CHEMISTRY
A European Journal

Supporting Information

Hydroxylated Fluorescent Dyes for Live-Cell Labeling: Synthesis, Spectra and Super-Resolution STED** Microscopy

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Structures of small molecule ligands used in live cell nanoscopy

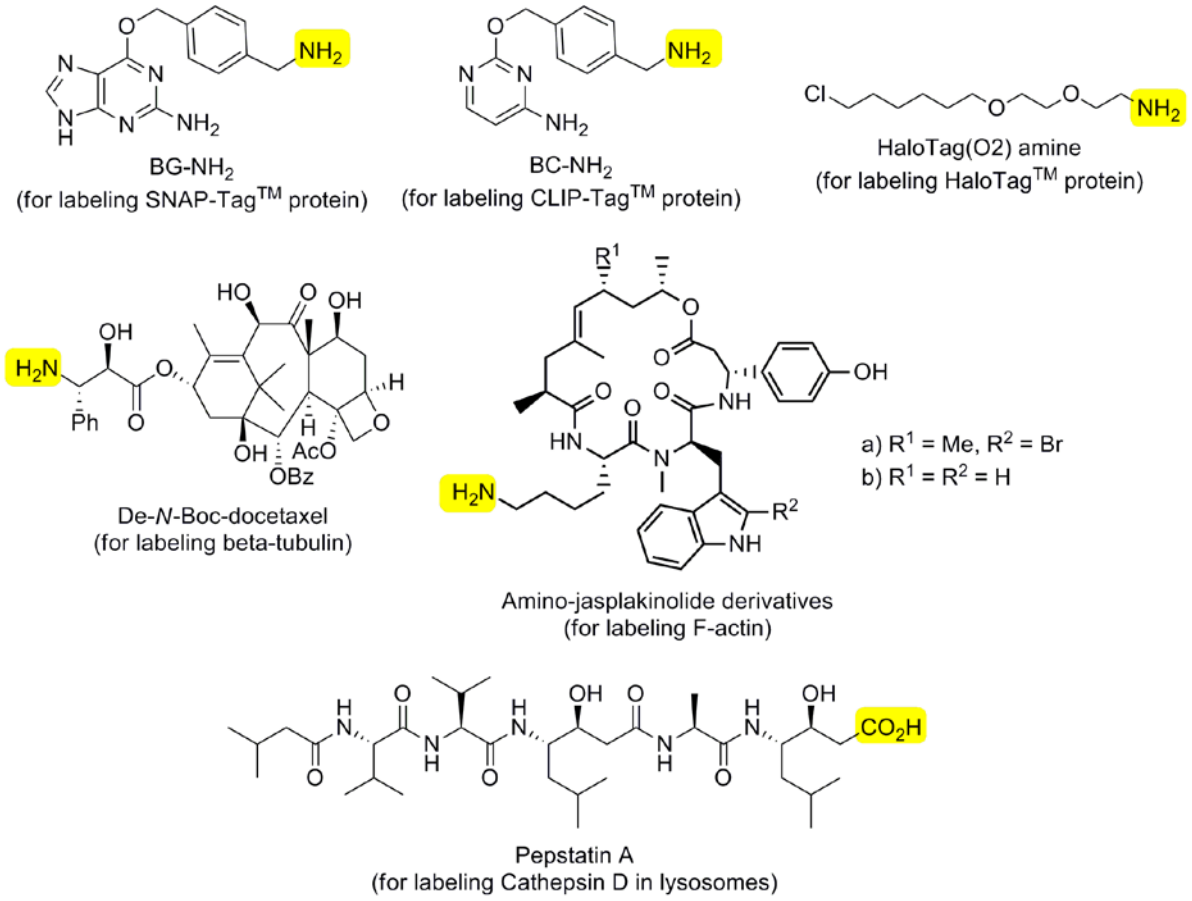


Figure S1. Structures of small molecule ligands used in live cell STED nanoscopy. The ligands are recognized by and bound to biological targets (covalently, in case of SNAP-, CLIP- and HaloTags; or non-covalently with native proteins). Cell-permeant fluorescent dyes are attached (through an appropriate linker) using peptide coupling methodology to the aliphatic amino groups in BG-NH_2, BC-NH_2, Halo-Tag amine, de-*N*-Boc-docetaxel and jasplakinolides, or to the C-terminal carboxylate of pepstatin A, and applied as fluorescent probes in living cells (see main text for references).

Previously reported rhodamines and silicon-rhodamines used for live-cell imaging, and Ge-containing fluorescent dyes

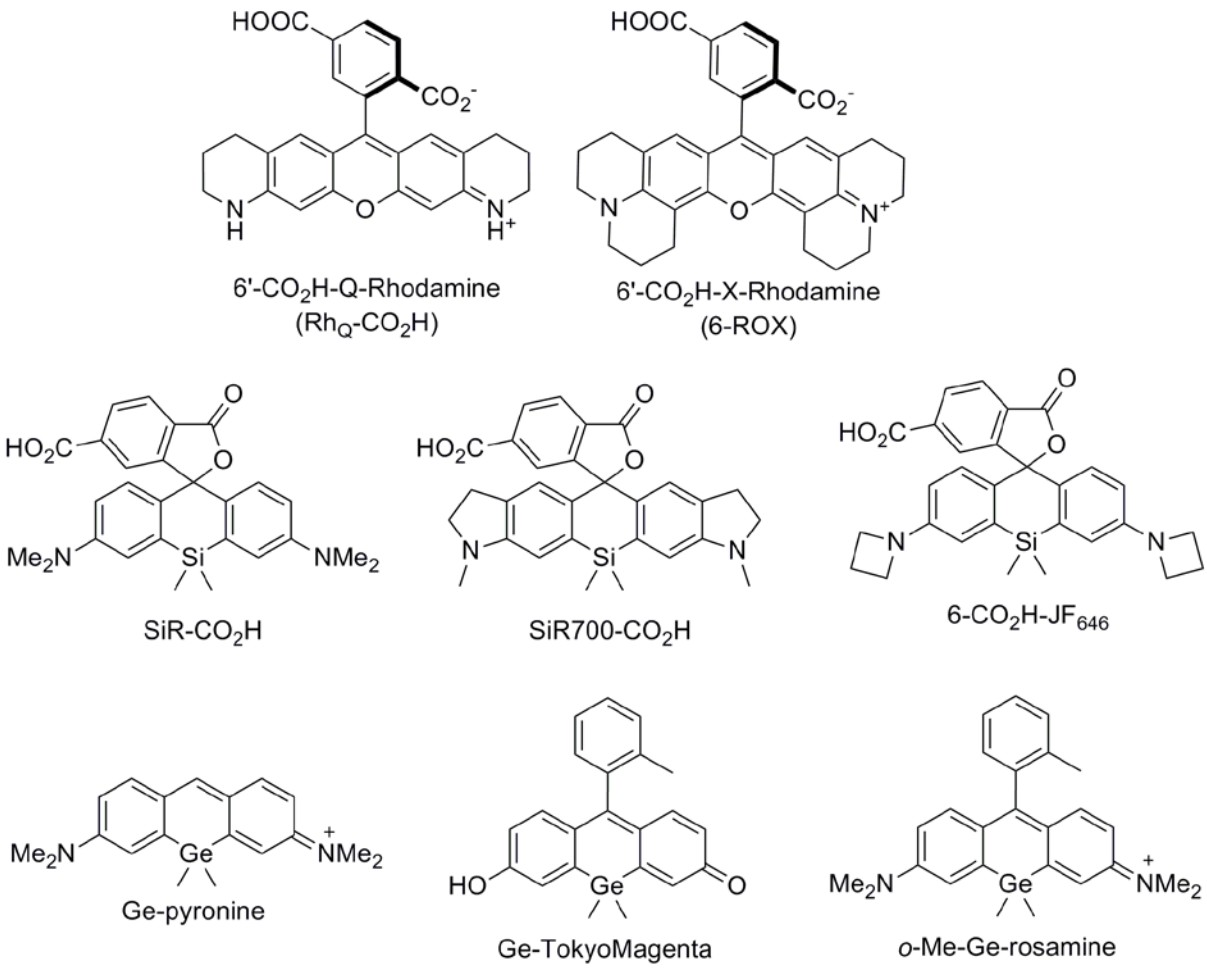


Figure S2. Previously reported rhodamines and silicon-rhodamines used for live-cell imaging, and Ge-containing fluorescent dyes appearing outside of patent literature (see main text for references).

Normalized absorption and emission spectra of the fluorescent dyes

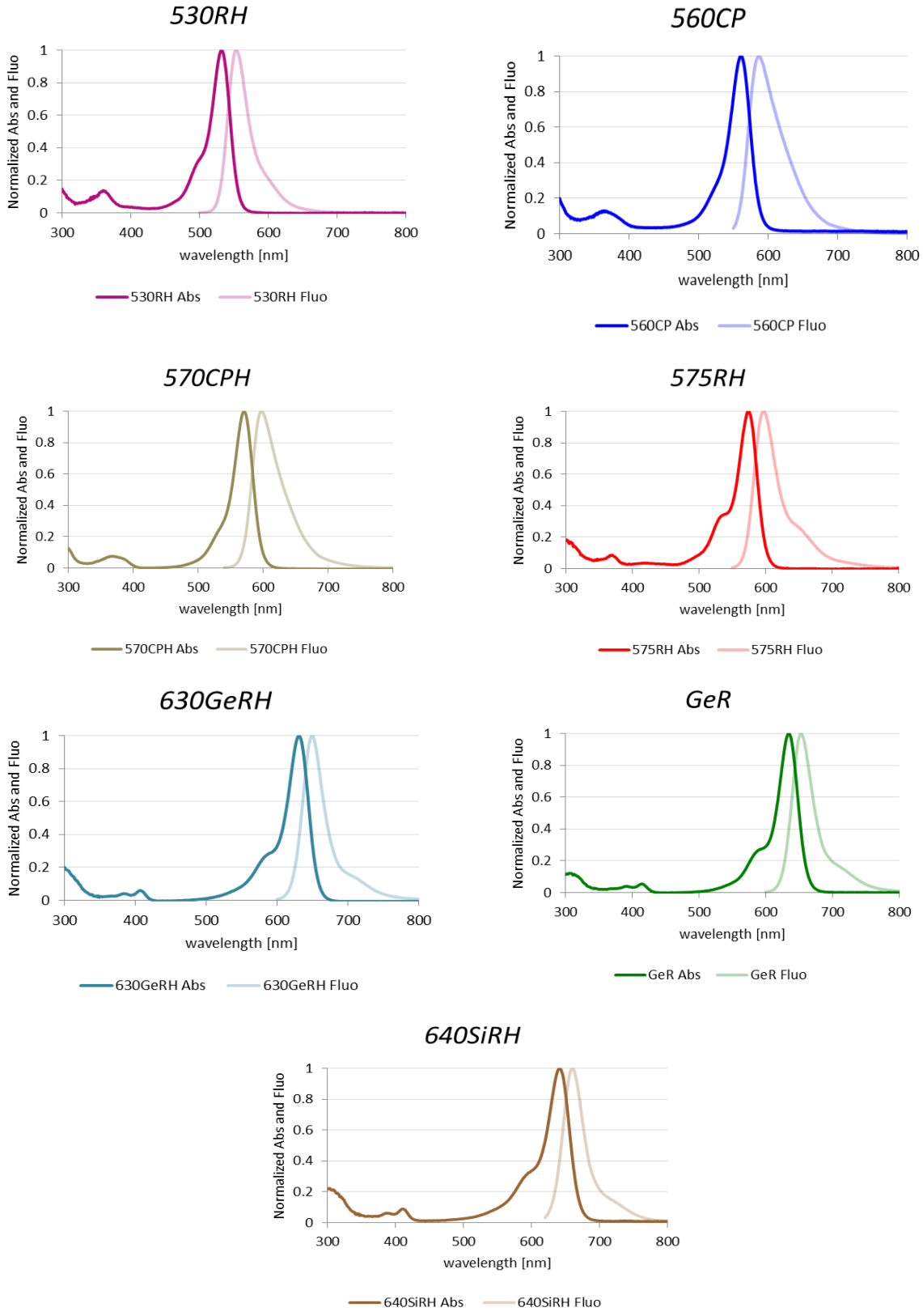


Figure S3. Normalized absorption and emission spectra of the fluorescent dyes of the present study (recorded in PBS, pH 7.4).

STED image of a fixed cell stained with *560CP*

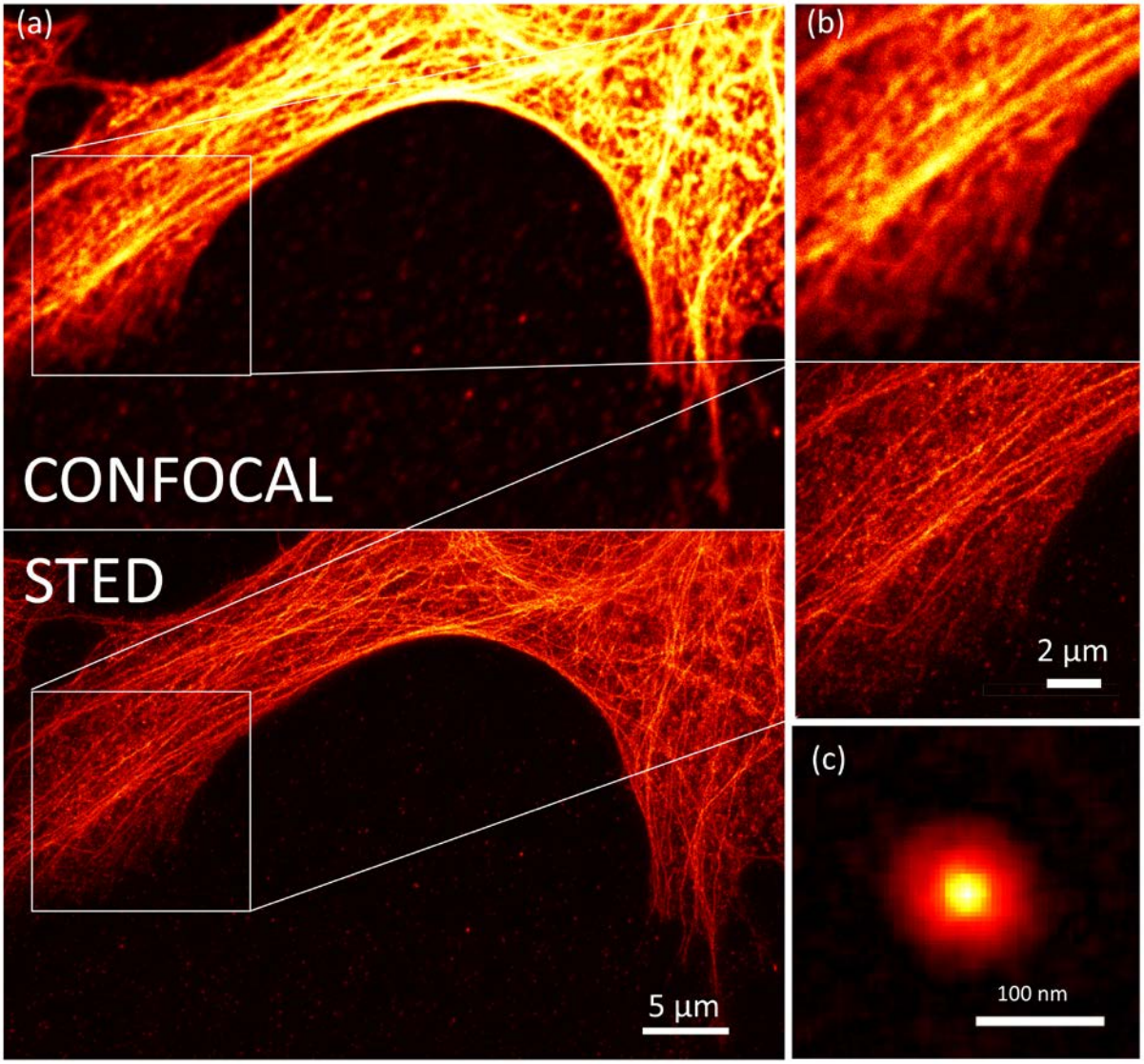
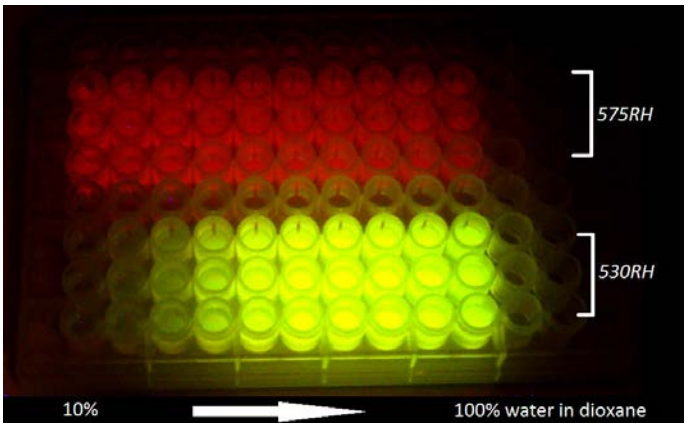


Figure S4. (a) Confocal and STED example images recorded from Hela Cells stained with ***560CP*-NHS**-labeled secondary (sheep anti-mouse) antibody; primary antibody – mouse anti-tubulin. Imaging parameters: 561 nm excitation laser line, 70% STED 660 nm line, HyD with gate from 1 to 6 ns applied. (b) Zoom in onto microtubuli clearly showing strong resolution increase. (c) Overlay of > 400 objects outside the cells as a representation of the STED PSF for estimation of the resolution. The FWHM (full width at half maximum) of the overlay is 44 nm in X and Y direction (recorded with a Leica STED confocal system, by LEICA Microsystems GmbH).

Spirolactone-zwitterion equilibria of the dyes *530RH* and *575RH* in dioxane-water mixtures



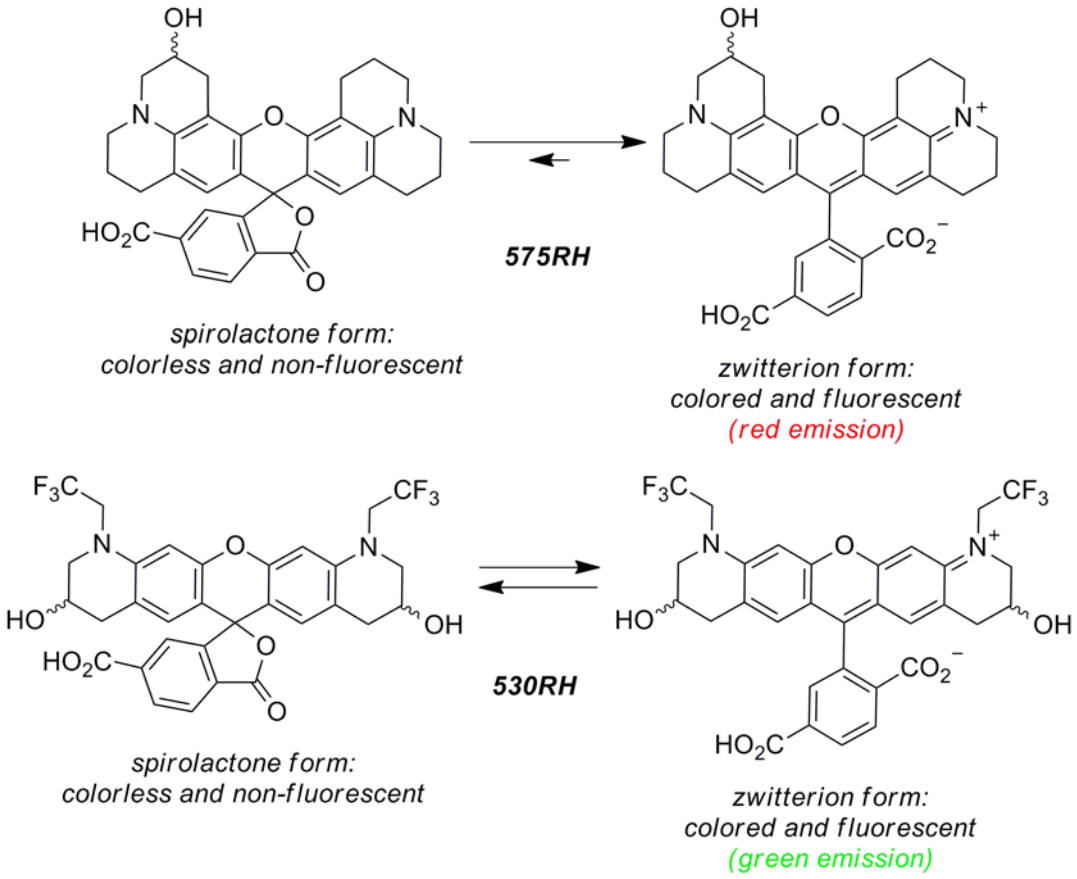


Figure S5. Fluorescence of dyes ***530RH*** and ***575RH*** under UV irradiation (360 nm) in 1,4-dioxane solutions with 10 … 100% v/v of water (in 10% increments). While the dye ***530RH*** shows little visible fluorescence in the mixtures with water content <30% (v/v), the emission intensity of ***575RH*** (and other ROX dyes) is nearly independent on water content.

Fluorescence intensity changes of HaloTag ligands in the presence of a HaloTag fusion protein

In 1.5 mL test tubes (Eppendorf), 2 µL of 25 µM stock solution of a dye-HaloTag ligand conjugate in DMSO was added to 100 µL of

1) 10% FBS (FBS Superior, available from Biochrom AG, cat. No. S 0615) in DMEM ("high glucose" (4.5 g/L), no *l*-glutamine, no pyruvate, no Phenol Red; by Gibco®, available from Life Technologies, cat. No. 31053-028) *or*
2) 810 nM HaloTag® Standard Protein (HaloTag protein fused to GST (glutathione *S*-transferase), M = 61 kDa; available from Promega, cat. No. G4491), prepared by dissolution of a 30 µg sample in 607 µL of 10% FBS in DMEM

and incubated at 37 °C for 2 h (all samples prepared in duplicates; protein/dye ratio = 1.62). The samples were aliquoted (45 µL each) onto a 96-well microplate, and fluorescence intensity was measured on a Spark 20M (Tecan) microplate reader in 10 min intervals over 1 h (excitation and emission bandwidths set to 10 nm). The excitation and emission wavelengths were selected as follows:

Dye	*Rh_QCO_2H*	*530RH*	*560CP*	*570CPH*	*6-ROX*	*575RH*	***13b***	*GeR*	*630GeRH*	*SiR*	*640SiRH*
Excitation wavelength, nm	535	525	555	565	570	570	570	630	625	640	635
Detection wavelength, nm	561	553	588	600	602	597	597	650	651	670	662

The last three readings of two samples per dye (6 values total) were averaged, and the results presented as fluorescence intensity ratios for solutions containing HaloTag protein/solutions containing FBS in DMEM only.

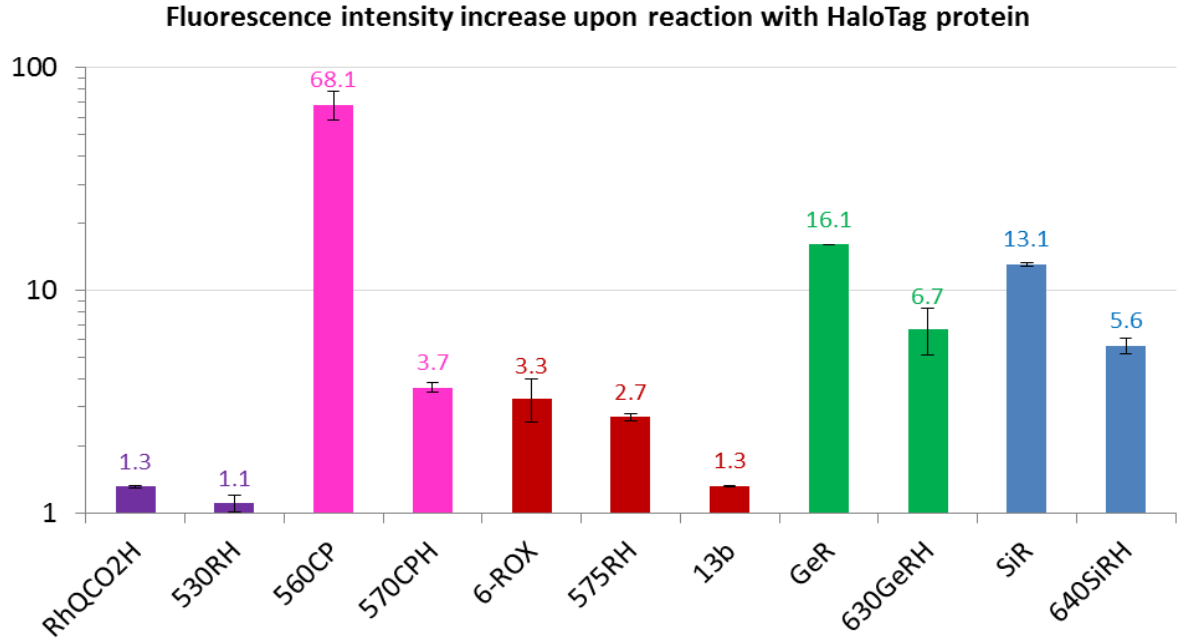


Figure S6. Fluorescence intensity changes upon reaction of dye-HaloTag ligand conjugates with HaloTag protein in DMEM + 10% FBS (average of 2 experiments).

Live cell STED nanoscopy images

Cell staining and washing protocol

The following procedure applies to the preparation of all following samples. The coverslip with the adherent cells was placed in a small Petri dish (35 mm diameter), with the cells facing up. 500 µL of the staining solution (dye diluted in HDMEM to the final concentration indicated, at 37 °C) was applied on top. The Petri dish was closed with a lid and placed in a cell incubator (37 °C, humidified 5% CO_2 atmosphere). After 20 minutes of incubation the dye solution was discarded. Fresh HDMEM (2-3 ml, at 37 °C) was poured in the dish to briefly wash the cells (repeated three times), which were then left for 10 minutes in the incubator at 37°C with fresh HDMEM (3 mL) before mounting them in a coverslip holder with fresh HDMEM for imaging.

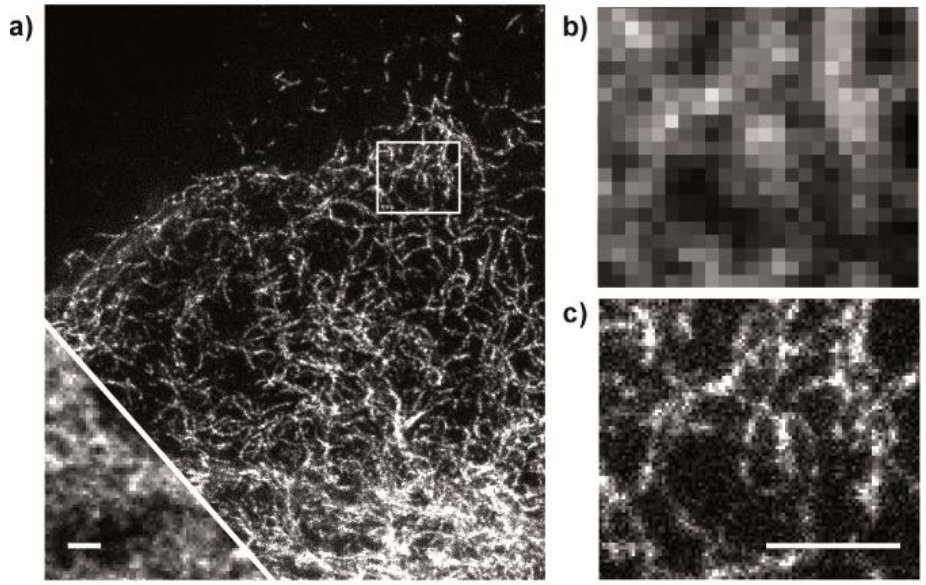


Figure S7. a) STED image of a transfected HeLa cell expressing vimentin-HaloTag fusion protein live-labeled with ***530RH*-Halo** (1 µM, 20 minutes at 37°C, washed, and imaged at room temperature), counterpart confocal image in the lower left corner.
b) and **c)** Close-ups of the boxed region from a) in confocal (b) and STED (c) mode. ***530RH*-Halo** was excited with a pulsed 532 nm laser and fluorescence detection was at 560-580 nm. The STED laser was pulsed at 631 nm. For the STED image the fluorescence counts of 5 scans per line were accumulated with a pixel dwell time of 17 µs. Scale bars: 1 µm.

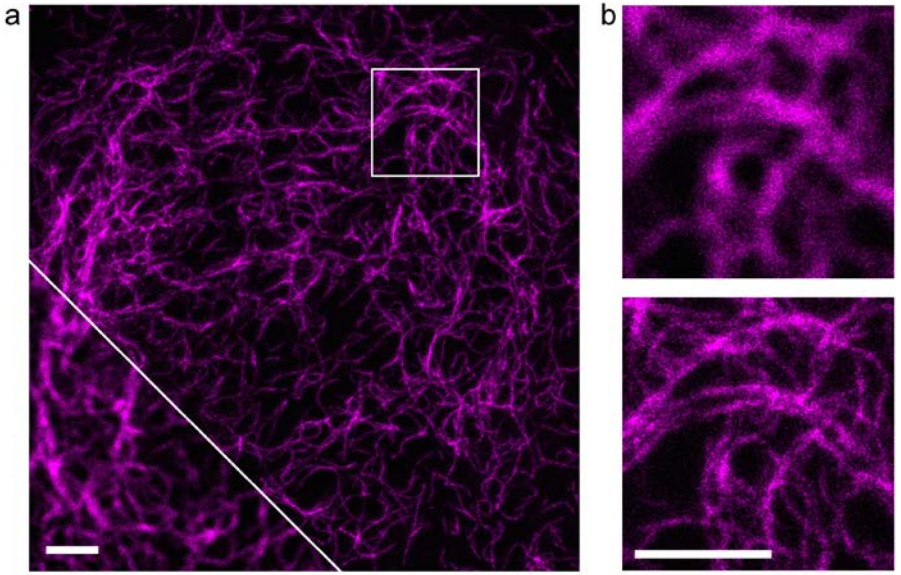


Figure S8. Confocal and STED images (raw data) of vimentin filaments in a living HeLa cell that expresses the vimentin–HaloTag fusion protein after incubation with ***570CPH*-Halo** (1 µM for 20 min, 10 min wash).
a) STED image with the corresponding confocal data in the bottom left corner.
b) Magnified views of the region marked in (a) of the confocal and STED images, respectively. Scale bars: 2 µm; pixel dwell time: 10 µs; pixel size: 26 nm for both the STED and confocal image. Each line was scanned three times in the STED mode, and counts were accumulated.

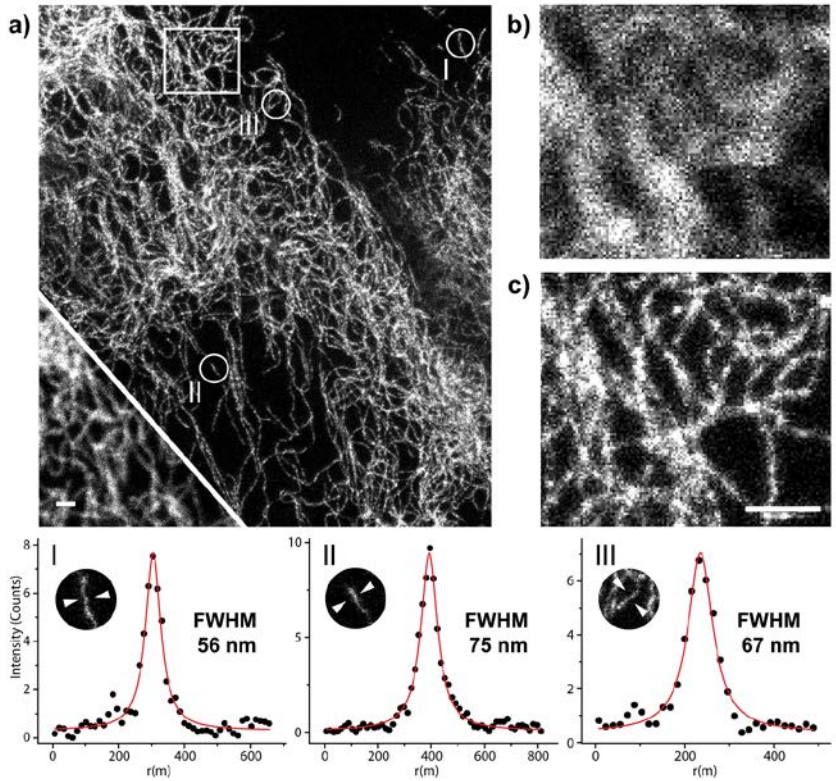


Figure S9. a) STED image of a U2OS cell, stable expression of vimentin-HaloTag fusion protein, live-labeled with ***575RH*-Halo** (1 µM, 20 minutes at 37°C, washed, and imaged at room temperature), counterpart confocal image in the lower left corner and line profiles of the corresponding regions indicated in the STED image are shown below.

b) and **c)** Close-ups of the boxed region from a) in confocal (b) and STED (c) mode. ***575RH*-Halo** was excited with a pulsed 561 nm laser and fluorescence was detected in both detection windows: 605-625 nm and 650-720 nm. The STED laser was pulsed at 775 nm. For the STED image the fluorescence counts of 3 scans per line were accumulated with a pixel dwell time of 15 µs. Scale bars: 1 µm.

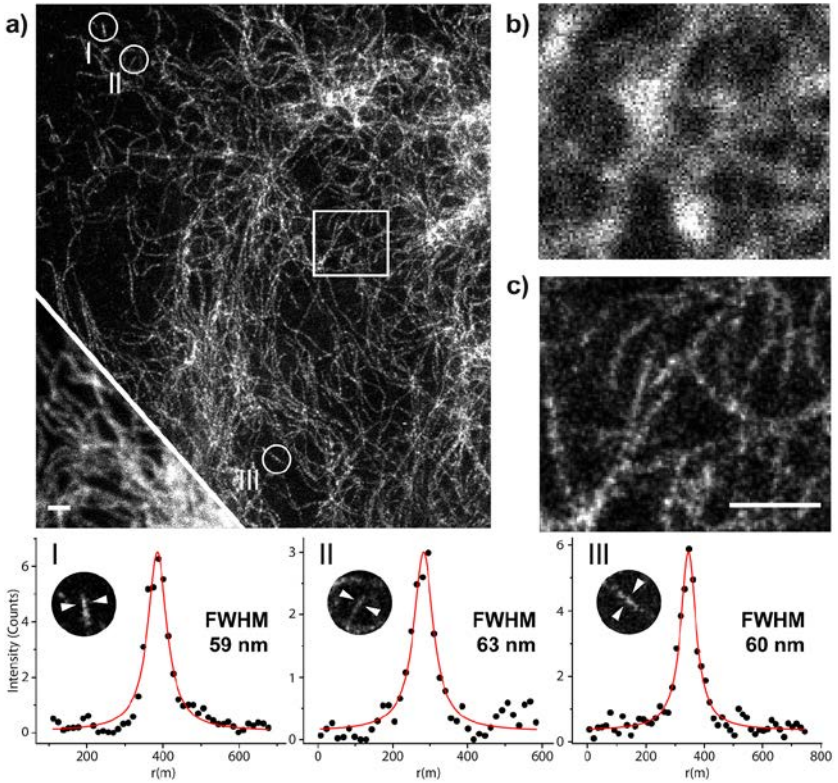


Figure S10. a) STED image of a U2OS cell, stable expression of vimentin-HaloTag fusion protein, live-labeled with ***6-ROX*-Halo** (1 µM, 20 minutes at 37°C, washed, and imaged at room temperature), counterpart confocal image in the lower left corner and line profiles of the corresponding regions indicated in the STED image are shown below.

b) and **c)** Close-ups of the boxed region from a) in confocal (b) and STED (c) mode. ***6-ROX*-Halo** was excited with a pulsed 561 nm laser and fluorescence was detected in two detection windows: 605-625 nm and 650-720 nm. The STED laser was pulsed at 775 nm. For the STED image the fluorescence counts of 3 scans per line were accumulated with a pixel dwell time of 15 µs. The STED image was smoothed with a lowpass Gaussian filter within the software Imspector. Scale bars: 1 µm.

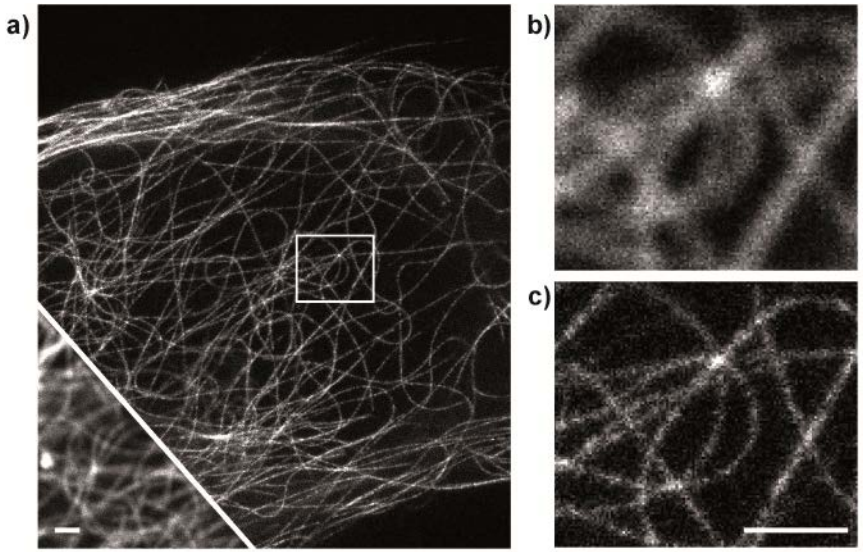


Figure S11. a) STED image of a transfected HeLa cell live-labeled with ***630GeRH*-tubulin** (5 µM, 20 minutes at 37°C, washed, and imaged at room temperature), counterpart confocal image in the lower left corner.
b) and **c)** Close-ups of the boxed region from a) in confocal (b) and STED (c) mode. ***630GeRH*-tubulin** was excited with a pulsed 640 nm laser and fluorescence was detected from 650-720 nm. The STED laser was pulsed at 775 nm. The STED image was recorded with a pixel dwell time of 15 µs. Scale bars: 1 µm.

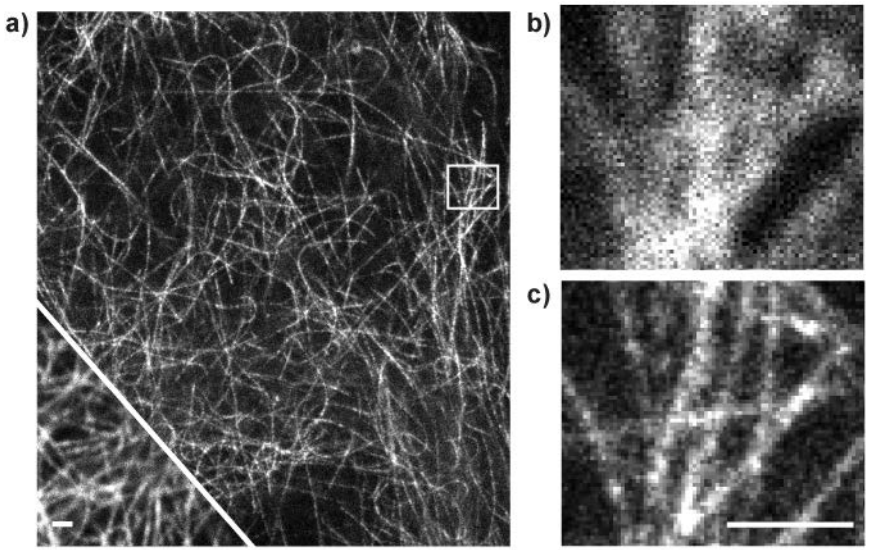


Figure S12. a) STED image of a transfected HeLa cell live-labeled with ***GeR*-tubulin** (Scheme 7, 1 µM, 20 minutes at 37°C, washed, and imaged in HDMEM at room temperature), counterpart confocal image in the lower left corner.
b) and **c)** Close-ups of the boxed region from a) in confocal (b) and STED (c) mode. ***GeR*-tubulin** was excited with a pulsed 640 nm laser and fluorescence was detected from 650-720 nm. The STED laser was pulsed at 775 nm. For the STED image the fluorescence counts of 4 scans per line were accumulated with a pixel dwell time of 7 µs. The STED image was smoothed with a lowpass Gaussian filter. Scale bars: 1 µm.

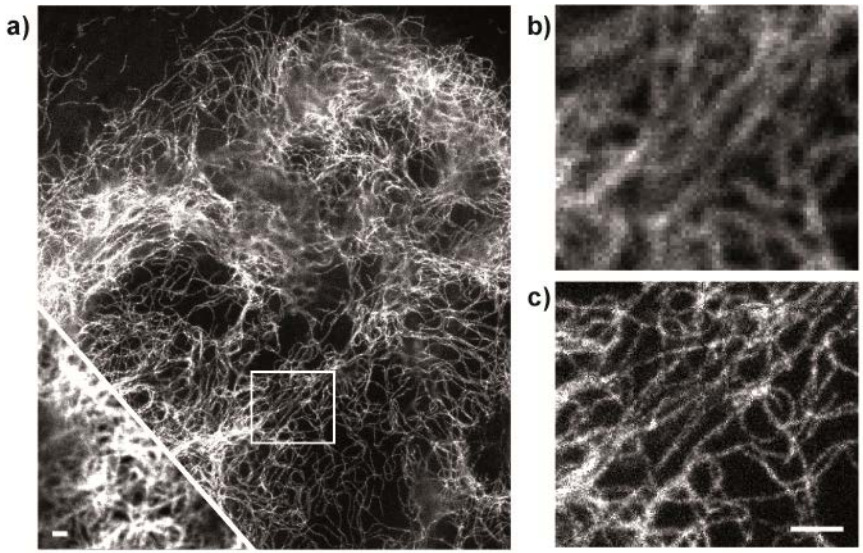


Figure S13. a) STED image of a U2OS cell, stable expression of vimentin-HaloTag fusion protein, live-labeled with ***630GeRH*-Halo** (1 µM, 20 minutes at 37°C, washed, and imaged at room temperature), counterpart confocal image in the lower left corner.
b) and **c)** Close-ups of the boxed region from a) in confocal (b) and STED (c) mode. ***630GeRH*-Halo** was excited with a pulsed 640 nm laser and fluorescence was detected from 650-720 nm. For the STED image the fluorescence counts of 3 scans per line were accumulated with a pixel dwell time of 5 µs. The STED laser was pulsed at 775 nm. Scale bars: 1 µm.

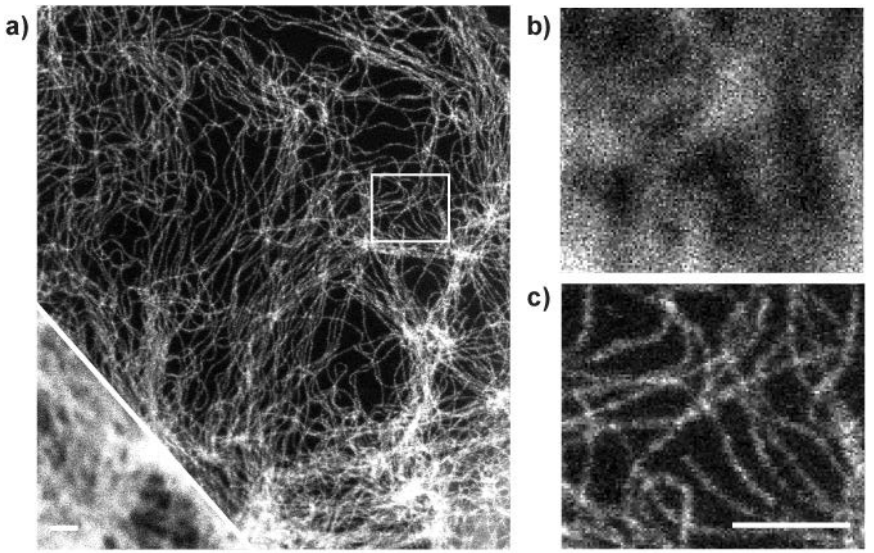


Figure S14. a) STED image of a U2OS cell, stable expression of vimentin-HaloTag fusion protein, live-labeled with ***GeR*-Halo** (1 µM, 20 minutes at 37°C, washed, and imaged at room temperature), counterpart confocal image in the lower left corner.
b) and **c)** Close-ups of the boxed region from a) in confocal (b) and STED (c) mode. ***GeR*-Halo** was excited with a pulsed 640 nm laser and fluorescence was detected from 650-720 nm. The STED laser was pulsed at 775 nm. The STED image was recorded with a pixel dwell time of 15 µs and smoothed with a lowpass Gaussian filter. Scale bars: 1 µm.

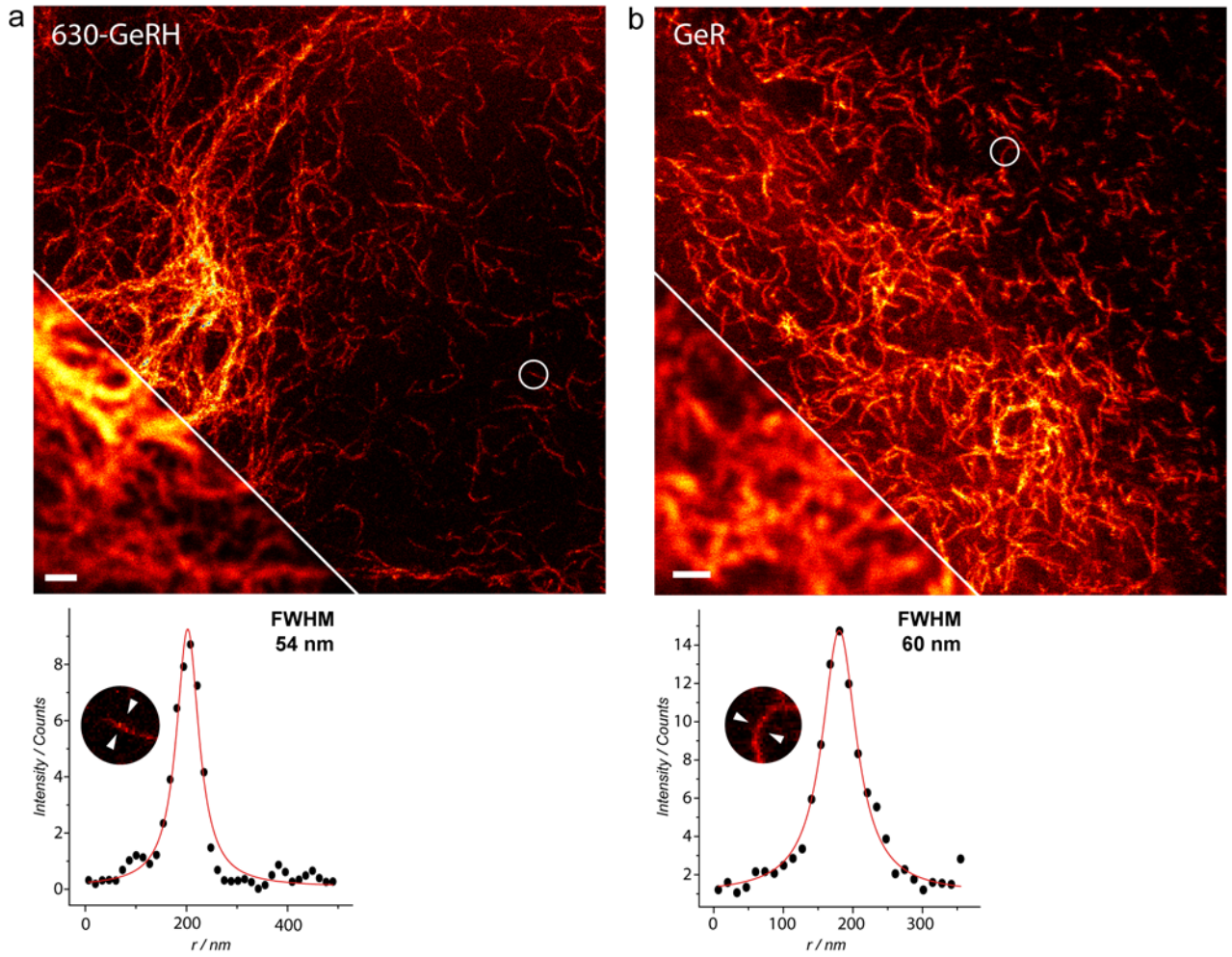


Figure S15. STED images of a transfected HeLa cells expressing vimentin-HaloTag fusion protein live-labeled with ***630GeRH*-Halo (a)** and ***GeR*-Halo (b)** (2 µM, 20 minutes at 37°C, washed 10 minutes, and imaged at room temperature), counterpart confocal image in the lower left corner and corresponding line profiles of the indicated regions are shown below the images. Both dyes were imaged with the same image parameters and laser powers using a pulsed 640 nm laser for excitation, and fluorescence detection was at 650-720 nm. The STED laser was pulsed at 775 nm. For the STED image, the fluorescence counts of 3 scans per line were accumulated with a pixel dwell time of 6 µs and pixel size of 25 nm. Scale bars: 1 µm.

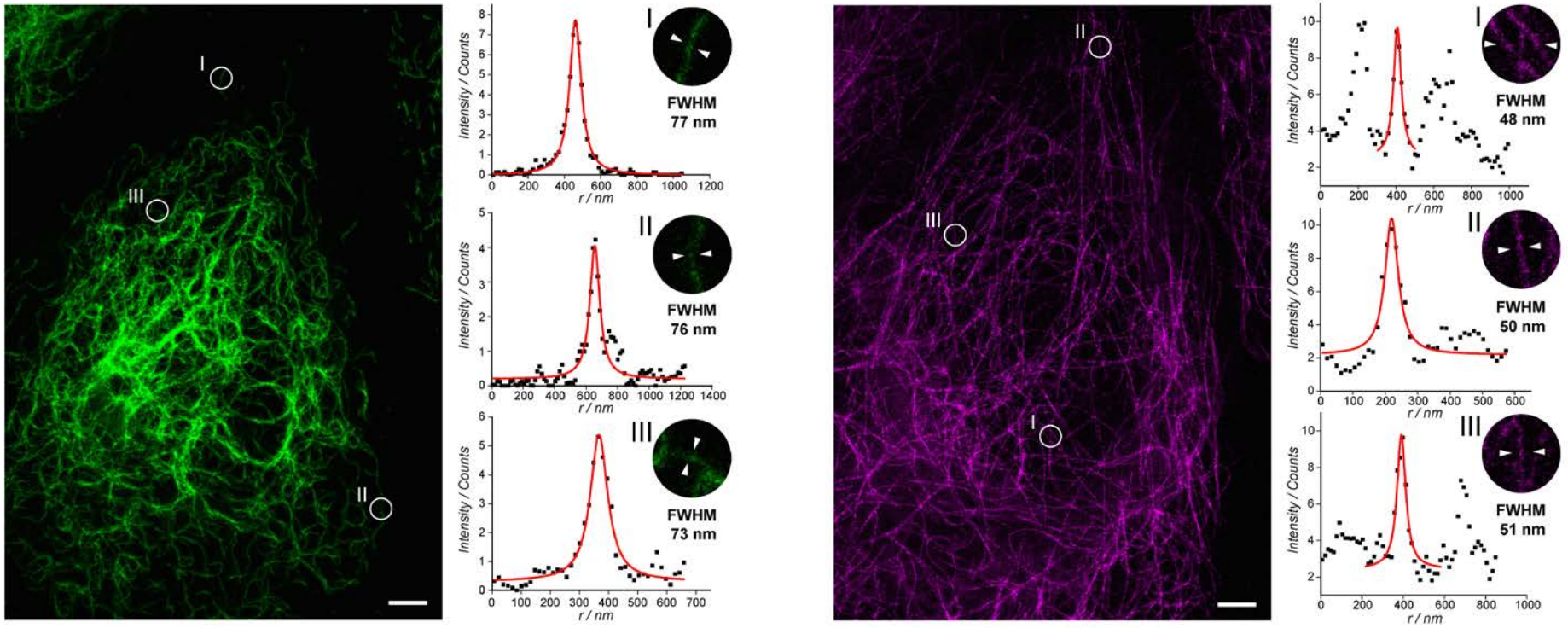


Figure S16. Larger field of view of the two-color STED image (raw data) of vimentin (green) and tubulin (magenta) from Figure 3 in the main text and the corresponding line profiles for each color channel (I-III). Line profiles were drawn perpendicular to the indicated filaments and counts were averaged over five pixels along the direction of the filament and fitted to the Lorentzian function. Resulting FWHM (full-width at half maximum) values are given for each fit, yielding values of ~75 nm for vimentin labeled via HaloTag fusion protein with ***575RH*-Halo** (1 µM) and ~50 nm for endogenous tubulin directly labeled using ***GeR*-tubulin** probe (5 µM, 20 min incubation in a solution of both markers, followed by 10 min washing). Scale bars 2 µm; pixel dwell time: 12 µs for both color channels; pixel size: 28 nm for STED and confocal image. Color channels were recorded linewise; each line was scanned two times for the ***575RH***-vimentin (green, left) and five times for ***GeR***-tubulin (magenta, right) channel, and counts were accumulated.

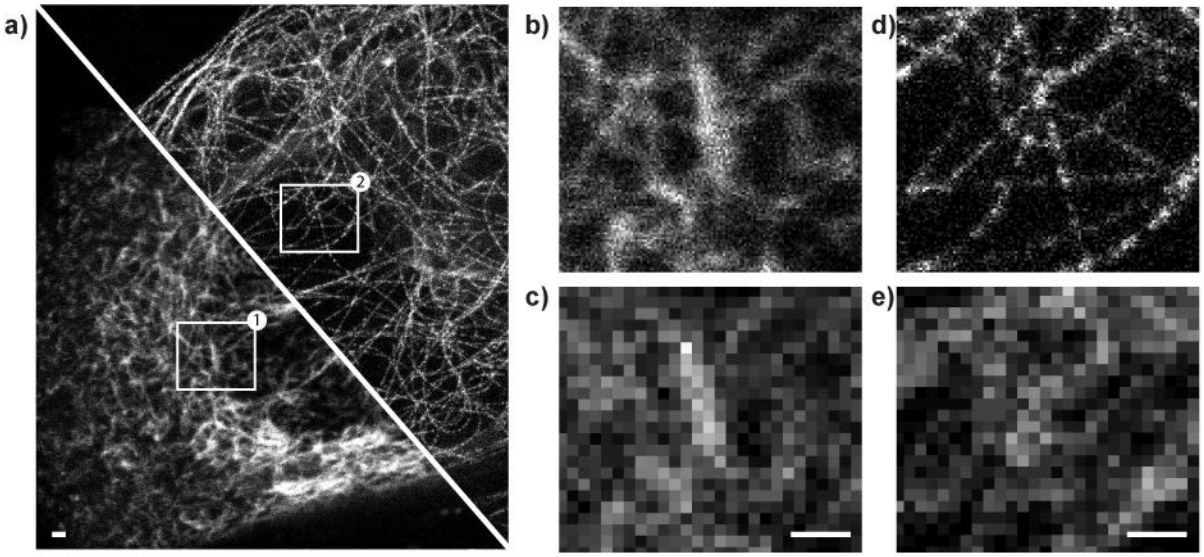


Figure S17. a) STED image of a transfected HeLa cell expressing vimentin-HaloTag fusion protein live-labeled simultaneously with ***575RH*-Halo** (vimentin staining, lower left image, 1 µM) and ***630GeRH*-tubulin** (upper right image, 5 µM, 20 minutes at 37°C, washed, and imaged at room temperature).

b) and **c)** Close-ups of the boxed region from a1) in STED (b) and confocal (c) mode for vimentin-HaloTag fusion protein labeled with ***575RH*-Halo**.

d) and **e)** Close-ups of the boxed region from a2) in STED (d) and confocal (e) mode for ***630GeRH*-tubulin**.

***575RH*-Halo** was excited with a pulsed 561 nm laser and fluorescence was detected in the range of 605-625 nm. ***630GeRH*-tubulin** was excited with a pulsed 640 nm laser and fluorescence was detected in the range of 650-720 nm. For the STED image the fluorescence of both color channels were recorded linewise with a pixel dwell time of 15 µs. The STED laser was pulsed at 775 nm. Scale bars: 1 µm.

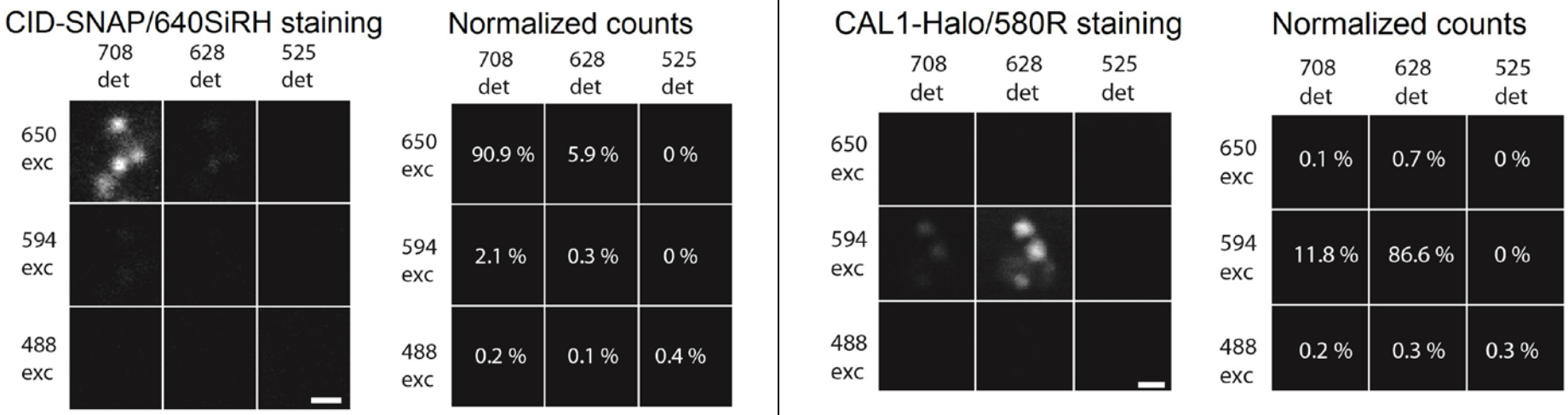


Figure S18. Illustration of color separation between the dyes ***640SiRH*** and ***580R***.[1] Either the cells with labelled CID (centromere histone, via SNAP-Tag fusion with ***640SiRH*-BG**, left panels), or the cells with labelled CAL1 (CID assembly factor, via HaloTag fusion with ***580R*-Halo**, right panels) were imaged. STED microscope with three excitation lasers (488, 594, and 650 nm) and three detectors (used bandpass filters are 525/50, 628/32 and 708/75 nm, Semrock Brightline single-band) was used. For each excitation laser individually, the signals in all three detection channels were measured. The raw data are shown in the "staining" panels (same scale, no background subtraction). The photon count of the region of interest in each image was compared with the sum of the intensities of all regions. The signals of both dyes are so well separated (~2% crosstalk from ***640SiRH*** in the 594 excitation/708 detection frame and <12% crosstalk from ***580R*** in the 594 excitation/708 detection frame) that it is possible to excite and detect both dyes at the same time without the need for spectral unmixing. The images were taken with 50 µs dwell time, 30 nm pixel size, no line or frame accumulation.

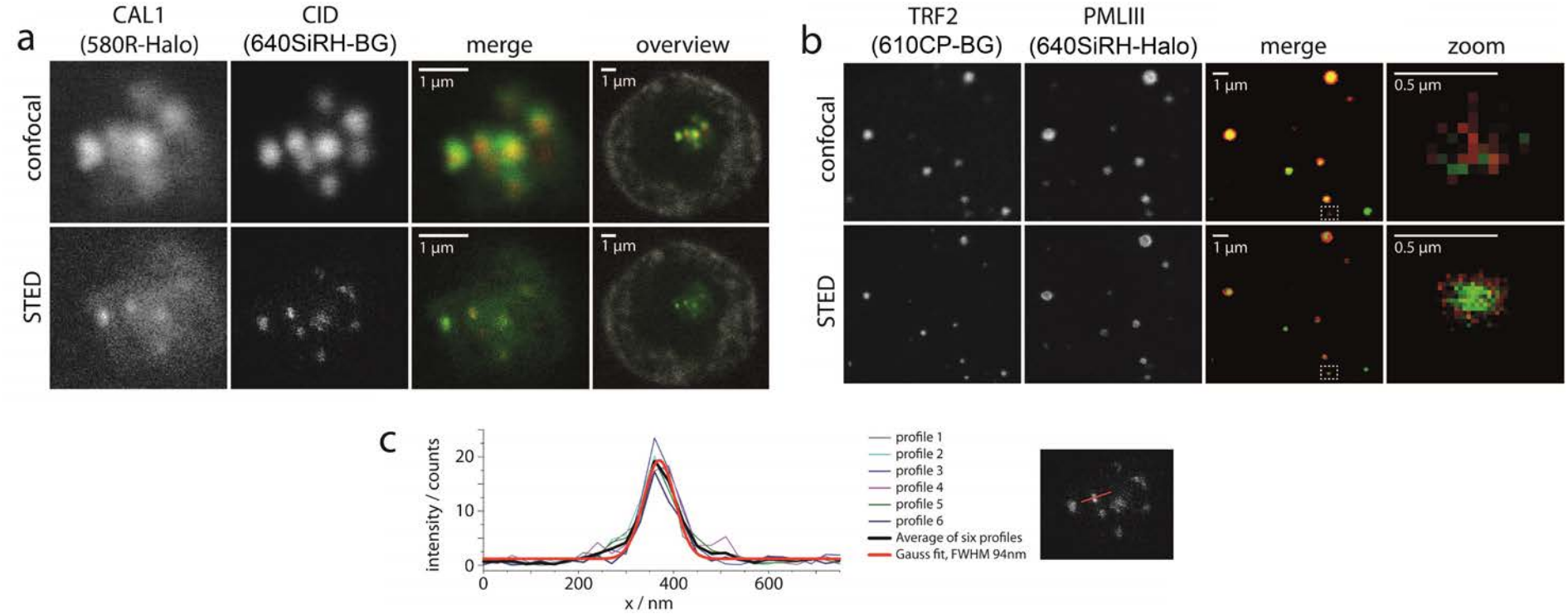


Figure S19. Live cell STED imaging of nuclear proteins in *Drosophila* S2 cells and osteosarcoma U2OS cells. The different color channels were recorded line-wise (no line or frame accumulation) without any crosstalk (as shown for ***580R*** and ***640SiRH*** in Figure S18), raw data is shown (no spectral unmixing, background subtraction, deconvolution or other post-processing methods were performed). Cells were incubated for 30 min in a staining solution of both dyes.

a) Confocal and STED images of nuclear proteins in a living *Drosophila* S2 cell. CAL1, the Drosophila-CID assembly factor, is labelled via HaloTag fusion with ***580R*-Halo**[1] (1 µM, red). CID, the Drosophila centromere histone, is labelled via Snap-Tag fusion with ***640SiRH*-BG** (1 µM, green). In the overview image Tubulin is shown in grey (labelled with Tubulin Tracker Green, 0.5 µM, 10 min, Oregon green 488 Taxol, Bis-Acetate, *ThermoFisher* T34075). Both the nucleus and the cytoplasm are free from nonspecific background. The images were taken with 30 µs dwell time, 30nm pixel size. Only CAL1 and CID are imaged with STED, during the imaging of Tubulin the STED beam was turned off.

b) Confocal and STED image of ALT-associated promyelocytic nuclear bodies (APBs) in a living U2OS cell. The telomeric repeat binding factor 2 (TRF2) is labeled via SNAP-Tag fusion with ***610CP*-BG**[1] (1 µM, green). The PML (promyelocytic leukemia protein) shell encasing some telomeres is labeled via HaloTag fusion to PMLIII with ***640SiRH*-Halo** (1 µM, red). The images were taken with 30 µs dwell time, 20 nm pixel size for STED and 50 nm pixel size for confocal.

Dye	*575RH* (Figure S9)	*6-ROX* (Figure S10)	*630GeRH* (Figure S15,a)	*GeR* (Figure S15,b)
FWHM (full width at half-maximum), *nm*	67±9	59±4	52±5	54±7
Average signal/noise ratio	35	26	14	13

Table S1. Comparative imaging performance of hydroxylated and non-hydroxylated dyes on examples of *575RH*/*6-ROX* and *630GeRH*/*GeR* pairs. All dyes were applied as HaloTag(O2) amine ligands. Optical resolutions are represented by apparent visible vimentin-Halo fusion filament thickness (expressed as FWHM) in living HeLa cells. The FWHM values and signal-to-noise ratios were evaluated by analyzing multiple line profiles on different cells across the filaments in Figures S9, S10 and S15.

Prediction of the positions of absorption and emission bands (examples)

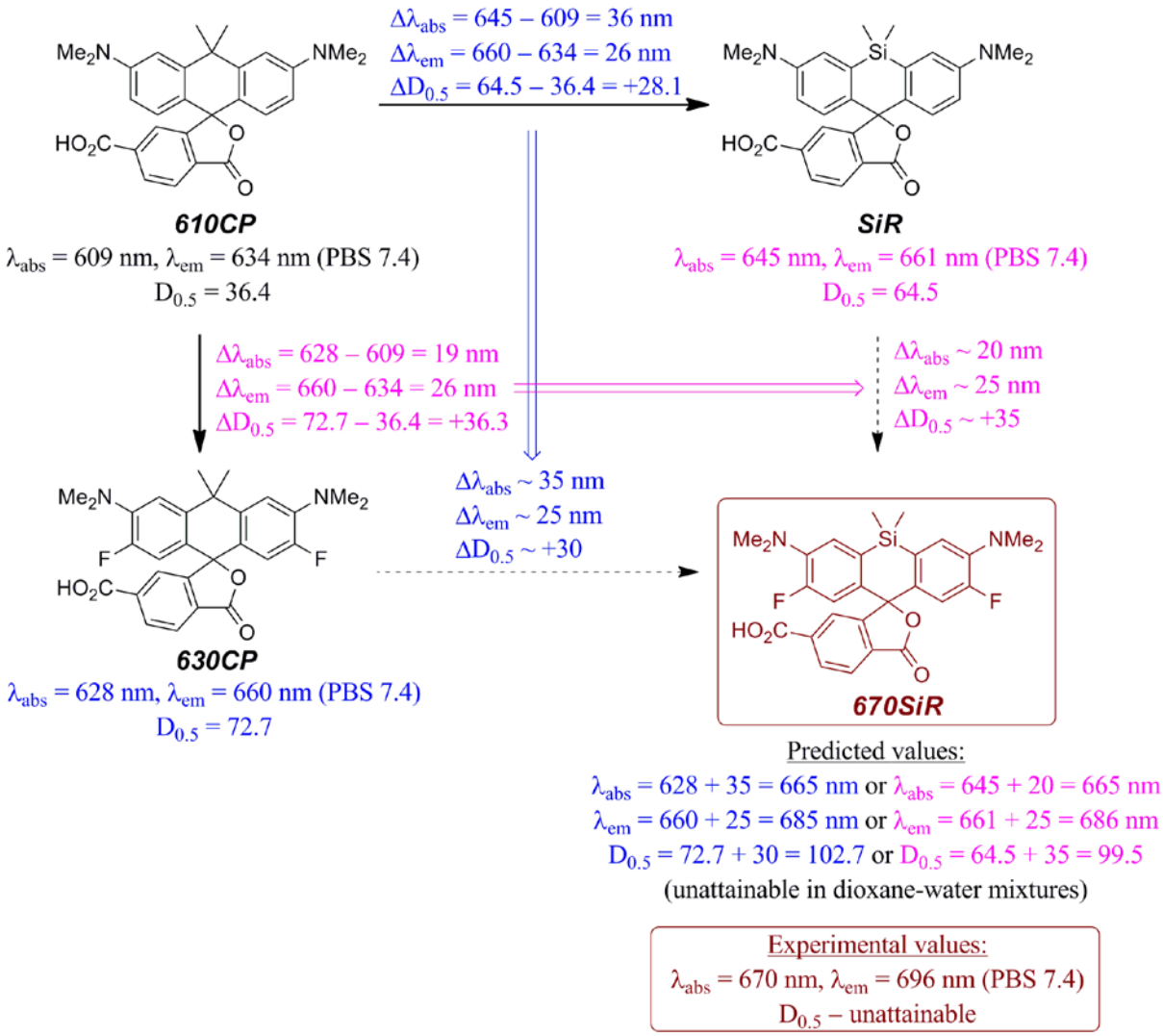


Figure S20. Prediction of the positions of absorption and emission bands, as well as $D_{0.5}$ value, for a sample dye ***670SiR***[1] is based on the available data for ***610CP***, ***630CP***[1] and ***SiR***. In this case, ***SiR*** is an analog of carborhodamine ***610CP*** with a dimethylsilylene bridge, and ***630CP*** is a difluorinated analog of ***610CP***. Transition from ***610CP*** to ***SiR*** is accompanied with bathochromic shift of 36 nm and bathofluoric shift of 26 nm, and $D_{0.5}$ value is increased by 28.1. Assuming the same increments (rounded to within 5 units) are approximately valid for the transition from ***630CP*** to the dye of interest (in this case, ***670SiR***), its estimated λ_{abs}, λ_{em} and $D_{0.5}$ can be obtained by applying the same increments to the parameters of dye ***630CP*** (values highlighted in blue). Similarly, the increments obtained from the transition ***610CP*** → ***630CP***, applied to the parameters of dye ***SiR***, provide comparable values (highlighted in purple). In particular, the very high $D_{0.5}$ value suggests the dye ***670SiR*** will exist nearly exclusively in non-fluorescent spirolactone form in aqueous buffers and will be impractical for imaging (which was indeed confirmed upon its synthesis).

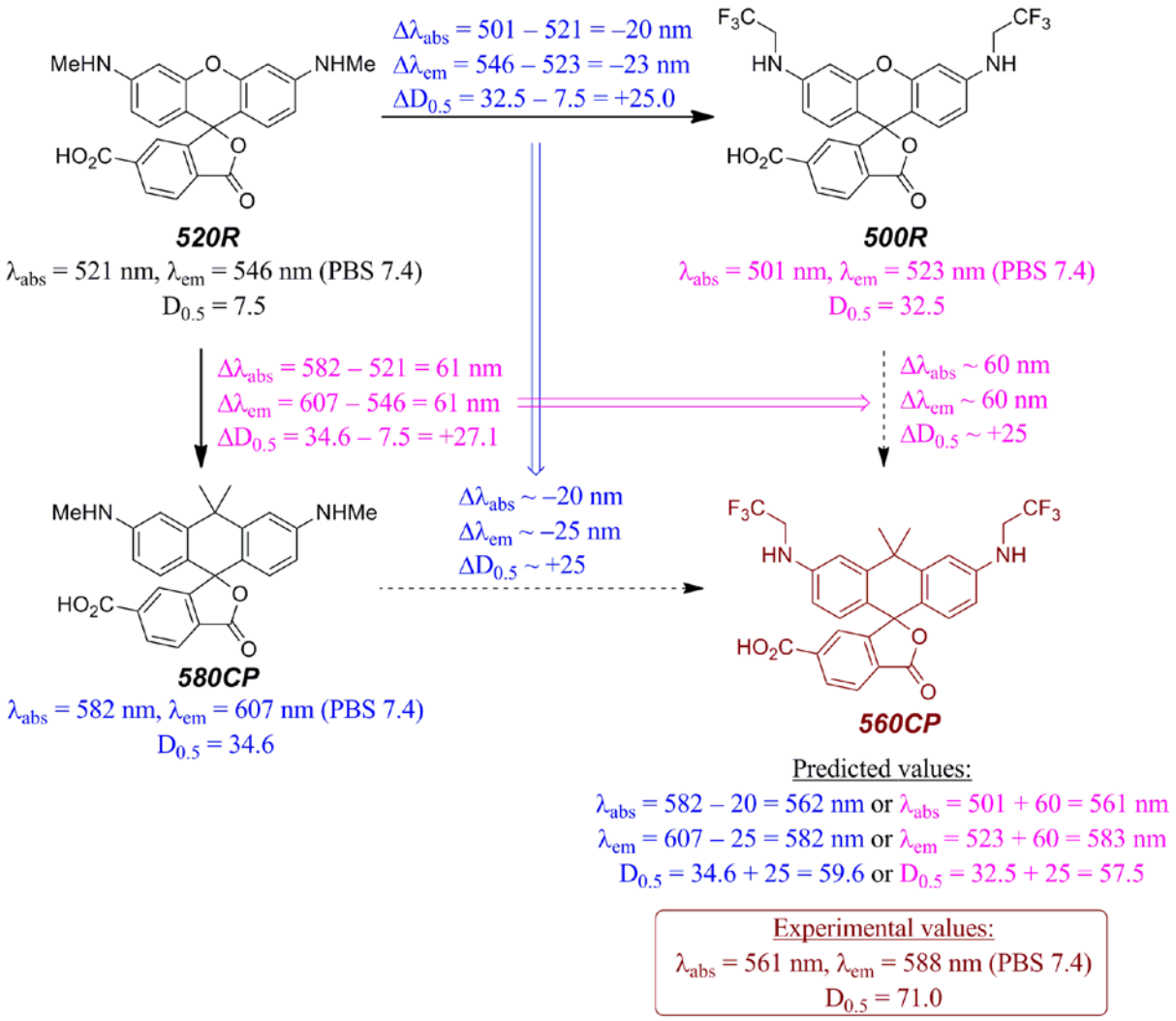
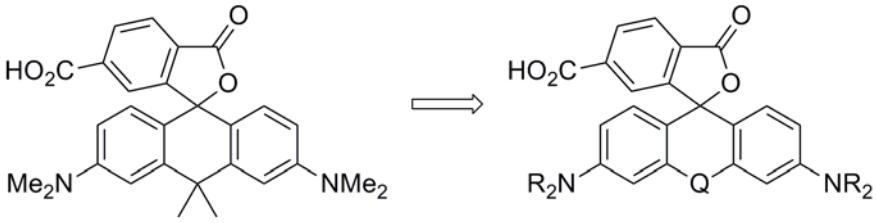
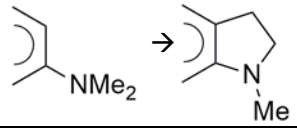
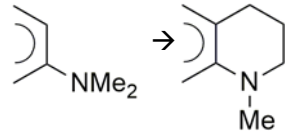
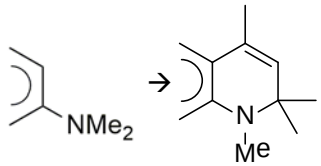
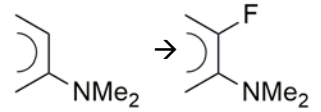


Figure S21. Prediction of the positions of absorption and emission bands, as well as $D_{0.5}$ value, for a new dye ***560CP*** is based on the available data for ***500R***, ***520R*** and ***580CP***.[1] In this case, ***500R*** is a bis(mono-trifluoroethylamino) analog of ***520R***, and ***580CP*** is an analog of rhodamine ***520R*** with an isopropylidene bridge. Transition from ***520R*** to ***500R*** is accompanied with hypsochromic shift of –20 nm and hypsofluoric shift of –23 nm, and $D_{0.5}$ value is increased by 25.0. Assuming the same increments (rounded to within 5 units) are approximately valid for the transition from ***580CP*** to the dye of interest (in this case, ***560CP***), its estimated λ_{abs}, λ_{em} and $D_{0.5}$ can be obtained by applying the same increments to the parameters of dye ***580CP*** (values highlighted in blue). Similarly, the increments obtained from the transition ***520R*** → ***580CP***, applied to the parameters of dye ***500R***, provide comparable values (highlighted in purple).

In both examples, use of the additive schemes provides the values of λ_{abs} and λ_{em} within 10 nm and a good evaluation of $D_{0.5}$ (which is a rather arbitrarily chosen parameter). The degree of precision of the proposed additive schemes using the measured values for structural analogs is particularly evident when compared to reported computational results (DFT, TD-DFT), which still allow a large margin of error for the red-emitting fluorophores.[2] Further accumulation of experimental photophysical data, including those appearing in the patent literature, will lead to better understanding of the structure-properties relationship for a variety of cell-permeant fluorophores.



reference dye **A** (e.g., *610CP*) → analog **X** (amine substitution pattern or Q bridge varied)

Structure alterations (**A** → **X**): amine substitutions	Increments		Notes
	Δλ(AX), nm	$\Delta D_{0.5}$(AX)	
—NMe_2 → —NHMe	-30	~0	A. N. Butkevich et al., *Angew. Chem. Int. Ed.* **2016**, *55*, 3290
—NMe_2 → —$NHCH_2CF_3$	-50…-60	+25	V. N. Belov et al., *Chem. Eur. J.* **2010**, *16*, 4477; A. N. Butkevich et al., *Angew. Chem. Int. Ed.* **2016**, *55*, 3290
—NMe_2 → —$NHCH_2CF_2CH_2OH$	-40	+20	this work (for CP)
—NMe_2 → 	0	(unchanged)*	L. Lavis et al., WO 2015/153813
—NMe_2 → 	-10	(increase)*	L. Lavis et al., WO 2015/153813
—NMe_2 → 	-25	(increase)*	L. Lavis et al., WO 2015/153813
—NMe_2 → 	-5	+10	this work
	+50 (SiR) +10 (R)	-20 (SiR) ~0 (R)	G. Lukinavičius et al., *J. Am. Chem. Soc.* **2016**, *138*, 9365
	+30 (SiR) +10 (R)	(decrease)*	Y. Koide et al., *J. Am. Chem. Soc.* **2012**, *134*, 5029 (for SiR)
	+80 (SiR) +25 (R)	(decrease)*	Y. Koide et al., *J. Am. Chem. Soc.* **2012**, *134*, 5029 (for SiR)
	+20…+35 (SiR, CP) -5 (R)	+35 (SiR) +10 (R)	A. N. Butkevich et al., *Angew. Chem. Int. Ed.* **2016**, *55*, 3290; V. N. Belov et al., *Chem. Eur. J.* **2010**, *16*, 4477

* not determined: expected behavior in brackets. All increments are listed for double substitution in symmetric fluorophores with an ortho-substituent (CO_2H or CH_3) in the pendant phenyl ring.

Structure alterations (**A** → **X**): bridge substitutions	Increments		Notes
	Δλ(AX), nm	$\Delta D_{0.5}$(AX)	
Q = CMe_2 → Q = O	-60	-30	A. N. Butkevich et al., *Angew. Chem. Int. Ed.* **2016**, *55*, 3290
Q = CMe_2 → Q = $GeMe_2$	+20 … +25	+30	this work
Q = CMe_2 → Q = $SiMe_2$	+30 … +35	+30	A. N. Butkevich et al., *Angew. Chem. Int. Ed.* **2016**, *55*, 3290

Table S2. Increments for the absorption and emission maxima (λ_{max}) and $D_{0.5}$ values in aqueous buffers (pH 7), compiled from the literature and authors' data on cell-permeant dyes and close structural analogs; rounded to ±5 nm (for Δλ) or ±5 dielectric constant units (for $\Delta D_{0.5}$).

General experimental information and synthesis

NMR spectra were recorded at 25 °C with Agilent 400-MR spectrometer at 400.06 MHz (^{1}H), 376.40 MHz (^{19}F) and 100.60 MHz (^{13}C). All ^{1}H spectra are referenced to tetramethylsilane (δ = 0 ppm) using the signals of the residual protons of $CHCl_3$ (7.26 ppm) in $CDCl_3$, acetone-d_5 (2.05 ppm) in acetone-d_6, CHD_2OD (3.31 ppm) in CD_3OD or DMSO-d_5 (2.50 ppm) in DMSO-d_6. ^{13}C spectra are referenced to tetramethylsilane (δ = 0 ppm) using the signals of the solvent: $CDCl_3$ (77.16 ppm), acetone-d_6 (CD_3, 29.84 ppm), CD_3OD (49.00 ppm) or DMSO-d_6 (39.52 ppm). Multiplicities of signals are described as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet or overlap of non-equivalent resonances; br = broad signal. Coupling constants (*J*) are given in Hz. Multiplicities in the ^{13}C NMR spectra were determined by APT (attached proton test) or gHSQCad (heteronuclear single-quantum one-bond *J*-correlation with adiabatic 180° C-nuclei pulses and gradient coherence selection) experiments.

ESI-MS were recorded on a Varian 500-MS spectrometer (Agilent). ESI-HRMS were recorded on a MICROTOF spectrometer (Bruker) equipped with ESI ion source (Apollo) and direct injector with LC autosampler Agilent RR 1200.

Liquid chromatography: HPLC was performed with Knauer Azura liquid chromatography system, equipped with a diode array detector (DAD 6.1L), a mixing chamber and an injection valve with 20 and 100 µL loops for the analytical and semi-preparative columns, respectively; two automatic 6-port-3-channel switching valves were used for switching between the columns. Analytical column: Eurospher II 100 C18, 5 µm, 250×4 mm or 150×4 mm (as indicated); semi-preparative column: Eurospher 100 C18, 5 µm, 250×8 mm. Eluents: solvent A – acetonitrile + 0.1% v/v TFA, solvent B – H_2O + 0.1% v/v TFA (unless stated otherwise). Analytical TLC was performed on MERCK ready-to-use plates with silica gel 60 (F_{254}). High performance silica gel precoated thin-layer plates with silica gel for high performance TLC (HPTLC Kieselgel 60, 10×10 cm) were purchased from MERCK (Darmstadt, Germany). Automated flash purifications on silica gel using cartridges from Biotage (SNAP Ultra, 25 µm silica), Interchim (PF-SIHC, 15 µM silica) or Teledyne Isco (RediSep® Rf, 35 µM silica) and on reversed phase using cartridges Biotage SNAP Ultra C_{18} were performed on a Biotage Isolera One instrument with variable UV-Vis 200-800 nm wavelength detector. Alternatively, reversed-phase chromatography was done manually on Polygoprep 60-50 C_{18} (Macheray-Nagel, Düren, Germany).

All photophysical parameters were measured in PBS (pH 7.4) solutions at ambient temperature (25 °C). The absorption spectra were recorded on a Varian Cary 4000 UV-Vis spectrophotometer (Agilent). The emission spectra were recorded on a Varian Cary Eclipse fluorescence spectrophotometer (Agilent). The fluorescence quantum yields (absolute values) were obtained on a Quantaurus-QY absolute PL quantum yield spectrometer (model C11347-12, Hamamatsu). Fluorescence lifetimes were measured on a Quantaurus-Tau fluorescence lifetime spectrometer (model C11367-32, Hamamatsu).

For microwave synthesis, Biotage Initiator+ synthesizer equipped with Robot Eight was used. The reactions were run in standard 0.5-2 mL or 2-5 mL glass reaction vials with 30 sec pre-stirring and fixed hold time.

***N,N'*-Bis-(2,2,2-trifluoroethyl)-6'-carboxy-Q-rhodamine-β,β'-diol** (*530RH)*

Dye *530RH* (Figure S22) exists as a mixture of 3 diastereomers due to the possible orientation of its two hydroxyl groups in *syn*- and *anti*-positions to each other during the condensation step. The *syn*-isomer of compound *530RH* has a symmetry plane and *anti-530RH* has a C_2 rotation axis. Because of the hindered rotation around the single C–C bond between the pendant phenyl ring and the fluorophore core, *syn-530R* exists in the form of two diastereoisomers (with the carboxyl group in the *ortho*-position of the phenyl ring on the same side of the molecular plane as the hydroxyl groups in the xanthene core, or on the opposite side). All 3 diastereomers of *530RH* were detected by HPLC analysis, and 2 diastereomers were isolated and demonstrated identical optical properties, making the separation of diastereomeric labels unnecessary for any imaging application.

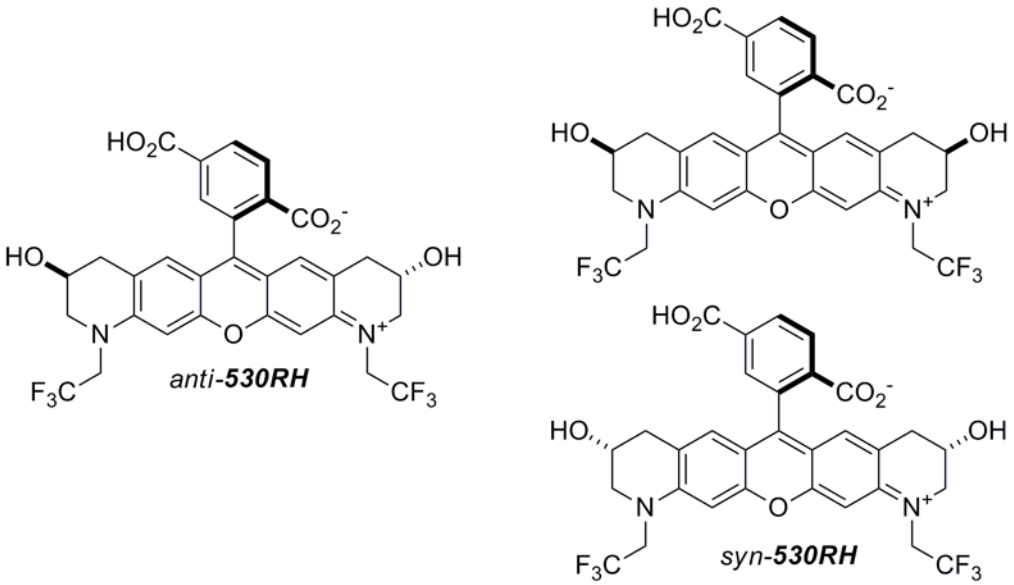
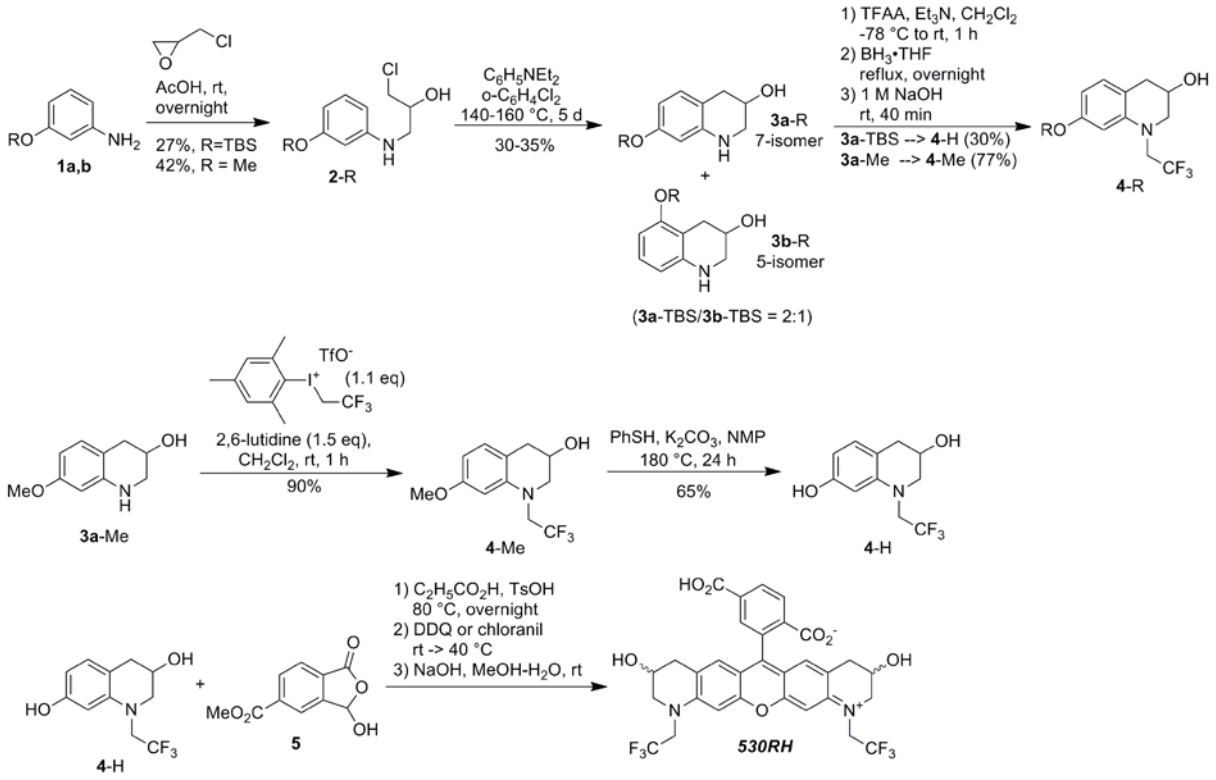


Figure S22. Diastereomeric froms of the dye *530RH* (one diastereomer of *anti-530RH* and two diastereomers of *syn-530RH*).



Compound 2-TBS. A mixture of 3-(*tert*-butyldimethylsilyloxy)aniline[3] (2.24 g, 10.0 mmol) and epichlorohydrin (0.94 g, 10.0 mmol) in acetic acid (20 mL) was stirred overnight at rt. The mixture was poured into aq. $NaHCO_3$ (30 g in 300 mL water), extracted with ethyl acetate (3 × 50 mL), and the combined organic layers were washed with brine and dried over $MgSO_4$. The product **2-TBS** was isolated by flash column chromatography (Biotage SNAP Ultra 100 g; gradient 0% to 5% ethyl acetate – CH_2Cl_2) as light yellow oil, yield 0.84 g (27%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.02 (t, *J* = 8.0 Hz, 1H), 6.29–6.23 (m, 2H), 6.16 (t, *J* = 2.2 Hz, 1H), 4.09 – 4.03 (m, 1H), 3.68 (dd, *J* = 11.3, 4.5 Hz, 1H), 3.63 (dd, *J* = 11.2, 6.1 Hz, 1H), 3.35 (dd, *J* = 13.3, 4.4 Hz, 1H), 3.21 (dd, *J* = 13.3, 7.1 Hz, 1H), 0.98 (s, 9H), 0.19 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 156.8, 149.1, 129.9, 110.1, 106.8, 105.2, 69.9, 47.7, 47.1, 25.7, 18.2, -4.4. ESI-MS, positive mode: *m/z* (rel. int., %) = 316.3 (100) $[M+H]^+$.

Compound 2-Me. A mixture of 3-methoxyaniline (*m*-anisidine; 2.46 g, 20.0 mmol) and epichlorohydrin (2.03 g, 22.0 mmol, 1.1 eq) in acetic acid (7 mL) was stirred overnight at rt. The mixture was poured into aq. $NaHCO_3$ (15 g in 200 mL water), extracted with ethyl acetate (2 × 50 mL), the combined organic layers were washed with brine and dried over $MgSO_4$. The product **2-Me** was isolated by flash column chromatography (Teledyne Isco RediSep Rf 120 g; gradient 0% to 5% ethyl acetate – CH_2Cl_2) as light yellow oil, yield 1.66 g (38%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.10 (t, *J* = 8.1 Hz, 1H), 6.32 (ddt, *J* = 8.2, 2.5, 0.7 Hz, 1H), 6.27 (ddt, *J* = 8.1, 2.3, 0.6 Hz, 1H), 6.22 (t, *J* = 2.3 Hz, 1H), 4.06 (ddtd, *J* = 7.2, 6.1, 4.5, 0.5 Hz, 1H), 3.77 (s, 3H), 3.69 – 3.58 (m, 2H), 3.36 (dd, *J* = 13.3, 4.4 Hz, 1H), 3.21 (dd, *J* = 13.3, 7.2 Hz, 1H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 160.9, 149.2, 130.2, 106.3, 103.3, 99.4, 69.8, 55.2, 47.7, 47.1.

Compounds 3a-TBS and 3b-TBS. A solution of **2-TBS** (343 mg, 1.09 mmol) and *N*,*N*-diethylaniline (810 mg, 5.43 mmol, 5 eq) in 1,2-dichlorobenzene (15 mL) was heated at 140 °C (bath temperature) in a sealed vessel for 5 days. Upon cooling, 1 M NaOH (10 mL) was

added, the organic layer was separated, and the aqueous layer containing viscous residue was extracted with CH_2Cl_2 (20 mL). The combined organic layers were washed with 1 M NaOH, water and dried over $MgSO_4$. TLC control (SiO_2 / 20% ethyl acetate in CH_2Cl_2): R_f (product) = 0.3. The product was isolated by flash column chromatography (Teledyne Isco RediSep Rf 24 g; gradient 0% to 20% ethyl acetate – CH_2Cl_2) as light yellow oil, yield 0.84 g (27%), as a 2:1 mixture of regioisomers **3a**-TBS and **3b**-TBS, used in the next step without separation. Major 7-isomer (**3a**-TBS): ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.81 (d, *J* = 8.3 Hz, 1H), 6.23–6.17 (m, 1H), 6.04 (d, *J* = 2.3 Hz, 1H), 4.23–4.18 (m, 1H), 3.32–3.28 (m, 1H), 3.23–3.20 (m, 1H), 2.97 (ddt, *J* = 16.1, 4.3, 1.3 Hz, 1H), 2.75–2.67 (m, 1H), 0.97 (s, 9H), 0.18 (s, 6H). Minor 5-isomer (**3b**-TBS): ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.87 (t, *J* = 8.0 Hz, 1H), 6.23–6.17 (m, 2H), 4.27–4.22 (m, 1H), 3.28–3.24 (m, 1H), 3.20–3.17 (m, 1H), 2.85 (dd, J = 17.4, 4.7 Hz, 1H), 2.80–2.73 (m, 1H), 1.00 (s, 9H), 0.23 (s, 3H), 0.22 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$; **3a-TBS/3b-TBS**): δ 155.0, 154.8, 145.1, 144.3, 131.1, 126.9, 111.5, 110.3, 108.1, 107.3, 105.5, 63.6, 63.4, 47.6, 47.3, 34.9, 30.7, 25.8, 25.7, 18.3, 18.2, -4.2, -4.4. ESI-MS, positive mode: *m/z* (rel. int., %) = 250.2 (100) $[M+H]^+$.

Compound 3a-Me *(7-isomer).* A solution of **2**-Me (1.61 g, 7.46 mmol) and *N*,*N*-diethylaniline (5.56 g, 37.3 mmol, 5 eq) in 1,2-dichlorobenzene (50 mL) was heated at 160 °C (bath temperature) in a sealed vessel for 5 days. Upon cooling, the volatiles were removed on a rotavapor (bath temperature 75 °C), the residue was dissolved in CH_2Cl_2 (100 mL), washed with 1 M NaOH and water (50 mL each). The organic layer was dried over $MgSO_4$, filtered and dried in vacuo at 85 °C to remove most of *N*,*N*-diethylaniline. TLC control (SiO_2 / 20% ethyl acetate in CH_2Cl_2): R_f (product) = 0.3. The product was isolated by flash column chromatography (Teledyne Isco RediSep Rf 80 g; gradient 0% to 30% ethyl acetate – CH_2Cl_2) as light yellow oil, yield 468 mg (35%) of **3a**-Me (7-isomer, nearly free from 5-isomer). ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.88 (d, *J* = 8.3 Hz, 1H), 6.28 (dd, *J* = 8.3, 2.5 Hz, 1H), 6.10 (d, *J* = 2.5 Hz, 1H), 4.22 (qd, *J* = 4.6, 2.3 Hz, 1H), 3.74 (s, 3H), 3.31 (dtd, *J* = 11.3, 1.6, 0.8 Hz, 1H), 3.22 (ddd, *J* = 11.3, 5.1, 2.0 Hz, 1H), 3.02 – 2.92 (m, 1H), 2.78 – 2.67 (m, 1H), 2.32 (br.s, 1H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 159.2, 144.6, 131.4, 111.1, 104.2, 99.6, 63.7, 55.3, 47.7, 34.9. ESI-MS, positive mode: *m/z* (rel. int., %) = 180.2 (100) $[M+H]^+$.

Compound 4-Me. *Method A:* A solution of **3a-Me** (205 mg, 1.14 mmol) and triethylamine (289 mg, 2.86 mmol, 2.5 eq) in CH_2Cl_2 (10 mL) was cooled in dry ice-acetone bath, and trifluoroacetic anhydride (TFAA; 365 µL, 551 mg, 2.62 mmol, 2.3 eq) was added. The resulting mixture was stirred for 10 min, allowed to warm up to r.t. and stirred for 1 h. The mixture was diluted with CH_2Cl_2 (10 mL), washed sequentially with water, 10% aq. $KHSO_4$, sat. aq. $NaHCO_3$, brine (15 mL of each) and dried over $MgSO_4$. The filtrate was evaporated, the residue was dissolved in THF (10 mL) and BH_3·THF (1 M in THF, 8 mL, 8 mmol) was added. The reaction mixture was refluxed under argon overnight, cooled in ice-water bath and quenched by careful addition of methanol. The solution was evaporated, 1 N NaOH (15 mL) was added to the residue and the mixture was stirred for 30 min at rt. The reaction mixture was extracted with diethyl ether (3×20 mL), the organic layer was washed with sat. aq. $NaHCO_3$, brine (20 mL of each) and dried over $MgSO_4$. The product was isolated by flash column chromatography (Büchi Sepacore Silica HP 12 g; gradient 0% to 10% ethyl acetate – CH_2Cl_2), yielding 230 mg (77% over 2 steps) of **4-Me** as yellowish oil.

Method B: Solid 2,2,2-trifluoroethyl(mesityl)iodonium trifluoromethanesulfonate[4] was added portionwise over 2-3 min to a solution of **3a-Me** (90 mg, 0.5 mmol) and 2,6-lutidine (87 µL, 80 mg, 0.75 mmol, 1.5 eq) in dry CH_2Cl_2 (3 mL), and the resulting clear solution was stirred at r.t. for 1 h. TLC control (silica, 60% EtOAc – hexane, stained with vanillin): R_f = 0.14 (starting material, yellow-orange), 0.36 (product, red). The mixture was diluted with sat. aq. $NaHCO_3$ (10 mL), extracted with CH_2Cl_2 (3×20 mL), the combined extracts were washed with brine and dried over Na_2SO_4. The product was isolated by flash column chromatography (Büchi Sepacore Silica HP 12 g; gradient 20% to 100% ethyl acetate – hexane), fractions containing the product were evaporated and dried in vacuo to remove any residual 2,6-lutidine. Yield 118 mg (90%) of **4-Me** as viscous yellowish oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.92 (dt, *J* = 8.2, 1.0 Hz, 1H), 6.33 (dd, *J* = 8.2, 2.4 Hz, 1H), 6.28 (d, *J* = 2.3 Hz, 1H), 4.21 (qd, *J* = 5.4, 2.7 Hz, 1H), 3.96 – 3.83 (m, 1H), 3.82 – 3.72 (m, 1H), 3.77 (s, 3H), 3.50 (dt, *J* = 11.7, 2.1 Hz, 1H), 3.29 (ddd, *J* = 11.7, 5.7, 1.8 Hz, 1H), 3.05 – 2.95 (m, 1H), 2.74 (ddt, *J* = 15.9, 5.6, 1.2 Hz, 1H), 2.14 (br.s, 1H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 159.5, 144.3, 131.4, 125.6 (q, *J* = 283.3 Hz), 112.0, 103.4, 98.6 (q, J = 1.8 Hz), 63.3, 56.5, 55.4, 53.5 (q, *J* = 32.8 Hz), 35.6. ^{19}F NMR (376 MHz, $CDCl_3$): δ -69.8. ESI-MS, positive mode: *m/z* (rel. int., %) = 262.1 (100) $[M+H]^+$.

Compound 4-H *(from **3a,b-TBS**).* A solution of mixed isomers **2a,b-TBS** (169 mg, 0.605 mmol) and triethylamine (153 mg, 1.51 mmol, 2.5 eq) in CH_2Cl_2 (10 mL) was cooled in dry ice-acetone bath, and trifluoroacetic anhydride (TFAA; 193 µL, 292 mg, 1.40 mmol, 2.3 eq) was added. The resulting mixture was stirred at -78 °C for 10 min, allowed to warm up to r.t. and stirred for 1 h. The mixture was diluted with CH_2Cl_2 (10 mL), washed sequentially with water, 10% aq. $KHSO_4$, sat. aq. $NaHCO_3$, brine (15 mL of each) and dried over $MgSO_4$. The filtrate was evaporated, the residue was dissolved in THF (10 mL) and BH_3·THF (1 M in THF, 4.5 mL, 4.5 mmol) was added. The reaction mixture was refluxed under argon overnight, cooled in ice-water bath and quenched by careful addition of methanol. The solution was evaporated, the residue was treated with 1 M NaOH (20 mL), extracted with diethyl ether (10 mL), the organic layer was discarded, and the aqueous layer was carefully neutralized with 1 M HCl and then with phosphate buffer to pH 7.5. The resulting aqueous solution was saturated with NaCl and extracted with diethyl ether (3×20 mL), the combined extracts were dried over $MgSO_4$. The products were isolated by flash column chromatography (Interchim Puriflash 15 µm 25 g; gradient 0% to 40% ethyl acetate – CH_2Cl_2), yielding 45 mg (30%) of **4-H** (less polar 7-isomer, eluted first) as yellowish oil, along with 12 mg (~8%) of impure 5-regioisomer.

Compound 4-H *(from **3-Me**).* A solution of **3-Me** (650 mg, 3.0 mmol), thiophenol (0.40 g, 3.6 mmol) and K_2CO_3 (20 mg) was heated in *N*-methylpyrrolidone at 180 °C (bath temperature) for 24 h. The reaction mixture was cooled down, diluted with diethyl ether (100 mL) and washed with 1 M NaOH (20 mL). The organic layer was discarded, and the aqueous layer was neutralized to pH ~ 8 with 5% aq. citric acid. The title product (and, partially, thiophenol) was extracted with ether (3 × 20 mL). Combined organic solutions were dried (Na_2SO_4) and evaporated. The residue was subjected to chromatography on 25 cm^3 SiO_2 (Isolera), and the title compound (0.4 g, 65%) isolated by elution with a CH_2Cl_2 - EtOAc mixture (10 – 50% EtOAc). ^{1}H NMR (400 MHz, CD_3CN): δ 6.78 (dt, *J* = 8.0, 1.0 Hz, 1H), 6.63 (s, 1H), 6.19 (br.d, *J* = 2.6 Hz, 1H), 6.14 (dd, *J* = 8.0, 2.3 Hz, 1H), 4.05 (dddd, *J* = 11.9,

7.1, 4.7, 3.1 Hz, 1H), 4.00 – 3.81 (m, 2H), 3.42 (ddd, *J* = 11.6, 3.3, 1.9 Hz, 1H), 3.21 – 3.14 (m, 1H), 3.05 (d, *J* = 5.1 Hz, 1H), 2.87 (ddt, *J* = 15.5, 4.7, 1.3 Hz, 1H), 2.58 (ddt, *J* = 15.5, 7.1, 1.2 Hz, 1H). ^{19}F NMR (376 MHz, CD_3CN): δ -70.7. ^{13}C NMR (101 MHz, CD_3CN): δ 157.2, 145.7, 131.9, 127.2 (q, *J* = 282.7 Hz), 113.1, 105.9, 99.6 (q, *J* = 1.6 Hz), 63.7, 57.3, 53.7 (q, *J* = 32.2 Hz), 36.3. ESI-MS, positive mode: *m/z* (rel. int., %) = 248.1 (100) $[M+H]^+$.

Compound 5 was prepared according to the published procedure.[5]

N,N'*-Bis-(2,2,2-trifluoroethyl)-6'-carboxy-Q-rhodamine-β,β'-diol** ***(530RH). Compound **4-H** (25 mg, 0.1 mmol), compound **5** (15 mg, 0.072 mmol) and *p*-toluenesulfonic acid monohydrate (5 mg) were heated in propionic acid (1 mL) at 80 °C overnight. DDQ (16 mg, 0.07 mmol) was added to the reaction mixture at room temperature, it was heated to 40 °C, and stirred at this temperature for 1 h. All volatile materials have been removed in vacuo, and the residue dissolved in CH_2Cl_2 (100 mL). The organic solution was washed with water and sat. aq. $NaHCO_3$. Combined aqueous solutions were extracted with CH_2Cl_2 (20 mL). Combined organic solutions were dried (Na_2SO_4), filtered, and dichloromethane was evaporated in vacuo. The residue was dissolved in MeOH (3 mL), and 2 M aq. NaOH (0.5 mL) was added to the methanolic solution. The reaction mixture was kept overnight at room temperature, and all volatile materials were evaporated in vacuo. The residue was dissolved in aqueous MeCN and acidified with aq. TFA. TLC on reversed phase (VWR International, TLC Siliga gel 60 RP-18 F_{254}s) in the solvent system MeCN/H_2O = 1:2 (+0.3% HCOOH) revealed a brightly fluorescent orange spot. The title dye was isolated by RP chromatography on Polygoprep 60-50 C_{18} (Macherey Nagel). Elution with aqueous acetonitrile (MeCN/H_2O = 1:2 +0.1% TFA) followed by direct lyophilization of the pooled fractions containing diastereomers of dye ***530RH*** afforded 8 mg of red voluminous solid (mixture of 3 diastereomers). TLC control (silica, dichloromethane/methanol/water, 60:30:2): R_f = 0.06 (isomer 1), 0.18 (isomer 2), 0.10 (isomer 3). HPLC: analytical column *Kinetex C18 100,* 5 µm, 250×4.6 mm, 1.2 mL/min; solvent A: water + 0.1% v/v trifluoroacetic acid (TFA); solvent B: MeCN + 0.1% v/v TFA. Analytical method: A/B: 70/30 → 0/100 in 20 min, UV-VIS detection with dioden array. t_R = 5.4 min (isomers 1+2 are inseparable under these conditions), 5.6 min (isomer 3) in ratio ca. 2.5:1. Substances with t_R = 5.4 min and t_R = 5.6 min were isolated by preparative HPLC; preparative column: *Kinetex C18 100*, 5 µm, 250×20 mm, 10 mL min/mL, solvent A: water + 0.05% v/v trifluoroacetic acid (TFA); solvent B: MeCN. By analytical HPLC using another buffer [solvent A: water + 0.05 M $Et_3N{*}H_2CO_3$ buffer with pH = 8; solvent B: MeCN; method: A/B: 80/20 → 0/100 in 20 min, UV-VIS detection with dioden array], it turned out that the substance with t_R = 5.4 min (see above) was a mixture of two isomers (1+2) with t_R = 5.0 min and 5.2 min (~2:1). Isomers 1+2 were used in the preparation of an NHS ester and an amide from Halo-Tag-amine (see below). Isomers differ in mutual orientations of hydroxyl groups (*syn* and *anti*; see Figure S22). For the *syn*-isomer, an additional pair of diastereomers is possible, due to the hindered rotation of the phenyl ring with 2 carboxylic acid groups (see Figure S22). Isomers can be separated by repeated chromatography on RP (using aqueous acetonitrile with 0.05 M $Et_3N{*}H_2CO_3$ buffer; pH = 8) followed by chromatography on regular SiO_2 using acetonitrile – dichloromethane solvent system (10/1) with increasing content of water (5 – 12%). ^{1}H NMR (400 MHz, CD_3OD, isomer 3): δ = 8.37 (m, 2H), 7.93 (d, *J* = 1.2 Hz, 1H), 7.22 (s, 2H), 6.95 (s, 2H), 4.58 (dd, $^2J_{HH}$ = 16.6, $^3J_{HF}$ = 8.6 Hz, 2H), 4.37 (dd, $^2J_{HH}$ = 16.6, $^3J_{HF}$ = 8.6 Hz, 2H), 4.22 (m, 2H,

CHOH), 3.79 (m, 2H), 3.55 (ddd, J = 10.6, 5.8 and 3.1 Hz, 2H), 2.98 (m, 2H), 2.77 (dd, J = 16.3, 5.8, 2H) ppm. ^{19}F NMR (376 MHz, CD_3OD, isomer 3): δ = – 70.2 (t, $^3J_{HF}$ = 8.8 Hz) ppm. The following spectral data mere obtained for aqueous solutions. Isomer 3: absorption λ_{max}, nm (ε, M^{-1} cm^{-1}) = 532 (56000), emission λ_{max}, nm (Φ_{fl} –fluorescence quantum yield) = 553 (0.89 – absolute value; excitation at 488 nm). $C_{31}H_{24}F_6N_2O_7$ (650.1488). HR-MS, ESI (positive mode): *m/z* = 651.1549 (found $[M+H]^+$), 651.1560 (calc.); ESI (negative mode): *m/z* (rel. int., %) = 649.1408 (100) (found $[M-H]^-$); 649.1415 (calc.).

***N*-Hydroxysuccinimidyl ester of *N,N'*-bis-(2,2,2-trifluoroethyl)-6'-carboxy-Q-rhodamine-β,β'-diol (*530RH-NHS*) and its conjugate with HaloTag amine (*530RH-Halo*)**

Three stock solutions (A, B and C) were prepared. Solution A: 22 mg HATU (1-[bis(dimethylamino)-methylene]-1*H*-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate) in dry DMF (0.10 mL); solution B: Et_3N (10 mg) in dry DMF (0.10 mL); solution C: *N*-hydroxysuccinimide (19 mg) in dry DMF (0.10 mL). To a solution of dye **5**-OH (1.7 mg, 2.6 μmol) in dry DMF (0.10 mL), solutions A, B and C were added successively (14 μL of each solutions) under argon, and the mixture was stirred for 30 min at room temperature. HPLC analysis indicated the conversion degree of 94–95%. (*Eurospher-100 C18* 5 μm 250×4 mm, 1.2 mL/min; solvent A: water + 0.1% v/v trifluoroacetic acid (TFA); solvent B: MeCN + 0.1% v/v TFA. A/B: 30/70 – 100/0, 25 min, t_R = 6.7 min (compound **5**-OH), 8.3/8.6 min (mono-NHS esters, mixture of diastereomers). The product (6'-NHS ester of dye ***530RH***, a stable compound ***530RH-NHS***) was used *in situ*. For that, HaloTag-amine ($H_2N(CH_2)_2O(CH_2)_2O(CH_2)_6Cl$ = $C_{10}H_{22}ClNO_2$, 1.5 mg, 6.7 μM) was added to the reaction mixture as a solution in DMF (20-40 μL), and the reaction mixture was stirred at 40 °C. After several hours, HPLC control indicated that the conversion to new products with t_R = 11.9/12.2 min (***530RH-Halo***, mixture of dye diastereomers) was nearly complete (93%; HPLC area). After removing DMF in vacuo, amide ***530RH-Halo*** was isolated by preparative HPLC (~1 mg, 46%), dissolved in DMSO (400 μL), and this stock-solution was used (after dilution with aqueous PBS to 1 – 5 μM) in labeling experiments with living cells (see section with imaging results). ESI-MS ($C_{41}H_{44}ClF_6N_3O_8$, 855.27), positive mode: *m/z* (rel. int., %) = 856 (100) $[M+H]^+$. HR-MS, ESI (positive mode): *m/z* = 878.2591 (found $[M+Na]^+$), 878.2613 (calc.).

<u>Hydroxylated ROX dyes</u> (13a = *575RH* and **13b)**

Because of the hindered rotation around the single C–C bond between the pendant phenyl ring and the fluorophore core, dye **13a** exists in the form of two diastereoisomers (Figure S23). Dye **13b** exists as a mixture of 3 diastereomers due to the possible orientation of its two hydroxyl groups in *syn*- and *anti*-positions to each other during the condensation step. As with the compound **5**-OH (*530RH*), the *syn*-isomer of compound **13b** has a symmetry plane and *anti*-**13b** has a C_2 rotation axis. Because of the hindered rotation around the single C–C bond between the pendant phenyl ring and the fluorophore core, *syn*-**13b** exists in the form of two diastereoisomers (with the carboxyl group in the *ortho*-position of the phenyl ring on the same side of the molecular plane as the hydroxyl groups in the xanthene core, or on the opposite side). Indeed, all three isomers of **13b** were detected by HPLC analysis (see below). The remarkable feature of the synthesis in Scheme 2 is that it creates a separable mixture of 5 diastereomeric dyes in one step. The identical optical properties of isomeric dyes **13a** allow using the *mono*-hydroxylated ROX labels **13a** without separation of individual diastereoisomers (as dye *575RH*).

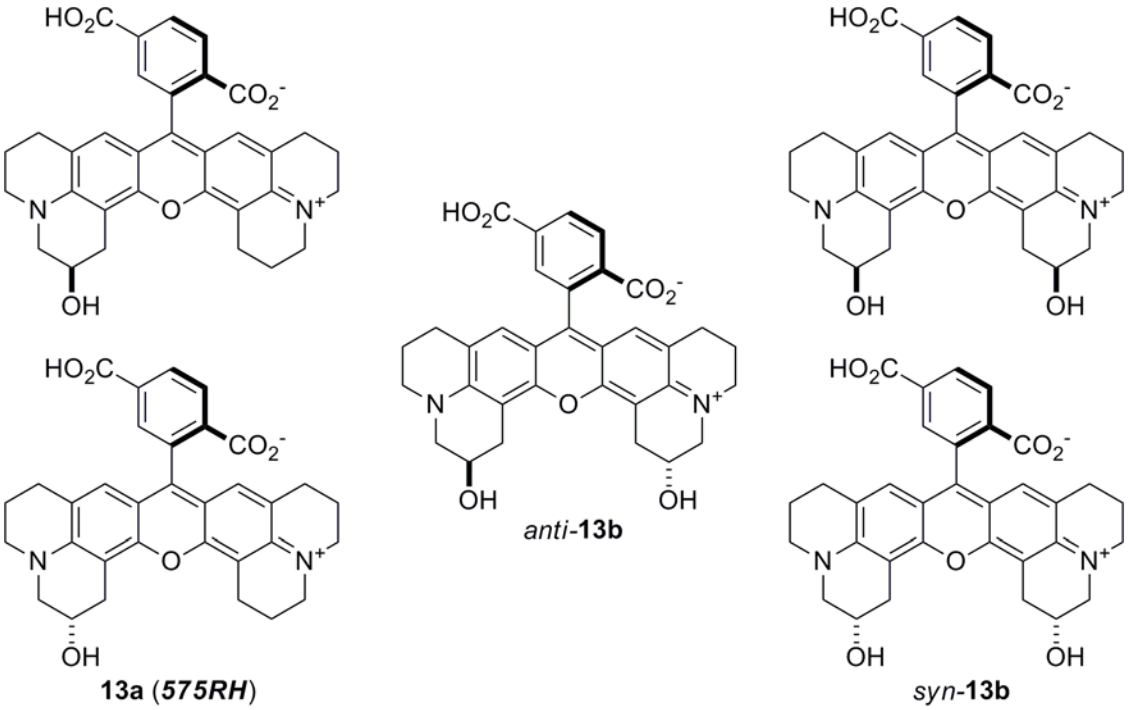
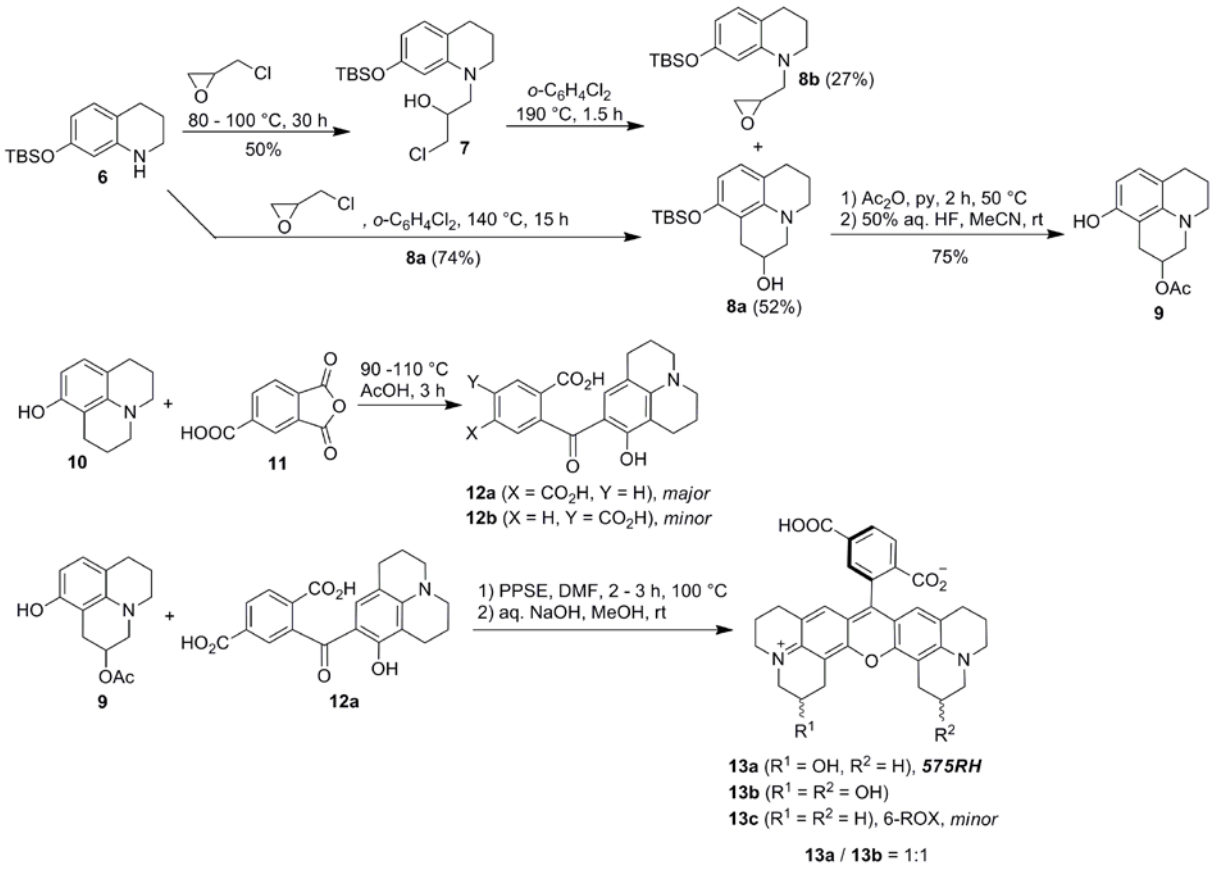


Figure S23. Diastereomeric forms of the dyes **13a** (*575RH, mono*-hydroxylated 6-ROX) and **13b** (dihydroxylated 6-ROX).



7-(*tert*-Butyldimethylsilyloxy)-1,2,3,4-tetrahydroquinoline (6) was prepared according to the published method[6] with modifications.[7]

Compound 7. Compound **6** (90 mg) was dissolved in neat epichlorohydrin (1 mL), and the solution was heated at 80 °C for 30 h. The reaction mixture was applied onto column with SiO_2 (10 g) packed in hexane – dichloromethane mixture (3:1). Elution with hexane – dichloromethane (3:1 → 1:1) afforded 60 mg of compound **7** (oil). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 6.80 (dd, J = 8.1, 0.9 Hz, 1H), 6.16 (d, J = 2.3 Hz, 1H), 6.13 (dd, J = 8.1, 2.3 Hz, 1H), 4.15 (m, 1H), 3.70 (dd, J = 11.2, 4.3 Hz, 1H), 3.62 (dd, J = 11.3, 5.4 Hz, 1H), 3.35 (dd, J = 6.4, 4.0 Hz, 1H), 3.30 (dd, J = 6.4, 4.0 Hz, 1H), 3.30 (td, J = 5.3, 1.3 Hz, 2H), 2.70 (t, J = 6.4 Hz, 2H), 2.49 (d, J = 4.6 Hz, 1H), 1.92 (m, 2H), 0.98 (s, 9H), 0.20 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$): δ = 156.8, 149.0, 129.9, 110.1, 106.8, 105.2, 69.9, 47.7, 47.1, 25.70, 18.2, -4.4. MS (ESI): m/z (positive mode, %) = 356 (100) $[M+H]^+$.

Alcohol 8a and oxirane 8b (*tert*-butyl-dimethyl-[[1-(oxiran-2-ylmethyl)-3,4-dihydro-2*H*-quinolin-7-yl]-oxy]silane. Amine **6** (1.60 g, 6.08 mmol) was dissolved in a mixture of epichlorohydrine (7.28 g, 79.1 mmol, 6.15 mL) and 1,2-dichlorobenzene (15 mL) The mixture was heated at 140°C (oil bath temperature) for 18 h in a thick-walled screw-cup test tube. Then all volatile materials were distilled-off under reduced pressure (0.5–1 mbar, bath temperature 50–100°C) leaving 2.64 g of colorless oil. TLC (hexane - ethyl acetate, 3:1, or dichloromethane - ethyl acetate, 10:1) indicated complete consumption of the starting material (compound **6**) and formation of two new compounds: R_f = 0.26 (**8a**), 0.50 (**8b**). The residual oil was subjected to chromatography over regular silica gel (Isolera, RediSep®Rf, 35 μM, cartridge with 40 g SiO_2, hexane - ethyl acetate, from 95:5 to 70:30). Alcohol **8a** was isolated

as a red-brown viscous oil (1.02 g, 52%); oxirane **8b** (0.52 g, 27%) – as a light yellow viscous oil.

Compound **8a**. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 6.70 (dt, J = 8.1, 0.9 Hz, 1H), 6.16 (d, J = 8.1 Hz, 1H), 4.36 (dddd, J = 14.4, 7.7, 6.8, 4.9 Hz, 1H), 3.16 – 2.99 (m, 3H), 2.90 – 2.61 (m, 4H), 2.37 (d, J = 9.3 Hz, 1H), 2.10 – 1.92 (m, 2H), 0.99 (s, 9H), 0.20/0.21 (2×s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$): δ = 152.7, 143.3, 126.8, 115.1, 110.4, 107.4, 63.3, 55.4, 50.4, 31.50, 27.1, 25.8, 25.6, 22.3, 18.2, -4.1. MS (ESI): *m*/*z* (positive mode, %) = 320.2 (100) $[M+H]^+$. HR-MS ($C_{18}H_{29}NO_2Si$): 320.2048 (found M+H), 320.2040 (calc.); 342.1864 (found for M+Na), 342.1860 (calc.).

Compound **8b**. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 6.77 (ddt, J = 7.8, 1.0, 0.5 Hz, 1H), 6.16 – 6.03 (m, 2H), 3.52 (dd, J = 12 and 3.2 Hz, 1H), 3.39 – 3.25 (m, 3H), 3.15 (dddd, J = 4.6, 4.0, 3.2, 2.7 Hz, 1H), 2.81 – 2.75 (m, 1H), 2.68 (t, J = 6.4 Hz, 2H), 2.58 (dd, J = 5.0, 2.7 Hz, 1H), 2.00 – 1.80 (m, 2H), 0.97 (s, 9H), 0.19 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$): δ = 154.9, 146.1, 129.5, 115.6, 107.7, 103.0, 52.9, 50.4, 50.3, 45.4, 27.3, 25.8, 22.4, 18.2, -4.4. MS (ESI): *m*/*z* (positive mode, %) = 320 (100) $[M+H]^+$. HR-MS ($C_{18}H_{29}NO_2Si$): 320.2041 (found M+H), 320.2040 (calc.).

8a-Acetate. In an argon atmosphere, alcohol **8a** (0.66 g, 2.1 mmol) was dissolved in pyridine (2.22 g, 28.0 mmol, 2.26 mL) in a screw-cup tube containing a stirring bar. Acetic anhydride (1.19 g, 11.6 mmol, 1.10 mL) was added at room temperature, and the reaction mixture was stirred for 18 h. The course of the reaction was monitored by TLC (silica) using dichloromethane – hexane mixture (3:1) with 10% v/v of ethyl acetate: R_f = 0.20 (**8a-acetate**). The reaction mixture was concentrated in vacuo to ca. 3/10 of its initial volume and diluted with ethyl acetate (100 mL). The organic solution was shaken with saturated aq. $KHSO_4$ (20 mL); an aqueous phase was separated and extracted with EtOAc (100 mL). The combined organic solutions were washed with water, saturated aqueous $NaHCO_3$ and brine. The organic phase was dried over Na_2SO_4, and the solvent was evaporated in vacuo. The residue – reddish-brown viscous oil – was subjected to chromatography on regular SiO_2. Elution with dichloromethane – hexane (1:1 to 1:0) gave acetate **8a-acetate** (0.564 g, 75%) as a red-orange viscous oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 6.68 (dt, J = 8.1, 0.9 Hz, 1H), 6.11 (d, J = 8.1 Hz, 1H), 5.26 (m_c, 1H), 3.26 (ddd, J = 11.3, 3.5, 1.7 Hz, 1H), 3.17–2.92 (m, 4H), 2.76–2.62 (m, 3H), 2.07 (s, 3H), 2.02–1.91 (m, 2H), 0.99 (s, 9H), 0.21 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$): δ = 170.8, 152.1, 143.0, 126.8, 114.6, 109.5, 106.8, 66.9, 52.3, 50.1, 27.9, 27.1, 25.8, 22.2, 21.4, 18.3, -4.1. MS (ESI): *m*/*z* (positive mode, %) = 362.2 (100) $[M+H]^+$, HR-MS ($C_{20}H_{31}NO_3Si$): 384.1964 (found for M+Na), 384.1965 (calc. for M+Na).

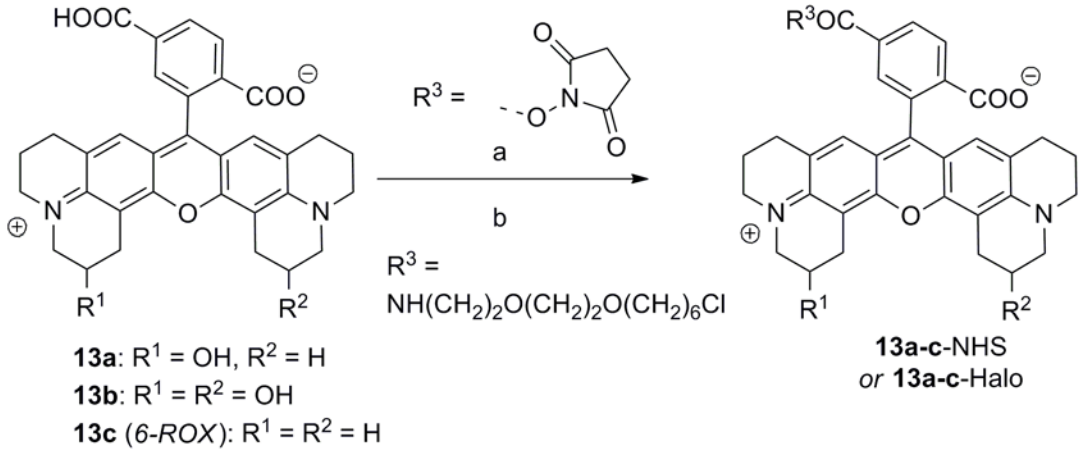
Phenol 9. A solution of **8a-acetate** (0.560 g, 1.55 mmol) in acetonitrile (14 mL) was prepared in a polyethylene screw-cap bottle and cooled with ice. An aqueous HF solution (0.2 mL of 50-55% solution) was added; the ice bath was removed, and the reaction mixture was stirred at room temperature for 20 h. TLC (silica, dichloromethane/ethyl acetate, 10:1) indicated the full conversion of the staring material: R_f = 0.56 (**9**). The reddish reaction solution was diluted with H_2O (50 mL) and extracted four times with ethyl acetate (50 mL and 3 × 25 mL). The combined organic solutions were washed with brine and dried over Na_2SO_4. The solvents were removed under reduced pressure, and the residue (0.46 g of a green viscous semi-solid) was subjected to chromatography (PuriFlash HC, 25 g SiO_2, 15 μm, dichloromethane/ethyl acetate 95: 5 → 80:20). Yield - 0.314 g (82%) of phenol **9** as a white solid. ^{1}H NMR (400

MHz, $CDCl_3$): δ = 7.26 (s, 1H), 6.69 (d, *J* = 8.0, 1H), 6.07 (d, *J* = 8.0 Hz, 1H), 5.26 (tdd, *J* = 6.8, 5.8, 3.5 Hz, 1H), 4.48 (s, 1H), 3.28 (ddd, *J* = 11.4, 3.5, 1.7 Hz, 1H), 3.19–2.98 (m, 4H), 2.78–2.66 (m, 3H), 2.07 (s, 3H), 2.11–1.89 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$): δ = 170.7, 152.0, 142.9, 127.1, 114.3, 105.2, 103.3, 66.6, 52.2, 50.1, 27.1, 22.2, 21.3. MS (ESI): *m*/*z* (positive mode, %) = 248.1 (100) $[M+H]^+$. HR-MS ($C_{14}H_{17}NO_3$): 270.1105 (found for M+Na), 270.1101 (calc.).

Benzophenone 12a. In a screw-cap bottle, 8-hydroxyjulolidine **10** (0.60 g, 3.17 mmol) and trimellitic anhydride **11** (1.83 g, 9.51 mmol) were suspended in acetic acid (15 mL) and flushed with argon. Conc. H_2SO_4 (1 drop) was added, and the reaction mixture was gradually heated in an oil bath (up to 100 °C) and stirred for 2.5 h. The precipitate dissolved at about 80–90 °C, and a red solution formed. After about 0.5 h, the product began to precipitate. TLC control (silica, acetonitrile/water/dichloromethane, 9:1:1): R_f = 0.23 (**12a**). After cooling to room temperature, the purple suspension was transferred into centrifuge test-tubes, sonicated for several seconds in an ultrasonic bath, centrifuged, and the purple supernatant solution was discarded. The precipitate was washed with acetic acid (5 mL); the same sonication – centrifugation cycles were repeated 5 – 6 times, until the supernatant solution became colorless. The solid residue was dried in vacuo to obtain 0.48 g (40%) of benzophenone **12a** with traces of **12b** (>95:<5). Beige solid; TLC: ACN/H_2O/DCM, 9:1:1. HPLC (*Eurospher-100 C18* 5 µm 250×4 mm, 1.2 mL/min; solvent A: water + 0.1% v/v trifluoroacetic acid (TFA); solvent B: MeCN + 0.1% v/v TFA). A/B: 30/70 – 100/0, 25 min, t_R = 9.7 min (**12a**), 10.7 min (**12b**). ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.82 (s, 1H), 8.09 (dd, *J* = 8.1, 1.7 Hz, 1H), 8.02 (d, *J* = 8.1 Hz, 1H), 7.76 (d, *J* = 1.7 Hz, 1H), 6.39 (s, 1H), 3.24 (td, *J* = 7.4, 4.8 Hz, 4H), 2.58 (t, *J* = 6.4 Hz, 2H), 2.40 (t, *J* = 6.2 Hz, 2H), 1.91–1.78 (m, 2H), 1.80–1.69 (m, 2H). $C_{21}H_{19}NO_6$ (381.12124). MS (ESI): *m*/*z* (negative mode, %) = 380 (100) $[M-H]^-$.

Dyes 13a (*575RH*), 13b and 13c (*6-ROX*). Benzophenone **12a** (23 mg, 0.060 mmol) and acetate **9** (15 mg, 0.061 mmol) were dissolved in 2.3 mL DMF, trimethylsilyl polyphosphate (PPSE; 170 mg) in DMF (0.4 mL) was added, and the reaction mixture was heated under argon for 3 h at 100 °C (or 2 h at 120 °C). TLC control (silica, dichloromethane/methanol/water, 60:30:2): R_f = 0.60 (**13c**), 0.33 (**13b**), 0.18 (**13a**). After cooling down to room temperature, the reaction mixture was diluted with 40 mL of the half-saturated brine and transferred into a separation funnel with CH_2Cl_2 (40 mL) and 5 mL acetonitrile. After shaking and separation of the organic layer, the aqueous phase was extracted with CH_2Cl_2 – acetonitrile mixture (40 mL + 5 mL) and with CH_2Cl_2 (20 mL). Combined organic solutions were washed with water (20 mL), and the aqueous extract was washed with CH_2Cl_2 (40 mL). HPLC analysis of the reaction mixture (the sample was hydrolyzed with aq. NaOH and then acidified): column - *Eurospher-100 C18* 5 µm, 250×4 mm, 1.2 mL/min; solvent A: water + 0.1% v/v trifluoroacetic acid (TFA); solvent B: MeCN + 0.1% v/v TFA A/B = 70/30 – 0/100 in 20 min, detection at 580 nm. Retention times (t_R), min (peak areas, %, compounds): 10.2 (30, **13b**), 11 (46, **13a**), 12.3 (24, **13c**). Combined organic solutions were evaporated in vacuo, and the residue was applied onto a column with regular SiO_2 (55 g). Elution with acetonitrile – water mixture (10:1 → 5:1 + 5% v/v CH_2Cl_2) afforded magenta-colored fractions: first containing the compound **13c**, followed by the inseparable mixture of mono- and diacetates of dyes **13a** and **13b** (displaying a single spot on TLC). These fractions were combined, evaporated in vacuo, and the residue was dissolved in 10 mL

of 0.1 M aq. NaOH. After keeping overnight at room temperature, the reaction mixture was acidified with TFA (0.2 mL). Then the reaction mixture saturated with NH_4Cl, and extracted twice with CH_2Cl_2 – acetonitrile mixtures (5:1 and 5:2, 60 mL and 70 mL, respectively). Combined organic solutions were evaporated in vacuo, and the residue was subjected to chromatography on regular SiO_2 using the solvent system CH_2Cl_2 – MeOH – H_2O (75/30/2). Dyes **13a** and **13b** were isolated in approximately equal amounts and additionally purified by RP chromatography on RP-C_{18} (Macherey Nagel, Polygoprep 60-50) using acetonitrile – water mixture (1/5 → 1/1; +0.2% v/v HCOOH). The homogeneous fractions were pooled, evaporated and afforded **13a**, **13b** and **13c** (in ratio ca. 3:2:1; HPLC conditions as above). ^{1}H NMR (600 MHz, CD_3CN + D_2O, **13a**, isomer 1): δ = 8.20 (s, 2H), 7.76 (s, 1H), 6.59 / 6.57 (s×2, 2H), 4.32 (m, 1H), 3.56 (m, 1H), 3.42 (t, J = 5.7 Hz, 2H), 3.37 (dt, J = 13.6 and 5.9 Hz, 3H), 3.30 (dd, J = 13.7, 4.9 Hz, 1H), 3.05 (m, 3H), 2.95 (q, J = 6.1 Hz, 2H), 2.55 (q, J = 6.7 Hz, 4H), 1.97 (m, 2H), 1.82 (m, 4H). ^{1}H NMR (600 MHz, CD_3CN + D_2O, **13a**, isomer 2): δ = 8.24 (s, 2H), 7.78 (s, 1H), 6.59 / 6.57 (s×2, 2H), 4.33 (p, J = 4.6 Hz, 1H), 3.56 (m, 1H), 3.48 – 3.36 (m, 6H), 3.33 (m, 1H), 3.07 (dd, J = 5.8, 2.2 Hz, 2H), 2.96 (m, 2H), 2.56 (q, J = 6.1 Hz, 4H), 1.99 (h, J = 5.9 Hz, 2H), 1.91 – 1.77 (m, 4H). ^{1}H NMR (400 MHz, CD_3OD, compound **13b**; main diastereomer): δ = 8.16 (dd, J = 8.1 and 1.7 Hz, 1H), 8.04 (d, J = 8.0 Hz, 1H), 7.73 (m, 1H), 6.85 (s, 2H), 4.36 (m, 2H, C<u>H</u>OH), 3.65 (br. d, J = 13.5 Hz, 2H), 3.48 (m, J = 5.6 Hz, 4H), 3.38 (dd, J = 13.3 and 5.2 Hz, 2H), 3.18 (m, 4 H), 2.71 (m, 4H), 1.96 (m, 4H) ppm. The following spectral data mere obtained for aqueous solutions containing PBS buffer (pH 7.4). **13a**: absorption λ_{max}, nm (ε, M^{-1} cm^{-1}) = 574 (55000), emission λ_{max}, nm (Φ_{fl} – fluorescence quantum yield) = 597 (0.74 – absolute value; excitation at 540–550 nm). **13b**: absorption λ_{max}, nm (ε, M^{-1} cm^{-1}) = 564 (60000), emission λ_{max}, nm (Φ_{fl} –fluorescence quantum yield) = 588 (0.63 – absolute value; excitation at 550 nm). **13a** ($C_{33}H_{30}N_2O_6$, M = 550). HR-MS (ESI): *m*/*z* (positive mode) = 551.2181 (found $[M+H]^+$), 551.2177 (calculated). MS (ESI): *m*/*z* (negative mode, %) = 549 (100) $[M-H]^-$. **13b** ($C_{33}H_{30}N_2O_7$, M = 566). HR-MS (ESI): *m*/*z* (negative mode) = 565.1976 (found $[M–H]^-$), 565.1980 (calculated).



Scheme S24. Derivatives of rhodamine dyes **13a** (*575RH*), **13b** and **13c** (*6-ROX*) used as labeling reagents: a) *N*,*N*′-disuccinimidyl carbonate (DSC), Et_3N, DMF, CH_2Cl_2; b) DSC, Et_3N, DMF, CH_2Cl_2, then $Cl(CH_2)_6O(CH_2)_2O(CH_2)_2NH_2$

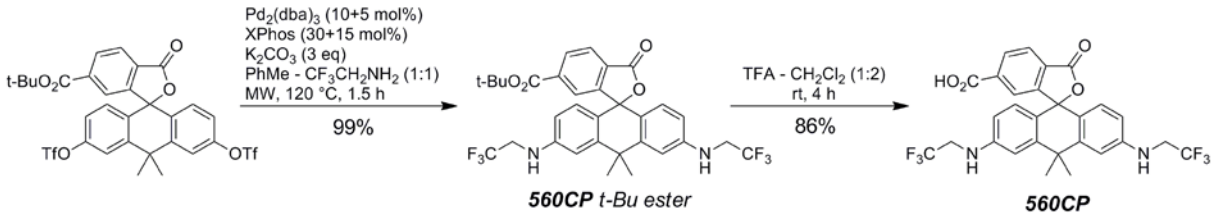
General method for the preparation of *mono*-NHS esters 13a-c-NHS (Scheme S24)**.** A dye (**13a**, **13b** or **13c**) in amount of ca. 3.5 mg (ca. 6.3 μM) was dissolved in the mixture of

DMF (1.5 mL) and CH_2Cl_2 (0.5 mL) and treated with Et_3N (portions of 2.5 μL) and disuccinimidyl carbonate (DSC, portions of 5 mg). After addition of each portion, the course of the reaction is monitored by HPLC [column - *Eurospher-100 C18* 5 μm, 250×4 mm, 1.2 mL/min; solvent A: water + 0.1% v/v trifluoroacetic acid (TFA); solvent B: acetonitrile + 0.1% v/v TFA A/B = 70/30 – 0/100 in 20 min, detection at 580 nm] and/or TLC (regular SiO_2, acetonitrile/H_2O, 5:1). The reaction was usually complete, when ca. 20 mg of *N*,*N'*-disuccinimidyl carbonate (DSC) and 15 μL of Et_3N were added. The spot of the double NHS ester was also detected (with highest R_f; blue color), and (sometimes) the spot of the mono-NHS ester of the sterically more hindered carboxylic acid group. The latter had the lowest R_f-value (and also gave the bluish spot, in contrary to the magenta-colored (main) spots of **13a-c-NHS**). The reaction mixture was diluted with the equal volume of dry acetonitrile and applied onto a column with regular SiO_2 (10 g). Elution with acetonitrile/CH_2Cl_2/H_2O (10:1:1) followed by acetonitrile/H_2O (5:1) afforded the required *mono*-NHS esters **13a-c**-NHS as magenta-colored solutions. They were concentrated, filtered through the Rotilabo membrane filters (attached to syringes; in order to remove SiO_2) and lyophilized. **13a**-NHS ($C_{37}H_{33}N_3O_8$, M = 647). MS (ESI): *m*/*z* (positive mode, %) = 648 (100) $[M+H]^+$; **13b**-NHS ($C_{37}H_{33}N_3O_9$, M = 663.2217). HR-MS (ESI): *m*/*z* (positive mode, %) = 664.2252 (found $[M+H]^+$), 664.2290 (calculated).

General method for obtaining *mono*-amides 13a-c-Halo. *Mono*-NHS esters **13a-c-NHS** in amounts of ca. 1 mg (ca. 2 μM) were dissolved in ca. 200 μL of the solution of $Cl(CH_2)_6O(CH_2)_2O(CH_2)_2NH_2$ (HaloTag(O2) amine) in DMF (20 mg/mL), and Et_3N (10 μL) was added with stirring. The course of the reaction was monitored by TLC. When the reaction was complete (ca. 2 h), the reaction mixture was diluted with CH_2Cl_2 – acetonitrile mixture (1:3) and extracted with equal volume of the saturated aqueous $NaHCO_3$ solution (lower phase). The upper organic layer was separated and extracted with half-saturated aqueous NH_4Cl acidified with trifluoroacetic acid (TFA; in order to remove excess of the amine). The organic solution was evaporated in vacuo, and the residue applied onto a column with regular SiO_2 (5 g). Elution with CH_2Cl_2 – methanol (10:1 → 8:1) afforded the title amides which were additionally purified by chromatography on regular SiO_2 (5 g) using MeCN – H_2O mixture (10:1 → 5:1). Pure fractions were pooled, concentrated, filtered through the Rotilabo syringe filters (0.22 μm), and lyophilized. **13a-Halo** (*575RH*-Halo, $C_{43}H_{50}N_3O_7Cl$): HR-MS (ESI): *m*/*z* (positive mode, %) = 756.3384 (found $[M+H]^+$), 756.3410 (calculated). HPLC [column - *Kinetex* 5 μm C18 100, 250×4 mm, 1.2 mL/min; solvent A: 0.05 M $Et_3N{\cdot}H_2CO_3$ buffer (TEAB); solvent B: MeCN, A/B = 70/30 – 0/100 in 20 min, diode-array detection at 600 nm]; t_R = 9.7 and 10.5 min (in ratio 1:1) – 2 diastereomers differing in the mutual position of OH and COOH groups; see Figure S17. The following spectral data were obtained for aqueous solutions containing PBS buffer (pH 7.4). Absorption λ_{max}, nm (ε, M^{-1} cm^{-1}) = 578 (55000), emission λ_{max}, nm (Φ_{fl}) = 601 (0.74 - relative value obtained with Rhodamine 101 as a reference dye with Φ_{fl} = 0.96 in ethanol; excitation at 540 nm). **13b-Halo** ($C_{43}H_{50}N_3O_8Cl$): HR-MS (ESI): *m*/*z* (positive mode, %) = 772.3329 (found $[M+H]^+$), 772.3359 (calculated). HPLC [column - *Kinetex* 5 μm C18 100, 250×4 mm, 1.2 mL/min; solvent A: 0.05 M $Et_3N{\cdot}H_2CO_3$ buffer (TEAB); solvent B: MeCN, A/B = 70/30 – 0/100 in 20 min, diode array detection in the range 200–700 nm]; t_R = 8.0 and 8.0 min (in ratio 1:1) – 2 diastereomers differing in the mutual position of OH and COOH groups; see Figure S17. The following

spectral data were obtained for aqueous solutions containing PBS buffer (pH 7.4). Absorption λ_{max}, nm (ε, M^{-1} cm^{-1}) = 575 (63000), emission λ_{max}, nm (Φ_{fl}) = 598 (0.55 - relative value obtained with Rhodamine 101 as a reference dye with Φ_{fl} = 0.96 in ethanol; excitation at 540 nm). **13c-Halo** (*6-ROX*-Halo). The following spectral data were obtained for aqueous solutions containing PBS buffer (pH 7.4). Absorption λ_{max}, nm (ε, M^{-1} cm^{-1}) = 582 (55000), emission λ_{max}, nm (Φ_{fl}) = 605 (0.64 - relative value obtained with Rhodamine 101 as a reference dye with Φ_{fl} = 0.96 in ethanol; excitation at 540 nm).

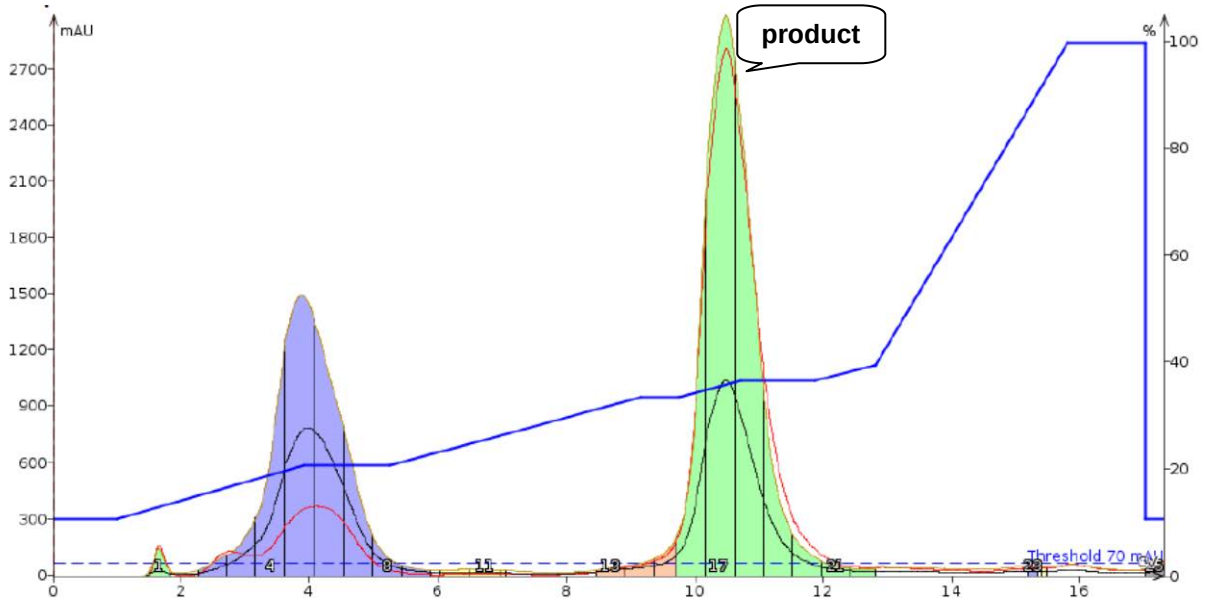
Dyes *560CP* and *570CPH*



The starting material, 6'-carboxycarbofluorescein *tert*-butyl ester ditriflate, was prepared according to the published procedure.[1]

Dye *560CP*. A mixture of 6'-carboxycarbofluorescein *tert*-butyl ester ditriflate (191 mg, 0.26 mmol), $Pd_2(dba)_3$ (24 mg, 0.026 mmol, 10 mol%), XPhos (37 mg, 0.078 mmol, 30 mol%) and K_2CO_3 (108 mg, 0.78 mmol, 3 eq) in toluene (1.5 mL) was sealed in a 5 mL microwave vial, degassed on a Schlenk line, and 2,2,2-trifluoroethylamine (1.5 mL) was injected. The mixture was irradiated in a microwave reactor at 120 °C (150 W) for 1 h and was checked by TLC (silica/30% ethyl acetate-hexane, stained by heating with 1M NaOH) upon cooling, showing incomplete conversion: R_f(ditriflate) = 0.54 (pink), R_f(monotriflate) = 0.37 (pink-purple), R_f(product) = 0.22 (purple). Another portion of $Pd_2(dba)_3$ (12 mg, 0.013 mmol, 5 mol%) and XPhos (19 mg, 0.039 mmol, 15 mol%) was added, the headspace was flushed with argon, the vial was resealed, and the heating was continued at 120 °C (150 W) for 30 min. TLC check showed disappearance of both the starting ditriflate and the monotriflate intermediate. The mixture was diluted with brine (30 mL) and extracted with ethyl acetate (3×30 mL), the combined organic layers were dried over Na_2SO_4. The filtrate was evaporated on Celite and the product was isolated by flash column chromatography on silica (RediSep Rf 24 g; gradient 10% to 60% ethyl acetate – hexane) as viscous yellowish oil, which was freeze-dried from dioxane to yield 154 mg (99%) of ***560CP t*-butyl ester** as yellowish fluffy solid.

Flash LC trace:

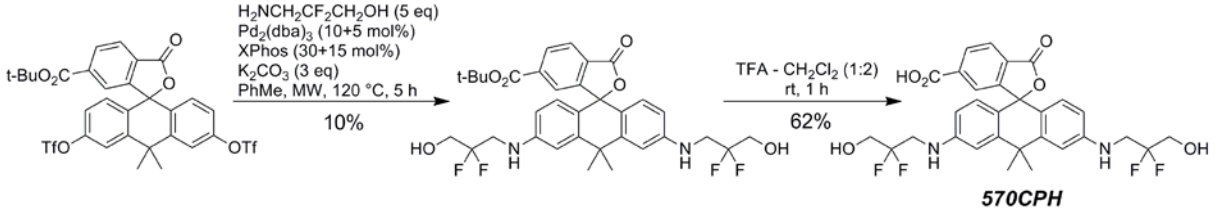


^{1}H NMR (400 MHz, $CDCl_3$): δ 8.16 (dd, *J* = 8.0, 1.3 Hz, 1H), 8.02 (dd, *J* = 8.0, 0.7 Hz, 1H), 7.61 (dd, *J* = 1.3, 0.8 Hz, 1H), 6.88 (d, *J* = 2.5 Hz, 2H), 6.56 (d, *J* = 8.6 Hz, 2H), 6.45 (dd, *J* =

8.6, 2.5 Hz, 2H), 4.23 (td, *J* = 7.0, 2.5 Hz, 2H), 3.77 (qd, *J* = 8.9, 6.9 Hz, 4H), 1.80 (s, 3H), 1.70 (s, 3H), 1.54 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 170.0, 164.4, 155.3, 147.0, 146.9, 138.1, 130.3, 129.9, 129.3, 125.0 (q, $^1J_{C–F}$ = 280.1 Hz), 124.96, 124.95, 121.6, 112.4, 110.2, 87.7, 82.5, 45.9 (q, $^2J_{C–F}$ = 33.8 Hz), 38.4, 35.1, 32.9, 28.2. ^{19}F NMR (376 MHz, $CDCl_3$): δ 72.2. ESI-MS, positive mode: m/z (rel. int., %) = 621.2 $[M+H]^+$. HR-MS (ESI, positive mode): 621.2183 $[M+H]^+$ (found), 621.2183 (calculated for $C_{32}H_{31}N_2O_4F_6$, $[M+H]^+$).

The intermediate ***560CP t*-butyl ester** (75 mg, 0.126 mmol) was dissolved in CH_2Cl_2 (2 mL), the solution was cooled in ice-water bath, and trifluoroacetic acid (1 mL) was added quickly dropwise. An intense fuchsine-red color appeared immediately. The solution was stirred at rt for 4 h, checking the completion of the reaction by TLC (silica/50% ethyl acetate-hexane: R_f(starting material) = 0.38, R_f(product) = 0. The reaction mixture was evaporated and chased twice with toluene to get rid of excess trifluoroacetic acid, and the product was isolated by reversed-phase chromatography (10 g RP-C_{18}, gradient 70% to 40% H_2O – acetonitrile). Fractions containing the product were evaporated, the residue was dissolved in dioxane (12 mL), centrifuged, microfiltered through a 0.2 μm PTFE syringe filter and lyophilized giving the dye ***560CP*** as purple fluffy solid, yield 61 mg (86%). ^{1}H NMR (400 MHz, DMSO-d_6): δ 13.56 (br.s, 1H), 8.16 (dd, *J* = 7.9, 1.3 Hz, 1H), 8.07 (d, *J* = 8.0 Hz, 1H), 7.44 (d, *J* = 1.3 Hz, 1H), 7.04 (d, *J* = 2.4 Hz, 2H), 6.57 (dd, *J* = 8.7, 2.4 Hz, 2H), 6.46 (t, *J* = 6.9 Hz, 2H), 6.41 (d, *J* = 8.6 Hz, 2H), 4.07 – 3.94 (m, 4H), 1.76 (s, 3H), 1.66 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6): δ 169.0, 166.1, 155.3, 148.1, 146.2, 137.2, 130.2, 129.1, 128.3, 125.8 (q, $^1J_{C–F}$ = 281.3 Hz), 125.1, 123.9, 118.9, 112.2, 109.3, 87.3, 43.7 (q, $^2J_{C–F}$ = 33.1 Hz), 37.7, 34.35, 33.2. ^{19}F NMR (376 MHz, DMSO-d_6): δ -70.5. ESI-MS, positive mode: m/z (rel. int., %) = 565.2 $[M+H]^+$. HR-MS (ESI, positive mode): 565.1555 $[M+H]^+$ (found), 565.1557 (calculated for $C_{28}H_{23}N_2O_4F_6$, $[M+H]^+$).

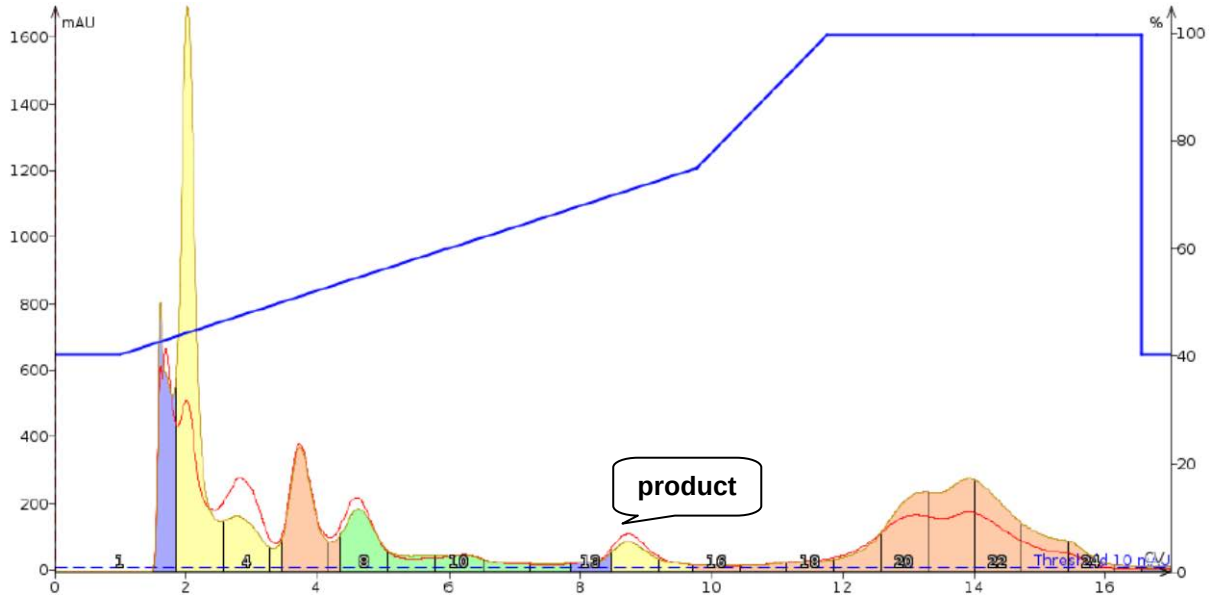
For antibody labeling, the dye was converted into NHS ester as follows: TSTU (*N*,*N*,*N*′,*N*′-tetramethyl-*O*-(*N*-succinimidyl)uronium tetrafluoroborate; 20 μL of 20 mg/100 μL DMF stock solution, 13.31 μmol, 1.5 eq) was added to a solution of ***560CP*** (5 mg, 8.87 μmol) and DIEA (*N*-ethyldiisopropylamine; 11 μL) in DMF (200 μL). The yellow reaction mixture was stirred at rt for 30 min, the solvents were evaporated *in vacuo* at rt, and the product ***560CP*-NHS** was isolated by preparative TLC (silica, 50% ethyl acetate – hexane) as white fluffy solid, yield 5.5 mg (94%), purity (HPLC) ~90%. HPLC (20/80−100/0 over 15 min, column 4×150 mm, 1.2 mL/min, detection at 254 nm): t_R = 10.4 min. ESI-MS, positive mode: *m/z* (rel. int., %) = 662.2 $[M+H]^+$. HR-MS (ESI, positive mode): 662.1715 (found), 662.1720 (calculated for $C_{32}H_{26}N_3O_6F_6$, $[M+H]^+$).



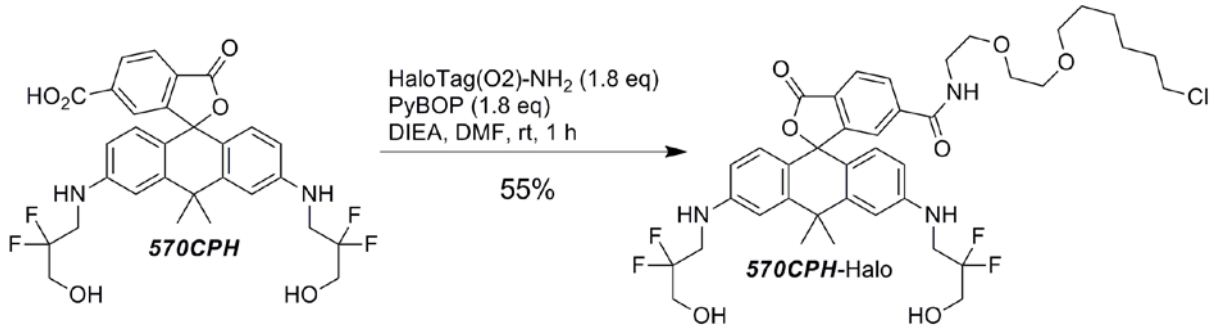
Dye *570CPH*. A mixture of 6'-carboxycarbofluorescein *tert*-butyl ester ditriflate (73 mg, 0.1 mmol), 3-amino-2,2-difluoro-1-propanol (34 mg, 0.3 mmol, 3 eq), $Pd_2(dba)_3$ (9 mg, 0.01 mmol, 10 mol%), XPhos (14 mg, 0.03 mmol, 30 mol%) and K_2CO_3 (41 mg, 0.3 mmol, 3 eq)

in toluene (0.8 mL) was sealed in a 2 mL microwave vial, degassed on a Schlenk line, and irradiated in a microwave reactor at 120 °C for 1 h. TLC control (silica/30% ethyl acetate-hexane, stained by heating with 1M HCl) showed incomplete conversion: R_f(ditriflate) = 0.80 (colorless), R_f(product) = 0.57 (purple), with multiple spots above and below the product. Another portion of $Pd_2(dba)_3$ (4.5 mg, 0.005 mmol, 5 mol%), XPhos (7 mg, 0.015 mmol, 15 mol%) and 3-amino-2,2-difluoro-1-propanol (23 mg, 0.2 mmol, 2 eq) was added, the headspace was flushed with argon, the vial was resealed, and the heating was continued at 120 °C for 4 h. The mixture was diluted with brine (10 mL) and sat. aq. $NaHCO_3$ (10 mL), extracted with ethyl acetate (3×20 mL), and the combined organic layers were dried over Na_2SO_4. The filtrate was evaporated on Celite and the product was isolated by flash column chromatography on silica (Interchim Puriflash 15 µm 12 g; gradient 40% to 100% ethyl acetate – hexane) as violet solid (6.5 mg, 10%), which was used in the next step without further characterization. ESI-MS, positive mode: m/z (rel. int., %) = 645.3 $[M+H]^+$. HR-MS (ESI, positive mode): 645.2576 $[M+H]^+$ (found), 645.2582 (calculated for $C_{34}H_{37}N_2O_6F_4$, $[M+H]^+$).

Flash LC trace:

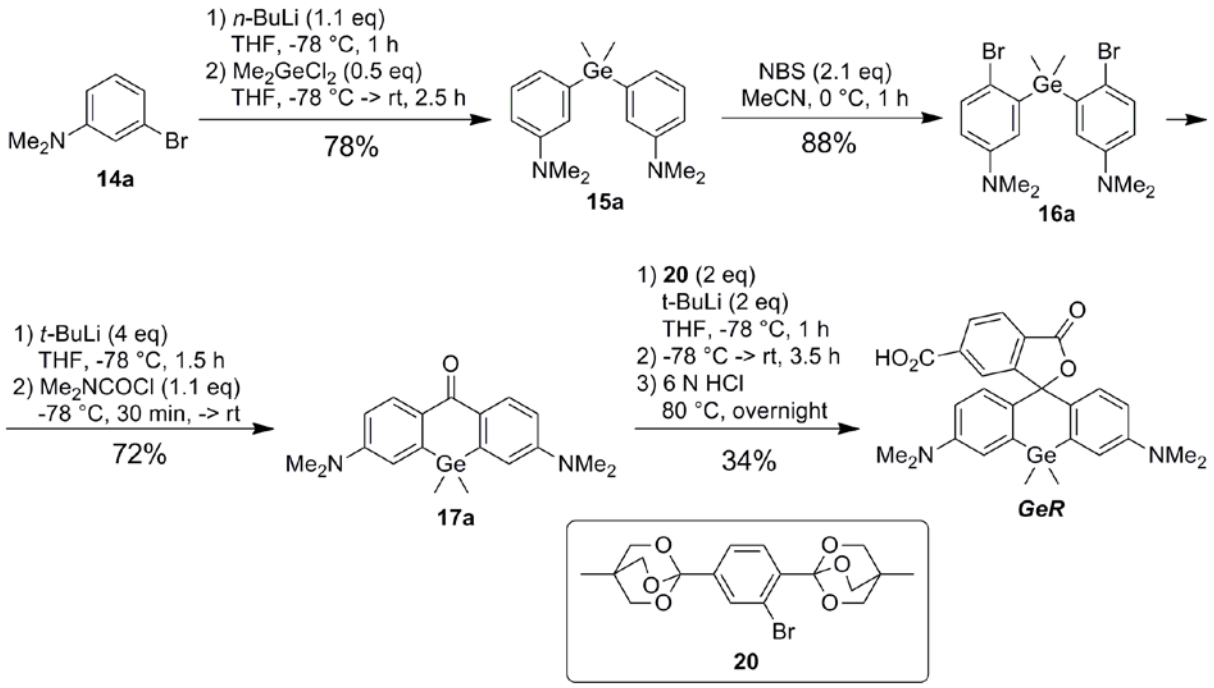


The entire amount of the intermediate was dissolved in CH_2Cl_2 (1 mL), trifluoroacetic acid (0.5 mL) was added quickly dropwise, and the reaction mixture was stirred at rt for 1 h (TLC control). The reaction mixture was evaporated and chased twice with toluene to get rid of excess trifluoroacetic acid, the product was isolated by preparative HPLC (gradient gradient 70% to 30% A:B, A – 0.1% v/v trifluoroacetic acid in H_2O, B – acetonitrile) and lyophilized from aqueous dioxane to give 3.7 mg (62%) of ***570CPH*** (trifluoroacetate salt) as dark violet fluffy solid. ^{1}H NMR (400 MHz, CD_3CN): δ 8.23 (dd, *J* = 8.2, 1.4 Hz, 1H), 8.15 (d, *J* = 8.2 Hz, 1H), 7.70 (s, 1H), 7.13 (d, *J* = 2.3 Hz, 2H), 6.74 (d, *J* = 8.8 Hz, 1H), 6.61 (dd, *J* = 8.8, 2.4 Hz, 2H), 3.78 (t, *J* = 14.7 Hz, 4H), 3.74 (t, *J* = 13.1 Hz, 4H), 1.80 (s, 3H), 1.68 (s, 3H). ^{13}C NMR (101 MHz, CD_3CN): δ 133.6 (+), 131.0 (+), 128.7 (+), 113.8 (+), 111.3 (+), 67.3 (–), 61.8 (–), 45.0 (–), 34.6 (+), 32.2 (+) (indirect detection from a gHSQC experiment, only H-coupled carbons are detected). ^{19}F NMR (376 MHz, CD_3CN): δ -76.6, -113.1. ESI-MS, positive mode: m/z (rel. int., %) = 589.2 $[M+H]^+$. HR-MS (ESI, positive mode): 589.1954 $[M+H]^+$ (found), 589.1956 (calculated for $C_{30}H_{29}N_2O_6F_4$, $[M+H]^+$).



Compound *570CPH*-Halo. PyBOP (28 µL of 20 mg/100 µL stock solution in DMF, 10.69 µmol, 1.8 equiv) was added to a solution of ***570CPH*** (3 mg, 5.68 µmol), HaloTag Amine (O2) (2-(2-((6-chlorohexyl)oxy)ethoxy)ethanamine; 2.4 mg, 10.69 µmol, 1.8 equiv) and DIEA (*N*-ethyldiisopropylamine; 10 µL) in DMF (200 µL). After 1 h, the solvents were evaporated *in vacuo* at rt, and the product ***570CPH*-Halo** was isolated by preparative TLC (silica, 100% ethyl acetate). Yield 2.6 mg (55%), purity (HPLC) >98%. HPLC (30/70−100/0 over 15 min, column 4×150 mm, 1.2 mL/min, detection at 254 nm): t_R = 8.4 min. ESI-MS, positive mode: *m/z* (rel. int., %) = 794.3 $[M+H]^+$. HR-MS (ESI, positive mode): 794.3208 (found), 794.3190 (calculated for $C_{40}H_{49}ClF_4N_3O_7$, $[M+H]^+$).

Germanorhodamine dye *GeR*



Compound 15a. A solution of 3-bromo-*N*,*N*-dimethylaniline **14a** (2.32 g, 11.6 mmol, 2 eq) in anhydrous THF (40 mL) was degassed on a Schlenk line and cooled to -78 °C under argon. *n*-Butyllithium (5.1 mL of 2.5 M solution in hexanes, 12.8 mmol, 2.2 eq) was injected with a syringe quickly dropwise, and the mixture was stirred at -78 °C for 1 h. Dimethylgermanium dichloride (1.01 g, 5.8 mmol), dissolved in anhydrous THF (3 mL), was injected dropwise with a syringe. The mixture was allowed to warm up to rt and stirred for 2.5 h. Brine (50 mL) was then added, the mixture was extracted with ethyl acetate (3 × 40 mL), and the combined organic layers were dried over Na_2SO_4. TLC control (SiO_2 / 10% ethyl acetate in hexane): R_f (product) = 0.37. The product **15a** was isolated by flash column chromatography (Biotage SNAP Ultra 50 g, gradient 1% to 10% ethyl acetate – hexane) as colorless oil, yield 1.56 g (78%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.27 (ddd, *J* = 8.3, 7.1, 0.5 Hz, 2H), 6.93 (br.d, *J* = 2.9 Hz, 2H), 6.90 (dt, *J* = 7.1, 1.0 Hz, 2H), 2.96 (s, 8H), 0.66 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 150.3, 141.1, 128.7, 122.2, 117.9, 113.1, 40.8, -2.7. ESI-MS, positive mode: *m/z* (rel. int., %) = 345.2 (100) $[M+H]^+$. HR-MS (ESI, positive mode): 345.1387 $[M+H]^+$ (found), 345.1384 (calculated for $C_{18}H_{27}N_2Ge$, $[M+H]^+$).

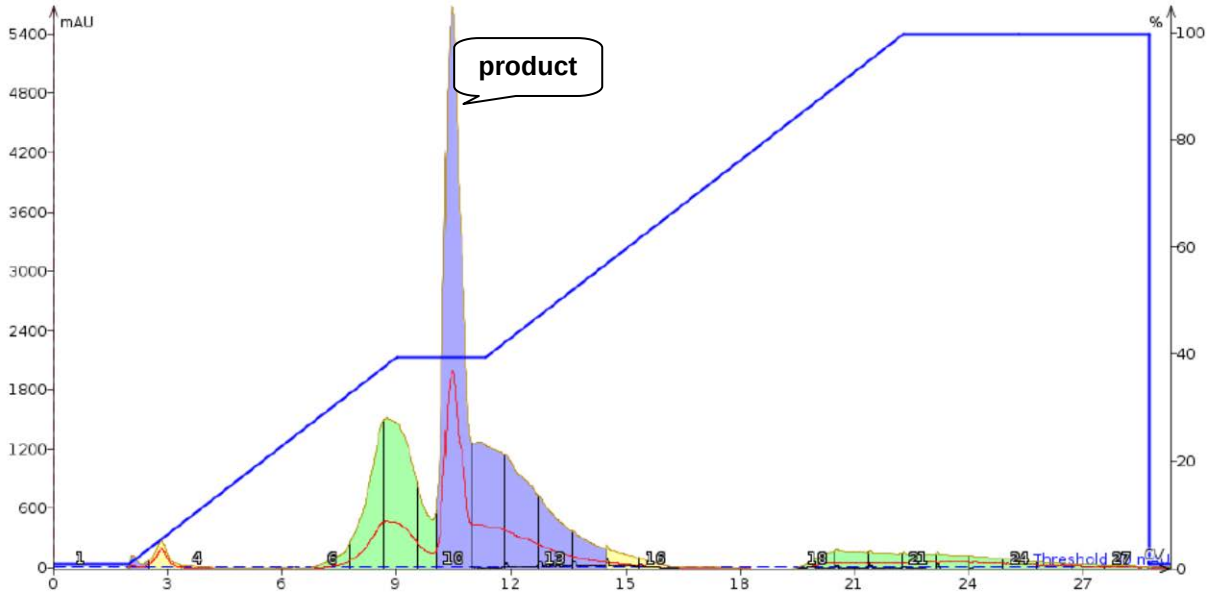
Compound 16a. *N*-Bromosuccinimide (NBS; 1.69 g, 9.51 mmol, 2.1 eq) was added portionwise to a solution of **15a** (2.61 g, 4.34 mmol) in acetonitrile (25 mL), cooled in ice-water bath. Yellow color appeared first, followed by precipitation. The addition of NBS was terminated when yellow color of the suspension abruptly turned brownish, and the mixture was left stirring in ice-water bath for 1 h. TLC control (SiO_2 / CH_2Cl_2): R_f (product) = 0.65, R_f (starting material) = 0.55. Sat. aqueous $NaHCO_3$ (40 mL) was added, the mixture was extracted with CH_2Cl_2 (3 × 40 mL), the combined extracts were washed with brine and dried over Na_2SO_4. The product **16a** was isolated by flash column chromatography (Biotage SNAP Ultra 50 g, gradient 10% to 100% CH_2Cl_2 – hexane) as yellowish solid, yield 2.00 g (88%).

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.36 (d, *J* = 8.7 Hz, 2H), 6.75 (d, *J* = 3.2 Hz, 2H), 6.58 (dd, *J* = 8.8, 3.2 Hz, 2H), 2.87 (s, 12H), 0.87 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 149.1, 141.5, 132.8, 120.9, 116.8, 115.0, 40.7, -0.1. ESI-MS, positive mode: *m/z* (rel. int., %) = 501.0 (100) $[M+H]^+$. HR-MS (ESI, positive mode): 500.9590 $[M+H]^+$ (found), 500.9581 (calculated for $C_{18}H_{25}N_2GeBr_2$, $[M+H]^+$).

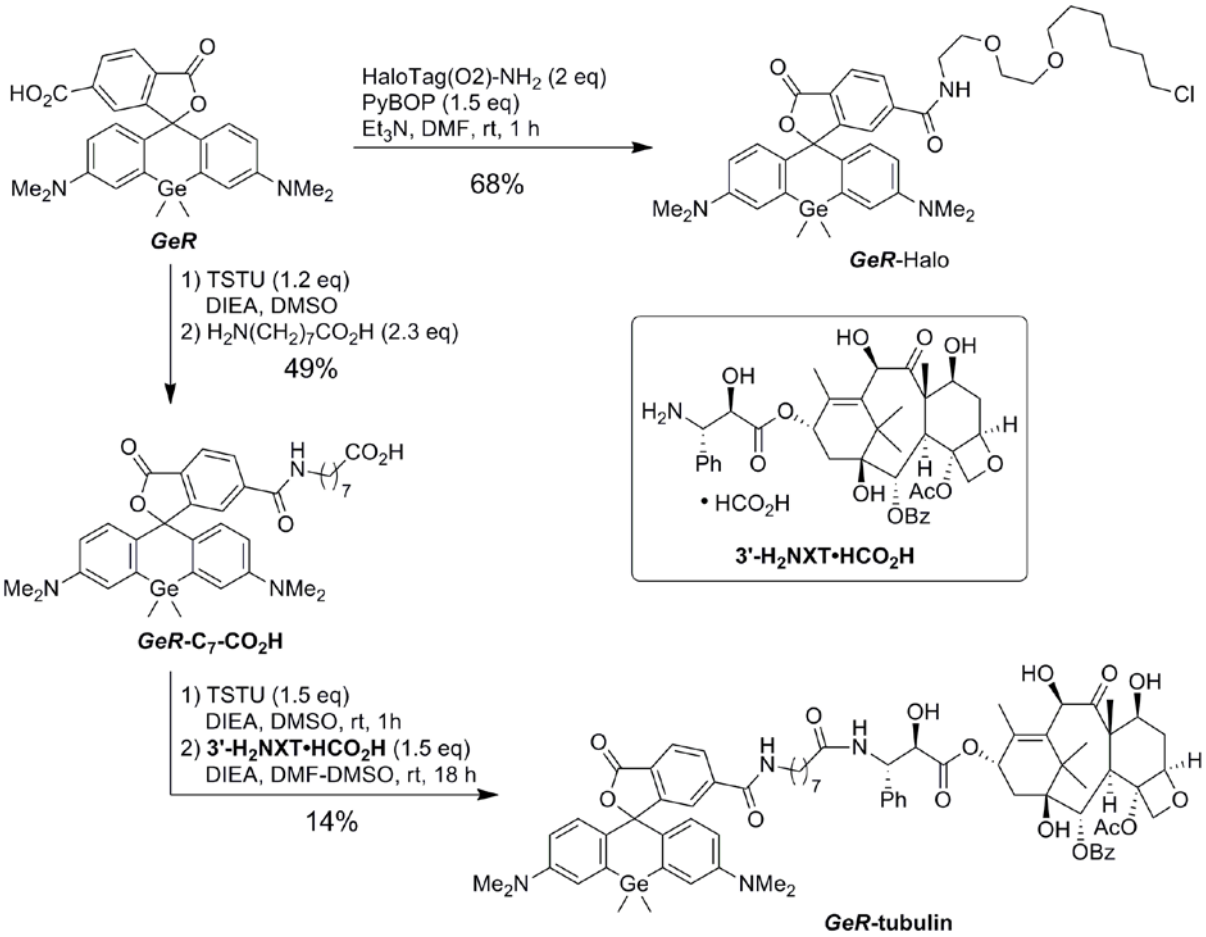
Compound 17a. A solution of **16a** (500 mg, 0.994 mmol) in anhydrous THF (20 mL) was degassed on a Schlenk line and cooled to -78 °C under argon. *tert*-Butyllithium (2.4 mL of 1.7 M solution in pentane, 3.98 mmol, 4 eq) was added dropwise, and the resulting bright yellow solution was stirred at -78 °C for 1.5 h. Dimethylcarbamyl chloride (100 µL, 1.09 mmol) was then injected dropwise with a Hamilton syringe. The mixture was stirred at -78 °C for 30 min, allowed to warm up to rt and quenched with sat. aq. NH_4Cl (10 mL). TLC control (SiO_2 / ethyl acetate): R_f (product) = 0.50 (bright yellow), R_f (impurity) = 0.71 (colorless). The mixture was extracted with ethyl acetate (3 × 20 mL) and the combined organic layers were dried over Na_2SO_4 The product **17a** was isolated by flash column chromatography (Sepacore Silica HP 12 g; gradient 10% to 100% ethyl acetate – hexane) as bright yellow solid, quickly turning greenish, yield 264 g (72%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.42 (d, *J* = 9.0 Hz, 2H), 6.80 (dd, *J* = 9.1, 2.8 Hz, 2H), 6.72 (d, *J* = 2.8 Hz, 2H), 3.08 (s, 12H), 0.60 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 185.2, 151.5, 143.3, 132.1, 129.7, 114.3, 112.7, 40.1, -1.3. ESI-MS, positive mode: *m/z* (rel. int., %) = 371.1 (100) $[M+H]^+$. HR-MS (ESI, positive mode): 371.1175 $[M+H]^+$ (found), 371.1177 (calculated for $C_{19}H_{25}N_2OGe$, $[M+H]^+$).

Dye *GeR*. In a 100 mL round-bottom flask, a degassed solution of bis-orthoester **20** (223 mg, 0.54 mmol, 2 eq)[1] in anhydrous THF (10 mL) was cooled to -78 °C. *tert*-Butyllithium (0.32 mL of 1.7 M solution in pentane, 0.54 mmol, 2 eq) was added dropwise, and the resulting yellow-orange solution was stirred at -78 °C for 1 h. A solution of ketone **17a** (100 mg, 0.27 mmol) in THF (8 mL) was added quickly dropwise, the light orange mixture was allowed to warm up to rt and stirred for 3.5 h. The mixture was then cooled in ice-water bath and acetic acid (1.4 mL) was added. The resulting blue solution was evaporated to a viscous residue, 6 N HCl (16 mL) was added, and the mixture was stirred at 80 °C (bath temperature) overnight. TLC control (SiO_2 / 10% methanol – CH_2Cl_2): R_f (product) = 0.30 (blue). The mixture was adjusted to pH 1-2 with $NaHCO_3$, extracted with CH_2Cl_2 (4×50 mL), washed with 0.1 HCl (2×50 mL), brine and dried over Na_2SO_4. Combined organic layers were washed with brine and dried over Na_2SO_4. The product was isolated by flash column chromatography (Sepacore Silica HP 12 g; gradient 0% to 10% methanol – CH_2Cl_2) and freeze-dried from dioxane to give ***GeR*** dye as blue fluffy solid (47 mg, 34% yield).

Flash LC trace:



UV-Vis (PBS 7.4): λ_{max} (ε) = 634 nm (97000 $M^{-1}cm^{-1}$); fluorescence (PBS 7.4): λ_{excit} = 590 nm, λ_{em} = 655 nm; Φ_{fl}(relative to Oxazine 1, Φ_{fl} = 0.14 in ethanol) = 0.43. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.29 (dd, *J* = 7.9, 1.3 Hz, 1H), 8.23 (dd, *J* = 1.3, 0.8 Hz, 1H), 8.05 (dd, *J* = 8.0, 0.7 Hz, 1H), 6.98 (d, *J* = 2.9 Hz, 2H), 6.86 (d, *J* = 8.9 Hz, 2H), 6.52 (dd, *J* = 8.9, 2.9 Hz, 2H), 2.96 (s, 12H), 0.81 (s, 3H), 0.80 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 170.4, 169.5, 153.4, 149.6, 140.4, 134.1, 131.6, 131.2, 130.7, 127.5, 127.1, 126.3, 117.5, 112.7, 40.5, 0.8, -2.2. ESI-MS, positive mode: *m/z* (rel. int., %) = 519.1 (100) $[M+H]^+$. HR-MS (ESI, positive mode): 519.1330 $[M+H]^+$ (found), 519.1339 (calculated for $C_{27}H_{28}N_2O_4Ge$, $[M+H]^+$).



Scheme S25. Derivatives of ***GeR*** dye used as labeling reagents.

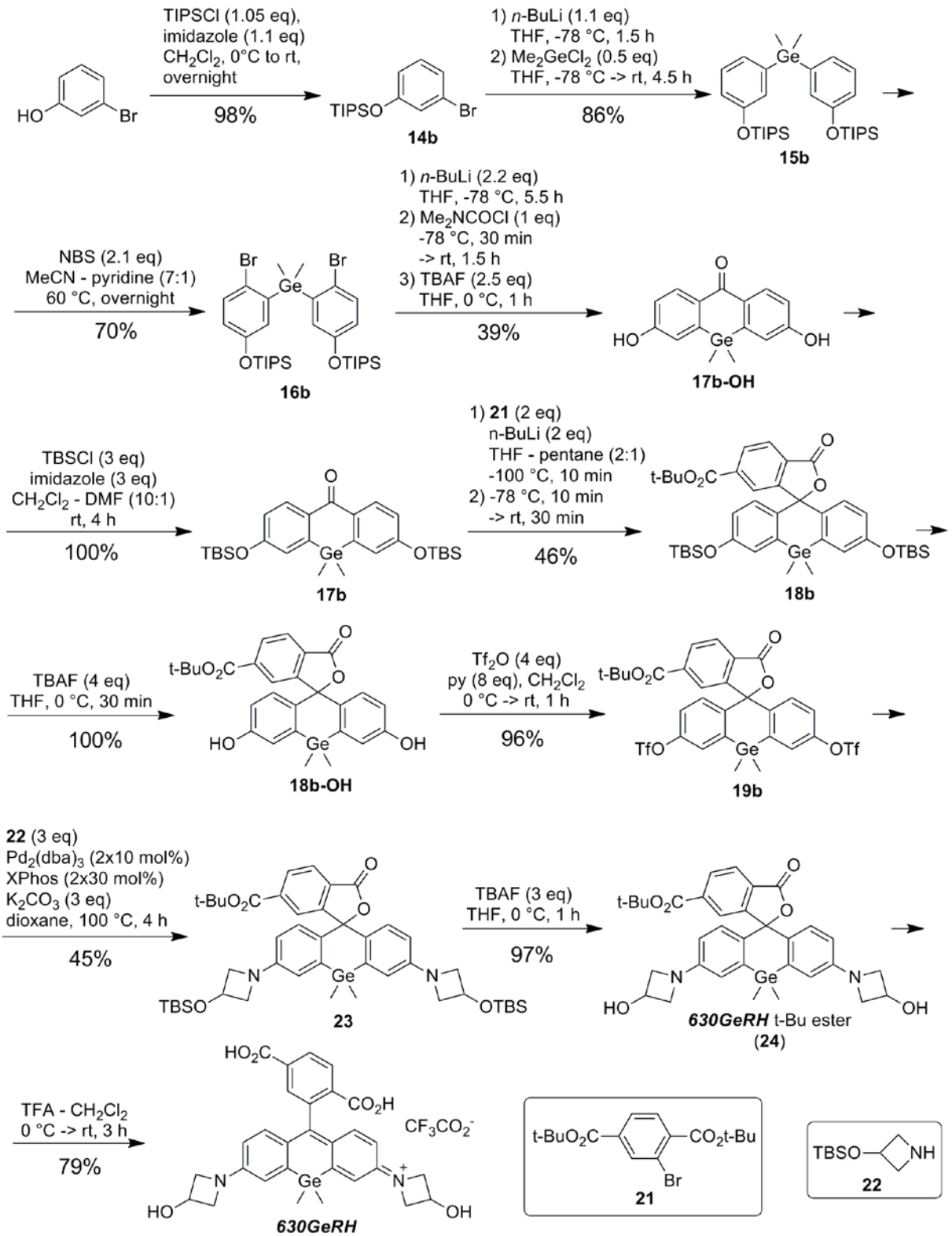
Compound *GeR*-Halo. PyBOP (30 µL of 20 mg/100 µL DMF stock solution, 11.6 µmol, 1.5 eq) was added to a solution of ***GeR*** (4 mg, 7.74 µmol), HaloTag(O2) amine (2-(2-((6-chlorohexyl)oxy)ethoxy)ethanamine; 3.5 mg, 15.63 µmol, 2 eq) and trimethylamine (12 µL) in DMF (100 µL). After 1 h, the solvent was evaporated *in vacuo* at rt, and the product was isolated by preparative TLC (silica, 3% methanol – CH_2Cl_2, then CH_2Cl_2) giving an impure material, which was repurified by preparative HPLC (Kinetex 5µm C18 100, 21mm×25cm, 11 mL/min, isocratic 65/35 A/B, A – acetonitrile, B – water + 0.05% TFA). Yield 3.8 mg (68%), purity (HPLC) ~95%. HPLC (30/70−100/0 A/B over 20 min, Kinetex 5 µm C18 100 4.6×250 mm, 1.2 mL/min, detection at 254 nm or 636 nm): t_R = 12.5 min. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.99 (dd, *J* = 7.9, 0.7 Hz, 1H), 7.93 (dd, *J* = 7.9, 1.4 Hz, 1H), 7.91 (dd, *J* = 1.4, 0.8 Hz, 1H), 6.96 (d, *J* = 2.9 Hz, 2H), 6.84 (d, *J* = 8.9 Hz, 2H), 6.78 (br.s, 1H), 6.50 (dd, *J* = 8.9, 2.9 Hz, 2H), 3.71 – 3.63 (m, 6H), 3.59 – 3.55 (m, 2H), 3.49 (t, *J* = 6.7 Hz, 2H), 3.41 (t, *J* = 6.7 Hz, 2H), 2.96 (s, 12H), 1.71 (m, 2H + H_2O), 1.57 – 1.25 (m, 6H), 0.79 (s, 3H), 0.79 (s, 3H). ESI-MS, positive mode: *m/z* (rel. int., %) = 724.3 $[M+H]^+$. HR-MS (ESI, positive mode): 724.2572 (found), 724.2571 (calculated for $C_{37}H_{49}ClGeN_3O_5$, $[M+H]^+$).

Compound *GeR*-C$_7$-CO$_2$H. TSTU (*N*,*N*,*N*′,*N*′-tetramethyl-*O*-(*N*-succinimidyl)uronium tetrafluoroborate; 20 µL of 14 mg/100 µL DMSO stock solution, 9.1 µmol, 1.2 eq) was added to a solution of ***GeR*** (4 mg, 7.74 µmol) and DIEA (*N*-ethyldiisopropylamine; 12 µL) in DMSO (200 µL). After stirring for 5 min, 8-aminooctanoic acid (20 µL of 15 mg/100 µL DMSO stock suspension, prepared by sonication; 18.2 µmol, 2.3 eq) was added, the resulting

suspension was sonicated at rt for 10 min followed by vigorous stirring for 15 min. Water (50 µL) was then added, and the mixture was stirred for further 30 min. Acetic acid (50 µL) was added, and the solvents were evaporated *in vacuo* at rt. The product ***GeR*-C_7-CO_2H** was isolated by preparative HPLC (Kinetex 5 µm C18 100, 21 mm×25 cm, 11 mL/min, isocratic 50/50 A/B, A – acetonitrile, B – water + 0.05% TFA). Yield 2.5 mg (49%). HPLC (30/70−100/0 A/B over 20 min, Kinetex 5 µm C18 100 4.6×250 mm, 1.2 mL/min, detection at 254 nm or 636 nm): t_R = 8.6 min. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.75 (t, *J* = 5.6 Hz, 1H), 8.11 (dd, *J* = 8.0, 1.4 Hz, 1H), 8.03 (br.d, *J* = 8.2 Hz, 1H), 7.86 (br.s, 1H), 7.05 (br.s, 2H), 6.71 (br.d, *J* = 9.0 Hz, 2H), 6.61 (br.d, *J* = 8.1 Hz, 2H), 3.24 (q, J = 6.7 Hz, 2H), 2.94 (br.s, 12H), 2.17 (t, *J* = 7.4 Hz, 2H), 1.49 (m, 4H), 1.27 (m, 6H), 0.77 (s, 3H), 0.68 (s, 3H). ESI-MS, positive mode: *m/z* (rel. int., %) = 660.4 (100) $[M+H]^+$.

Compound *GeR*-tubulin. TSTU (*N*,*N*,*N*′,*N*′-tetramethyl-*O*-(*N*-succinimidyl)uronium tetrafluoroborate; 10 µL of 17 mg/100 µL DMF stock solution, 5.7 µmol, 1.5 eq) was added to a solution of ***GeR*-C_7-CO_2H** (2.5 mg, 3.8 µmol) and DIEA (*N*-ethyldiisopropylamine; 10 µL) in DMSO (200 µL), and the reaction mixture was stirred for 1 h. DIEA (20 µL) followed by **3'-H_2NXT·HCO_2H** (de-*N*-Boc-docetaxel, or "3'-aminodocetaxel formate";[8] 4.3 mg, 5.7 µmol, 1.5 eq, dissolved in 100 µL DMF + 100 µL DMSO) were added, and the reaction mixture was left stirring at rt overnight. The solvents were evaporated *in vacuo* at rt, and the product was isolated by preparative HPLC (Kinetex 5µm C18 100, 10mm×25cm, gradient 30/70–80/20 A/B over 20 min; A – acetonitrile, B – water + 0.05% TFA). Yield 0.73 mg (14%), determined spectrophotometrically.[8] HPLC (20/80−80/20 A/B over 20 min, Kinetex 5µm C18 100 4.6×250 mm, 1.2 mL/min, detection at 650 nm): t_R = 14.9 min. ESI-MS, positive mode: *m/z* (rel. int., %) = 1371.5 $[M+Na]^+$. HR-MS (ESI, positive mode): 1371.5111 (found), 1371.5165 (calculated for $C_{73}H_{86}N_4O_{16}GeNa$, $[M+Na]^+$).

Hydroxylated germanorhodamine dye *630GeRH*



Compound 14b. A solution of 3-bromophenol (8.1 g, 46.8 mmol) and imidazole (3.5 g, 51.5 mmol, 1.1 eq) in CH_2Cl_2 (55 mL) was cooled in ice-water bath. To the cold mixture, a solution of triisopropylsilyl chloride (TIPSCl; 10.4 mL, 49.2 mmol, 1.05 eq) in CH_2Cl_2 (40 mL) was added dropwise over ~20 min. The resulting suspension was removed from ice-

water bath and stirred at rt overnight. TLC control (SiO_2 / 10% EtOAc in hexane): R_f (product) = 0.70, R_f (3-bromophenol) = 0.18. The mixture was diluted with water (150 mL), extracted with CH_2Cl_2 (3 × 50 mL), the combined organic layers were washed with brine and dried over Na_2SO_4. The product **14b** was isolated by flash chromatography on a 1 L glass Büchner funnel with ~200 g silica (3.5 cm layer), eluting with 0% → 5% → 10% CH_2Cl_2 – hexane in 400 mL portions. Colorless oil, yield 15.07 g (98%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.08 – 7.06 (m, 2H), 7.05 – 7.03 (m, 1H), 6.84 – 6.77 (m, 1H), 1.32 – 1.18 (m, 3H), 1.10 (d, *J* = 7.2 Hz, 18H).

Compound 15b. A solution of TIPS-protected bromophenol **14b** (3.54 g, 10.76 mmol, 2 eq) in anhydrous THF (40 mL) was degassed on a Schlenk line and cooled to -78 °C under argon. *n*-Butyllithium (4.7 mL of 2.5 M solution in hexanes, 11.84 mmol, 2.2 eq) was injected with a syringe quickly dropwise, and the mixture was stirred at -78 °C for 1 h, turning into a thin suspension. Dimethylgermanium dichloride (0.62 mL, 5.38 mmol), dissolved in anhydrous THF (2.5 mL), was injected dropwise with a syringe. The mixture was allowed to warm up to rt (the solids dissolved), and the resulting solution was stirred at rt for 4.5 h. Brine (40 mL) was then added, the mixture was extracted with ethyl acetate (3 × 40 mL), and the combined organic layers were dried over Na_2SO_4. TLC control (SiO_2 / 10% CH_2Cl_2 in hexane): R_f (product) = 0.34, R_f (impurity) = 0.50. The product **22** was isolated by flash column chromatography (Teledyne Isco RediSep Rf 80 g, isocratic in pure hexane for 5 CV, then gradient 0% to 20% CH_2Cl_2 – hexane over 8 CV) as colorless oil, yield 2.79 g (86%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.23 – 7.17 (m, 2H), 7.01 (dt, *J* = 7.1, 1.1 Hz, 2H), 6.98 – 6.96 (m, 2H), 6.85 (ddd, *J* = 8.1, 2.6, 1.1 Hz, 2H), 1.27 – 1.16 (m, 6H), 1.08 (d, *J* = 7.2 Hz, 36H), 0.60 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 155.9, 141.6, 129.1, 126.2, 125.0, 120.2, 18.1, 12.8, -3.0. ESI-MS, positive mode: *m/z* (rel. int., %) = 625.3 (100) $[M+Na]^+$. HR-MS (ESI, positive mode): 625.2897 $[M+Na]^+$ (found), 625.2930 (calculated for $C_{32}H_{56}O_6Si_2GeNa$, $[M+Na]^+$).

Compound 16b. *N*-Bromosuccinimide (1.62 g, 9.11 mmol, 2.1 eq) was added portionwise over 5 min to a solution of **15b** (2.61 g, 4.34 mmol) in the mixture of acetonitrile (60 mL) and pyridine (8.5 mL). The resulting mixture was stirred at 60 °C (bath temperature) overnight (22 h), turning into a brown solution. TLC control (SiO_2 / 5% CH_2Cl_2 in hexane): R_f (product) = 0.27, R_f (starting material) = 0.19. The solvents were removed on a rotary evaporator; the residue was redissolved in CH_2Cl_2 (120 mL) and the solution was washed with sat. aqueous $NaHCO_3$, water, brine (100 mL each) and dried over Na_2SO_4. The product **23** was isolated by flash column chromatography (Teledyne Isco RediSep Rf 80 g, gradient 0% to 25% CH_2Cl_2 – hexane over 15 CV) as viscous colorless oil with purity ~85%, yield 2.64 g (70% considering purity). The material was used in the next step without further purification. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.35 (d, *J* = 8.6 Hz, 2H), 6.85 (d, *J* = 3.0 Hz, 2H), 6.74 (dd, *J* = 8.6, 3.0 Hz, 2H), 1.23 – 1.12 (m, 6H), 1.05 (d, *J* = 7.0 Hz, 36H), 0.84 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 155.1, 142.4, 133.5, 127.5, 122.5, 120.9, 18.0, 12.8, -0.3. ESI-MS, positive mode: *m/z* (rel. int., %) = 783.1 (100) $[M+Na]^+$. HR-MS (ESI, positive mode): 783.1103 $[M+Na]^+$ (found), 783.1119 (calculated for $C_{32}H_{54}O_2Si_2GeBr_2Na$, $[M+Na]^+$).

Compound 17b-OH. A solution of **16b** (2.9 g, 3.82 mmol) in anhydrous THF (60 mL) was degassed on a Schlenk line and cooled to -78 °C under argon. *n*-Butyllithium (3.2 mL of 2.5 M solution in hexanes, 8.02 mmol, 2.1 eq) was added dropwise, and the resulting light

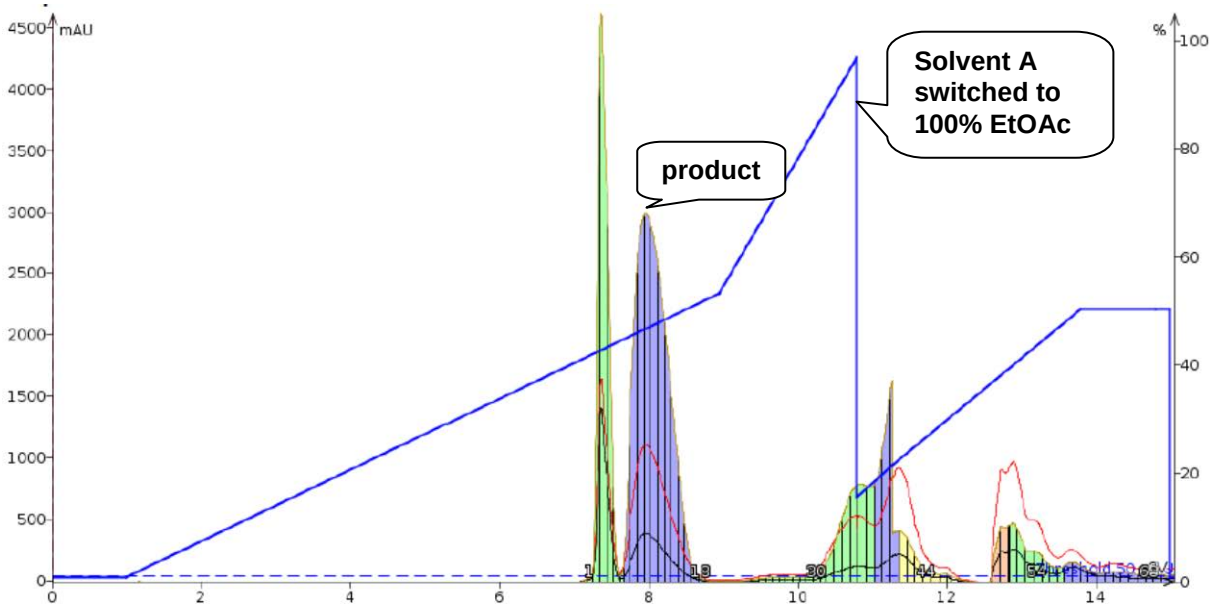
yellow solution was stirred at -78 °C for 5.5 h. Dimethylcarbamyl chloride (0.36 mL, 3.82 mmol) was then injected dropwise with a syringe. The mixture was stirred at -78 °C for 30 min, turning nearly colorless, and was then allowed to warm up to rt and stirred for further 1.5 h. TLC control (SiO_2 / 10% ethyl acetate in hexane): R_f (product) = 0.62 (yellow upon heating with NaOH), R_f (starting material) = 0.72. The reaction mixture was quenched with sat. aq. NH_4Cl (30 mL), extracted with ethyl acetate (3 × 30 mL), and the combined organic layers were dried over Na_2SO_4 The product was isolated by flash column chromatography (Teledyne Isco RediSep Rf 80 g, gradient 0% to 100% A:B, A = 10% ethyl acetate – hexane, B = hexane) as light yellow viscous oil with purity ~70 %, yield 2.13 g (62% considering purity). The impure material (bis-TIPS ether of **17b-OH**) was used directly for deprotection. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.37 (d, *J* = 8.7 Hz, 2H), 7.02 (d, *J* = 2.6 Hz, 2H), 6.98 (dd, *J* = 8.7, 2.6 Hz, 2H), 1.36 – 1.21 (m, 6H), 1.13 (d, *J* = 7.3 Hz, 36H), 0.59 (s, 6H). ESI-MS, positive mode: *m/z* (rel. int., %) = 629.3 (100) $[M+H]^+$. HR-MS (ESI, positive mode): 629.2898 $[M+H]^+$ (found), 629.2903 (calculated for $C_{33}H_{55}GeO_3Si_2$, $[M+H]^+$).

Tetrabutylammonium fluoride trihydrate (2.68 g, 8.5 mmol, 2.5 equiv), dissolved in THF (20 mL), was added a solution of crude di-TIPS ether from the previous step (2.13 g, ~70% purity) in THF (25 mL), cooled in ice-water bath. The resulting bright yellow solution was stirred at 0 °C for 1 h. TLC control (SiO_2 / 50% ethyl acetate in hexane): R_f (product) = 0.26 (yellow with NaOH), R_f (impurity) = 0.48, R_f (starting material) = 0.76. Sat. aq. NH_4Cl (50 mL) was added followed by minimal amount of water necessary to dissolve the solids, the mixture was extracted with ethyl acetate (3×40 mL), the combined organic layers were washed with brine and dried over Na_2SO_4. The product was isolated by flash column chromatography (Teledyne Isco RediSep Rf 80 g; gradient 20% to 80% ethyl acetate – hexane) to give pure **17b-OH** as white solid. Yield 475 mg (39% over 2 steps). ^{1}H NMR (400 MHz, acetone-d_6): δ 9.09 (s, 2H), 8.31 (d, *J* = 8.7 Hz, 2H), 7.12 (d, *J* = 2.6 Hz, 2H), 7.00 (dd, *J* = 8.8, 2.6 Hz, 2H), 0.60 (s, 6H). ^{13}C NMR (101 MHz, acetone-d_6): δ 161.1, 144.7, 134.1, 133.3, 119.9, 117.6, -1.9. ESI-MS, positive mode: *m/z* (rel. int., %) = 339.0 (100) $[M+Na]^+$. HR-MS (ESI, positive mode): 339.0052 $[M+Na]^+$ (found), 339.0050 (calculated for $C_{15}H_{14}GeO_3Na$, $[M+Na]^+$).

Compound 17b. A solution of **17b-OH** (475 mg, 1.51 mmol) and imidazole (308 mg, 4.53 mmol, 3 eq) in CH_2Cl_2 (30 mL) and DMF (3 mL) was cooled in ice-water bath. To the cold mixture, a solution of *tert*-butyldimethylsilyl chloride (TBSCl; 684 mg, 4.53 mmol, 3 eq) in CH_2Cl_2 (5 mL) was added quickly dropwise. The resulting white suspension was removed from ice-water bath and stirred at rt for 4 h. TLC control (SiO_2 / 10% EtOAc in hexane): R_f (product) = 0.56, R_f (starting material) = 0. The mixture was diluted with water (300 mL), extracted with CH_2Cl_2 (3 × 50 mL), the combined organic layers were washed with water (200 mL), sat. aq. $NaHCO_3$, brine (100 mL each) and dried over Na_2SO_4. The product **17b** was isolated by flash column chromatography (Teledyne Isco RediSep Rf 40 g; gradient 0% to 100% A:B, A = 10% ethyl acetate – hexane, B = hexane). White solid, yield 817 g (100%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.37 (d, *J* = 8.7 Hz, 2H), 6.98 (d, *J* = 2.6 Hz, 2H), 6.95 (dd, *J* = 8.7, 2.6 Hz, 2H), 1.01 (s, 18H), 0.60 (s, 6H), 0.26 (s, 12H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 186.2, 158.8, 143.8, 134.8, 132.7, 123.9, 121.3, 25.8, 18.5, -1.7, -4.2. ESI-MS, positive mode: *m/z* (rel. int., %) = 545.2 (100) $[M+H]^+$. HR-MS (ESI, positive mode): 545.1958 $[M+H]^+$ (found), 545.1962 (calculated for $C_{27}H_{43}O_3Si_2Ge$, $[M+H]^+$).

Compound 18b. In a 100 mL round-bottom flask, a degassed solution of **21**[1] (1.04 g, 2.9 mmol, 2 eq) in anhydrous THF (10 mL) and pentane (5 mL) was cooled to -100 °C (bath temperature, diethyl ether – liquid N_2). *n*-Butyllithium (1.2 mL of 2.5 M solution in hexanes, 2.9 mmol, 2 eq) was carefully introduced through a needle along the wall of the flask. Clear solution quickly turned orange and then brown-orange; it was stirred at -100 °C for 10 min, and the solution of ketone **17b** (787 mg, 1.45 mmol) in THF (8 mL) was injected over 1-2 min along the wall of the flask. The flask was then placed into a -78° bath (dry ice – acetone) and the brown solution was stirred for 10 min. The cooling bath was removed, the mixture was allowed to warm up to rt and stirred for further 30 min. TLC control (SiO_2 / 10% ethyl acetate in hexane): R_f (product) = 0.35 (purple upon heating with NaOH), R_f (starting material) = 0.47. The reaction mixture was quenched with water (50 mL), adjusted to pH ~ 4 with acetic acid, extracted with ethyl acetate (3×30 mL), the combined organic layers were washed with brine and dried over Na_2SO_4. The product was isolated by flash column chromatography (Biotage SNAP Ultra 100 g; gradient 0% to 100% A:B, A = 15% ethyl acetate – hexane, B = hexane) and freeze-dried from dioxane to give **18b** as white solid (501 mg, 46% yield).

Flash LC trace:



^{1}H NMR (400 MHz, $CDCl_3$): δ 8.18 (dd, *J* = 8.0, 1.3 Hz, 1H), 8.08 (dd, *J* = 1.3, 0.7 Hz, 1H), 7.99 (dd, *J* = 8.0, 0.7 Hz, 1H), 7.10 (d, *J* = 2.7 Hz, 2H), 6.96 (d, *J* = 8.7 Hz, 2H), 6.65 (dd, *J* = 8.7, 2.7 Hz, 2H), 1.60 (s, 9H), 0.98 (s, 18H), 0.795 (s, 3H), 0.790 (s, 3H), 0.19 (s, 12H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 169.4, 164.5, 155.5, 153.0, 140.6, 137.0, 136.2, 130.4, 129.7, 127.8, 126.2, 125.9, 125.5, 120.2, 91.2, 82.6, 28.3, 25.8, 18.4, 0.5, -1.9, -4.21, -4.23. ESI-MS, positive mode: *m/z* (rel. int., %) = 749.3 (100) $[M+H]^+$. HR-MS (ESI, positive mode): 749.2732 $[M+H]^+$ (found), 749.2753 (calculated for $C_{39}H_{55}O_6GeSi_2$, $[M+H]^+$).

Compound 18b-OH. Tetrabutylammonium fluoride trihydrate (850 mg, 2.68 mmol, 4 eq), dissolved in THF (5 mL), was added a solution of **18b** (500 mg, 0.67 mmol) in THF (7 mL), cooled in ice-water bath. The resulting blue solution with intense red fluorescence was stirred at 0 °C for 30 min. TLC control (SiO_2 / 10% ethyl acetate in CH_2Cl_2): R_f (product) = 0.31, R_f (starting material) = 0.83. Sat. aq. NH_4Cl (20 mL) was added followed by minimal amount of water necessary to dissolve the solids, the mixture was extracted with ethyl acetate (3×25

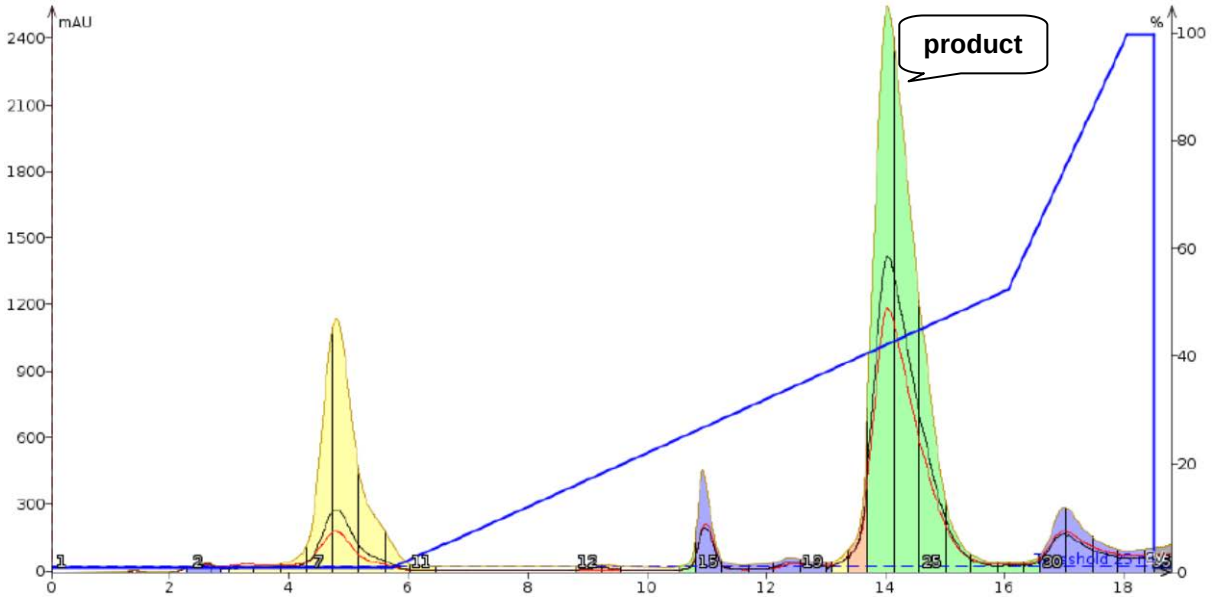
mL), the combined organic layers were washed with brine and dried over Na_2SO_4. The product was isolated by flash column chromatography (Teledyne Isco RediSep Rf 24 g; gradient 0% to 30% ethyl acetate – CH_2Cl_2), evaporated to viscous colorless oil and freeze-dried from dioxane to give **18b-OH** as white solid (393 mg, contains 0.65 mol dioxane per mol of product, 100% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.18 (dd, *J* = 8.0, 1.3 Hz, 1H), 8.03 (dd, *J* = 1.3, 0.8 Hz, 1H), 7.99 (dd, *J* = 8.0, 0.8 Hz, 1H), 7.08 (d, *J* = 2.8 Hz, 2H), 6.86 (d, *J* = 8.7 Hz, 2H), 6.60 (dd, *J* = 8.7, 2.8 Hz, 2H), 6.40 (d, *J* = 4.0 Hz, 2H), 1.59 (s, 9H), 0.67 (s, 3H), 0.63 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 170.6, 164.7, 155.7, 153.4, 140.8, 137.2, 134.9, 130.5, 129.4, 127.9, 126.3, 125.8, 120.9, 115.9, 92.1, 83.1, 28.3, 0.3, -1.9. ESI-MS, positive mode: *m/z* (rel. int., %) = 543.1 (100) $[M+Na]^+$. HR-MS (ESI, positive mode): 543.0838 $[M+Na]^+$ (found), 543.0839 (calculated for $C_{27}H_{26}GeO_6Na$, $[M+Na]^+$).

Compound 19b. Trifluoromethanesulfonic anhydride (Tf_2O; 155 μL, 0.92 mmol, 4 eq) was added dropwise to a solution of **18b-OH** (133 mg of material containing 0.65 mol dioxane per mol of **18b-OH**, 0.23 mmol) and pyridine (150 μL, 1.84 mmol, 8 eq) in dry CH_2Cl_2 (5 mL), cooled in ice-water bath. Pink color appeared upon addition of each drop and vanished immediately. The flask was then removed from the cooling bath, and the mixture was stirred at rt for 1 h. TLC control (SiO_2 / 10% ethyl acetate in hexane): R_f (product) = 0.18 (pink with NaOH). The mixture was diluted with cold water (20 mL), extracted with CH_2Cl_2 (3×20 mL), the combined extracts were washed with brine and dried over Na_2SO_4. The product was isolated by flash column chromatography (Sepacore Silica HP 12 g; gradient 0% to 40% ethyl acetate – hexane) and freeze-dried from dioxane to give **19b** as white fluffy solid, yield 173 mg (96%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.26 (dd, *J* = 8.0, 1.2 Hz, 1H), 8.16 (dd, *J* = 1.3, 0.7 Hz, 1H), 8.05 (dd, *J* = 8.0, 0.8 Hz, 1H), 7.54 (d, *J* = 2.7 Hz, 2H), 7.31 (d, *J* = 8.8 Hz, 2H), 7.16 (dd, *J* = 8.8, 2.7 Hz, 2H), 1.62 (d, *J* = 0.8 Hz, 9H), 0.93 (s, 3H), 0.92 (s, 3H). ^{19}F NMR (376 MHz, $CDCl_3$): δ -72.78. ^{13}C NMR (101 MHz, $CDCl_3$): δ 168.1, 164.0, 150.8, 149.5, 143.3, 142.5, 137.7, 131.4, 129.2, 128.4, 127.0, 126.7, 125.7, 122.1, 118.8 (q, *J* = 320.8 Hz), 89.2, 83.2, 28.3, 0.9, -1.7. ESI-MS, positive mode: *m/z* (rel. int., %) = 807.0 (100) $[M+Na]^+$. HR-MS (ESI, positive mode): 806.9805 $[M+Na]^+$ (found), 806.9824 (calculated for $C_{29}H_{24}O_{10}GeS_2F_6Na$, $[M+Na]^+$).

Compound 23. A mixture of **19b** (170 mg, 0.22 mmol), 3-(*tert*-butyldimethylsilyloxy)azetidine **22** (123 mg, 0.66 mmol, 3 eq), $Pd_2(dba)_3$ (20 mg, 0.022 mmol, 10 mol%), XPhos (31 mg, 0.066 mmol, 30 mol%) and K_2CO_3 (91 mg, 0.66 mmol, 3 eq) in dry dioxane (2 mL) was degassed on a Schlenk line and stirred at 100 °C under argon (bath temperature) in a sealed flask for 2 h. TLC control showed incomplete conversion (SiO_2 / hexane – CH_2Cl_2 – ethyl acetate 70:25:5, stained by heating with 1 M NaOH): R_f (product) = 0.2 (blue), R_f (monosubstitution product) = 0.33 (violet), R_f (starting material) = 0.38 (pink). Additional portions of $Pd_2(dba)_3$ (20 mg, 0.022 mmol, 10 mol%) and XPhos (31 mg, 0.066 mmol, 30 mol%) were added, the mixture was degassed stirred at 100 °C for further 2 h (4 h total time). The consumption of both the starting material and the monotriflate intermediate was verified by TLC, and upon cooling the brown mixture was diluted with water (20 mL), extracted with CH_2Cl_2 (3×20 mL), the combined organic layers were washed with brine and dried over Na_2SO_4. The filtrate was evaporated on Celite and the product was isolated by flash column chromatography (Interchim Puriflash 15 μm 25 g; isocratic in pure CH_2Cl_2 until the first peak is eluted (6 CV), then gradient 0% to 100% A:B, A = 10% ethyl acetate in

CH_2Cl_2, B = CH_2Cl_2) and freeze-dried from 1,4-dioxane to yield 84 mg (45%) of **23** as light yellow fluffy solid.

Flash LC trace:

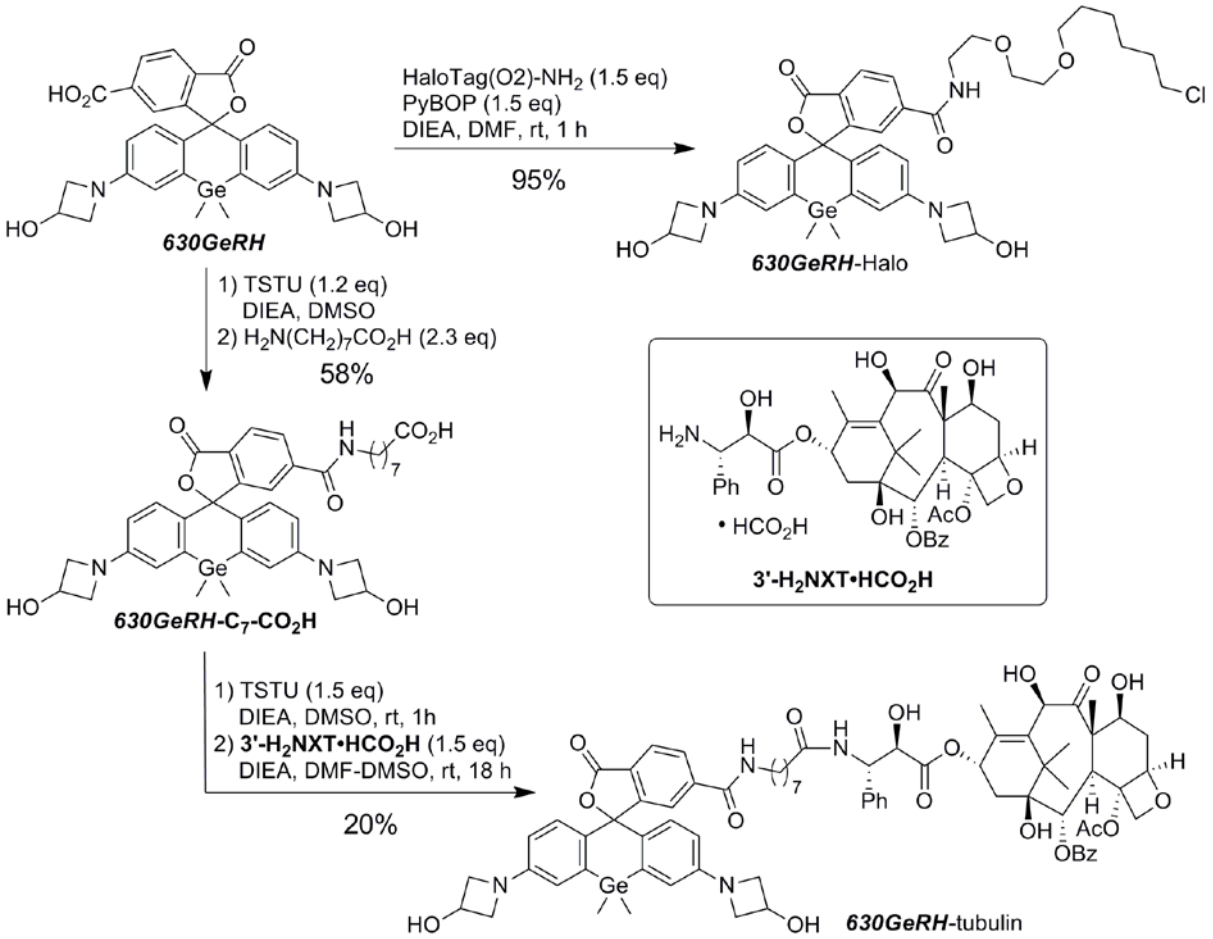


^{1}H NMR (400 MHz, $CDCl_3$): δ 8.16 (dd, *J* = 8.0, 1.3 Hz, 1H), 8.03 (t, *J* = 1.0 Hz, 1H), 7.97 (dd, *J* = 8.0, 0.7 Hz, 1H), 6.88 (d, *J* = 8.6 Hz, 2H), 6.67 (d, *J* = 2.6 Hz, 2H), 6.26 (dd, *J* = 8.7, 2.6 Hz, 2H), 4.74 (tt, *J* = 6.3, 5.0 Hz, 2H), 4.15 (td, *J* = 6.5, 1.3 Hz, 4H), 3.65 (dd, *J* = 7.6, 5.0 Hz, 4H), 1.59 (s, 9H), 0.89 (s, 18H), 0.78 (s, 3H), 0.78 (s, 3H), 0.07 (s, 12H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 169.7, 164.6, 153.6, 150.9, 139.8, 136.8, 132.3, 130.1, 130.0, 127.2, 125.94, 125.92, 116.6, 112.0, 92.2, 82.4, 62.5, 62.2, 62.1, 28.3, 25.9, 18.1, 0.6, -1.8, -4.8. ESI-MS, positive mode: *m/z* (rel. int., %) = 859.4 (100) $[M+H]^+$. HR-MS (ESI, positive mode): 859.3586 $[M+H]^+$ (found), 859.3599 (calculated for $C_{45}H_{65}N_2O_6Si_2Ge$, $[M+H]^+$).

Compound 24 (*630GeRH t*-butyl ester). Tetrabutylammonium fluoride trihydrate (93 mg, 294 µmol, 3 equiv), dissolved in THF (5 mL), was added a solution of **23** (84 mg, 98 µmol) in THF (10 mL), cooled in ice-water bath. The reaction mixture was stirred at 0 °C for 1 h. Water (20 mL), acetic acid (0.5 mL) and brine (20 mL) were then added, and the mixture was extracted with ethyl acetate (3×20 mL), the combined organic layers were dried over Na_2SO_4, filtered, evaporated and re-evaporated twice with ethyl acetate (2×20 mL). The product was isolated by flash column chromatography (Sepacore Silica HP 12 g; gradient 20% to 100% ethyl acetate – CH_2Cl_2) and freeze-dried from 1,4-dioxane to give **24** as light green fluffy solid. Yield 60 mg (97%). ^{1}H NMR (400 MHz, CD_3OD): δ 8.16 (dd, *J* = 8.1, 1.3 Hz, 1H), 7.96 (dd, *J* = 8.0, 0.7 Hz, 1H), 7.90 (t, *J* = 1.0 Hz, 1H), 6.79 (d, *J* = 8.7 Hz, 2H), 6.73 (d, *J* = 2.6 Hz, 2H), 6.28 (ddd, *J* = 8.7, 2.7, 0.9 Hz, 2H), 4.85 (s, 2H + H_2O), 4.63 (tt, *J* = 6.4, 4.8 Hz, 2H), 4.16 – 4.07 (m, 4H), 3.67 – 3.56 (m, 4H), 1.55 (s, 9H), 0.76 (s, 3H), 0.70 (s, 3H). ^{13}C NMR (101 MHz, CD_3OD): δ 171.5, 165.6, 155.7, 152.5, 140.3, 138.3, 133.0, 131.1, 130.5, 128.1, 126.9, 126.5, 117.6, 113.4, 83.6, 63.0, 62.5, 28.3, 0.1, -1.7. ESI-MS, positive mode: *m/z* (rel. int., %) = 631.2 (100) $[M+H]^+$. HR-MS (ESI, positive mode): 631.1851 $[M+H]^+$ (found), 631.1865 (calculated for $C_{33}H_{37}N_2O_6Ge$, $[M+H]^+$).

Dye *630GeRH*. Trifluoroacetic acid (1.6 mL) was added to a solution of **24** (50 mg, 79.5 µmol) in CH_2Cl_2 (8 mL), cooled in ice-water bath. The cooling bath was removed, and the reaction mixture was stirred at rt for 3 h. The solvents were evaporated, and the residue was

re-evaporated twice with toluene (2×20 mL). The product was isolated by flash column chromatography (20 g silica, acetonitrile – CH_2Cl_2 – water 10:1:0.5, then 10:1:1), followed by chromatography on a reversed phase (RP-C_{18} 10 g; gradient 67% to 33% water – acetonitrile). Fractions containing the product were evaporated, dissolved in 1,4-dioxane with addition of minimal amount of water, a small amount of viscous insoluble material was centrifuged off, the supernatant was microfiltered and freeze-dried to give 51 mg (79%) blue fluffy solid (trifluoroacetate salt, containing 1.4 mol of 1,4-dioxane per mol of the dye). HPLC (10/90−100/0 over 25 min, column 4×250 mm, 1.2 mL/min, detection at 254 nm): τ = 10.32 min. UV-Vis (PBS 7.4): λ_{max} (ε) = 631 nm (61000 $M^{-1}cm^{-1}$); fluorescence (PBS 7.4): λ_{excit} = 590 nm, λ_{em} = 651 nm; Φ_{fl}(abs.) = 0.60. ^{1}H NMR (400 MHz, CD_3OD): δ 8.22 (br.d, *J* = 7.0 Hz, 1H), 8.07 (br.s, 1H), 7.92 (d, *J* = 7.0 Hz, 1H), 6.78 (d, *J* = 8.6 Hz, 2H), 6.73 (d, *J* = 2.5 Hz, 2H), 6.25 (dd, *J* = 8.6, 2.6 Hz, 2H), 4.63 (tt, *J* = 6.2, 4.8 Hz, 2H), 4.16 – 4.08 (m, 4H), 3.62 – 3.57 (m, 4H), 0.74 (s, 3H), 0.71 (s, 3H). ^{19}F NMR (376 MHz, CD_3OD): δ -76.95. ^{13}C NMR (101 MHz, CD_3OD): δ 171.7, 154.1, 152.5, 141.4, 133.4, 130.1, 128.3, 126.4, 117.7, 112.9, 94.8, 63.0, 62.5, 0.5, -2.6. ESI-MS, positive mode: *m/z* (rel. int., %) = 575.2 (100) $[M+H]^+$. HR-MS (ESI, positive mode): 575.1225 (found), 575.1238 (calculated for $C_{29}H_{29}N_2O_6Ge$, $[M+H]^+$).



Scheme S26. Derivatives of hydroxylated germanorhodamine ***630GeRH*** used as labeling reagents.

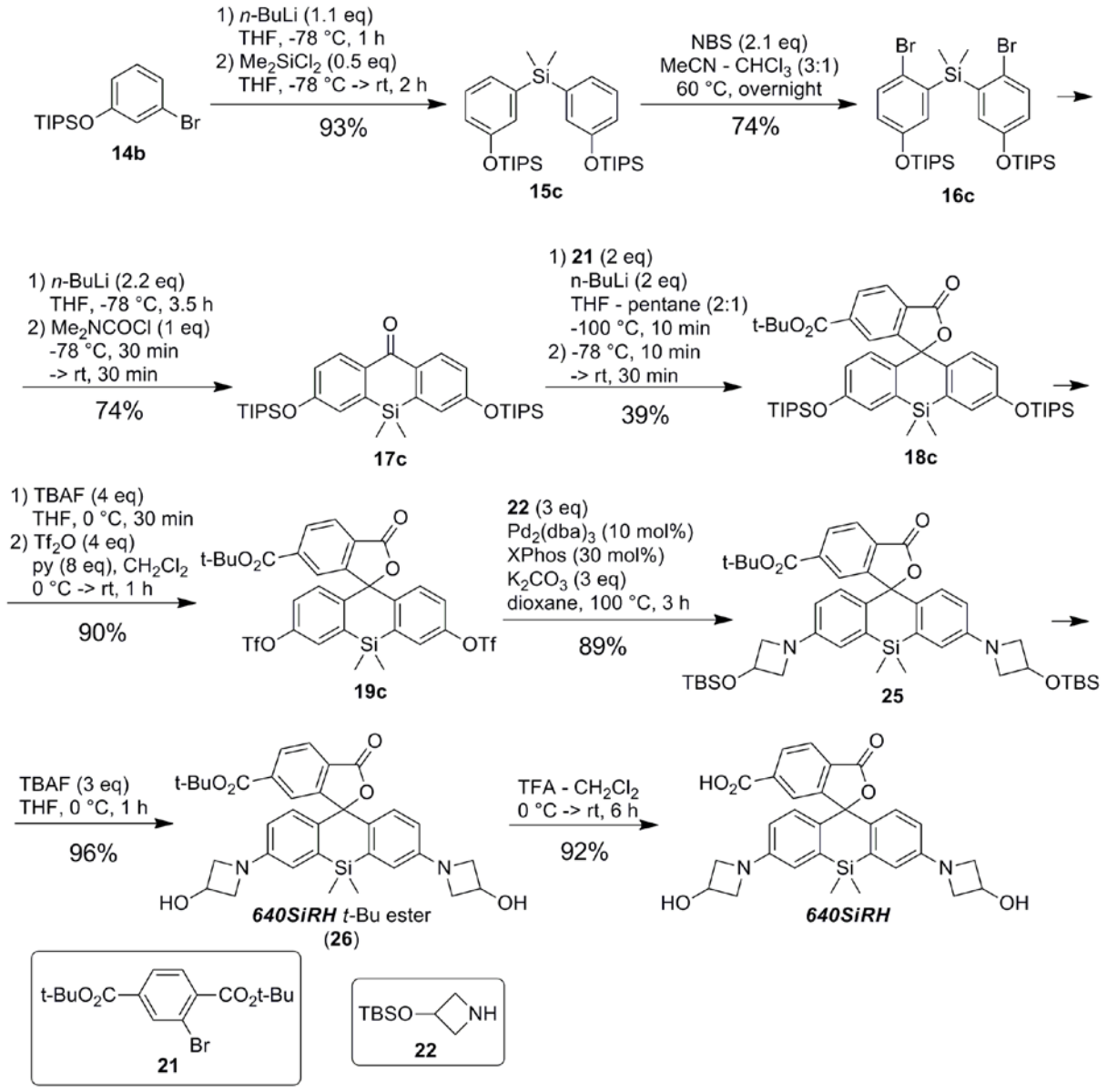
Compound *630GeRH*-Halo. PyBOP (20 µL of 20 mg/100 µL stock solution, 7.41 µmol, 1.5 equiv) was added to a solution of ***630GeRH*** (4 mg, 5.82 µmol), HaloTag(O2) amine (2-(2-((6-chlorohexyl)oxy)ethoxy)ethanamine; 2.0 mg, 8.73 µmol, 1.5 equiv) and DIEA (*N*-ethyldiisopropylamine; 10 µL) in DMF (100 µL). After 1 h, the solvent was evaporated *in vacuo* at rt, and the product ***630GeRH* -Halo** was isolated by preparative TLC (silica, 5% methanol – CH_2Cl_2, then 10% methanol – CH_2Cl_2). Yield 4.3 mg (95%), purity (HPLC) ~95%. HPLC (10/90−100/0 A/B over 25 min, column 4×250 mm, 1.2 mL/min, detection at 254 nm or 640 nm): τ = 14.60 min. UV-Vis (PBS 7.4): λ_{max} = 639 nm; fluorescence (PBS 7.4): λ_{excit} = 610 nm, λ_{em} = 660 nm. ESI-MS, positive mode: *m/z* (rel. int., %) = 780.3 $[M+H]^+$. HR-MS (ESI, positive mode): 780.2471 (found), 780.2470 (calculated for $C_{39}H_{49}ClGeN_3O_7$, $[M+H]^+$).

Compound *630GeRH*-C_7-CO_2H. TSTU (*N*,*N*,*N*′,*N*′-tetramethyl-*O*-(*N*-succinimidyl)uronium tetrafluoroborate; 20 µL of 18 mg/100 µL DMSO stock solution, 12 µmol, 1.2 eq) was added to a solution of ***630GeRH*** (6 mg, 10 µmol) and DIEA (*N*-ethyldiisopropylamine; 12 µL) in DMSO (250 µL). After stirring for 5 min, 8-aminooctanoic acid (20 µL of 18 mg/100 µL DMSO stock suspension, prepared by sonication; 23 µmol, 2.3 eq) was added, the resulting suspension was sonicated at rt for 10 min followed by vigorous stirring for 15 min. Water (50 µL) was then added, and the mixture was stirred for further 30 min. Acetic acid (50 µL) was added, and the solvents were evaporated *in vacuo* at rt. The product was isolated by

preparative HPLC (Kinetex 5µm C18 100, 21mm×25cm, 11 mL/min, gradient 20/75–70/30 A/B over 20 min, A – acetonitrile, B – water + 0.05% TFA). Yield 2.5 mg (49%). HPLC (20/80−70/30 A/B over 20 min, Kinetex 5µm C18 100 4.6×250 mm, 1.2 mL/min, detection at 254 nm): τ = 10.90 min. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.75 (t, *J* = 5.6 Hz, 1H), 8.11 (dd, *J* = 8.0, 1.4 Hz, 1H), 8.03 (br.d, *J* = 8.2 Hz, 1H), 7.86 (br.s, 1H), 7.05 (br.s, 2H), 6.71 (br.d, *J* = 9.0 Hz, 2H), 6.61 (br.d, *J* = 8.1 Hz, 2H), 3.24 (q, J = 6.7 Hz, 2H), 2.94 (br.s, 12H), 2.17 (t, *J* = 7.4 Hz, 2H), 1.49 (m, 4H), 1.27 (m, 6H), 0.77 (s, 3H), 0.68 (s, 3H). ESI-MS, positive mode: *m/z* (rel. int., %) = 716.2 $[M+H]^+$. HR-MS (ESI, positive mode): 716.2374 (found), 716.2394 (calculated for $C_{37}H_{44}N_3O_7Ge$, $[M+H]^+$).

Compound *630GeRH*-tubulin. TSTU (*N*,*N*,*N*′,*N*′-tetramethyl-*O*-(*N*-succinimidyl)uronium tetrafluoroborate; 13 µL of 20 mg/100 µL DMF stock solution, 8.67 µmol, 1.5 eq) was added to a solution of ***630GeRH*-C_7-CO_2H** (5.78 µmol in 500 µL DMSO) and DIEA (*N*-ethyldiisopropylamine; 16 µL), and the reaction mixture was stirred for 1 h. DIEA (30 µL) followed by **3'-H_2NXT·HCO_2H** (de-*N*-Boc-docetaxel, or "3'-aminodocetaxel formate";[8] 6.5 mg, 8.7 µmol, 1.5 eq, dissolved in 150 µL DMF) were added, and the reaction mixture was left stirring at rt overnight. The solvents were evaporated *in vacuo* at rt, and the product was isolated by preparative HPLC (Kinetex 5µm C18 100, 10mm×25cm, isocratic 50/50 A/B, A – acetonitrile, B – water + 0.05% TFA). Yield 1.59 mg (20%), determined spectrophotometrically. HPLC (10/90−80/20 A/B over 2 min, column 4×250 mm, 1.2 mL/min, detection at 254 nm): t_R = 15.8 min. ESI-MS, positive mode: *m/z* (rel. int., %) = 1405.5 (100) $[M+H]^+$. HR-MS (ESI, positive mode): 1405.5237 (found), 1405.5244 (calculated for $C_{75}H_{87}N_4O_{18}Ge$, $[M+H]^+$).

Hydroxylated Si-rhodamine dye JF_{646} (*640SiRH*)



Compound 15c. A solution of TIPS-protected bromophenol **14b** (5.27 g, 16.02 mmol, 2 eq) in anhydrous THF (60 mL) was degassed on a Schlenk line and cooled to -78 °C under argon. *n*-Butyllithium (7.05 mL of 2.5 M solution in hexanes, 17.62 mmol, 2.2 eq) was injected with a syringe quickly dropwise, and the mixture was stirred at -78 °C for 1 h, turning into a thick suspension. Dichlorodimethylsilane (0.97 mL, 8.01 mmol) was added dropwise with a syringe. The mixture was allowed to warm up to rt (the solids dissolved), and the resulting solution was stirred at rt for 2 h. Brine (50 mL) and water (20 mL) were then added, the mixture was extracted with ethyl acetate (3 × 50 mL), and the combined organic layers were dried over Na_2SO_4. TLC control (SiO_2 / 10% CH_2Cl_2 in hexane): R_f (product) = 0.36, R_f (starting material) = 0.55. The product **15c** was isolated by flash column chromatography (90 g silica, gradient 0% to 30% CH_2Cl_2 – hexane) as colorless oil, yield 4.12 g (93%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.23 – 7.18 (m, 2H), 7.06 (dt, *J* = 7.2, 1.1 Hz, 2H), 7.02 – 7.00 (m, 2H),

6.88 (ddd, *J* = 8.1, 2.6, 1.1 Hz, 2H), 1.29 – 1.16 (m, 6H), 1.08 (d, *J* = 7.1 Hz, 36H), 0.51 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 155.7, 139.8, 129.0, 126.8, 125.6, 120.8, 18.1, 12.8, -2.3. ESI-MS, positive mode: *m/z* (rel. int., %) = 595.3 (100) $[M+K]^+$. HR-MS (ESI, positive mode): 595.3215 $[M+K]^+$ (found), 595.3220 (calculated for $C_{32}H_{56}O_2Si_3K$, $[M+K]^+$).

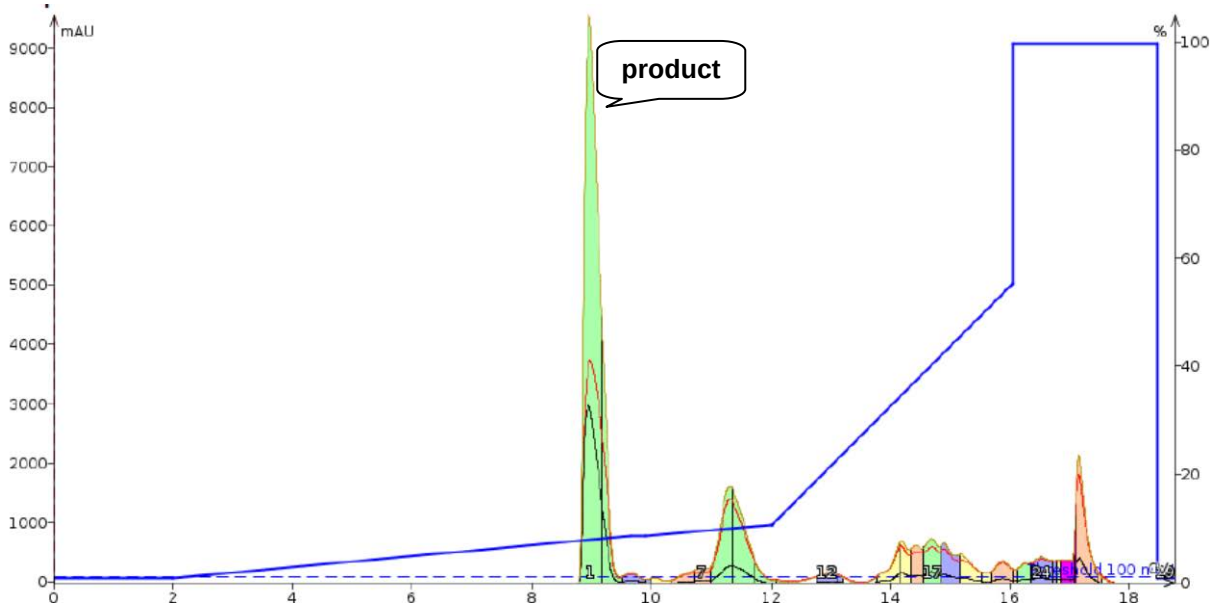
Compound 16c. *N*-Bromosuccinimide (2.33 g, 13.07 mmol, 2.1 eq) was added portionwise over 5 min to a solution of **15c** (3.46 g, 6.22 mmol) in the mixture of acetonitrile (85 mL) and chloroform (25 mL). The resulting solution was stirred at 60 °C (bath temperature) overnight (16 h), turning light yellow. TLC control (SiO_2 / 5% CH_2Cl_2 in hexane): R_f (product) = 0.44, R_f (starting material) = 0.36. The solvents were removed on a rotary evaporator; the residue was redissolved in CH_2Cl_2 (150 mL) and the solution was washed with sat. aqueous $NaHCO_3$, water, brine (100 mL each) and dried over Na_2SO_4. The product **36** was isolated by flash column chromatography (100 g silica, gradient 0% to 15% CH_2Cl_2 – hexane) as colorless oil, yield 3.27 g (74%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.34 (d, *J* = 8.6 Hz, 2H), 6.97 (d, *J* = 3.0 Hz, 2H), 6.76 (dd, *J* = 8.6, 3.0 Hz, 2H), 1.25 – 1.15 (m, 6H), 1.07 (d, *J* = 7.3 Hz, 36H), 0.72 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 155.0, 140.0, 133.8, 128.5, 123.0, 121.0, 18.0, 12.8, -1.0. ESI-MS, positive mode: *m/z* (rel. int., %) = 737.2 (100) $[M+Na]^+$. HR-MS (ESI, positive mode): 737.1664 $[M+Na]^+$ (found), 737.1673 (calculated for $C_{32}H_{54}Br_2O_2Si_3Na$, $[M+Na]^+$).

Compound 17c. A solution of **16c** (4.06 g, 5.69 mmol) in anhydrous THF (80 mL) was degassed on a Schlenk line and cooled to -78 °C under argon. *n*-Butyllithium (5 mL of 2.5 M solution in hexanes, 12.5 mmol, 2.2 eq) was added dropwise, and the mixture was stirred at -78 °C for 3.5 h, turning into a clear light yellow solution. Dimethylcarbamyl chloride (0.53 mL, 5.69 mmol, 1 eq) was then injected dropwise with a syringe. The mixture was stirred at -78 °C for 30 min, allowed to warm up to rt and stirred for further 30 min. TLC control (SiO_2 / 10% ethyl acetate in hexane): R_f (product) = 0.59 (yellow upon heating with NaOH), R_f (starting material) = 0.67. The reaction mixture was quenched with sat. aq. NH_4Cl (30 mL), extracted with ethyl acetate (3 × 40 mL), and the combined organic layers were dried over Na_2SO_4. The product **17c** was isolated by flash column chromatography (Teledyne Isco RediSep Rf 80 g, gradient 0% to 100% A:B, A = 10% ethyl acetate – hexane, B = hexane) as light yellow viscous oil, yield 2.46 g (74%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.36 (d, *J* = 8.7 Hz, 2H), 7.08 (d, *J* = 2.5 Hz, 2H), 7.02 (dd, *J* = 8.7, 2.6 Hz, 2H), 1.36 – 1.25 (m, 6H), 1.13 (d, *J* = 7.3 Hz, 36H), 0.45 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 186.1, 159.3, 141.2, 134.5, 132.4, 123.6, 121.8, 18.1, 12.9, -1.4. ESI-MS, positive mode: *m/z* (rel. int., %) = 583.3 $[M+H]^+$. HR-MS (ESI, positive mode): 583.3428 $[M+H]^+$ (found), 583.3454 (calculated for $C_{33}H_{55}O_3Si_3$, $[M+H]^+$).

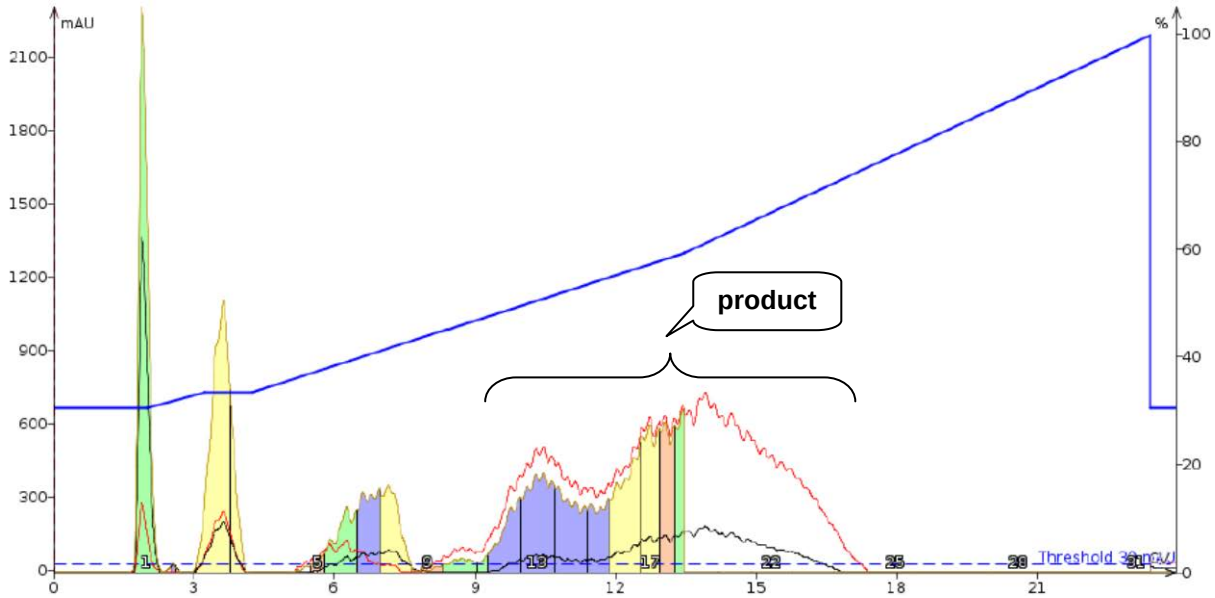
Compound 18c. In a 50 mL round-bottom flask, a degassed solution of **21**[1] (427 mg, 1.2 mmol, 2 eq) in anhydrous THF (4 mL) and pentane (2 mL) was cooled to -100 °C (bath temperature, diethyl ether – liquid N_2). *n*-Butyllithium (0.48 mL of 2.5 M solution in hexanes, 1.2 mmol, 2 eq) was carefully introduced through a needle along the wall of the flask. Clear solution quickly turned orange and then purple; it was stirred at -100 °C for 10 min, and the solution of ketone **17c** (349 mg, 0.6 mmol) in THF (2 mL) was injected over 1-2 min along the wall of the flask. The flask was then placed into a -78° bath (dry ice – acetone) and the stirring was continued for 10 min. The cooling bath was removed, the mixture was allowed to warm up to rt and stirred for further 30 min. TLC control (SiO_2 / 10% ethyl acetate in hexane): R_f (product) = 0.45 (purple upon heating with NaOH), R_f (starting material) = 0.62.

The reaction mixture was quenched with water (20 mL), adjusted to pH ~ 4 with acetic acid, extracted with ethyl acetate (3×20 mL), the combined organic layers were washed with brine and dried over Na_2SO_4. The product was isolated by flash column chromatography (Interchim Puriflash HP 15µm 25 g; gradient 0% to 20% ethyl acetate – hexane); fractions containing the product were repurified by flash column chromatography (Grace Reveleris HP 12 g; gradient 30% to 100% CH_2Cl_2 – pentane), and the product was freeze-dried from 1,4-dioxane to give **18c** as white solid. Yield 186 mg (39%).

Flash LC traces: 1st separation:



2nd separation (significant bubbling due to pentane boiling):



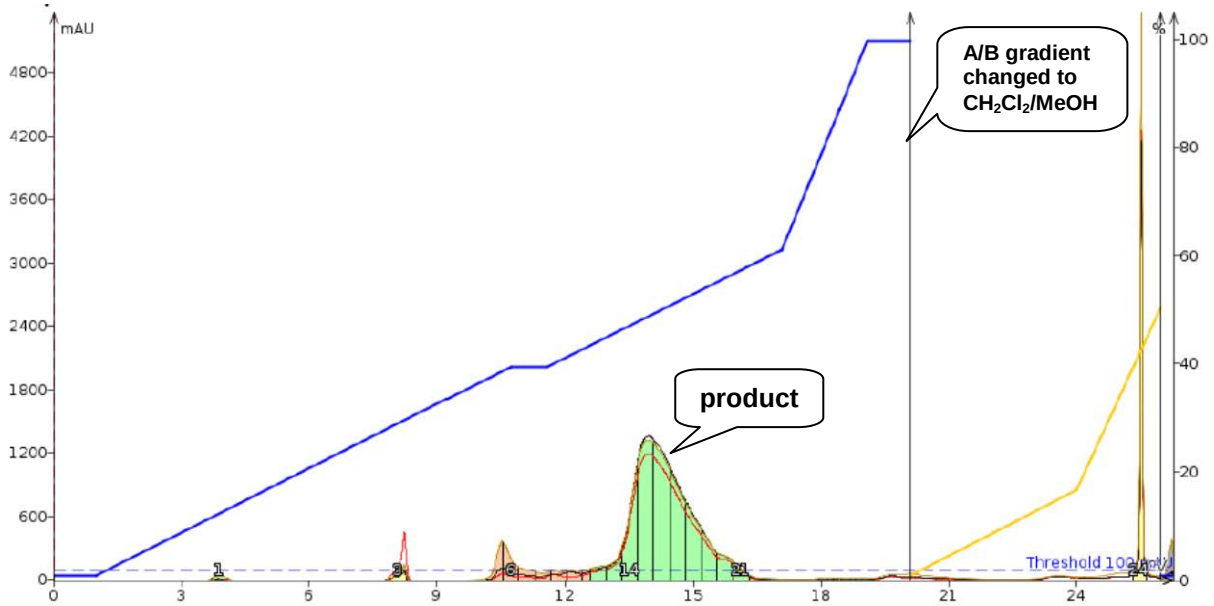
^{1}H NMR (400 MHz, acetone-d_6): δ 8.17 (dd, J = 8.0, 1.3 Hz, 1H), 8.04 (d, J = 8.0 Hz, 1H), 7.81 (dd, J = 1.3, 0.7 Hz, 1H), 7.35 (d, J = 2.7 Hz, 2H), 7.05 (d, J = 8.7 Hz, 2H), 6.93 (dd, J = 8.8, 2.7 Hz, 2H), 1.54 (s, 9H), 1.36 – 1.24 (m, 6H), 1.11 (d, J = 7.4 Hz, 36H), 0.74 (s, 3H), 0.61 (s, 3H). ^{13}C NMR (101 MHz, acetone-d_6): δ 170.0, 164.5, 156.53, 156.52, 138.4, 137.7, 137.0, 130.9, 130.0, 129.0, 128.7, 126.7, 125.7, 125.0, 122.6, 90.3, 82.9, 28.2, 18.3, 13.4, -0.3, -0.4. ESI-MS, positive mode: *m/z* (rel. int., %) = 787.5 (100) $[M+H]^+$. HR-MS (ESI, positive mode): 787.4224 $[M+H]^+$ (found), 787.4240 (calculated for $C_{45}H_{67}O_6Si_3$, $[M+H]^+$).

Compound 19c. Tetrabutylammonium fluoride trihydrate (290 mg, 0.92 mmol, 4 eq), dissolved in THF (3.5 mL), was added a solution of **18c** (180 mg, 0.23 mmol) in THF (3 mL), cooled in ice-water bath. An intense blue solution with red fluorescence formed. The solution was stirred at 0 °C for 30 min. TLC control (SiO_2 / 10% ethyl acetate in CH_2Cl_2): R_f (product) = 0.47 (orange; purple with NaOH). Sat. aq. NH_4Cl (20 mL) was added, the mixture was extracted with ethyl acetate (3×20 mL), the combined organic layers were washed with brine and dried over Na_2SO_4. The filtrate was evaporated to light orange oil, which was used directly in the next step.

Trifluoromethanesulfonic anhydride (Tf_2O; 155 μL, 0.92 mmol, 4 eq) was added dropwise to a solution of the crude material from the deprotection step and pyridine (150 μL, 1.84 mmol, 8 eq) in dry CH_2Cl_2 (5 mL), cooled in ice-water bath. Pink color appeared upon addition of each drop and vanished immediately. The flask was then removed from the cooling bath, and the yellow mixture was stirred at rt for 1 h. TLC control (SiO_2 / 10% ethyl acetate in hexane): R_f (product) = 0.22 (purple with NaOH). The mixture was diluted with cold water (30 mL), extracted with CH_2Cl_2 (3×20 mL), the combined extracts were washed with brine and dried over Na_2SO_4. The product was isolated by flash column chromatography (Sepacore Silica HP 12 g; gradient 0% to 20% ethyl acetate – pentane) and pure fraction were evaporated to give **19c** as white foam, yield 153 mg (90%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.21 (dd, *J* = 8.0, 1.3 Hz, 1H), 8.04 (dd, *J* = 8.0, 0.8 Hz, 1H), 7.91 (t, *J* = 1.0 Hz, 1H), 7.57 (d, *J* = 2.6 Hz, 2H), 7.28 (d, *J* = 8.9 Hz, 2H), 7.21 (dd, *J* = 8.9, 2.7 Hz, 2H), 1.58 (s, 9H), 0.81 (s, 3H), 0.71 (s, 3H). ^{19}F NMR (376 MHz, $CDCl_3$): δ -72.77. ESI-MS, positive mode: *m/z* (rel. int., %) = 739.3 (100) $[M+H]^+$.

Compound 25. A mixture of **19c** (150 mg, 0.2 mmol), 3-(*tert*-butyldimethylsilyloxy)azetidine **22** (112 mg, 0.6 mmol, 3 eq), $Pd_2(dba)_3$ (18 mg, 0.02 mmol, 10 mol%), XPhos (29 mg, 0.06 mmol, 30 mol%) and K_2CO_3 (83 mg, 0.6 mmol, 3 eq) in dry dioxane (2 mL) was degassed on a Schlenk line and stirred at 100 °C under argon (bath temperature) in a sealed flask for 3 h. TLC control (SiO_2 / pentane – CH_2Cl_2 – ethyl acetate 70:25:5, stained by heating): R_f (product) = 0.50 (blue), R_f (starting material) = 0.61 (colorless). Upon cooling, the reaction mixture was diluted with water (20 mL), extracted with CH_2Cl_2 (3×20 mL), the combined organic layers were washed with brine and dried over Na_2SO_4. The filtrate was evaporated on Celite, and the product was isolated by flash column chromatography (Interchim Puriflash 15 μm 25 g; gradient 0% to 100% A:B, A = hexane – CH_2Cl_2 – ethyl acetate 70:25:10, B = hexane – CH_2Cl_2 70:25) and freeze-dried from 1,4-dioxane to yield 145 mg (89%) of **25** as light yellow fluffy solid.

Flash LC trace:

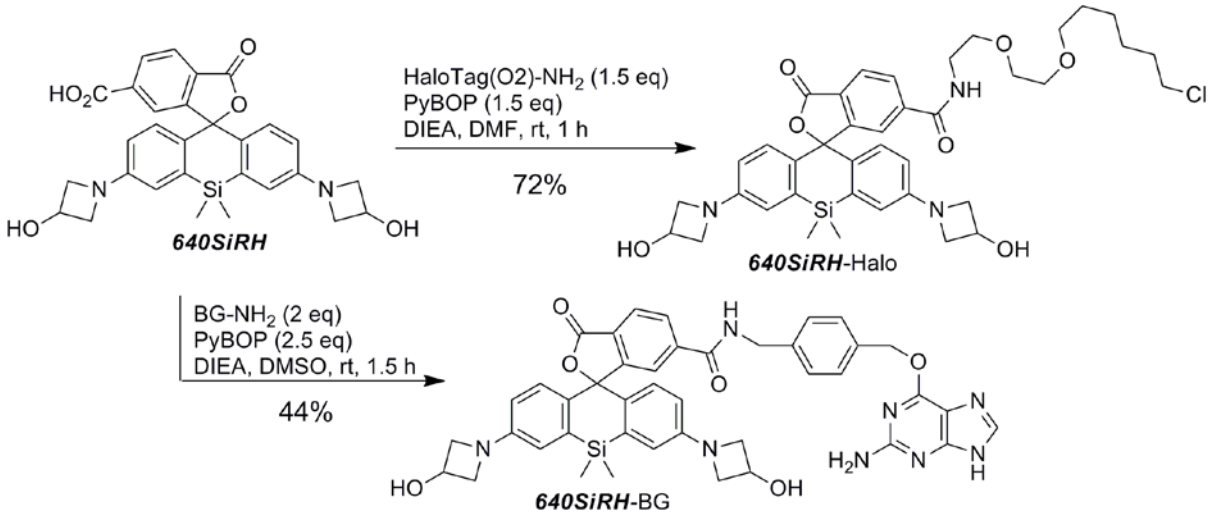


^{1}H NMR (400 MHz, $CDCl_3$): δ 8.11 (dd, *J* = 8.0, 1.3 Hz, 1H), 7.96 (dd, *J* = 8.0, 0.7 Hz, 1H), 7.81 (s, 1H), 6.85 (d, *J* = 8.7 Hz, 2H), 6.69 (d, *J* = 2.6 Hz, 2H), 6.33 (dd, *J* = 8.8, 2.6 Hz, 2H), 4.78 – 4.69 (m, 2H), 4.19 – 4.12 (m, 4H), 3.69 – 3.62 (m, 4H), 1.55 (s, 9H), 0.89 (s, 18H), 0.66 (s, 3H), 0.58 (s, 3H), 0.07 (s, 12H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 170.3, 164.4, 155.4, 150.7, 137.3, 136.1, 132.7, 129.9, 129.0, 127.7, 125.7, 125.1, 116.3, 113.3, 82.3, 62.5, 62.1, 28.2, 25.9, 18.1, 0.2, -0.6, -4.8. ESI-MS, positive mode: *m/z* (rel. int., %) = 813.4 (100) $[M+H]^+$. HR-MS (ESI, positive mode): 813.4120 $[M+H]^+$ (found), 813.4145 (calculated for $C_{45}H_{65}N_2O_6Si_3$, $[M+H]^+$).

Compound 26 (*640SiRH t*-butyl ester). Tetrabutylammonium fluoride trihydrate (142 mg, 0.45 mmol, 3 eq), dissolved in THF (10 mL), was added a solution of **25** (122 mg, 0.15 mmol) in THF (15 mL), cooled in ice-water bath. The reaction mixture was stirred at 0 °C for 1 h. Water (20 mL), acetic acid (1.5 mL) and brine (20 mL) were then added, and the mixture was extracted with ethyl acetate (3×20 mL), the combined organic layers were dried over Na_2SO_4, filtered, evaporated and re-evaporated twice with ethyl acetate (2×20 mL). The product was isolated by flash column chromatography (13 g silica; gradient 10% to 80% ethyl acetate – CH_2Cl_2) and freeze-dried from 1,4-dioxane to give **26** as light green fluffy solid. Yield 84 mg (96%). ^{1}H NMR (400 MHz, CD_3OD): δ 8.12 (dd, *J* = 8.0, 1.3 Hz, 1H), 7.97 (dd, *J* = 8.1, 0.7 Hz, 1H), 7.69 (t, *J* = 1.0 Hz, 1H), 6.77 (d, *J* = 2.8 Hz, 2H), 6.76 (d, *J* = 8.9 Hz, 2H), 6.35 (dd, *J* = 8.9, 2.8, 2H), 4.63 (tt, *J* = 6.5, 4.8 Hz, 2H), 4.17 – 4.09 (m, 4H), 3.64 – 3.57 (m, 4H), 1.51 (d, *J* = 0.8 Hz, 9H), 0.64 (s, 3H), 0.54 (s, 3H). ^{13}C NMR (101 MHz, CD_3OD): δ 172.0, 165.4, 157.4, 152.4, 138.7, 136.9, 133.4, 130.9, 129.7, 128.5, 126.7, 125.6, 117.3, 114.6, 93.2, 83.5, 68.1, 63.0, 62.5, 28.3, -0.1, -0.5. ESI-MS, positive mode: *m/z* (rel. int., %) = 585.3 (100) $[M+H]^+$. HR-MS (ESI, positive mode): 585.2429 $[M+H]^+$ (found), 585.2415 (calculated for $C_{33}H_{37}N_2O_6Si$, $[M+H]^+$).

Dye *640SiRH*. Trifluoroacetic acid (2.8 mL) was added to a solution of **26** (84 mg, 144 µmol) in CH_2Cl_2 (14 mL), cooled in ice-water bath. The cooling bath was removed, and the reaction mixture was stirred at rt for 6 h. The solvents were evaporated, and the residue was re-evaporated twice with toluene (2×20 mL) and freeze-dried from 1,4-dioxane, giving 101 mg of impure material. The product was isolated by chromatography on a reversed phase (RP-C_{18}

10 g; gradient 67% to 33% water – acetonitrile). Fractions containing the product were evaporated and freeze-dried from 1,4-dioxane to give 70 mg (92%) of ***640SiRH*** as blue fluffy solid (free base). HPLC (10/90−100/0 over 25 min, column 4×250 mm, 1.2 mL/min, detection at 254 nm): τ = 9.98 min. For comparison with non-hydroxylated *SiR*[9] dye, HPLC was performed with identical gradient (20/80−100/0 over 15 min, column 4×150 mm, 1.2 mL/min, detection at 630 nm):): τ = 7.2 min (***640SiRH***), 8.8 min (*SiR*). UV-Vis (PBS 7.4): λ_{max} (ε) = 641 nm (51000 $M^{-1}cm^{-1}$); fluorescence (PBS 7.4): λ_{excit} = 610 nm, λ_{em} = 662 nm; Φ_{fl} (relative to Oxazine 1, Φ_{fl} = 0.14 in ethanol) = 0.42. ^{1}H NMR (400 MHz, CD_3OD): δ 8.22 (dd, *J* = 7.9, 1.3 Hz, 1H), 8.00 (d, *J* = 8.0 Hz, 1H), 7.83 (d, *J* = 1.0 Hz, 1H), 6.78 (d, *J* = 2.6 Hz, 2H), 6.74 (d, *J* = 8.7 Hz, 2H), 6.39 (dd, *J* = 8.7, 2.7 Hz, 2H), 4.66 (tt, *J* = 6.4, 4.8 Hz, 2H), 4.16 (ddd, *J* = 7.4, 6.4, 1.0 Hz, 4H), 3.66 – 3.60 (m, 4H), 0.64 (s, 3H), 0.54 (s, 3H). ^{13}C NMR (101 MHz, CD_3OD): δ 171.7, 156.3, 152.5, 137.8, 133.5, 131.3, 130.5, 128.9, 126.8, 126.6, 117.5, 114.3, 63.0, 62.5, 0.1, -1.2. ESI-MS, positive mode: *m/z* (rel. int., %) = 529.2 (100) $[M+H]^+$. HR-MS (ESI, positive mode): 529.1787 (found), 529.1789 (calculated for $C_{29}H_{29}N_2O_6Si$, $[M+H]^+$).



Scheme S27. Preparation of the HaloTag and SNAP-tag ligand derivatives of ***640SiRH*** used as labeling reagents.

Compound *640SiRH*-Halo. PyBOP (20 µL of 22 mg/100 µL stock solution in DMF, 8.52 µmol, 1.5 equiv) was added to a solution of ***640SiRH*** (3 mg, 5.68 µmol), HaloTag Amine (O2) (2-(2-((6-chlorohexyl)oxy)ethoxy)ethanamine; 1.9 mg, 8.52 µmol, 1.5 equiv) and DIEA (*N*-ethyldiisopropylamine; 10 µL) in DMF (100 µL). After 1 h, the solvent was evaporated *in vacuo* at rt, and the product ***640SiRH*-Halo** was isolated by preparative TLC (silica, 10% methanol – CH_2Cl_2, then 100% CH_2Cl_2). Yield 3.0 mg (72%), purity (HPLC) ~96%. HPLC (10/90−100/0 over 25 min, column 4×250 mm, 1.2 mL/min, detection at 254 nm): t_R= 15.0 min. ESI-MS, positive mode: *m/z* (rel. int., %) = 734.3 $[M+H]^+$. HR-MS (ESI, positive mode): 734.3011 (found), 734.3023 (calculated for $C_{39}H_{49}ClSiN_3O_7$, $[M+H]^+$).

Compound *640SiRH*-BG. PyBOP (20 µL of 37 mg/100 µL stock solution in DMSO, 14.2 µmol, 2.5 equiv) was added to a solution of ***640SiRH*** (3 mg, 5.68 µmol), BG-NH_2 (6-[4-(aminomethyl)benzyloxy]-9*H*-purin-2-amine; 3 mg, 11.4 µmol, 2 eq) and DIEA (*N*-ethyldiisopropylamine; 10 µL) in DMSO (100 µL). After 1.5 h, the solvent was evaporated *in vacuo* at rt, and the product was isolated by preparative TLC (silica, 10% methanol – CH_2Cl_2). The resulting impure material was further purified by chromatography on a reversed

phase (RP-C_{18} 4 g; isocratic 50% water – acetonitrile) and lyophilized from dioxane to give 1.96 mg (44%) of ***640SiRH*-BG** as fluffy turquoise solid; purity (HPLC) ~95%. HPLC (10/90−100/0 over 25 min, column 4×250 mm, 1.2 mL/min, detection at 254 nm): t_R = 10.4 min. ESI-MS, positive mode: *m/z* (rel. int., %) = 781.3 $[M+H]^+$. HR-MS (ESI, positive mode): 781.2903 (found), 781.2913 (calculated for $C_{42}H_{41}SiN_8O_6$, $[M+H]^+$).

***610CP*-BG ligand**

Compound *610CP*-BG. PyBOP (29 µL of 20 mg/100 µL stock solution in DMSO, 10.95 µmol, 2.5 equiv) was added to a solution of dye ***610CP***[1] (2 mg, 4.38 µmol), BG-NH_2 (6-[4-(aminomethyl)benzyloxy]-9*H*-purin-2-amine; 2.4 mg, 8.89 µmol, 2 eq) and DIEA (*N*-ethyldiisopropylamine; 11 µL) in DMSO (100 µL). After 2 h, the solvent was evaporated *in vacuo* at rt, and the product was isolated by preparative TLC (silica, 15% methanol – CH_2Cl_2). Yield 1.5 mg (48%) of ***610CP*-BG** as fluffy blue solid; purity (HPLC) ~95%. HPLC (30/70−100/0 over 25 min, column 4×250 mm, 1.2 mL/min, detection at 254 nm): t_R = 6.81 min. ESI-MS, positive mode: *m/z* (rel. int., %) = 709.4 $[M+H]^+$. HR-MS (ESI, positive mode): 709.3237 (found), 709.3245 (calculated for $C_{41}H_{41}N_8O_4$, $[M+H]^+$).

References

[1] A. N. Butkevich, G. Y. Mitronova, S. C. Sidenstein, J. L. Klocke, D. Kamin, D. N. H. Meineke, E. D'Este, P.-T. Kraemer, J. G. Danzl, V. N. Belov, S. W. Hell, *Angew. Chem. Int. Ed.* **2016**, *55*, 3290–3294.

[2] S. Fleming, A. Mills, T. Tuttle, *Beilstein J. Org. Chem.,* **2011**, *7*, 432–441.

[3] M. S. Hossain, C. Q. Le, E. Joseph, T. Q. Nguyen, K. Johnson-Winters, F. W. Foss Jr. *Org. Biomol. Chem.* **2015**, *13*, 5082.

[4] a) T. Umemoto, Y. Gotoh, *J. Fluorine Chem.* **1986**, *31*, 231–236; b) G. L. Tolnai, A. Székely, Z. Makó, T. Gáti, J. Daru, T. Bihari, A. Stirling, Z. Novák, *Chem. Commun.* **2015**, *51*, 4488–4491.

[5] G. Mudd, I. Pérez Pi, N. Fethers, P. G. Dodd, O. R. Barbeau, M. Auer, *Methods Appl. Fluoresc.* **2015**, *3*, 045002.

[6] S. M. Pauff, S. C. Miller, *Org. Lett.* **2011**, *13*, 6196–6199.

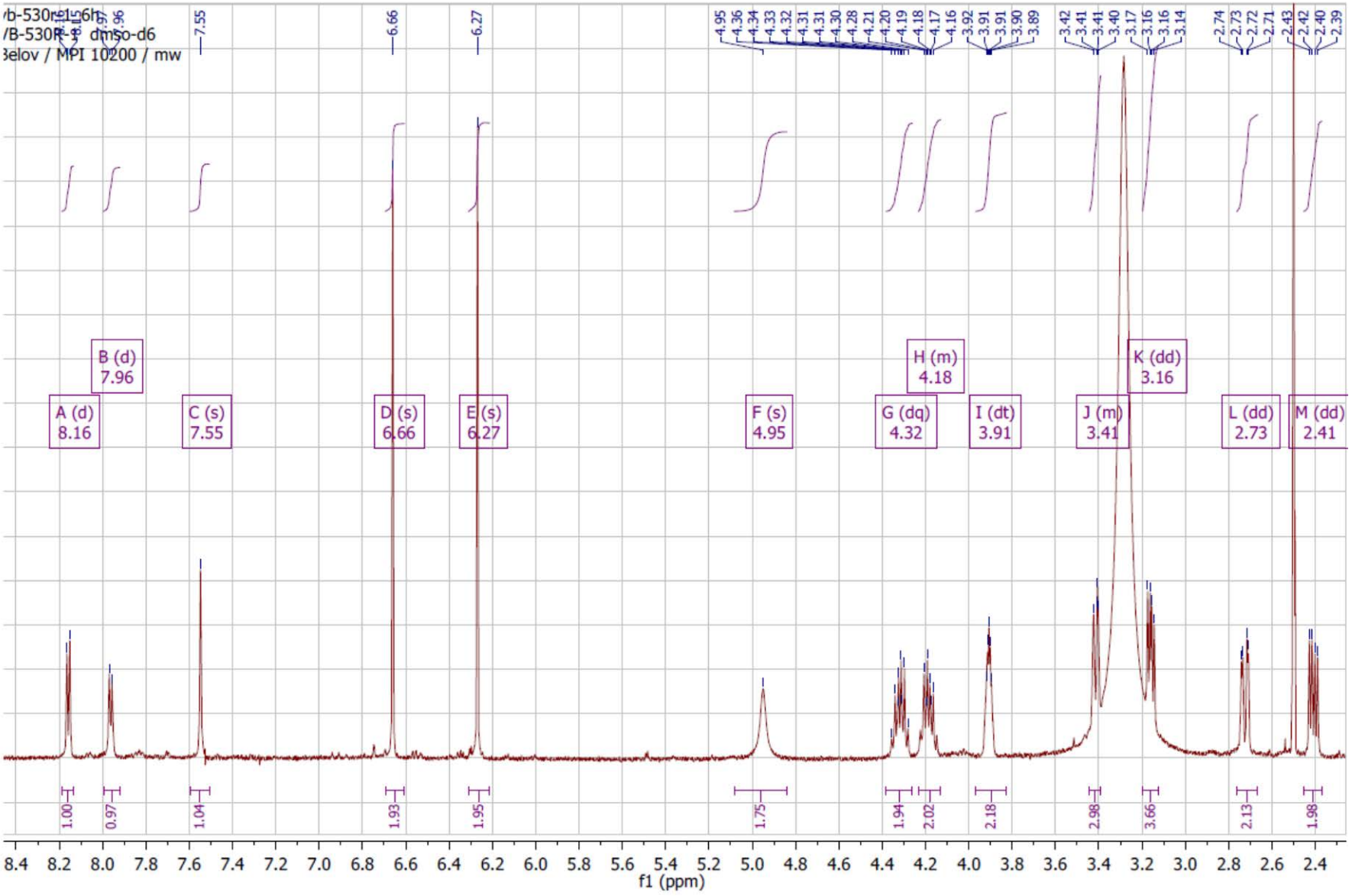
[7] K. Kolmakov, E. Hebisch, T. Wolfram, L. A. Nordwig, C A. Wurm, H. Ta, V. Westphal, V. N. Belov, S. W. Hell, *Chem. Eur. J.* **2015**, *21*, 13344–13356.

[8] G. Lukinavičius, L. Reymond, E. D'Este, A. Masharina, F. Göttfert, H. Ta, A. Güther, M. Fournier, S. Rizzo, H. Waldmann, C. Blaukopf, C. Sommer, D. W. Gerlich, H.-D. Arndt, S. W. Hell, K. Johnsson, *Nat. Methods* **2014**, *11*, 731–733.

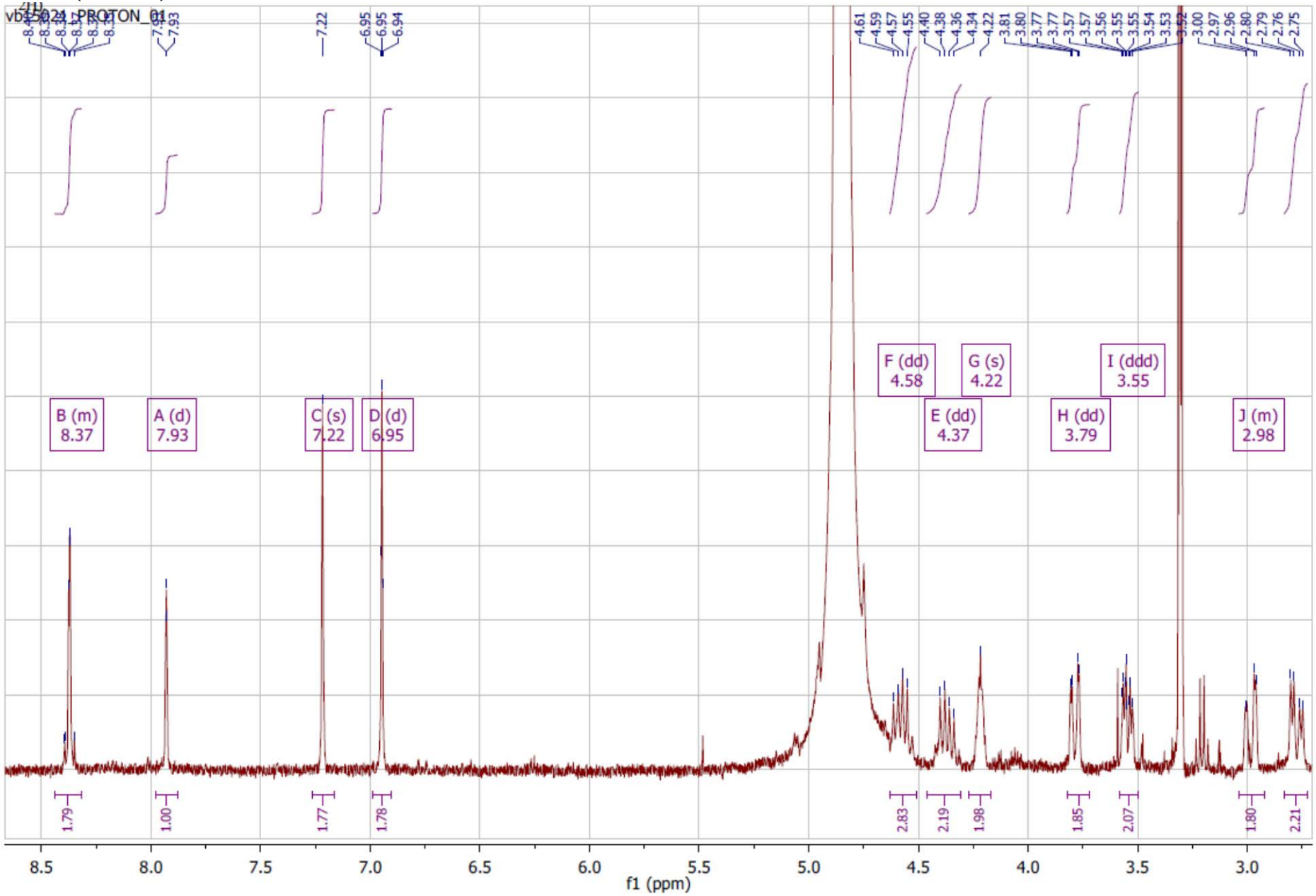
[9] G. Lukinavičius, K. Umezawa, N. Olivier, A. Honigmann, G. Yang, T. Plass, V. Mueller, L. Reymond, I. R. Corrêa, Jr., Z. G. Luo, C. Schultz, E. A. Lemke, P. Heppenstall, C. Eggeling, S. Manley, K. Johnsson, *Nat. Chem.* **2013**, *5*, 132–139.

Selected NMR spectra

530RH (isomer 1)

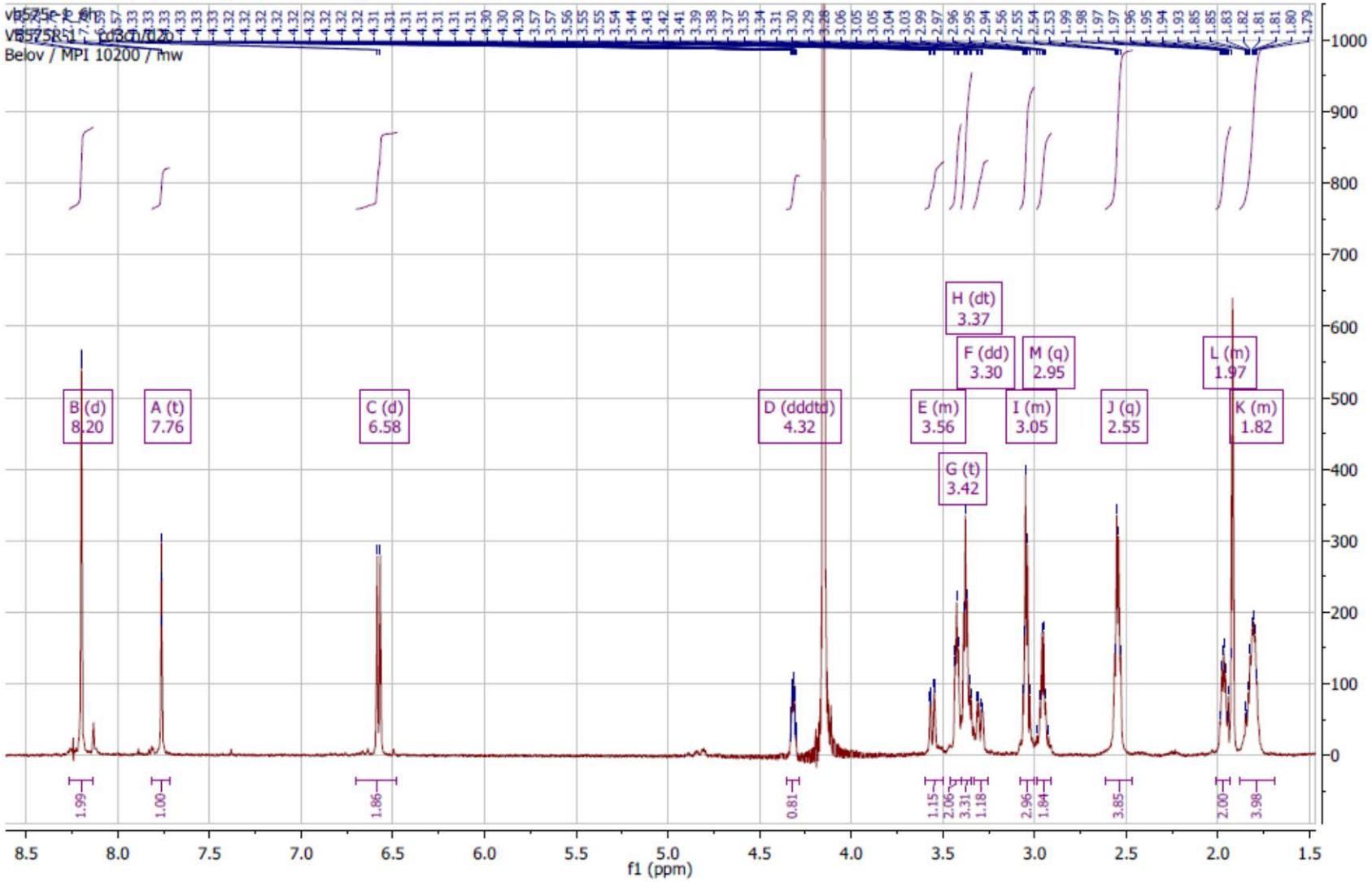


530RH (isomer 3)



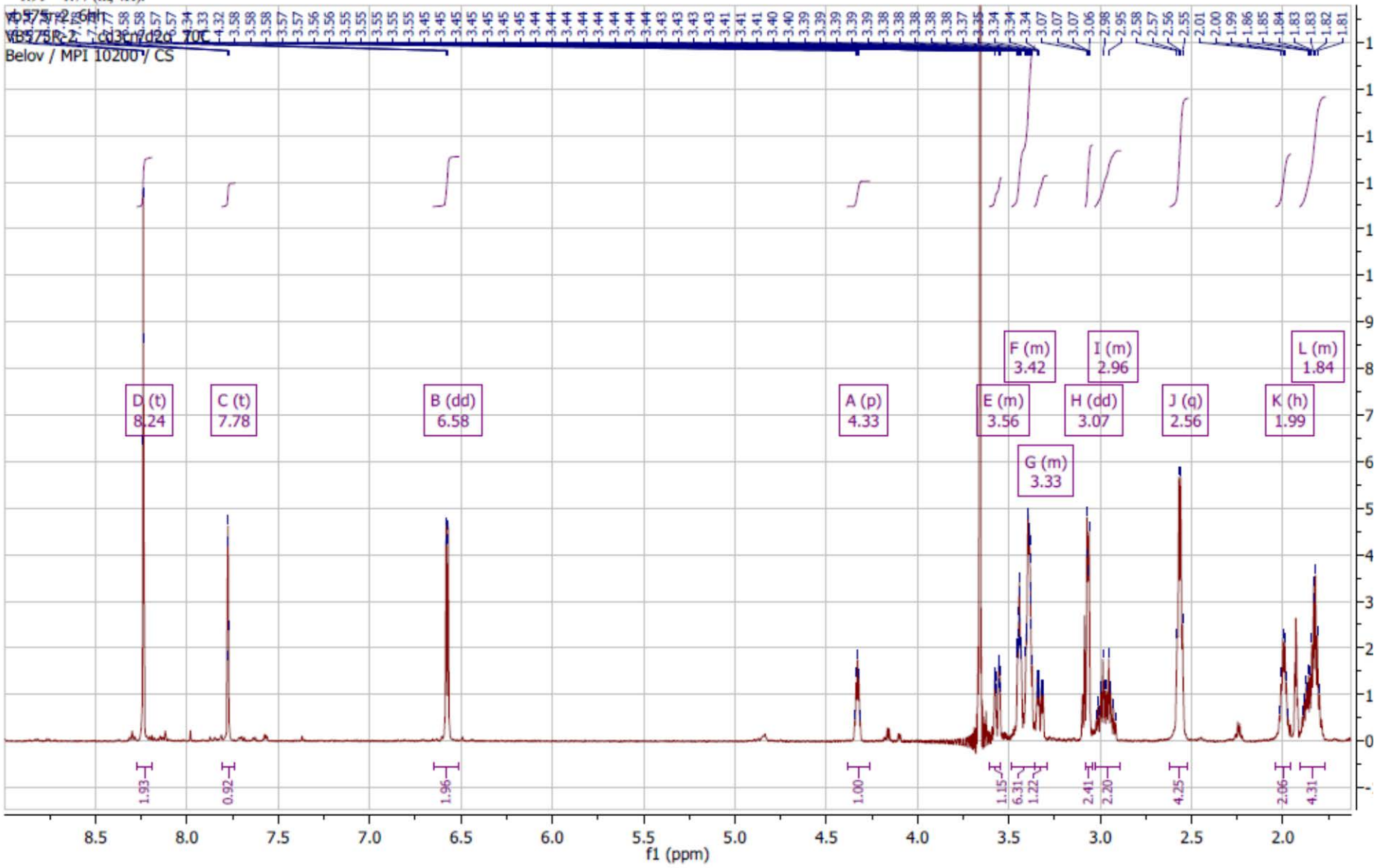
575RH (isomer 1)

^{1}H NMR (600 MHz, Acetonitrile-d_3) δ 8.20 (d, J = 1.3 Hz, 2H), 7.76 (t, J = 1.1 Hz, 1H), 6.58 (d, J = 10.0 Hz, 2H), 4.32 (dddtd, J = 6.6, 4.4, 2.9, 1.4, 0.7 Hz, 1H), 3.60 – 3.50 (m, 1H), 3.42 (t, J = 5.7 Hz, 2H), 3.37 (dt, J = 13.6, 5.9 Hz, 3H), 3.30 (dd, J = 13.7, 4.9 Hz, 1H), 3.08 – 3.00 (m, 3H), 2.95 (q, J = 6.1 Hz, 2H), 2.55 (q, J = 6.7 Hz, 4H), 2.01 – 1.93 (m, 2H), 1.88 – 1.69 (m, 4H).

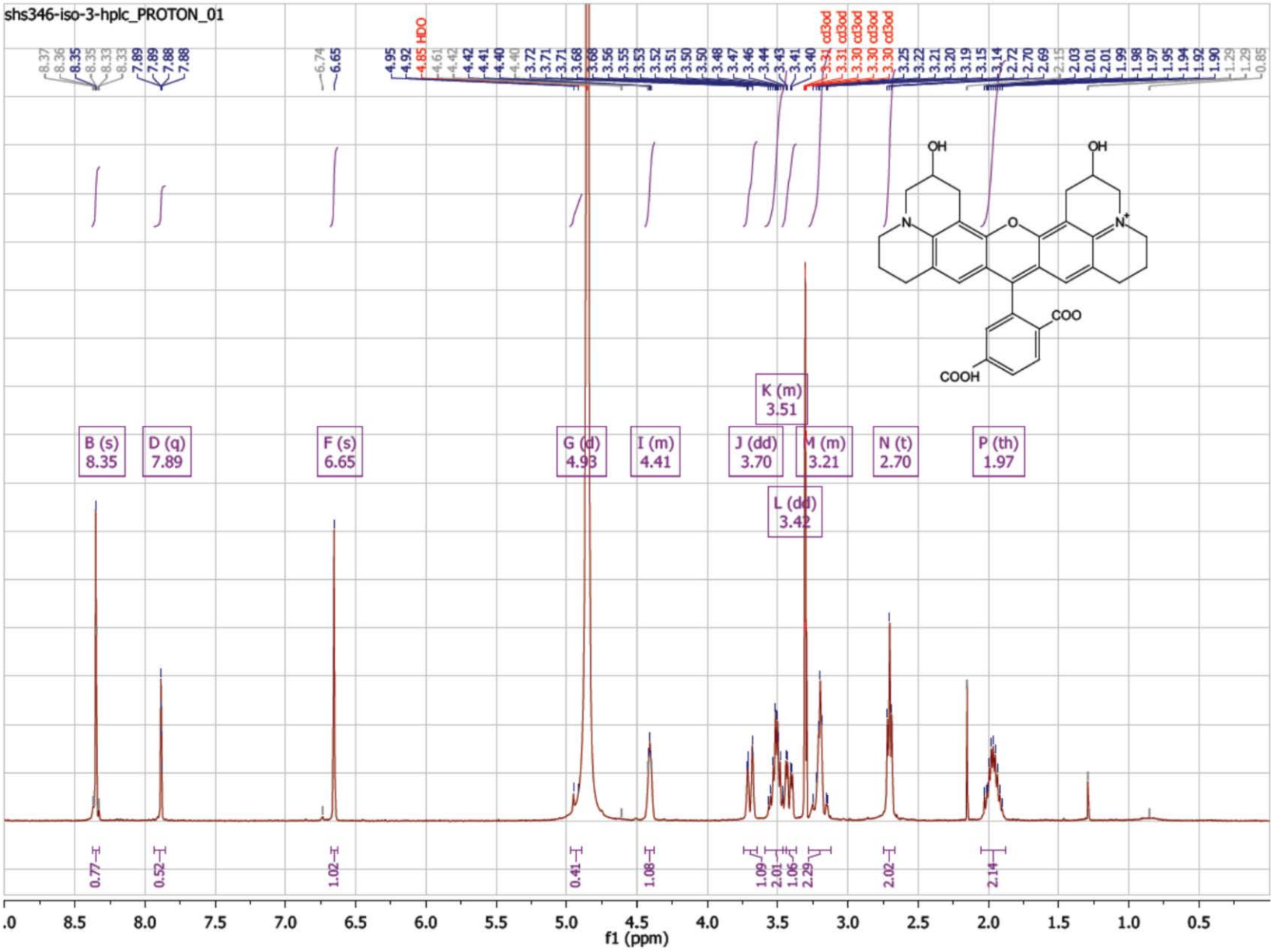


575RH (isomer 2)

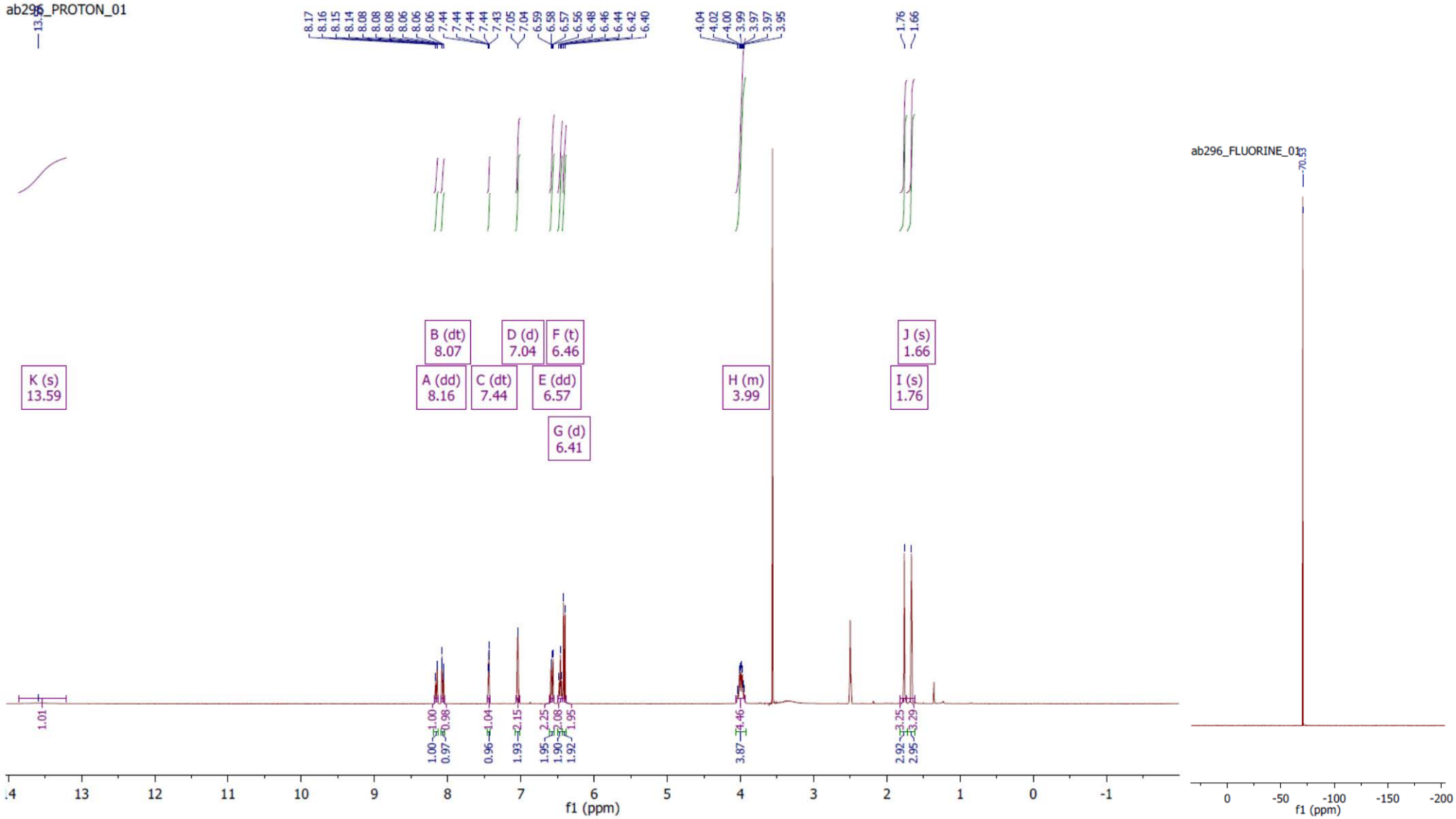
^{1}H NMR (600 MHz, Acetonitrile-d_3) δ 8.24 (t, J = 1.0 Hz, 2H), 7.78 (t, J = 1.2 Hz, 1H), 6.58 (dd, J = 6.5, 1.2 Hz, 2H), 4.33 (p, J = 4.6 Hz, 1H), 3.61 – 3.54 (m, 1H), 3.48 – 3.36 (m, 6H), 3.36 – 3.29 (m, 1H), 3.07 (dd, J = 5.8, 2.2 Hz, 2H), 3.03 – 2.89 (m, 2H), 2.56 (q, J = 6.1 Hz, 4H), 1.99 (h, J = 5.9 Hz, 2H), 1.91 – 1.77 (m, 4H).



Dye **13b** (major diastereoisomer)



560CP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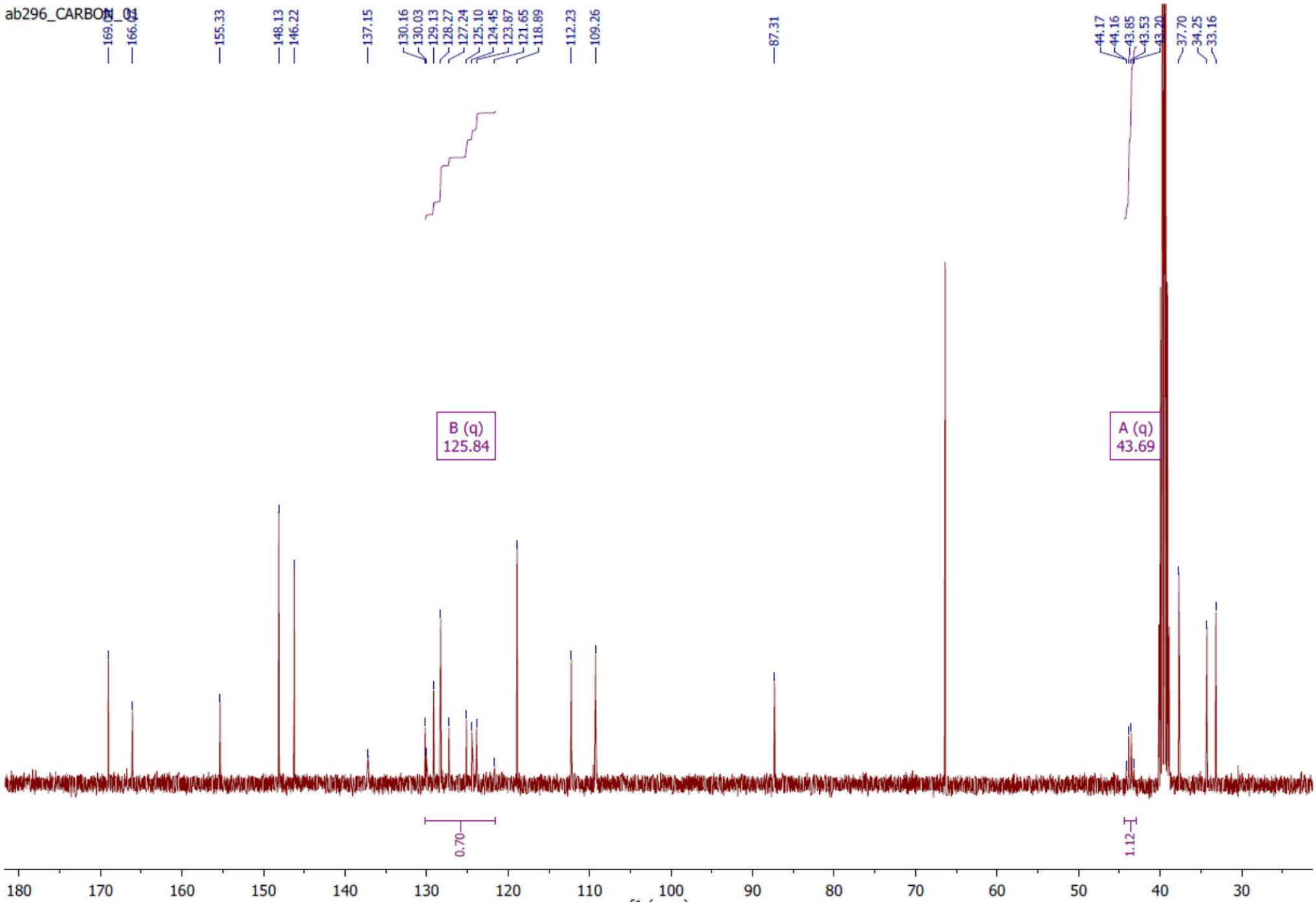
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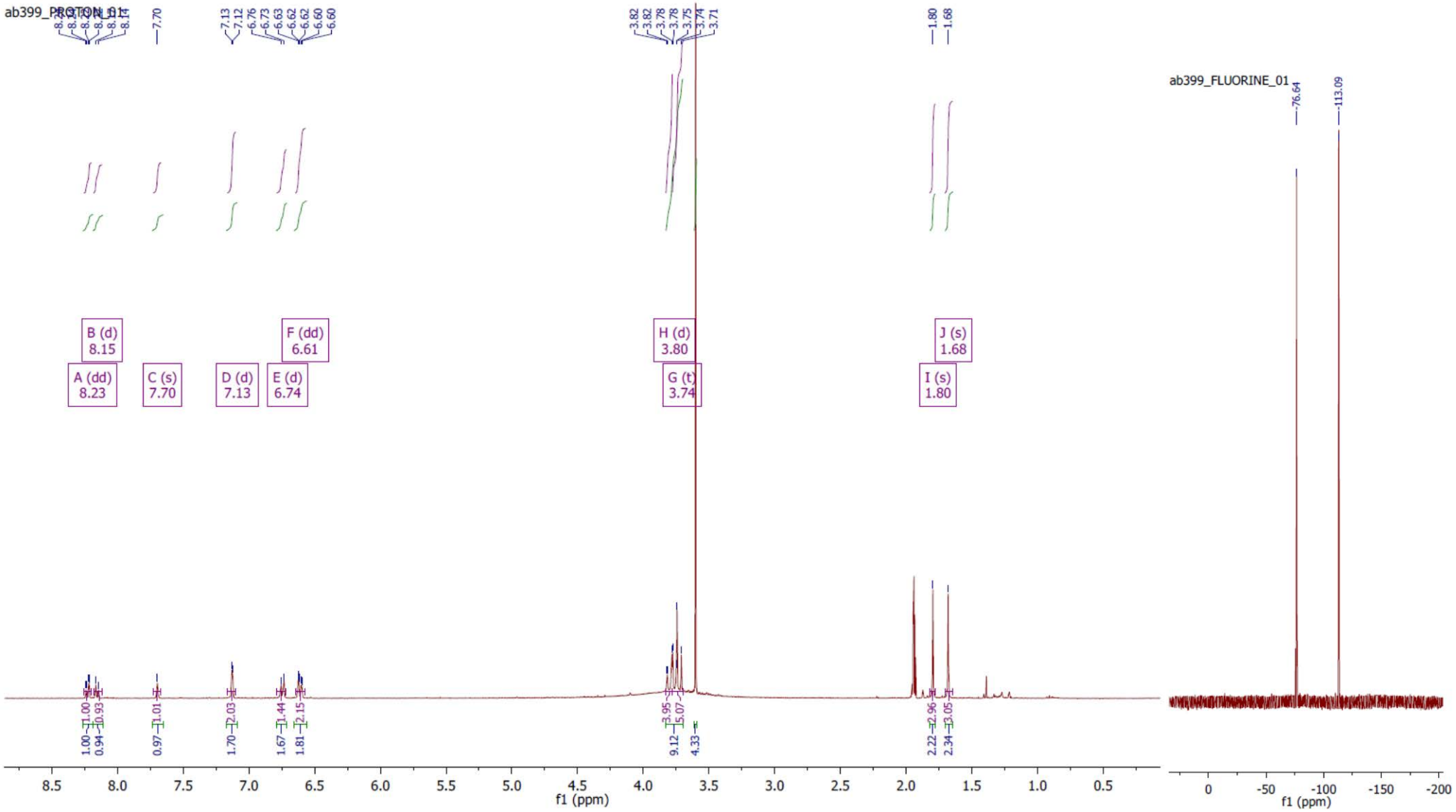
570CPH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G (t) 3.74
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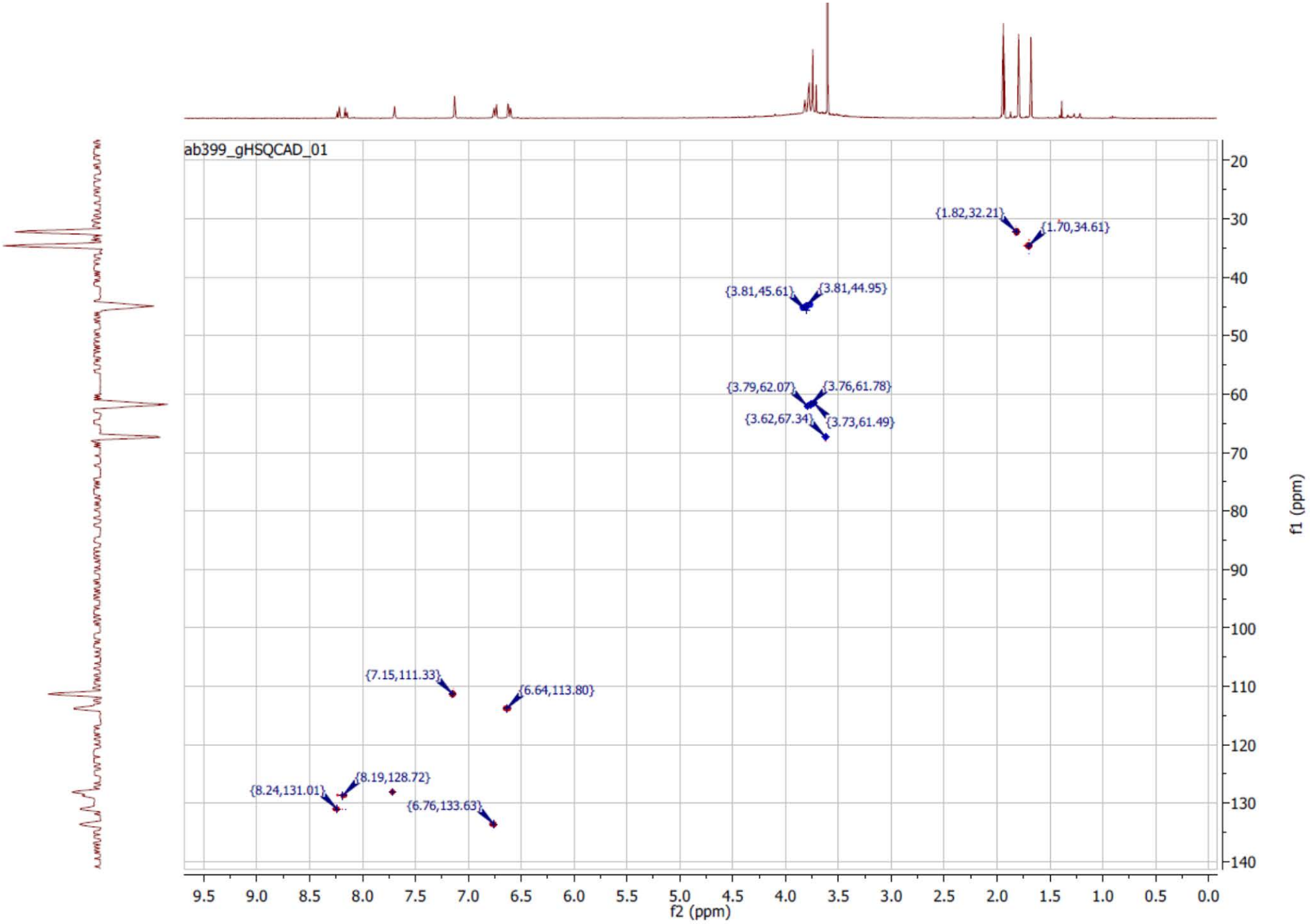
I (s) 1.80
f1 (ppm)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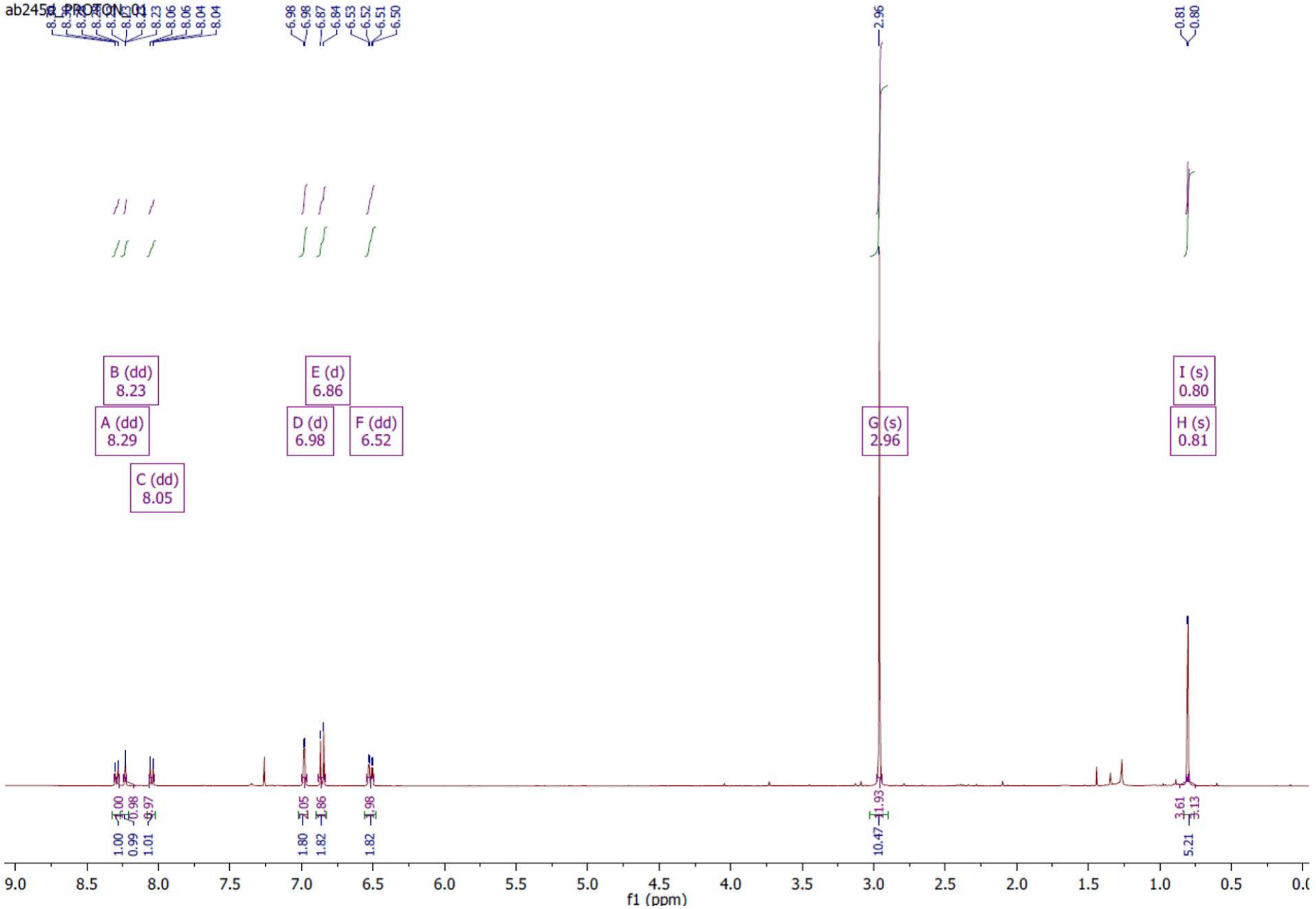
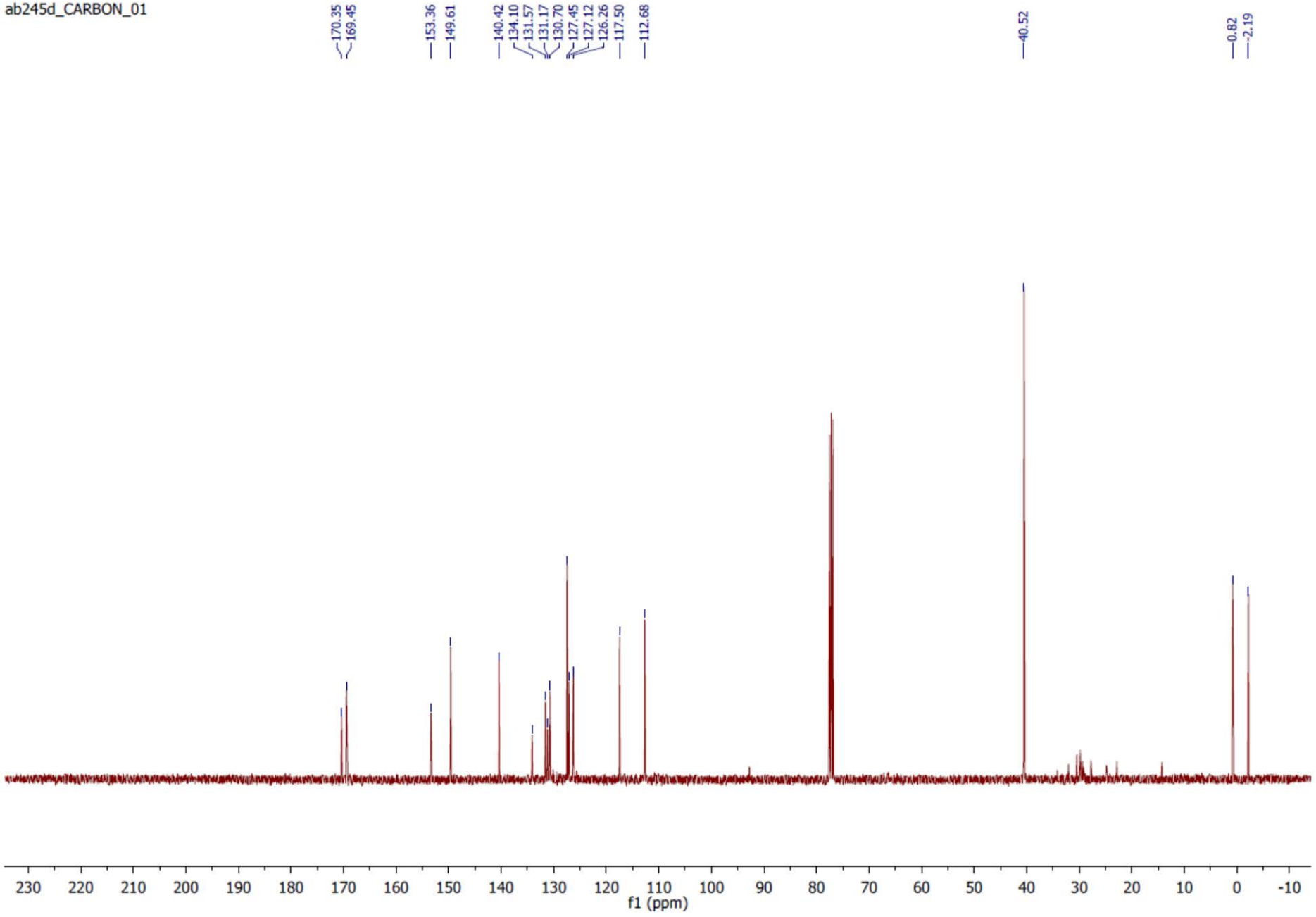
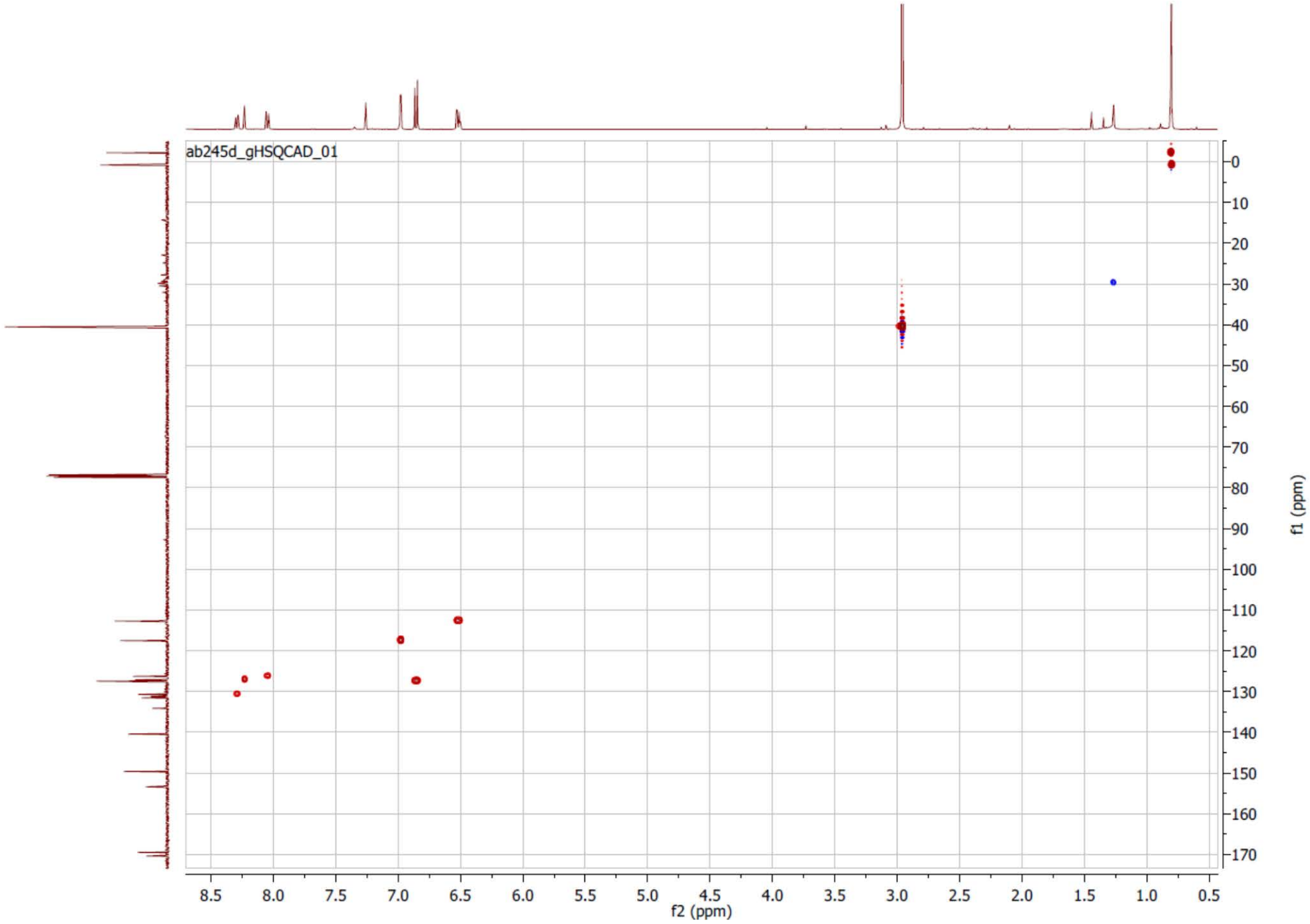
f2 (ppm)
f1 (ppm)

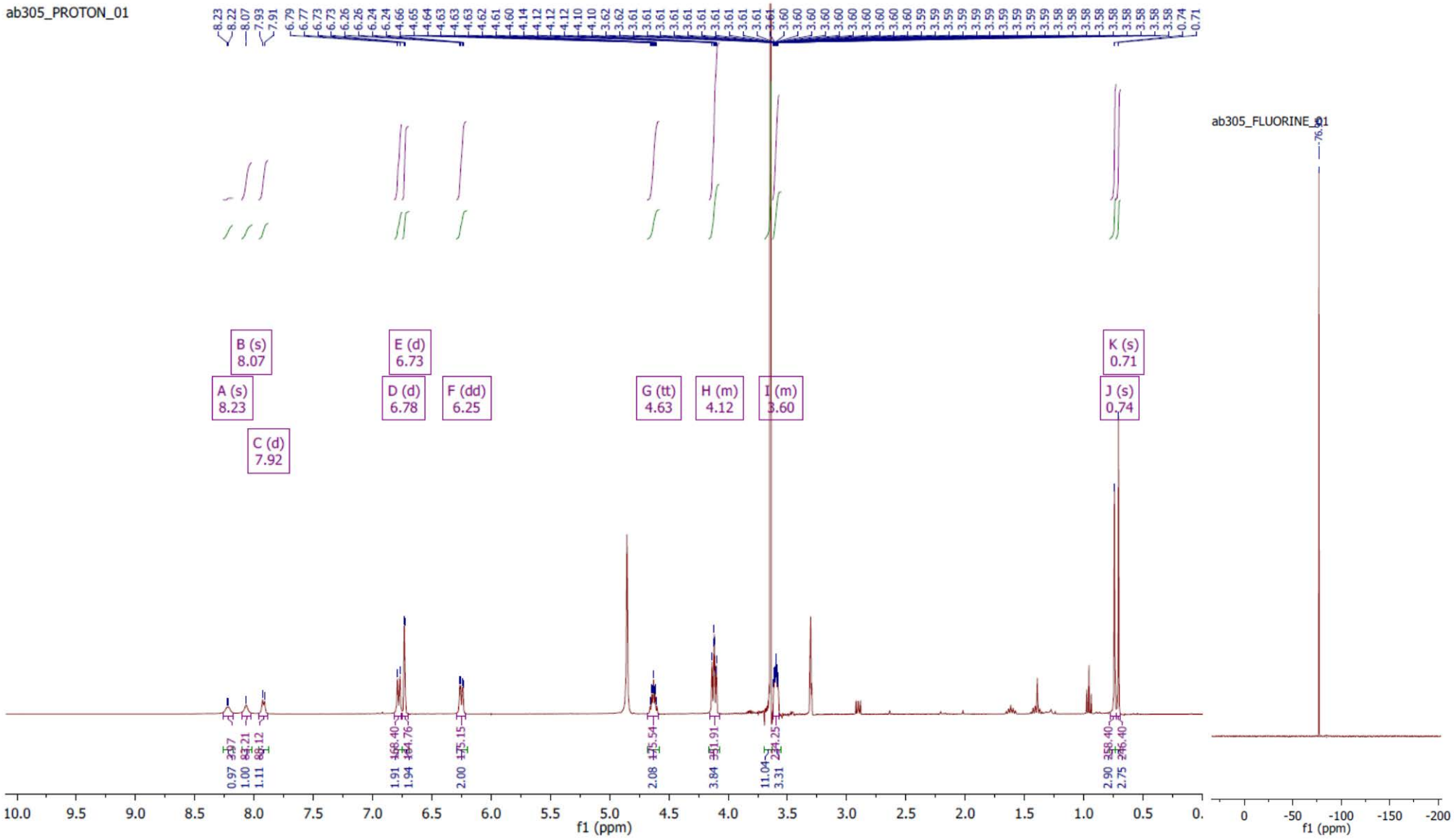
GeR

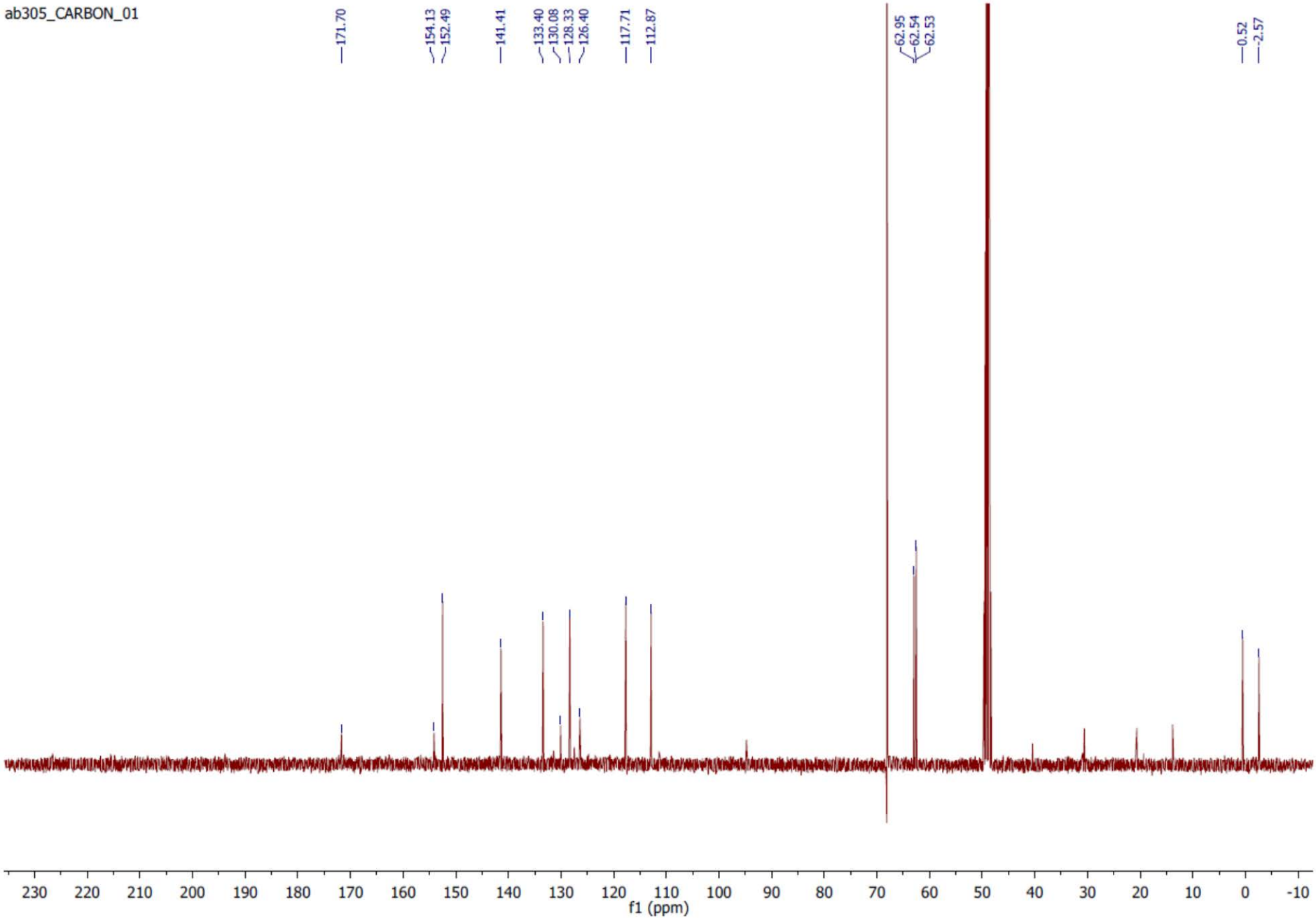

B (dd)
8.23
A (dd)
8.29
C (dd)
8.05
E (d)
6.86
D (d)
6.98
F (dd)
6.52
G (s)
2.96
I (s)
0.80
H (s)
0.81
f1 (ppm)


ab245d_CARBON_01
170.35
169.45
153.36
149.61
140.42
134.10
131.57
131.17
130.70
127.45
127.12
126.26
117.50
112.68
40.52
0.82
-2.19
230 220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10
f1 (ppm)

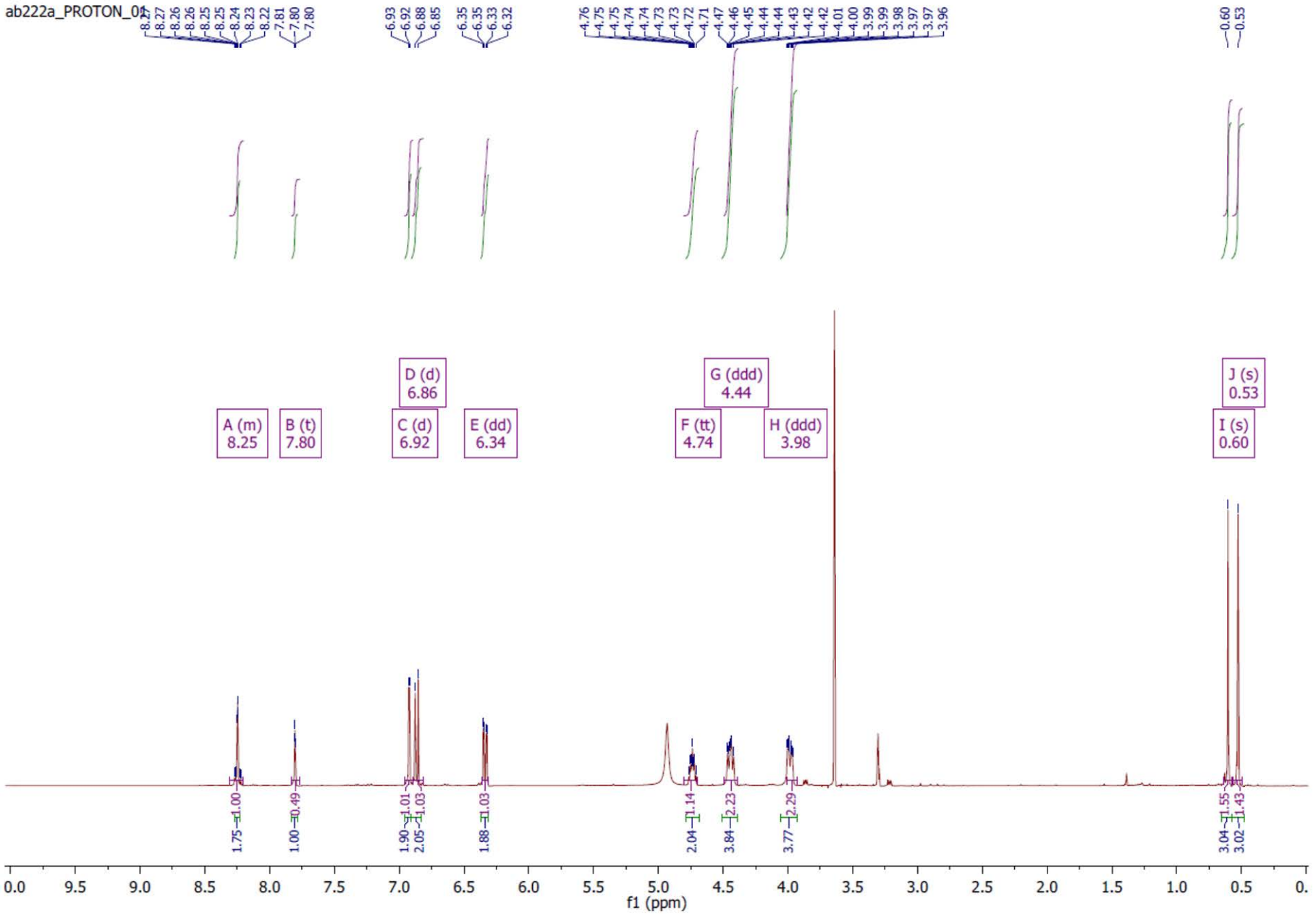

ab245d_gHSQCAD_01
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
f2 (ppm)
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
f1 (ppm)

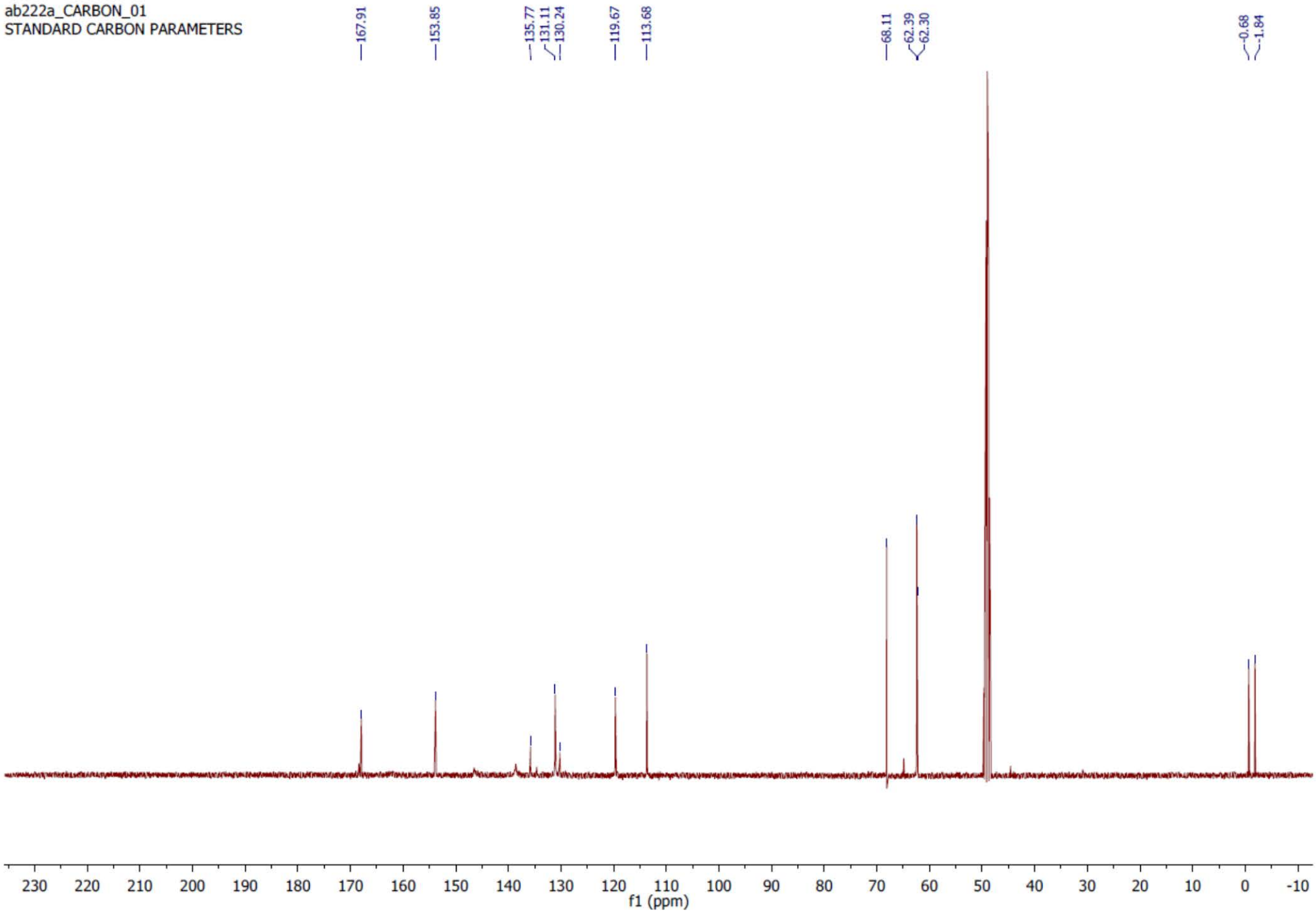
630GeRH


ab305_PROTON_01
B (s) 8.07
A (s) 8.23
C (d) 7.92
E (d) 6.73
D (d) 6.78
F (dd) 6.25
G (tt) 4.63
H (m) 4.12
I (m) 3.60
K (s) 0.71
J (s) 0.74
f1 (ppm)
ab305_FLUORINE_01
f1 (ppm)


ab305_CARBON_01
171.70
154.13
152.49
141.41
133.40
130.08
128.33
126.40
117.71
112.87
62.95
62.54
62.53
0.52
-2.57
230 220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10
f1 (ppm)

640SiRH


ab222a_PROTON_01
A (m) 8.25
B (t) 7.80
C (d) 6.92
D (d) 6.86
E (dd) 6.34
F (tt) 4.74
G (ddd) 4.44
H (ddd) 3.98
I (s) 0.60
J (s) 0.53
f1 (ppm)


ab222a_CARBON_01
STANDARD CARBON PARAMETERS
167.91
153.85
135.77
131.11
130.24
119.67
113.68
68.11
62.39
62.30
0.68
-1.84
230 220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10
f1 (ppm)