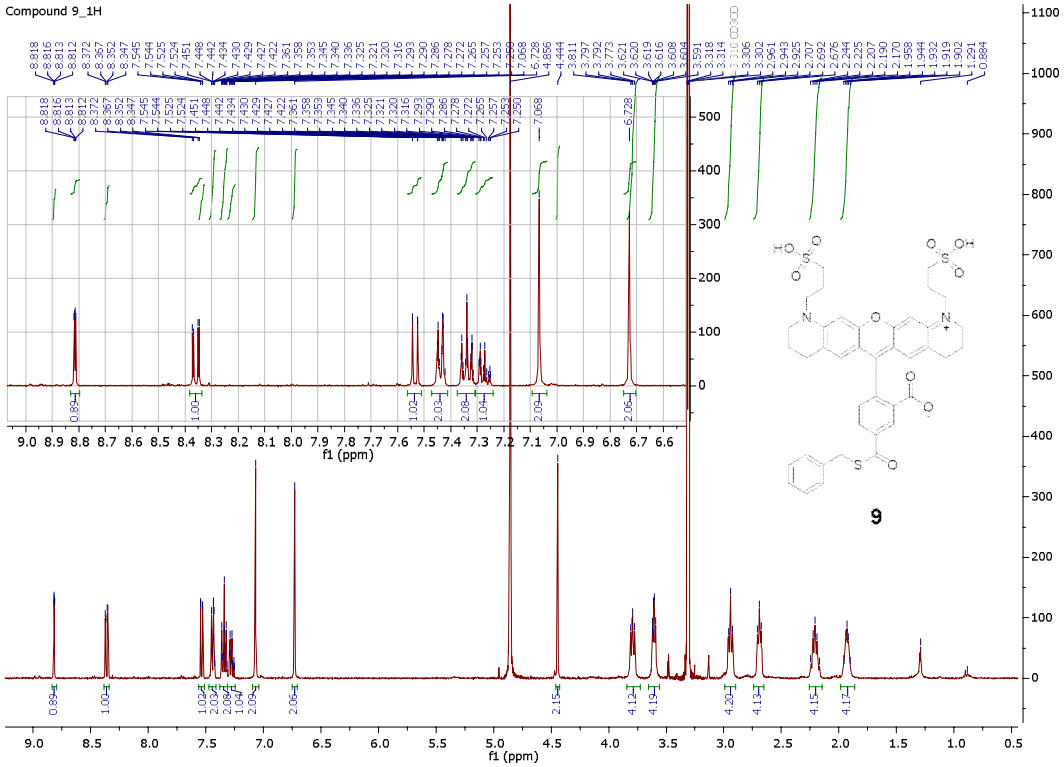


Figure S24. ^{1}H NMR spectrum (400 MHz, CD_3OD) of compound **9**.

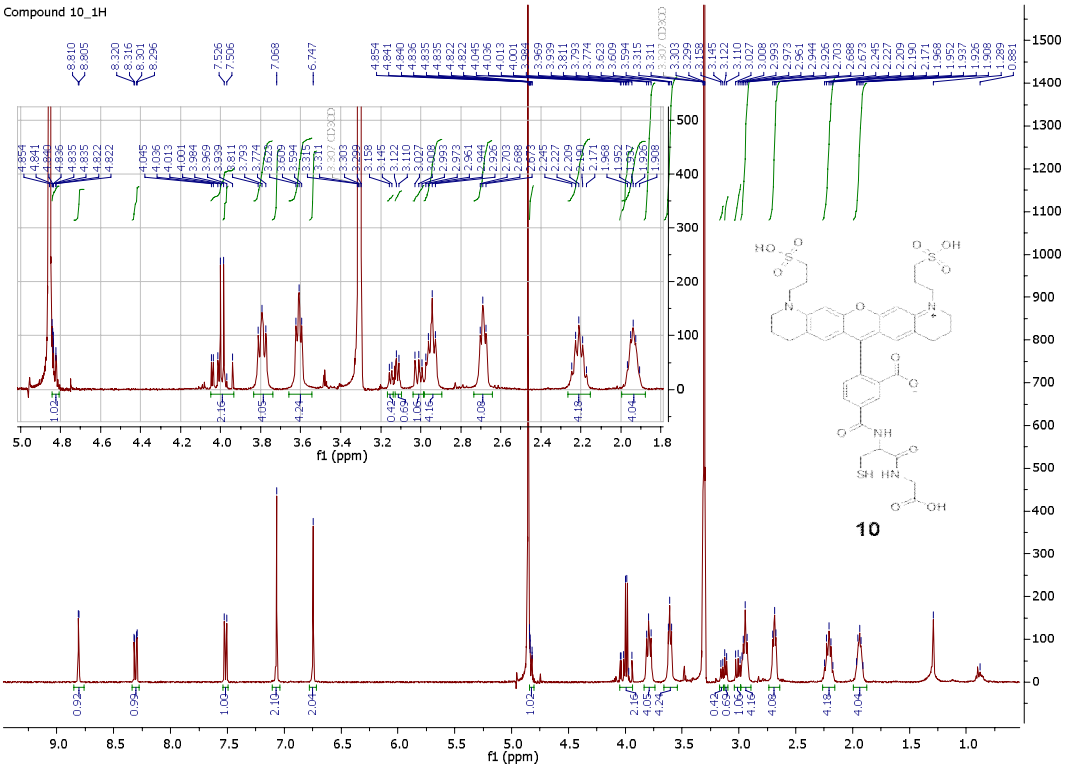


Figure S25. ^{1}H NMR spectrum (400 MHz, CD_3OD) of compound **10**.

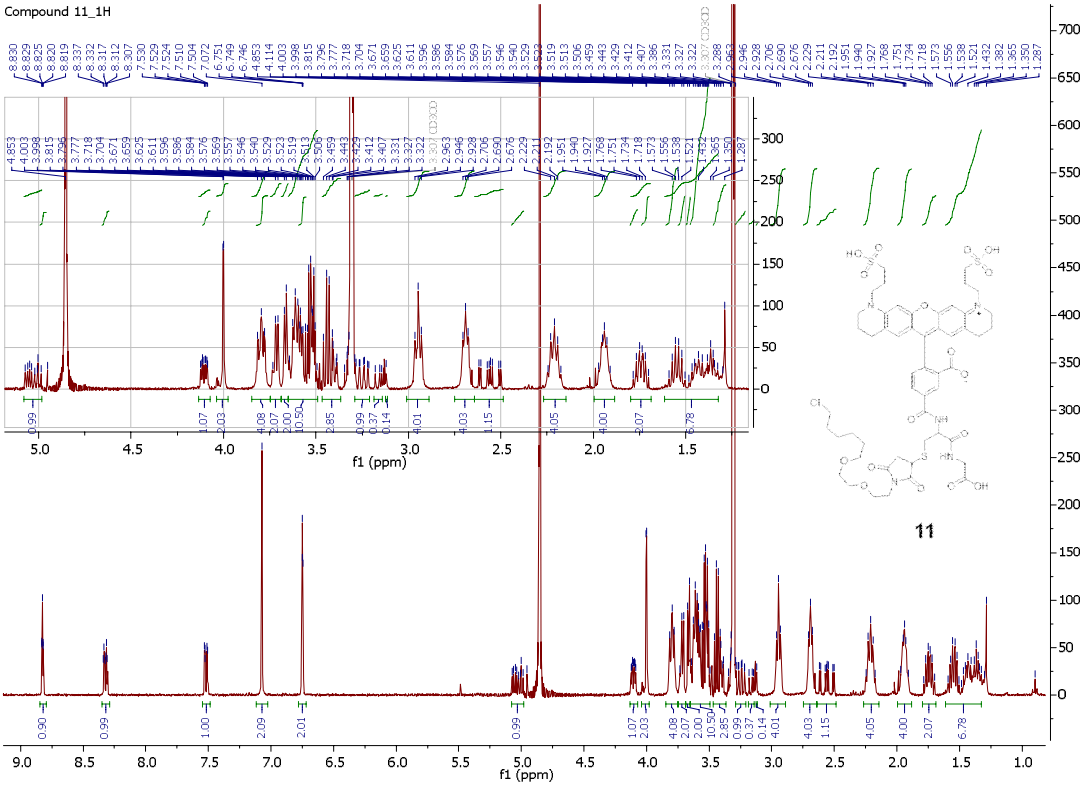


Figure S26. ^{1}H NMR spectrum (400 MHz, CD_3OD) of compound **11**.

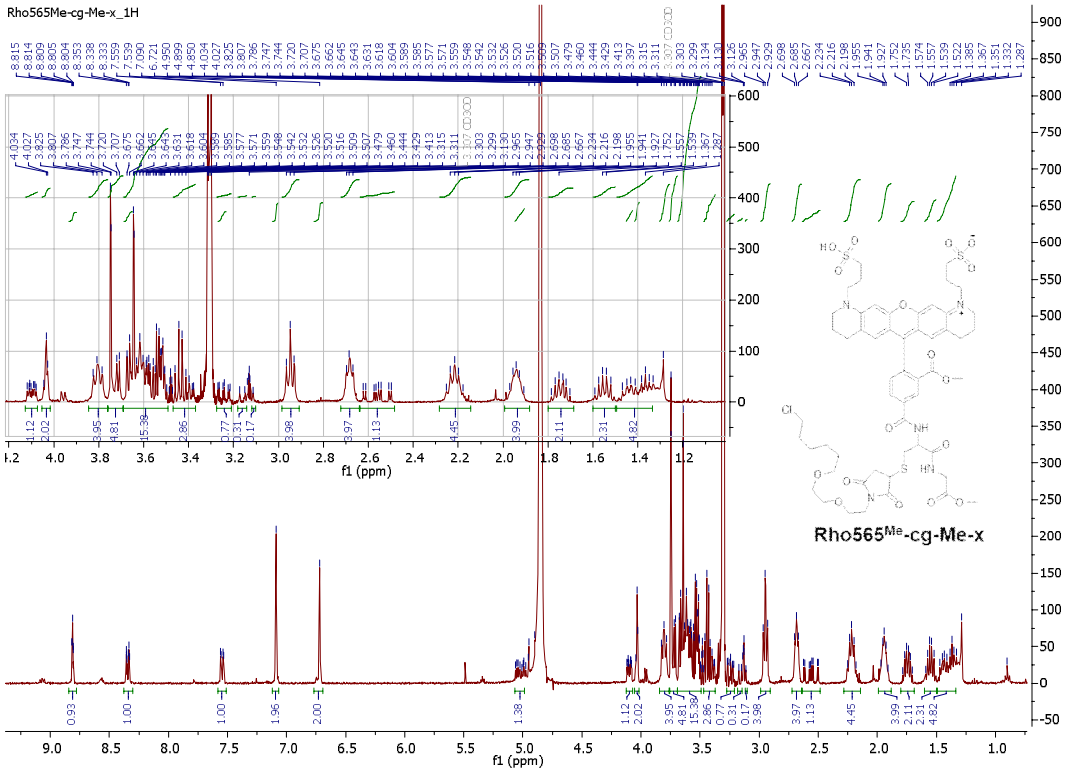


Figure S27. ^{1}H NMR spectrum (400 MHz, CD_3OD) of compound **Rho565^{Me}-cg-Me-x**.

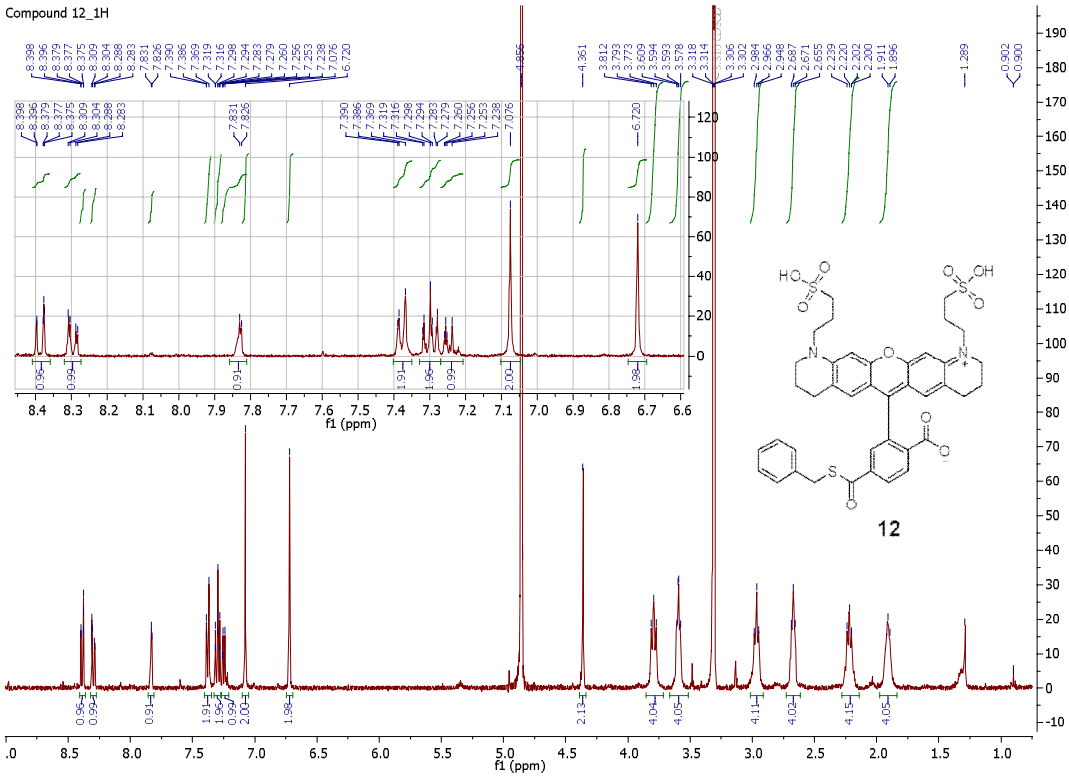


Figure S28. ^{1}H NMR spectrum (400 MHz, CD_3OD) of compound **12**.

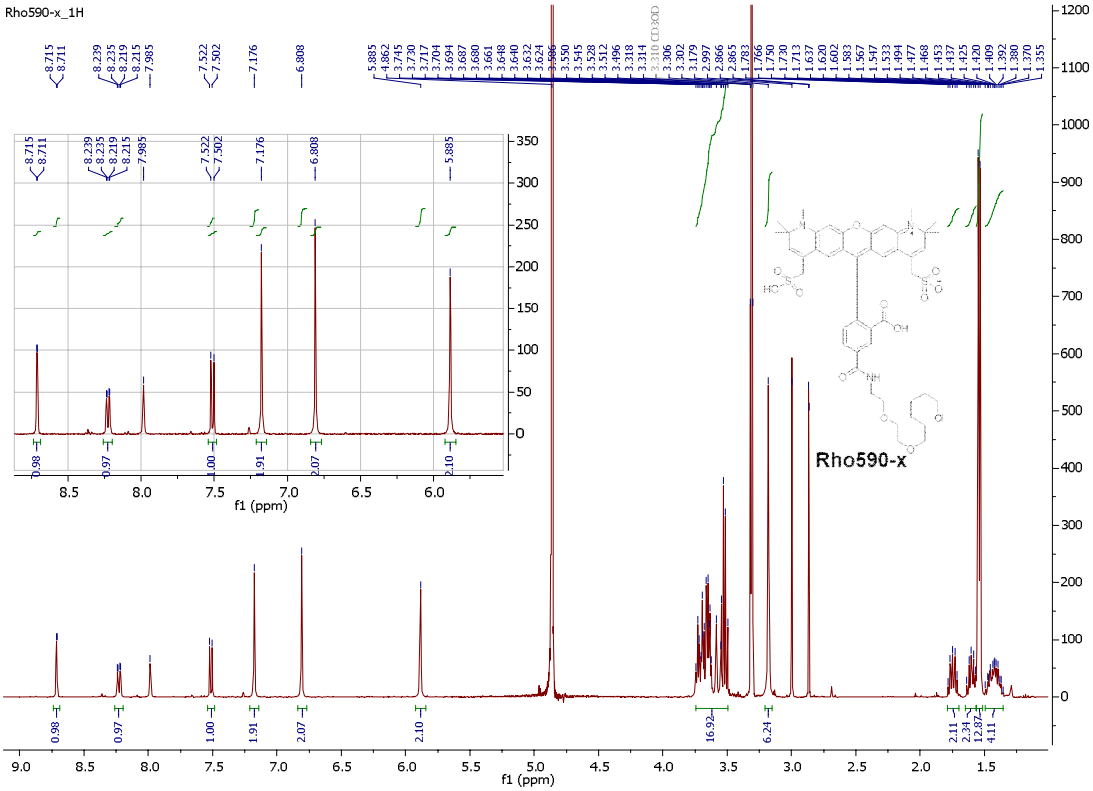


Figure S29. ^{1}H NMR spectrum (400 MHz, CD_3OD) of compound **Rho590-x**.

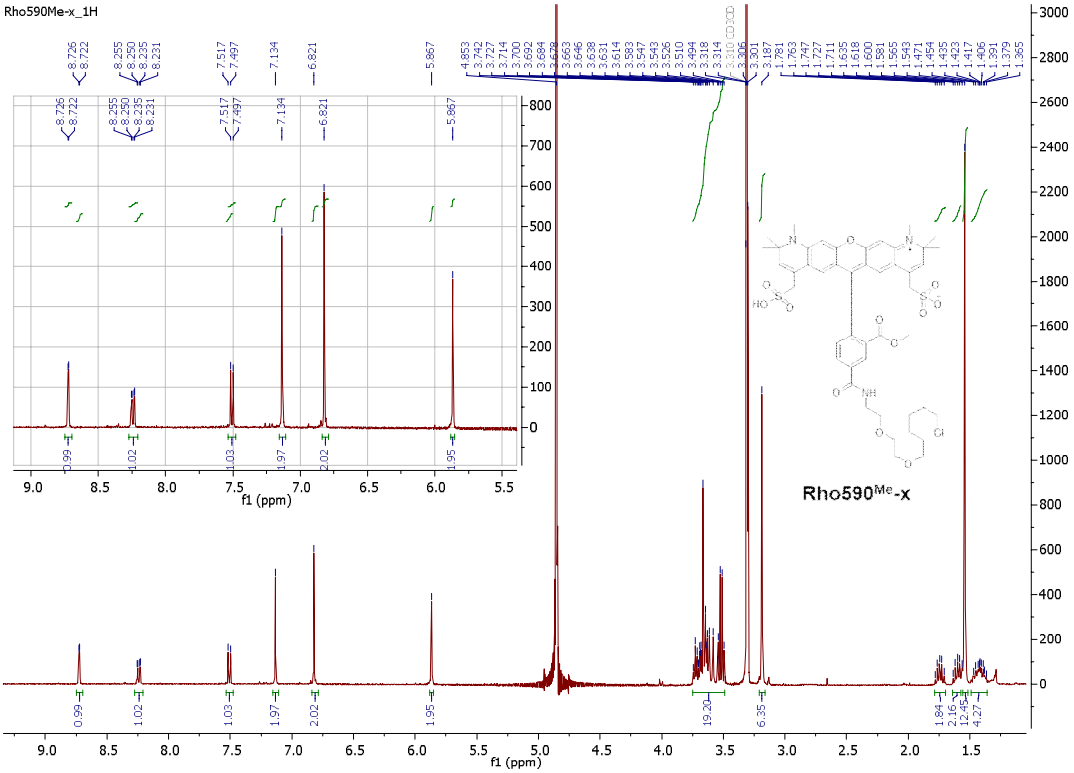


Figure S30. ^{1}H NMR spectrum (400 MHz, CD_3OD) of compound **Rho590^{Me}-x**.

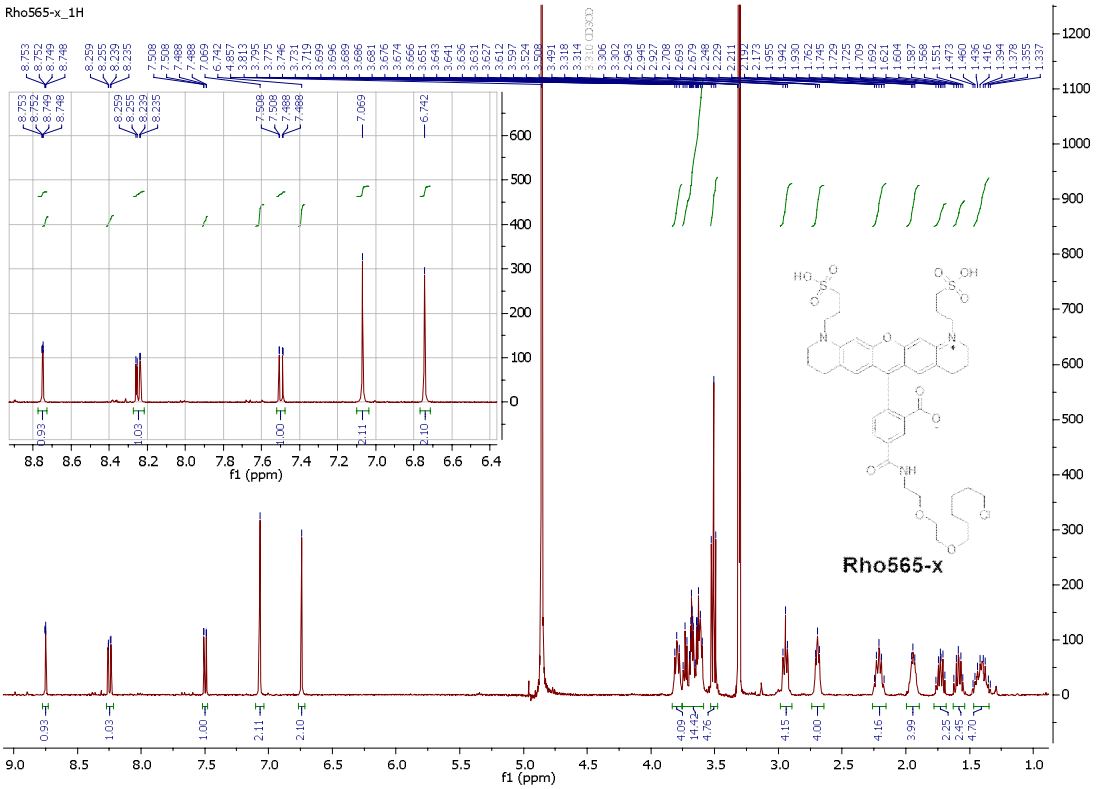


Figure S31. ^{1}H NMR spectrum (400 MHz, CD_3OD) of compound **Rho565-x**.

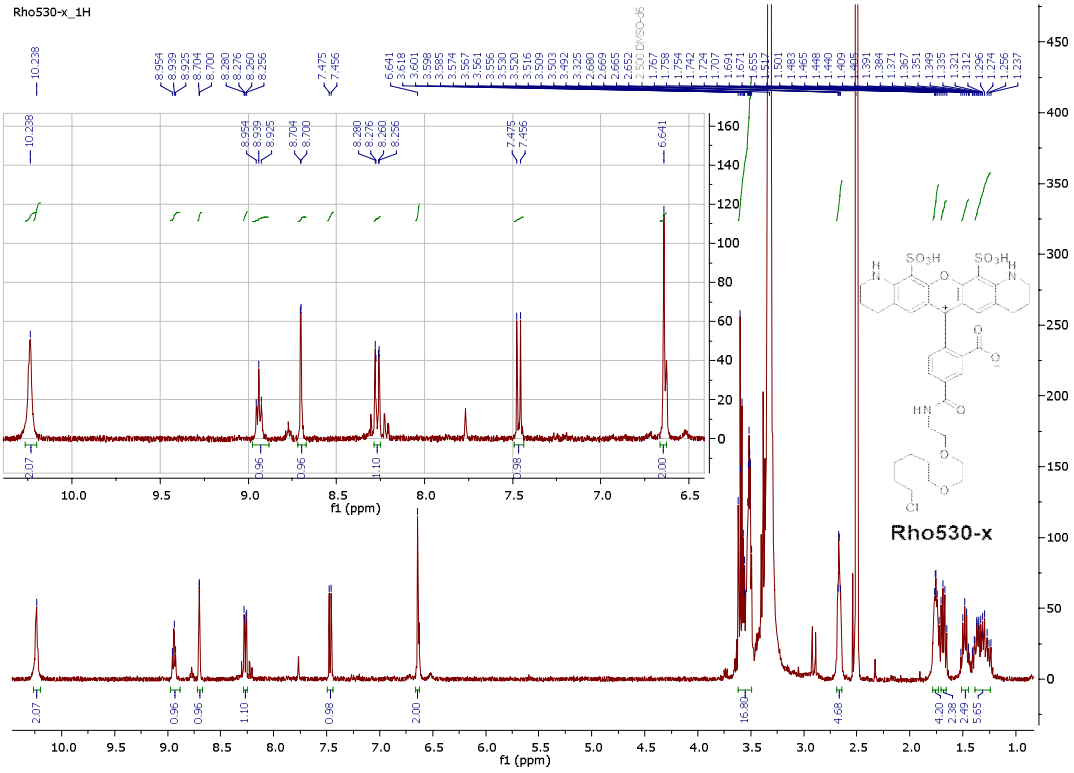


Figure S32. ^{1}H NMR spectrum (400 MHz, DMSO-d_6) of compound **Rho530-x**.

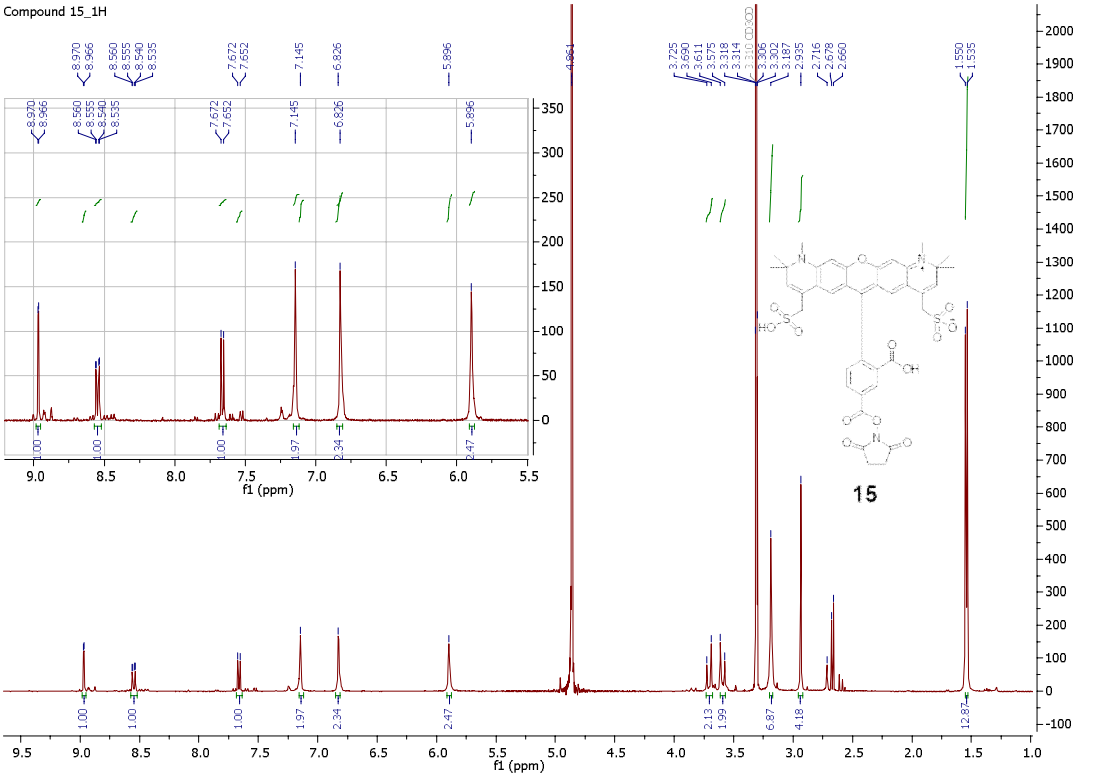


Figure S33. ^{1}H NMR spectrum (400 MHz, CD_3OD) of compound **15**.

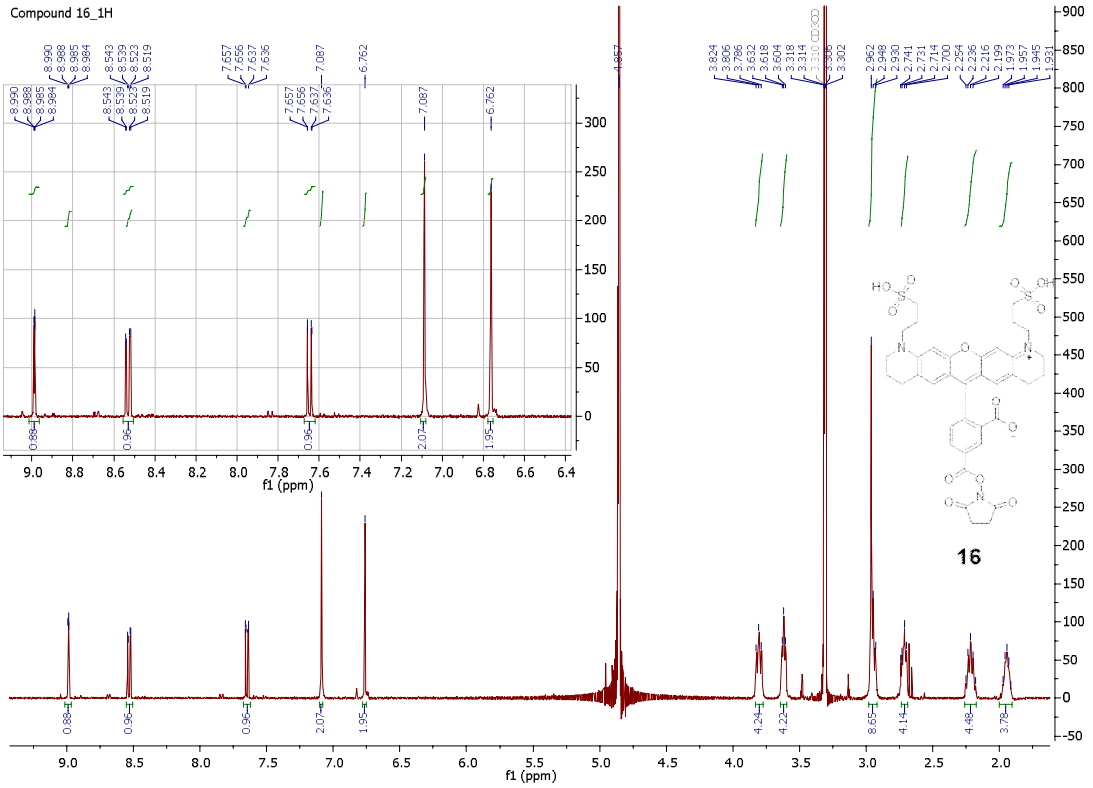


Figure S34. ^{1}H NMR spectrum (400 MHz, CD_3OD) of compound **16**.

Supplementary references

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