SUPPORTING INFORMATION

Rational design of fluorogenic and spontaneously blinking labels for super-resolution imaging

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Figure S1 (a) Absorption at $\lambda_{abs}$ for dyes 1 and 2 in 20 mM sodium phosphate buffer, pH 7.0 containing 5 mg/mL detergent, error bars show ± s.e.m.. (b,c) Absorption spectra for dyes 1 (b) and 2 (c) near $\lambda_{abs}$ in the presence (dashed line) or absence (solid line) of SDS. (d) Chemical structures and properties of HaloTag ligands $1_{HTL}$–$10_{HTL}$; $\Delta A/A_0$ indicates the change in absorption ($\Delta A$) and $\Delta F/F_0$ indicates the change in fluorescence ($\Delta F$) of the HaloTag ligands upon binding purified HaloTag protein divided by the basal absorption or fluorescence of the free ligand ($F_0$ or $A_0$). (e) $\Delta A/A_0$ of the HaloTag ligands $1_{HTL}$–$10_{HTL}$ vs. $K_L$ of the corresponding free dyes 1–10; the solid line indicates a linear fit ($R^2 = 0.93$). (f) $\Delta F/F_0$ vs. $\Delta A/A_0$ for the HaloTag ligands $1_{HTL}$–$10_{HTL}$; the solid line indicates a linear fit ($R^2 = 0.87$). (g) Normalized absorption (abs, solid line), normalized fluorescence excitation (ex, dashed line), and (h) normalized fluorescence emission (em) spectra for dye 8 in 10 mM HEPES, pH 7.3. (i) Normalized abs (solid line), normalized ex (dashed line), and (j) normalized em spectra for dyes 7 and 10 in 10 mM HEPES, pH 7.3; note: the normalized absorption spectra of dyes 8 and 10 exhibit higher noise due to relatively low visible absorption in aqueous buffer.
Figure S2 (a,b) Absorption spectra of HaloTag ligands 7HTL (a) and 10HTL (b) in the presence or absence of excess HaloTag protein (HT). (c) Absorption spectra of JF526–Hoechst conjugate (10HST, 5 μM) in PBS, pH 7.4 alone, with polyA-polyT DNA (DNAAT, 50 μM), or polyG-polyC DNA (DNAGC, 50 μM). (d) Fluorescence excitation and emission spectra of JF526–Hoechst conjugate (10HST, 500 nM) in PBS, pH 7.4 alone, with polyA-polyT DNA (DNAAT, 5 μM), or polyG-polyC DNA (DNAGC, 5 μM). (e) Chemical structures of ‘SiR–tubulin’ (1TXL) and JF525–Taxol (7TXL). (f–h) Normalized fluorescence excitation and emission spectra of 1TXL (f), 7TXL (g), or 10TXL (h) in the presence of polymerized tubulin (magenta or green) or bovine serum albumin (BSA; black). (i,j) Confocal image (i) and STED image (j) of live U2OS cells incubated with 10TXL (1 μM) and Verapamil (10 μM) for 1 h; scale bars: 5 μm. (k) Transverse profiles of fluorescence intensity corresponding to regions boxed in confocal (black) and STED (green). Solid lines indicate Gaussian fit; double-headed arrows and numbers indicate full width at half maximum (FWHM) determined by the Gaussian fit.
Scheme S1. Syntheses of JF526 (10) derivatives. (a) Synthesis of 10HTL and 10STL. (b) Synthesis of 10HST. (c) Synthesis of 10TXL. (d) Synthesis of 10PEP. (e) Synthesis of 7TXL.
SPECTROSCOPY AND FLUORESCENCE IMAGING METHODS

**UV-vis and fluorescence spectroscopy.** Fluorescent molecules for spectroscopy were prepared as stock solutions in DMSO and diluted such that the DMSO concentration did not exceed 1% v/v. Spectroscopy was performed using 1-cm path length, 3.5-mL quartz cuvettes or 1-cm path length, 1.0-ml quartz microcuvettes (Starna Cells). All measurements were taken at ambient temperature (22 ± 2 °C). Absorption spectra were recorded on a Cary Model 100 spectrometer (Agilent). Fluorescence spectra were recorded on a Cary Eclipse fluorometer (Varian). Maximum absorption wavelength (λ_{abs}), extinction coefficient (ε_{w}), and maximum emission wavelength (λ_{em}) were taken in 10 mM HEPES, pH 7.3 buffer unless otherwise noted; reported values for ε_{w} are averages (n = 3). Normalized spectra are shown for clarity.

**Determination K_{L-Z} and ε_{max}.** The lactone–zwitterion equilibrium constant (K_{L-Z}) was calculated as described previously\(^1,^2\) using equation 1:

\[
K_{L-Z} = (ε_{dw}/ε_{max})/(1 - ε_{dw}/ε_{max})
\]

where ε_{dw} is the extinction coefficient of the dyes in a 1:1 (v/v) dioxane:water solvent mixture containing 0.01% (v/v) triethylamine; the dioxane–water mixture was chosen to give a large range of K_{L-Z} values and the triethylamine additive ensures the rhodamines are in the net neutral form. The ε_{max} (maximal extinction coefficient) values were measured in 0.1% (v/v) trifluoroacetic acid (TFA) in 2,2,2-trifluoroethanol (TFE) for rhodamine 10, and 0.1% (v/v) TFA in ethanol for the Si-rhodamine 8.

**Quantum yield determination.** All reported absolute fluorescence quantum yield values (Φ) were measured in our laboratory under identical conditions using a Quantaurus-QY spectrometer (model C11374, Hamamatsu). This instrument uses an integrating sphere to determine photons absorbed and emitted by a sample. Measurements were carried out using dilute samples (A < 0.1) and self-absorption corrections were performed using the instrument software\(^3\) when appropriate. Reported values are averages (n = 3).

**Measurement of increase in absorption of HaloTag ligands 1_{HTL–10_{HTL}} following attachment to HaloTag protein.** HaloTag protein was prepared as a 100 µM solution in 75 mM NaCl, 50 mM TRIS-HCl, pH 7.4. Absorption measurements were performed in 1.0-mL quartz cuvettes. HaloTag ligands (5 µM) were dissolved in 10 mM HEPES, pH 7.3 containing 0.1 mg/mL CHAPS. An aliquot of HaloTag protein (75 µL, 7.5 µM, 1.5 equiv) or an equivalent volume of buffer (75 mM NaCl, 50 mM TRIS-HCl, pH 7.4) was added and the resulting mixture was incubated until a consistent absorption signal was observed (~60 min). Absorption scans are averages (n = 2).

**Measurement of increase in fluorescence of HaloTag ligands 1_{HTL–10_{HTL}} following attachment to HaloTag protein.** Fluorescence measurements were performed on an Infinite MI1000 Pro microplate reader (Tecan) and a 96-well quartz microplate (Hellma). HaloTag ligands (2 µM) were dissolved in 10 mM HEPES, pH 7.3 containing 0.1 mg/mL CHAPS. An aliquot of HaloTag protein (5 µM, 2.5 equiv) or an equivalent volume of buffer (75 mM NaCl, 50 mM Tris, pH 7.4) was added and the resulting mixture was incubated for 3 h followed by measurement of fluorescence. Fluorescence readings are averages (n = 2).
Measurement of increase in absorbance and fluorescence of 10HST following binding DNA. 10HST (5 µM) was added to phosphate buffered saline (PBS; pH 7.4) containing no DNA (blank), a DNA duplex with sequences 5′-A20-3′ and 5′-T20-3′ (polyA-polyT; i.e., DNAAT; 50 µM; Integrated DNA Technologies), or a DNA duplex with sequences 5′-G20-3′ and 5′-C20-3′ (polyG-polyC; i.e., DNAGC; 50 µM; Integrated DNA Technologies). These solutions were incubated for 2 h at ambient temperature, followed by measurement of absorption spectra. The solutions were then diluted to 500 nM in PBS for measurement of fluorescence spectra or diluted to 1.25 µM in PBS for measurement of quantum yield. Found: 10HST Φ = 0.009; 10HST-DNAAT Φ = 0.126; 10HST-DNAGC Φ = 0.040.

Measurement of increase in fluorescence of Taxol conjugates 1TXL, 7TXL, and 10TXL following binding to polymerized tubulin. In vitro assays were performed as previously described. Briefly, 1TXL, 7TXL, or 10TXL (3 µM) was added to a solution of monomeric tubulin (2 mg/mL) or bovine serum albumin (BSA, 2 mg/mL) in microtubule polymerization buffer. The microtubule polymerization buffer contained: 80 mM piperazine-N,N′-bis(2-ethanesulfonic acid) sequisodium salt (PIPES), 2 mM MgCl₂, 0.5 mM ethylene glycol-bis(β-aminoethyl ether) N,N,N′,N′-tetra-acetic acid (EGTA, pH 6.9), 1 mM GTP and 15% glycerol. Purified tubulin, BSA, and buffer are components of the tubulin polymerization assay kit (Cytoskeleton). The samples were incubated for 2–3 h at 37 °C and fluorescence spectra were measured on a Cary Eclipse fluorometer (Varian).

General cell culture. COS7 cells and U2OS cells were obtained from ATCC. To prepare cells stably expressing histone H2B–HaloTag, U2OS cells were integrated with a histone H2B–HaloTag expressing plasmid via the piggyback transposase. To prepare cells stably expressing histone H2B–SNAP-tag, COS7 cells were integrated with a histone H2B–SNAP-tag expressing plasmid via the piggyback transposase. Cells were cultured in Dulbecco’s modified Eagle medium (DMEM, phenol red-free; Life Technologies) supplemented with 10% v/v FBS (Life Technologies), 1 mM GlutaMAX (Life Technologies) and plated in 35-mm glass bottom dishes (MatTek). Cells were maintained at 37 °C in a humidified 5% (v/v) CO₂ environment. All cell lines undergo regular mycoplasma testing by the Janelia Cell Culture Facility.

Comparison of HaloTag ligands 6HTL and 9HTL in live yeast. A S. cerevisiae strain expressing a histone H2A.Z–HaloTag fusion under natural promoter control was created; the PDR5 transporter gene was also deleted for improved cellular retention of fluorescent ligands. Cells were grown to early log phase and incubated with either JF552–HaloTag ligand (9HTL; 10 nM) or JF549–HaloTag ligand (6HTL; 10 nM) for 2 h. Free ligand was removed by three washes in culture media and the live cells were imaged by wide-field fluorescence microscopy at ambient temperature.

Comparison of TMP ligands 6TMP and 9TMP in live cells. COS7 cells were transfected with a plasmid to express a histone H2B–E. coli dihydrofolate reductase (eDHFR) fusion protein using Amaxa Nucleofector (Lonza). Live cells were stained with ligands 6TMP or 9TMP (200 nM) for 4 h. Cells were washed twice before imaging with an LSM880 confocal microscope (Zeiss; excitation: 514 nm/emission: 526–615 nm). Signal to background (S/B) ratio was determined using the mean fluorescence of the nuclei relative to a region adjacent to each nuclei using Fiji.
Comparison of HaloTag ligands $7_{HTL}$ and $10_{HTL}$ in live cells. U2OS cells expressing histone H2B–HaloTag were incubated with 500 nM of $7_{HTL}$ or $10_{HTL}$ for 30 min and imaged with an LSM880 confocal microscope (Zeiss; excitation: 514 nm/emission: 526–615 nm) without intermediate washing steps. Signal to background (S/B) ratio was determined using the mean fluorescence of the nuclei relative to a region adjacent to each nuclei using Fiji.\(^5\)

Comparison of SNAP ligands $7_{STL}$ and $10_{STL}$ in live cells. COS7 cells expressing histone H2B–SNAP-tag were incubated with 1 µM $7_{STL}$ or $10_{STL}$ for 1 h and imaged with an LSM880 confocal microscope (Zeiss; excitation: 514 nm/emission: 526–615 nm) without intermediate washing steps. Signal to background (S/B) ratio was determined using the mean fluorescence of the nuclei relative to a region adjacent to each nuclei using Fiji.\(^5\)

Live cell staining with ligands $10_{HST}$, $10_{TXL}$, and $10_{PEP}$. For Figure 5b, U2OS cells were stained with JF526–Hoechst ($10_{HST}$, 4 µM). For Figure 5c, mouse primary hippocampal neurons were stained with JF526–Taxol ($10_{TXL}$, 1 µM) and JF646–Hoechst\(^6\) ($2_{HST}$, 1 µM). For Figure 5d, U2OS cells stably expressing histone H2B–HaloTag were stained with JF526–pepstatin A ($10_{PEP}$, 2 µM), JF585–HaloTag ligand\(^1\) ($5_{HTL}$, 100 nM), SiR–tubulin\(^4\) ($1_{HTL}$, Spirochrome, 1 µM), and Verapamil (10 µM). Verapamil was used to improve staining by preventing efflux of the Taxol conjugates.\(^4\) For all samples, cells were incubated with the stains for 1 h and imaged without intermediate washing steps. Imaging was performed on a LSM880 confocal microscope (Zeiss) using the following configuration: excitation: 514 nm/emission: 526–615 nm (JF526); excitation: 594 nm/emission: 599–734 nm (JF585); excitation: 633 nm/emission: 638–759 nm (JF646 or SiR).

Imaging with lattice light-sheet microscope. U2OS cells were stained with $10_{PEP}$ (2 µM) and $2_{HST}$ (4 µM) for 1 h before imaging on a custom-built lattice light sheet microscope (LLSM).\(^7\) Imaging was performed using 532 nm (for JF526) and 642 nm (for JF646) excitation, and a multi-band pass emission filter (NF03-405/488/532/635E, Semrock). Data were acquired by serial scanning the entire cell through the light sheet at 30 ms exposure per 2D image and 400 nm z-steps resulting in a 3D imaging rate of 6 s per volume. All acquired data were deconvolved by using a Richardson-Lucy algorithm adapted to run on a graphics processing unit, using an experimentally measured PSF for each emission wavelength\(^7\).

Imaging with structured illumination microscopy. Mouse primary hippocampal neurons were stained with $10_{PEP}$ (2 µM) and $2_{HST}$\(^6\) (4 µM, 1 h) before three-dimensional structured illumination microscopy (3D-SIM) using a custom-built microscope.\(^8,9\) Briefly, the 3D-SIM microscope was built on a Zeiss Axio Observer inverted microscope platform with an ASI motorized stage and a 100×/1.46 NA oil immersion Zeiss objective (Alpha Plan-APO). Two-color imaging was performed at 488 nm and 647 nm excitation for JF526 and JF646, respectively. Sequential excitation at the two wavelengths was enabled by a software-controlled filter wheel (Finger Lakes Instrumentation). Excitation grating patterns at each wavelength were generated by a spatial light modulator (Forth Dimension Display) as previously described.\(^8\) Fluorescence images were collected using an sCMOS camera (Hamamatsu Flash 4.0) with an exposure time of 30 ms. 3D reconstruction of the raw data was performed using a custom software as previously described.\(^8,9\)

Imaging with stimulated emission depletion microscopy (STED). For one-color STED, U2OS cells were stained with JF526–Taxol ($10_{TXL}$, 1 µM) and Verapamil (10 µM) for 1 h before imaging
using STED microscopy. For three-color STED, U2OS cells expressing TOMM20–HaloTag fusion protein were transfected with Sec61β–pSNAPf plasmids using Lipofectamine 2000 (ThermoFisher). Sec61β encodes an endoplasmic reticulum membrane protein translocator protein, and TOMM20 encodes an outer mitochondrial membrane protein as part of a protein translocase complex. Live cells were simultaneously stained with JF526–SNAP-tag ligand (3 µM with 0.2% (w/v) Pluronic F-127) and JF585–HaloTag ligand (100 nM) for 3 h. Cells were washed two times with DMEM media and stained with JF526–Taxol (100 µM) and Verapamil (10 µM) for 1 h before imaging with STED microscopy. STED images were acquired using a Leica TCS SP8 confocal laser scanning microscopy platform using the following configuration: 532 nm/emission: 540–592 nm (JF526); excitation: 594 nm/emission: 597–643 nm (JF585); excitation: 640 nm/emission: 650–699 nm (JF546). A 775 nm pulsed laser was used as the depletion source to generate STED images. Fluorescence images were collected using HyD detectors.

Labeling of secondary antibody with N-hydroxysuccinimide esters of HM-JF526. Goat anti-mouse IgG secondary antibody (IgG, Thermo Fisher) was used as the secondary antibody for immunofluorescence labeling. IgG was concentrated to 5 mg/mL using an Amicon Ultra 0.5 mL centrifugal filter. HM-JF526-NHS (37) was dissolved in DMSO to yield a 10 mM stock solution. IgG was incubated with HM-JF526-NHS (20 equiv.) in PBS pH 7.4 /1 M sodium bicarbonate pH 8.3 (9:1 v/v) at room temperature for 90 min. Proteins labeled with dyes were purified using a PD MiniTrapTM G-25 (GE Healthcare) with PBS (pH 7.4) as the eluent. The degree of labeling (DOL) ratio (dye/ protein) was determined by measuring the absorption of the labeled-IgG at pH 2, where the HM-JF526 exists in the open, colored form. The DOL of HM-JF526–IgG was 2.8.

Sample preparation for single-molecule localization microscopy. Tubulin was immunolabeled as previously described. U2OS cells were grown in 35-mm glass bottom dishes (MatTek) to appropriate density. Cells were washed twice with 60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM MgCl₂ (PHEM), followed by fixation with fresh 0.1% glutaraldehyde and 2% paraformaldehyde for 15 min at 37 °C, and three additional washes with PHEM for 10 min each. Cells were then permeabilized with 0.1% Triton X-100 for 2 min and washed 3 times with PHEM for 10 min each. Incubating in PBS with 1% bovine serum albumin (BSA) and 1 mg/mL lysine for 30 min blocked nonspecific protein binding sites. A primary monoclonal antibody against alpha-tubulin (DM1A, ThermoFisher) was bound to the cells at 4 µg/mL in PBS with 1% BSA and 0.05% Tween for 30 min with shaking. After three 10 min washes with PBS, the secondary antibody was applied at 3 µg/ml for 30 min with shaking. After another three 10 min washes with 0.05% Tween in PBS, cells were imaged in PBS.

TOMM20 proteins were immunolabeled as previously described. U2OS cells were fixed with 3.7% PFA solution with occasional gentle agitation before aspiration with minimal perturbation. Five PBS washes were administered lasting approximately 0.5, 1, 5, 10 and 15 minutes each. 0.1% Triton X-100 in PBS is used to permeabilize the cells. Following permeabilization, cells were again washed in PBS, once for 30 s and then twice for 5 min. Blocking was achieved using 1% BSA in PBS. A primary mouse monoclonal antibody against TOMM20 (F-10, Santa Cruz Biotech) was bound to the cells at 2 µg/mL in PBS with 1% BSA for 30 min with shaking. After three 5 min washes with PBS, the secondary antibody was applied at 3 µg/ml for 30 min with shaking. After three 5 min washes with PBS, cells were imaged in PBS.
**Single-molecule localization microscopy.** All single-molecule localization microscopy (SMLM) experiments were performed on an ELYRA system (Carl Zeiss) with a 100×/1.42 NA Plan-Apo objective, as previously described.\(^1\) For correction of sample drift, 100-nm TetraSpeck Microspheres (Invitrogen) were affixed to the cover-glass by incubation in the medium for 30 min at room temperature. Images were taken by a Highly Inclined and Laminated Optical sheet (HILO) microscope coupled to an EMCCD camera that can detect a single photon. Imaging was performed at room temperature in PBS buffer. SMLM images were collected with 10,000 cycles of excitation at a constant illumination power density of around 10 kW/cm\(^2\) for 561nm excitation laser. The exposure time for each cycle was 30 ms. The SMLM data were analyzed with Zeiss Zen software to reconstruct the SMLM images.
**EXPERIMENTAL INFORMATION FOR SYNTHESIS**

**General Synthetic Methods.** Commercial reagents were obtained from reputable suppliers and used as received. All solvents were purchased in septum-sealed bottles stored under an inert atmosphere. All reactions were sealed with septa through which a nitrogen atmosphere was introduced unless otherwise noted. Reactions were conducted in round-bottomed flasks or septum-capped crimp-top vials containing Teflon-coated magnetic stir bars. Heating of reactions was accomplished using an aluminum reaction block on top of a stirring hotplate equipped with an electronic contact thermometer to maintain the indicated temperatures unless otherwise indicated.

Reactions were monitored by thin layer chromatography (TLC) on precoated TLC glass plates (silica gel 60 F254, 250 µm thickness) or by tandem high pressure liquid chromatography mass spectrometry (Shimadzu 2020 LC–MS system; Phenomenex Kinetex 2.1 mm × 30 mm 2.6 µm C18 column; 5 µL injection; 5–98% MeCN/H2O, linear gradient, with constant 0.1% v/v HCO2H additive; 6 min run; 0.5 mL/min flow; ESI; positive ion mode). TLC chromatograms were visualized by UV illumination or developed with p-anisaldehyde, ceric ammonium molybdate, or KMnO4 stain. Reaction products were purified by flash chromatography on an automated purification system using pre-packed silica gel columns or by preparative HPLC (Phenomenex Gemini–NX 30 × 150 mm 5 µm C18 column). Analytical LC–MS analysis was performed on an Agilent 1200 LC–MS system equipped with an autosampler, diode array detector, and mass spectrometry detector using an Agilent Eclipse XDB 4.6 × 150 mm 5 µm C18 column under the indicated conditions with the retention time indicated as tR. The mass spectrograms for 7TXL and 10TXL were taken from experiments using LC–MS Shimadzu system.

NMR spectra were recorded on a 400 MHz spectrometer. 1H and 13C chemical shifts were referenced to TMS or residual solvent peaks, and 19F chemical shifts were referenced to CFCl3. Data for 1H NMR spectra are reported as follows: chemical shift (δ ppm), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, dd = doublet of doublets, m = multiplet, etc.), coupling constant (Hz), integration. Data for 13C NMR spectra are reported by chemical shift (δ ppm) with hydrogen multiplicity (C, CH, CH2, CH3) information obtained from DEPT spectra. Data for 19F NMR spectra are reported as follows: chemical shift (δ ppm), multiplicity, coupling constant (Hz).

**Boc2SiR110 (12):** A vial was charged with Si-fluorescein ditriflate12 (Tf2SiFl, 11, 90 mg, 141 µmol), tert-butyl carbamate (36 mg, 310 µmol, 2.2 eq), Pd2dba3 (12.9 mg, 14.1 µmol, 0.1 eq), Xantphos (24.5 mg, 42.3 µmol, 0.3 eq), and Cs2CO3 (129 mg, 395 µmol, 2.8 eq). The vial was sealed and evacuated/backfilled with nitrogen (3×). Dioxane (1 mL) was added, and the reaction was flushed again with nitrogen (3×). The reaction was stirred at 100 °C for 18 h. It was then cooled to room temperature, filtered through Celite with CH2Cl2, and concentrated under reduced pressure. The residue was purified by silica gel chromatography (0–30% EtOAc/hexanes, linear gradient) to afford 12 (62 mg, 77%) as a colorless solid. 1H NMR (CDCl3, 400 MHz) δ 7.99 – 7.93 (m, 1H), 7.71 (d, J = 2.2 Hz, 2H), 7.61 (td, J = 7.5, 1.1 Hz, 1H), 7.52 (td, J = 7.5, 0.8 Hz, 1H), 7.23 – 7.15 (m, 3H), 6.95 (d, J = 8.7 Hz, 2H), 6.59 (s, 2H), 1.50 (s, 18H), 0.65 (s, 3H), 0.59 (s, 3H); 13C
Si-Rhodamine 110 (SiR110, 8): Boc₂SiR₁₁₀ (12, 52 mg, 90.8 µmol) was taken up in CH₂Cl₂ (2.5 mL), and trifluoroacetic acid (0.5 mL) was added. The reaction was stirred at room temperature for 2 h. Toluene (3 mL) was added, and the reaction mixture was concentrated under reduced pressure. The residue was dissolved in EtOAc and washed with saturated NaHCO₃(aq) and brine. The organic layer was deposited onto silica gel and concentrated under reduced pressure. Flash chromatography (0–10% MeOH with 2 M NH₃/CH₂Cl₂, linear gradient; dry load with silica gel) afforded 31 mg (92%) of 8 as a blue solid.

1H NMR (DMSO-d₆, 400 MHz) δ 7.95–7.88 (m, 1H), 7.79 (td, J = 7.5, 1.1 Hz, 1H), 7.64 (td, J = 7.6, 0.8 Hz, 1H), 7.35–7.28 (m, 1H), 6.93–6.87 (m, 2H), 6.50–6.39 (m, 4H), 5.28 (s, 4H), 0.52 (s, 3H), 0.45 (s, 3H); 13C NMR (DMSO-d₆, 101 MHz) δ 169.7 (C), 153.9 (C), 148.0 (C), 136.1 (C), 134.4 (CH), 130.9 (C), 129.1 (CH), 127.6 (CH), 125.9 (C), 125.1 (CH), 124.6 (CH), 118.3 (CH), 114.8 (CH), 91.6 (C), 0.2 (CH₃), −1.8 (CH₃); Analytical LC–MS: tᵣ = 8.7 min, >99% purity (10–95% MeCN/H₂O, linear gradient, with constant 0.1% v/v TFA additive; 20 min run; 1 mL/min flow; ESI; positive ion mode; UV detection at 254 nm); HRMS (ESI) calcd for C₂₂H₂₁N₂O₂Si [M+H]+ 373.1367, found 373.1362.

6-Methoxycarbonyl-Boc₂SiR₁₁₀ (14): A vial was charged with 6-methoxycarbonyl-Si-fluorescein ditriflate ¹¹(Tf₂SiFl-6-CO₂Me, 13, 820 mg, 1.18 mmol), tert-butyl carbamate (331 mg, 2.83 mmol, 2.4 eq), Pd₂dba₃ (108 mg, 0.118 mmol, 0.1 eq), Xantphos (204 mg, 0.353 mmol, 0.3 eq), and Cs₂CO₃ (1.07 g, 3.30 mmol, 2.8 eq). The vial was sealed and evacuated/backfilled with nitrogen (3×). Dioxane (6 mL) was added, and the reaction vessel was flushed again with nitrogen (3×). The reaction was stirred at 100 °C for 3 h. It was then cooled to room temperature, filtered through Celite with CH₂Cl₂, and concentrated under reduced pressure. The residue was purified by silica gel chromatography (0–60% EtOAc/hexanes, linear gradient) to afford 14 (573 mg, 77%) as an off-white foam. ¹H NMR (CDCl₃, 400 MHz) δ 8.17 (dd, J = 8.0, 1.2 Hz, 1H), 8.01 (dd, J = 8.0, 0.8 Hz, 1H), 7.87 (t, J = 1.0 Hz, 1H), 7.73 (d, J = 2.0 Hz, 2H), 7.20 (dd, J = 8.7, 2.3 Hz, 2H), 6.98 (d, J = 8.7 Hz, 2H), 6.51 (s, 2H), 3.88 (s, 1H), 1.51 (s, 18H), 0.72 (s, 3H), 0.61 (s, 3H); ¹³C NMR (CDCl₃, 101 MHz) δ 169.9 (C), 155.1 (C), 152.7 (C), 138.3 (C), 138.2 (C), 136.2 (C), 135.6 (C), 130.4 (CH), 128.7 (C), 127.4 (CH), 126.2 (CH), 125.2 (CH), 123.4 (CH), 120.1 (CH), 90.2 (C), 81.0 (C), 52.8 (CH₃), 28.5 (CH₃), 0.1 (CH₃), −0.8 (CH₃); HRMS (ESI) calcd for C₃₄H₃₉N₂O₈Si [M+H]+ 631.2470, found 631.2480.
6-Carboxy-BocSiR_{110} (15): To a solution of 14 (560 mg, 0.888 mmol) in 1:1 MeOH/THF (8 mL) was added 1 M NaOH (1.78 mL, 1.78 mmol, 2 eq). The reaction was stirred at room temperature for 18 h. It was subsequently acidified with 1 M HCl (3 mL), diluted with water, and extracted with EtOAc (2×). The combined organic extracts were washed with brine, dried over anhydrous MgSO₄, filtered, and concentrated under reduced pressure to provide the carboxylic acid as a white solid (526 mg, 96%). ¹H NMR (DMSO-d₆, 400 MHz) δ 13.56 (s, 1H), 9.46 (s, 2H), 8.13 – 8.07 (dd, J = 8.0, 1.3 Hz, 1H), 8.07 (dd, J = 7.9, 0.6 Hz, 1H), 7.91 (d, J = 2.5 Hz, 2H), 7.71 – 7.65 (m, 1H), 7.38 (dd, J = 8.8, 2.4 Hz, 2H), 6.90 (d, J = 8.8 Hz, 2H), 1.46 (s, 18H), 0.63 (s, 3H), 0.52 (s, 3H); ¹³C NMR (DMSO-d₆, 101 MHz) δ 169.1 (C), 165.9 (C), 154.7 (C), 152.7 (C), 139.3 (C), 136.9 (C), 136.8 (C), 134.7 (C), 130.3 (CH), 127.2 (C), 126.7 (CH), 126.3 (CH), 123.9 (CH), 122.9 (CH), 120.0 (CH), 89.3 (C), 79.3 (C), 28.1 (CH₃), −0.4 (CH₃), −0.8 (CH₃); HRMS (ESI) calcd for C_{33}H_{37}N_{2}O_{8}Si [M+H]^+ 617.2314, found 617.2315.

SiR_{110}–HaloTag ligand (8_{HTL}): Acid 15 (48.4 mg, 78.5 µmol) was combined with TSTU (35.4 mg, 118 µmol, 1.5 eq) in DMF (5 mL). After adding DIEA (68 µL, 392 µmol, 5 eq), the reaction was stirred at room temperature for 1 h. A solution of HaloTag(O2)amine¹³ (16, 39.8 mg, 118 µmol, 1.5 eq) in DMF (250 µL) was then added. The reaction was stirred an additional 30 min at room temperature. It was subsequently diluted with saturated NaHCO₃(aq) and extracted with EtOAc (2×). The combined organic extracts were washed with brine, dried over anhydrous MgSO₄, filtered, and concentrated under reduced pressure. Silica gel chromatography (0–100% EtOAc/hexanes, linear gradient) afforded Boc₂SiR_{110}–HaloTag ligand as an off-white solid.

The dicarbamate intermediate was taken up in CH₂Cl₂ (2 mL), and trifluoroacetic acid (400 µL) was added. The reaction was stirred at room temperature for 2 h. Toluene (2 mL) was added, and the reaction mixture was concentrated under reduced pressure. The residue was diluted with saturated NaHCO₃(aq) and extracted with EtOAc (2×). The combined organic extracts were washed with brine, dried over anhydrous MgSO₄, filtered, and concentrated under reduced pressure. Flash chromatography (0–100% EtOAc/hexanes, linear gradient) followed by reverse phase HPLC (10–75% MeCN/H₂O, linear gradient, with constant 0.1% v/v TFA additive) afforded 8_{HTL} (TFA salt) as a blue-green solid. ¹H NMR (CD₃OD, 400 MHz) δ 8.72 (t, J = 5.5 Hz, 1H), 8.13 (d, J = 8.1 Hz, 1H), 8.07 (dd, J = 8.1, 1.6 Hz, 1H), 7.72 – 7.69 (m, 1H), 7.21 (d, J = 2.5 Hz, 2H), 6.82 (d, J = 8.9 Hz, 2H), 6.68 (dd, J = 8.9, 2.5 Hz, 2H), 3.66 – 3.53 (m, 8H), 3.51 (t, J = 6.6 Hz, 2H), 3.42 (t, J = 6.5 Hz, 2H), 1.75 – 1.64 (m, 2H), 1.54 – 1.44 (m, 2H), 1.42 – 1.27 (m, 4H), 0.62 (s, 3H), 0.55 (s, 3H); Analytical LC–MS: tᵣ = 10.7 min, >99% purity (10–95% MeCN/H₂O, linear gradient, with constant 0.1% v/v TFA additive; 20 min run; 1 mL/min flow; ESI; positive ion mode; detection at 254 nm); HRMS (ESI) calcd for C_{33}H_{41}ClN_{3}O_{5}Si [M+H]^+ 622.2499, found 622.2508.
3',6'-Dibromo-2',7'-difluoro-3H-spiro[isobenzofuran-1,9'-xanthene]-3-one (19). A round-bottom flask was charged with 3-bromo-4-fluorophenol (17, 5.0 g, 26 mmol, 2 equiv), phthalic anhydride (18, 1.9 g, 13 mmol 1 equiv), and 12.5 mL methanesulfonic acid. The flask was sealed and evacuated/backfilled with nitrogen (3×). The reaction was stirred at 140 °C for 21 h. The cooled mixture was poured into 7 volumes of stirred ice water and the precipitate was collected by filtration and dried to constant weight. Purification by silica gel chromatography (0–10% EtOAc/hexanes with constant 40% CH2Cl2, linear gradient) afforded 19 (630 mg, 10%) as a white solid. 1H NMR (CDCl3, 400 MHz) δ 8.10 – 8.04 (m, 1H), 7.76 – 7.66 (m, 2H), 7.55 (d, JHF = 5.7 Hz, 2H), 7.18 – 7.11 (m, 1H), 6.57 (d, JCF = 8.3 Hz, 2H). 13C NMR (CDCl3, 101 MHz) δ 168.4 (C), 155.6 (d, JCF = 245.4 Hz, CF), 152.0 (C), 147.2 (d, JCF = 2.6 Hz, C), 135.9 (CH), 130.9 (CH), 126.0 (CH), 125.5 (C), 123.7 (CH), 122.4 (CH), 118.8 (d, JCF = 6.4 Hz, C), 114.3 (d, JCF = 25.3 Hz, CH), 112.2 (d, JCF = 23.4 Hz, C), 80.8 (C). 19F NMR (CDCl3, 376 MHz) δ −112.44 (dd, JHF = 8.2, JFH = 5.5 Hz). Analytical LC–MS: tr = 17.5 min; >99% purity (10–95% MeCN/H2O, linear gradient, with constant 0.1% v/v TFA additive; 20 min run; 1 mL/min flow; ESI; positive ion mode; detection at 254 nm). HRMS (ESI) calcd for C20H9Br2F2O3 [M+H]⁺ 492.8886, found 492.8885.

JF552 (9). The following method for 9 is representative for preparation of rhodamines via C–N cross-coupling. A vial was charged with dibromide (19, 49 mg, 100 µmol), azetidine (20, 14 mg, 240 µmol, 2.4 equiv), Pd2dba3 (9 mg, 10 µmol, 0.1 equiv), XPhos (14 mg, 30 µmol, 0.3 equiv), and Cs2CO3 (157 mg, 484 µmol, 4.8 equiv). The vial was sealed and evacuated/backfilled with N2 (3×). Dioxane (1 mL) was added, and the reaction was flushed again with N2 (3×). The reaction was then stirred at 100 °C for 18 h. It was subsequently cooled to room temperature, diluted with MeOH, deposited onto Celite, and concentrated under reduced pressure. Purification by silica gel chromatography (0–10% MeOH (2 M NH3)/ CH2Cl2, linear gradient; dry load with Celite) afforded 9 (37 mg, 83%) as a purple solid. The 1H and 19F NMR spectra of 9 were identical to those previously reported.1

3',6'-Dibromo-2',7'-difluoro-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-6-carboxylic acid (22). A microwave vial was charged with 3-bromo-4-fluorophenol (17, 5.0 g, 26 mmol, 2 equiv), 1,2,4-benzenetricarboxylic anhydride (21, 2.5 g, 13 mmol 1 equiv), and 12.5 mL methanesulfonic acid. The vial was sealed and evacuated/backfilled with nitrogen (3×). The reaction was stirred at 140 °C in an Initiator microwave reactor (Biotage) for 12 h. The cooled
mixture was poured into 7 volumes of stirred ice water and the precipitate was collected by filtration and dried to constant weight. The precipitate contained 22 and its 5-carboxylic isomer; the desired product was isolated by crystallization from 140 mL 9:1 toluene:pyridine to afford crude 22 as an off-white solid. This was further purified by silica gel chromatography (2–15% MeOH/CH2Cl2; dry load with Celite) to give 22 (700 mg, 5%) as a white solid. 1H NMR (DMSO-d6, 400 MHz) δ 8.25 (dd, J = 8.0, 1.3 Hz, 1H), 8.14 (dd, J = 8.0, 0.7 Hz, 1H), 7.88 (d, 4JHF = 5.8 Hz, 2H), 7.86 (s, 1H), 7.05 (d, 3JHF = 8.8 Hz, 2H). 13C NMR (DMSO-d6, 101 MHz) δ 167.2 (C), 166.0 (C), 154.7 (d, 1JC = 242.2 Hz, CF), 151.3 (C), 146.8 (d, 4JC = 2.1 Hz, C), 137.6 (C), 131.5 (CH), 128.7 (C), 126.1 (CH), 124.6 (CH), 121.9 (CH), 118.3 (d, 3JC = 6.8 Hz, C), 114.9 (d, 2JC = 25.2 Hz, CH), 111.4 (d, 2JC = 23.3 Hz, C), 80.3 (C). 19F NMR (DMSO-d6, 376 MHz) δ −112.68 (dd, 3JFH = 8.1, 4JFH = 5.5 Hz). Analytical LC–MS: tR = 13.4 min; 97% purity (10–95% MeCN/H2O, linear gradient, with constant 0.1% v/v TFA additive; 20 min run; 1 mL/min flow; ESI; positive ion mode; detection at 254 nm). HRMS (ESI) calcd for C21H9Br2F2O5 [M+H]+ 536.8785, found 536.8786.

**tert-Butyl 3',6'-dibromo-2',7'-difluoro-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-6-carboxylate** (24). A suspension of 2',7'-difluoro-6-carboxyfluorescein dibromide (22, 340 mg, 0.669 mmol) in toluene (7 mL) was heated to 80 °C, and N,N-dimethylformamide di-tert-butyl acetal (23, 2 mL, 8.3 mmol, 12 equiv) was added dropwise over 5 min. The reaction was stirred at 80 °C for 15 min. After cooling the mixture to room temperature, it was diluted with saturated NaHCO3(aq) and extracted with CH2Cl2 (2×). The combined organic extracts were dried over MgSO4(s), filtered, and concentrated in vacuo. Purification by silica gel chromatography (75% CH2Cl2/hexanes) afforded 24 (305 mg, 81%) as a white solid. 1H NMR (CDCl3, 400 MHz) δ 8.28 (dd, J = 8.0, 1.3 Hz, 1H), 8.10 (dd, J = 8.0, 0.7 Hz, 1H), 7.70 (d, J = 1.0 Hz, 1H), 7.58 (d, 4JHF = 5.7 Hz, 2H), 6.54 (d, 3JHF = 8.2 Hz, 2H), 1.57 (s, 9H). 13C NMR (CDCl3, 101 MHz) δ 167.7 (C), 163.7 (C), 155.6 (d, 1JC = 245.6 Hz, CF), 152.0 (C), 147.1 (d, 4JC = 2.5 Hz, C), 139.3 (C), 131.9 (CH), 128.5 (C), 125.9 (CH), 124.7 (CH), 122.5 (CH), 118.2 (d, 3JC = 6.3 Hz, C), 114.3 (d, 2JC = 25.3 Hz, CH), 112.5 (d, 2JC = 23.4 Hz, C), 83.2 (C), 81.0 (C), 28.2 (CH3). 19F NMR (CDCl3, 376 MHz) δ −112.18 (dd, 3JFH = 8.1, 4JFH = 5.5 Hz). Analytical LC–MS: tR = 19.3 min; >99% purity (10–95% MeCN/H2O, linear gradient, with constant 0.1% v/v TFA additive; 20 min run; 1 mL/min flow; ESI; positive ion mode; detection at 254 nm). HRMS (ESI) calcd for C25H17Br2F2O5 [M+H]+ 592.9411, found 592.9419.

**6-tert-Butoxycarbonyl-JF552** (25). The title compound was prepared from dibromide 24 and azetidine (20) following the C–N cross-coupling method described for 9 (62%, purple solid). 1H NMR (CDCl3, 400 MHz) δ 8.22 (dd, J = 8.0, 1.3 Hz, 1H), 8.03 (dd, J = 8.0, 0.7 Hz, 1H), 7.73 (t, J = 1.0 Hz, 1H), 6.27 − 6.13 (m, 4H), 4.02 (td, J = 7.4 Hz, 5JHF = 2.1 Hz, 8H), 2.37 (p, J = 7.3 Hz, 95% MeCN/H2O, linear gradient, with constant 0.1% v/v TFA additive; 20 min run; 1 mL/min flow; ESI; positive ion mode; detection at 254 nm). HRMS (ESI) calcd for C25H17Br2F2O5 [M+H]+ 592.9411, found 592.9419.
4H), 1.57 (s, 9H). $^{13}$C NMR (CDCl$_3$, 101 MHz) $\delta$ 168.5 (C), 164.3 (C), 152.5 (C), 148.6 (d, $^4$J$_{CF}$ = 1.1 Hz, C), 148.6 (d, $^1$J$_{CF}$ = 239.3 Hz, CF), 142.3 (d, $^2$J$_{CF}$ = 13.2 Hz, C), 138.4 (C), 131.1 (CH), 130.2 (C), 125.2 (CH), 125.1 (CH), 113.6 (d, $^2$J$_{CF}$ = 21.3 Hz, CH), 106.0 (d, $^3$J$_{CF}$ = 6.3 Hz, C), 100.7 (d, $^3$J$_{CF}$ = 4.9 Hz, CH), 85.0 (C), 82.7 (C), 53.9 (d, $^4$J$_{CF}$ = 2.6 Hz, CH$_2$), 28.2 (CH$_3$), 17.8 (d, $^5$J$_{CF}$ = 2.4 Hz, CH$_2$). $^{19}$F NMR (CDCl$_3$, 376 MHz) $\delta$ −137.92 − −138.03 (m). Analytical LC–MS: $t_r$ = 13.27 min, 97% purity; (10–95% MeCN/H$_2$O, linear gradient, with constant 0.1% v/v TFA additive; 20 min run; 1 mL/min flow); ESI; positive ion mode; detection at 550 nm). HRMS (ESI) calcd for C$_{31}$H$_{29}$F$_2$N$_2$O$_5$ [M+H]$^+$ 547.2039, found 547.2030.

6-Carboxy-JF$_{552}$ (26). Ester 25 (23.2 mg, 30.4 µmol) was taken up in CH$_2$Cl$_2$ (2.5 mL) to which TFA (0.5 mL) was added. The reaction was stirred at room temperature for 6 h. Toluene (3 mL) was added; the reaction mixture was concentrated under reduced pressure and then azeotroped with MeOH (3 ×) to provide 25 as a dark pink solid (25.0 mg, 98%, TFA salt). Analytical LC–MS and NMR indicated that the material was >95% pure and thus was used without further purification. $^1$H NMR (CD$_3$OD, 400 MHz), $\delta$ 8.46 – 8.37 (m, 2H), 7.96 (d, $J = 1.4$ Hz, 1H), 6.77 (d, $^3$J$_{HF}$ = 12.6 Hz, 2H), 6.65 (d, $^4$J$_{HF}$ = 7.3 Hz, 2H), 4.50 (t, $J = 7.4$ Hz, 8H), 2.57 (p, $J = 7.5$ Hz, 4H). $^{13}$C NMR (CD$_3$OD, 101 MHz) $\delta$ 167.6 (C), 167.4 (C), 155.9 (C), 151.7 (d, $^1$J$_{CF}$ = 251.0 Hz, CF), 148.8 (d, $^2$J$_{CF}$ = 15.4 Hz, C), 136.2 (C), 135.0 (C), 133.0 (CH), 132.7 (CH), 132.0 (CH), 114.5 (d, $^3$J$_{CF}$ = 8.5 Hz, C), 113.7 (d, $^2$J$_{CF}$ = 21.6 Hz, CH), 97.8 (d, $^3$J$_{CF}$ = 5.5 Hz, CH), 55.6 (CH$_2$), 17.9 (CH$_3$). $^{19}$F NMR (CD$_3$OD, 376 MHz) $\delta$ −75.35 (s, 3F), −130.54 (dd, $^3$J$_{FH}$ = 12.8, $^4$J$_{FH}$ = 7.6 Hz, 2F). Analytical LC–MS: $t_r$ = 13.26 min, 96% purity (10–95% MeCN/H$_2$O, linear gradient, with constant 0.1% v/v TFA additive; 20 min run; 1 mL/min flow); ESI; positive ion mode; detection at 550 nm). HRMS (ESI) calcd for C$_{27}$H$_{21}$F$_2$N$_2$O$_5$ [M+H]$^+$ 491.1413, found 491.1420.

JF$_{552}$–HaloTag ligand (9$_{HTL}$). 6-Carboxy-JF$_{552}$ (26, 7 mg, 12 µmol) was combined with DSC (7 mg, 27 µmol, 2.2 equiv) in DMF (0.5 mL). After adding Et$_3$N (10 µL, 72 µmol, 6 equiv) and DMAP (0.2 mg, 1.2 µmol, 0.1 equiv), the reaction was stirred at room temperature for 1 h while shielded from light. HaloTag(O2)amine$^{13}$ (16, TFA salt, 7 mg, 30 µmol, 2.5 equiv, Promega) was then added. The reaction was stirred overnight at room temperature, then concentrated under reduced pressure. The crude material was purified by reverse phase HPLC (10–90% MeCN/H$_2$O, linear gradient, with constant 0.1% v/v TFA additive) to provide 7.3 mg (91%, TFA salt) of 9$_{HTL}$ as a pink solid. $^1$H NMR (CD$_3$OD, 400 MHz) $\delta$ 8.77 (t, $J = 5.5$ Hz, 1H), 8.41 (d, $J = 8.2$ Hz, 1H), 8.20 (dd, $J = 8.2$, 1.8 Hz, 1H), 7.78 (d, $J = 1.7$ Hz, 1H), 6.77 (d, $^3$J$_{HF}$ = 12.7 Hz, 2H), 6.66 (d, $^4$J$_{HF}$ = 7.3 Hz, 2H), 4.50 (t, $J = 7.5$ Hz, 8H), 3.70 – 3.55 (m, 8H), 3.53 (t, $J = 6.6$ Hz, 2H), 3.44 (t, $J = 6.5$ Hz, 2H), 2.58 (p, $J = 7.6$ Hz, 4H) 1.84 – 1.63 (m, 2H), 1.59 – 1.47 (m, 2H), 1.47 – 1.28 (m, 4H). $^{19}$F NMR (CD$_3$OD, 376 MHz) $\delta$ −76.55 (s, 3F), −131.84 (dd, $^3$J$_{FH}$ = 12.9, $^4$J$_{FH}$ = 7.4 Hz, 2F).
Analytical LC–MS: $t_R = 12.44$ min, >99% purity; (10–95% MeCN/H$_2$O, linear gradient, with constant 0.1% v/v TFA additive; 20 min run; 1 mL/min flow; ESI; positive ion mode; detection at 550 nm). HRMS (ESI) calcd for C$_{37}$H$_{41}$ClF$_2$N$_3$O$_6$ [M+H]$^+$ 696.2652, found 696.2656.

**JF$_{552}$–trimethoprim (JF$_{552}$–TMP; 9 TMP).** The following method for 9 TMP is representative for amidation to prepare HaloTag ligands, SNAP-tag ligands, TMP conjugates, Hoechst conjugates, and other amides unless otherwise noted. 6-Carboxy-JF$_{552}$ (26, 7 mg, 12 µmol) was combined with TSTU (8.1 mg, 27 µmol, 2.2 equiv) in DMF (0.5 mL). After adding DIEA (13 µL, 72 µmol, 6 equiv), the reaction was stirred at room temperature for 1 h while shielded from light. TMP amine$^{14}$ (28, 14 mg, 24 µmol, 2 equiv) was then added. The reaction was stirred overnight at room temperature, then concentrated under reduced pressure. The crude material was purified by reverse phase HPLC (10–90% MeCN/H$_2$O, linear gradient, with constant 0.1% v/v TFA additive) to provide 13.8 mg (44%, TFA salt) of 9 TMP as a pink solid. $^1$H NMR (CD$_3$OD, 400 MHz) δ 8.67 (t, $J = 5.6$ Hz, 1H), 8.38 (d, $J = 8.2$ Hz, 1H), 8.17 (dd, $J = 8.2$, 1.7 Hz, 1H), 7.22 (s, 1H), 6.77 (d, $J_{HF} = 12.6$ Hz, 2H), 6.65 (d, $J_{HF} = 7.3$ Hz, 2H), 6.55 (s, 2H), 4.49 (t, $J = 7.7$ Hz, 8H), 3.90 (t, $J = 6.1$ Hz, 2H), 3.66 (s, 2H), 3.54 – 3.45 (m, 6H), 3.23 (t, $J = 6.9$ Hz, 2H), 2.57 (p, $J = 7.7$ Hz, 4H), 2.23 (t, $J = 7.3$ Hz, 2H), 1.88 (p, $J = 6.5$ Hz, 2H), 1.82 – 1.64 (m, 6H). Analytical LC–MS: $t_R = 9.93$ min, >99% purity; (10–95% MeCN/H$_2$O, linear gradient, with constant 0.1% v/v TFA additive; 20 min run; 1 mL/min flow; ESI; positive ion mode; detection at 550 nm). HRMS (ESI) calcd for C$_{55}$H$_{65}$F$_2$N$_8$O$_{11}$ [M+H]$^+$ 1051.4741, found 1051.4737.

**JF$_{549}$–trimethoprim (JF$_{549}$–TMP; 6 TMP).** The title compound was prepared from 6-carboxy-JF$_{549}$ (27) and TMP amine (28) following the amidation method described for 9 TMP (65%, pink solid, TFA salt). $^1$H NMR (CD$_3$OD, 400 MHz) δ 8.66 (t, $J = 5.7$ Hz, 1H), 8.38 (d, $J = 8.2$ Hz, 1H), 8.18 (dd, $J = 8.2$, 1.8 Hz, 1H), 7.79 (d, $J = 1.7$ Hz, 1H), 7.23 (s, 1H), 7.06 (d, $J = 9.2$ Hz, 2H), 6.60 (dd, $J = 9.2$, 2.2 Hz, 2H), 6.55 (s, 2H), 6.54 (d, $J = 2.1$ Hz, 2H), 4.30 (t, $J = 7.6$ Hz, 8H), 3.90 (t, $J = 6.1$ Hz, 2H), 3.79 (s, 6H), 3.66 (s, 2H), 3.61 – 3.54 (m, 8H), 3.53 – 3.41 (m, 6H), 3.22 (t, $J = 6.9$ Hz, 2H), 2.56 (p, $J = 7.6$ Hz, 6H), 2.22 (t, $J = 7.3$ Hz, 2H), 1.87 (p, $J = 6.4$ Hz, 2H), 1.82 – 1.58 (m, 6H). Analytical LC–MS: $t_R = 9.07$ min, 99% purity; (10–95% MeCN/H$_2$O, linear gradient, with constant 0.1% v/v TFA additive; 20 min run; 1 mL/min flow; ESI; positive ion mode; detection at 550 nm). HRMS (ESI) calcd for C$_{55}$H$_{67}$N$_8$O$_{11}$ [M+H]$^+$ 1015.4929, found 1015.4934.
**JF₅₂₆ (10).** The title compound was prepared from dibromide 19 and 3,3-difluoroazetidine hydrochloride (29) following the C–N cross-coupling method described for 9 (65%, pink solid). ¹H NMR (CD₃CN, 400 MHz) δ 7.99 (dt, J = 7.5, 0.9 Hz, 1H), 7.77 (td, J = 7.5, 1.3 Hz, 1H), 7.71 (td, J = 7.5, 1.1 Hz, 1H), 7.22 (dt, J = 7.6, 0.9 Hz, 1H), 6.57 – 6.38 (m, 4H), 4.42 (td, ²J₉F = 12.1, ⁵J₉F = 2.1 Hz, 8H). ¹³C NMR (CDCl₃, 101 MHz) δ 169.0 (C), 152.3 (C), 148.7 (d, ¹JC₉F = 239.5 Hz, CF), 148.3 (C), 139.75 (d, ²JC₉F = 13.6 Hz, C), 135.4 (CH), 130.3 (CH), 126.8 (C), 125.5 (CH), 123.9 (CH), 116.1 (td, J = 272.7, 3.4 Hz, CF₂), 114.2 (d, ²JC₉F = 21.5 Hz, CH), 108.7 (d, ³JC₉F = 6.2 Hz, C), 102.0 (d, ³JC₉F = 3.7 Hz, CH), 83.1 (C), 64.5 (td, ²JC₉F = 26.9, ⁴JC₉F=2.3 Hz, CH₂). ¹⁹F NMR (CD₃CN, 376 MHz) δ −98.97 (p, ³JC₉F = 12.4 Hz, 4F), −135.77 − −136.03 (m, 2F). Analytical LC–MS: τᵣ = 13.47 min, >99% purity (10–95% MeCN/H₂O, linear gradient, with constant 0.1% v/v TFA additive; 20 min run; 1 mL/min flow; ESI; positive ion mode; detection at 550 nm). HRMS (ESI) calcd for C₂₆H₁₇F₆N₃O₢ [M+H]+ 519.1138, found 519.1146.

**6-tert-Butoxycarbonyl-JF₅₂₆ (30).** The title compound was prepared from dibromide 24 and 3,3-difluoroazetidine hydrochloride (29) following the C–N cross-coupling method described for 9 (69%, white solid). ¹H NMR (CDCl₃, 400 MHz) δ 8.24 (dd, J = 8.0, 1.3 Hz, 1H), 8.05 (dd, J = 8.0, 0.8 Hz, 1H), 7.73 (s, 1H), 6.33 – 6.29 (m, 4H), 4.36 (td, ³J₉F = 11.8, ⁵J₉F = 2.1 Hz, 8H), 1.57 (s, 9H). ¹³C NMR (CDCl₃, 376 MHz) δ −100.16 (p, ³JC₉F = 12.0 Hz, 4F), −137.31 (ddt, ³JC₉F = 12.4, ⁴JC₉F = 7.6, ⁵JC₉F = 2.2 Hz, 2F). ¹⁹F NMR (CDCl₃, 101 MHz) δ 168.2 (C), 164.1 (C), 152.4 (C), 148.7 (d, ¹JC₉F = 239.7 Hz, CF), 148.3 (d, ³JC₉F = 1.6 Hz, C), 140.0 (C), 138.7 (C), 131.3 (CH), 129.7 (C), 125.3 (CH), 125.0 (CH), 114.1 (d, ²JC₉F = 21.7 Hz, CH), 108.0 (d, ³JC₉F = 6.4 Hz, C), 102.0 (d, ³JC₉F = 3.8 Hz, CH), 83.5 (C), 82.9 (C), 64.5 (td, ²JC₉F = 26.9 Hz, ⁴JC₉F=2.2 Hz, CH₂), 28.2 (CH₃). Analytical LC–MS: τᵣ = 15.07 min, >99% purity (10–95% MeCN/H₂O, linear gradient, with constant 0.1% v/v TFA additive; 20 min run; 1 mL/min flow; ESI; positive ion mode; detection at 550 nm). HRMS (ESI) calcd for C₃₁H₂₅F₆N₃O₅ [M+H]+ 619.1662, found 619.1663.

**6-Carboxy–JF₅₂₆ (31):** Ester 30 (31.0 mg, 50 µmol) was taken up in CH₂Cl₂ (2.5 mL) to which TFA (0.5 mL) was added. The reaction was stirred at room temperature for 6 h. Toluene (3 mL) was added; the reaction mixture was concentrated under reduced pressure and then azeotroped with MeOH (3×) to provide 31 as a dark pink solid (33.3 mg, 98%, TFA salt). Analytical LC–MS and NMR indicated that the material was >95% pure and was used without further purification. ¹H
NMR (CD$_3$CN, 400 MHz) $\delta$ 8.31 (dd, $J = 8.1, 1.5$ Hz, 1H), 8.20 (dd, $J = 8.1, 0.6$ Hz, 1H), 7.87 – 7.80 (m, 1H), 6.68 (d, $^3J_{HF} = 12.5$ Hz, 2H), 6.59 (d, $^4J_{HF} = 7.6$ Hz, 2H), 4.58 (td, $^3J_{HF} = 12.0$, $^5J_{HF} = 2.1$ Hz, 8H). $^{13}$C NMR (CD$_3$OD, 101 MHz) $\delta$ 167.9 (C), 167.5 (C), 154.1 (C), 151.4 (d, $^1J_{CF} = 247.5$ Hz, CF), 146.3 (d, $^2J_{CF} = 11.8$ Hz, C), 137.1 (C), 134.1 (C), 132.9 (CH), 131.2 (CH), 130.1 (CH), 117.1 (t, $^1J_{CF} = 270.7$ Hz, $^2J_{CF} = 3.1$ Hz, CF$_2$), 114.4 (d, $^2J_{CF} = 22.0$ Hz, CH), 114.0 (d, $^3J_{CF} = 5.2$ Hz, C), 101.0 (d, $^3J_{CF} = 3.5$ Hz, CH), 66.0 (t, $^5J_{CF} = 28.7$ Hz, CH$_2$). $^{19}$F NMR (CD$_3$CN, 376 MHz,) $\delta$ -76.70 (s, 3F), -101.85 (p, $^3J_{FH} = 11.9$ Hz, 4F), -133.67 – -134.44 (m, 2F). Analytical LC–MS: $t_{R}$ = 11.70 min, 97% purity (10–95% MeCN/H$_2$O, linear gradient, with constant 0.1% v/v TFA additive; 20 min run; 1 mL/min flow; ESI; positive ion mode; detection at 550 nm). HRMS (ESI) calcd for C$_{27}$H$_{47}$F$_8$N$_5$O$_5$ [M+H]$^+$ 563.1042, found 563.1045.

**JF$_{526}$–HaloTag ligand** ($10_{HTL}$). The title compound was prepared from 6-carboxy-JF$_{526}$ (31) and HaloTag(O2)amine$^{13}$ (16) following the amidation procedure method described for 9$_{TMP}$ (53%, white solid, TFA salt). $^1$H NMR (CD$_3$OD, 400 MHz) $\delta$ 8.72 (d, $J = 4.6$ Hz, 1H), 8.22 (d, $J = 8.2$ Hz, 1H), 8.18 (dd, $J = 8.1, 1.5$ Hz, 1H), 7.68 (s, 1H), 6.69 (d, $^4J_{HF} = 7.6$ Hz, 2H), 6.60 (d, $^3J_{HF} = 12.3$ Hz, 2H), 4.55 (t, $J = 11.8$ Hz, 8H), 3.66 – 3.49 (m, 10H), 3.42 (t, $J = 6.5$ Hz, 2H), 1.77 – 1.64 (m, 2H), 1.50 (m, $J = 6.8$ Hz, 2H), 1.45 – 1.25 (m, 4H). $^{19}$F NMR (CD$_3$OD, 376 MHz) $\delta$ -75.35 (s, 3F), -100.75 (p, $^3J_{FH} = 11.9$ Hz, 4F), -134.45 – -136.80 (m, 2F). Analytical LC–MS: $t_{R}$ = 13.98 min, >99% purity; (10–95% MeCN/H$_2$O, linear gradient, with constant 0.1% v/v TFA additive; 20 min run; 1 mL/min flow; ESI; positive ion mode; detection at 254 nm). HRMS (ESI) calcd for C$_{37}$H$_{47}$ClF$_8$N$_3$O$_6$, [M+H]$^+$ 768.2275, found 768.2281.

**JF$_{526}$–SNAP-tag ligand** ($10_{STL}$). The title compound was prepared from 6-carboxy-JF$_{526}$ (31) and benzylguanine-amine (BG-NH$_2$, S1, New England Biolabs) following the amidation method described for 9$_{TMP}$ (58%, pink solid, TFA salt). $^1$H NMR (DMF-$d_7$, 400 MHz) $\delta$ 9.31 (t, $J = 5.8$ Hz, 1H), 8.36 (dd, $J = 8.1, 1.4$ Hz, 1H), 8.22 (s, 1H), 8.14 (d, $J = 7.9$ Hz, 1H), 7.94 (d, $J = 1.2$ Hz, 1H), 7.51 (d, $J = 8.1$ Hz, 2H), 7.39 (d, $J = 8.0$ Hz, 2H), 6.70 (d, $^3J_{HF} = 12.5$ Hz, 2H), 6.66 (d, $^4J_{HF} = 7.8$ Hz, 2H), 5.53 (s, 2H), 4.66 – 4.36 (m, 10H). $^{19}$F NMR (DMF-$d_7$, 376 MHz) $\delta$ -74.57 (s, 3F), -99.94 (p, $^3J_{FH} = 12.3$ Hz, 4F), -137.36 (dd, $^3J_{FH} = 12.7$, $^4J_{FH} = 7.9$ Hz, 2F). Analytical LC–MS: $t_{R}$ = 9.28 min, >99% purity (10–95% MeCN/H$_2$O, linear gradient, with constant 0.1% v/v TFA additive; 20 min run; 1 mL/min flow; ESI; positive ion mode; detection at 550 nm). HRMS (ESI) calcd for C$_{40}$H$_{29}$F$_{6}$N$_{8}$O$_{5}$ [M+H]$^+$ 815.2165, found 815.2164.
**JF₅₂₆-Hoechst (1₀HST).** The title compound was prepared from 6-carboxy-JF₅₂₆ (3₁) and Hoechst-PEG₂-NH₂₁₅ (S₂) following the amidation method described for 9TMP (76%, purple solid, TFA salt). ¹H NMR (CD₃OD, 400 MHz) δ 8.33 (d, J = 1.7 Hz, 1H), 8.22 – 8.16 (m, 2H), 8.07 – 7.98 (m, 2H), 8.01 (dd, J = 8.5, 1.8 Hz, 1H), 7.88 – 7.84 (m, 2H), 7.72 (d, J = 9.1 Hz, 1H), 7.40 (dd, J = 9.1, 2.3 Hz, 1H), 7.31 (d, J = 2.2 Hz, 1H), 7.13 – 7.06 (m, 2H), 6.63 – 6.44 (m, 4H), 4.47 (t, J = 12.0 Hz, 8H), 4.08 (t, J = 6.2 Hz, 2H), 3.65 – 3.50 (m, 20H), 3.67 (t, J = 5.4 Hz, 2H), 3.28 (t, J = 5.7 Hz, 2H), 3.02 (s, 3H), 2.39 (t, J = 7.2 Hz, 2H), 2.08 (p, J = 6.8 Hz, 2H). ¹⁹F NMR (CD₃OD, 376 MHz) δ −75.40 (s, 12F), −100.67 (p, ³JFH = 11.6 Hz, 4F), −133.47 – −134.98 (m, 2F). Analytical LC–MS: tᵣ = 9.16 min, >99% purity (10–95% MeCN/H₂O, linear gradient, with constant 0.1% v/v TFA additive; 20 min run; 1 mL/min flow; ESI; positive ion mode; detection at 254 nm). HRMS (ESI) calcd for C₉₂H₅₉F₆N₁₀O₈, [M+H]⁺ 1185.4422, found 1185.4430.

![Image of JF₅₂₆-Hoechst](image)

**4-((7-Carboxyheptyl)carbamoyl)-2-(3-(3,3-difluoro-1H4-azetidin-1-ylidene)-6-(3,3-difluoroazetidin-1-yl)-2,7-difluoro-3H-xanthen-9-yl)benzoate (S₄).** The title compound was prepared from 6-carboxy-JF₅₂₆ (3₁) and 8-aminoocytanoic acid (S₃) following the amidation method described for 9TMP (38%, pink solid, TFA salt). ¹H NMR (CD₃OD, 400 MHz) δ 8.70 (t, J = 5.3 Hz, 1H), 8.21 (d, J = 7.9 Hz, 1H), 8.15 (dd, J = 8.1, 1.5 Hz, 1H), 7.65 (s, 1H), 6.78 – 6.49 (m, 4H), 4.56 (m, 8H), 3.35 – 3.34 (m, 2H), 2.26 (t, J = 7.4 Hz, 2H), 1.64 – 1.50 (m, 4H), 1.41 – 1.23 (m, 6H). ¹⁹F NMR (CD₃OD, 376 MHz) δ −75.38 (s, 3F), −100.76 (d, ³JFH = 10.9 Hz, 4F), −135.03 – −138.56 (m, 2F). Analytical LC–MS: tᵣ = 12.43 min, >99% purity (10–95% MeCN/H₂O, linear gradient, with constant 0.1% v/v TFA additive; 20 min run; 1 mL/min flow; ESI; positive ion mode; detection at 550 nm). HRMS (ESI) calcd for C₃₅H₃₂F₆N₃O₆, [M+H]⁺ 704.2195, found 704.2193.

![Image of 4-((7-Carboxyheptyl)carbamoyl)-2-(3-(3,3-difluoro-1H4-azetidin-1-ylidene)-6-(3,3-difluoroazetidin-1-yl)-2,7-difluoro-3H-xanthen-9-yl)benzoate](image)
**JF\textsubscript{526}–Taxol (10\textsubscript{TXL}).** S4 (4 mg, 6 \textmu mol, Scheme S1) was combined with HATU (12 mg, 31 \textmu mol, 5.3 equiv) in DMSO (0.4 mL). After adding DIEA (50 \textmu L, 37 mg, 290 \textmu mol, 48 equiv), the reaction was stirred at ambient temperature for 5 min while shielded from light. 3′-aminodocetaxel\textsuperscript{4} (S5, 8 mg, 12 \textmu mol, 2 equiv, Scheme S1) was then added. The reaction was stirred for 2 days at room temperature, then concentrated under reduced pressure. The crude material was purified by reverse phase HPLC (10–90% MeCN/H\textsubscript{2}O, linear gradient, with constant 0.1% v/v TFA additive) to provide 2.7 mg of 10\textsubscript{TXL} (36%, TFA salt) as an off-white solid. \textsuperscript{1}H NMR (CD\textsubscript{3}OD, 400 MHz) \(\delta\) 8.66 (t, \(J = 5.7\) Hz, 1H), 8.40 (d, \(J = 9.1\) Hz, 1H), 8.24 (d, \(J = 8.1\) Hz, 1H), 8.14 (dd, \(J = 8.1, 1.6\) Hz, 1H), 8.10 – 8.05 (m, 2H), 7.68 (s, 1H), 7.66 – 7.59 (m, 1H), 7.54 (t, \(J = 7.5\) Hz, 2H), 7.44 – 7.30 (m, 4H), 7.30 – 7.19 (m, 1H), 6.73 (d, \(J_{HF} = 7.4\) Hz, 2H), 6.65 (d, \(J_{HF} = 12.2\) Hz, 2H), 6.22 – 6.09 (m, 1H), 5.63 (d, \(J = 7.2\) Hz, 1H), 5.46 (d, \(J = 4.3\) Hz, 1H), 5.26 (s, 1H), 4.99 (d, \(J = 9.6\) Hz, 1H), 4.69 – 4.44 (m, 9H), 3.86 (d, \(J = 7.1\) Hz, 1H), 2.44 (m, 1H), 2.34 (s, 3H), 2.31 – 2.18 (m, 2H), 2.08 – 2.00 (m, 2H), 1.88 (m, 3H), 1.86 – 1.76 (m, 1H), 1.68 (s, 2H), 1.57 (m, 4H), 1.30 (m, 6H), 1.15 (s, 3H), 1.10 (s, 3H). \textsuperscript{19}F NMR (CD\textsubscript{3}OD, 376 MHz) \(\delta\) –75.38 (s, 9F), –100.82 (p, \(J_{HF} = 12.0\) Hz, 4F), –132.20 – –134.93 (m, 2F). Analytical LC: \(t_r = 13.64\) min, >99% purity (10–95% MeCN/H\textsubscript{2}O, linear gradient, with constant 0.1% v/v TFA additive; 20 min run; 1 mL/min flow; detection at 550 nm). Analytical LC–MS: \(t_r = 3.68\) min, >99% purity (Phenomenex Kinetex 2.1 mm × 30 mm 2.6 \textmu m C18 column; 5 \textmu L injection; 5–98% MeCN/H\textsubscript{2}O, linear gradient, with constant 0.1% v/v HCO\textsubscript{2}H additive; 6 min run; 0.5 mL/min flow; ESI; positive ion mode; detection at 550 nm). HRMS (ESI) calcd for C\textsubscript{73}H\textsubscript{75}F\textsubscript{6}N\textsubscript{4}O\textsubscript{17} [M+H]\textsuperscript{+} 1393.5031, found 1393.5042.

![Diagram](image_url)

\(\text{N-(6-Aminohexyl)-3',6'-bis(3,3-difluorooazetidin-1-yl)-2',7'-difluoro-3-oxo-3\textsubscript{H}-spirop[isobenzofuran-1,9']-xanthene-6-carboxamide (S7).}\) The title compound was prepared from 6-carboxy-JF\textsubscript{526} (31) and hexane-1,6-diamine (S6, Scheme S1) following the amidation method described for 9\textsubscript{TMP} (41%, pink solid, TFA salt). \textsuperscript{1}H NMR (CD\textsubscript{3}OD, 400 MHz) \(\delta\) 8.22 (d, \(J = 8.1\) Hz, 1H), 8.15 (dd, \(J = 8.1, 1.6\) Hz, 1H), 7.66 (d, \(J = 1.3\) Hz, 1H), 6.69 (d, \(J_{HF} = 7.5\) Hz, 2H), 6.60 (d, \(J_{HF} = 12.2\) Hz, 2H), 4.55 (t, \(J_{HF} = 11.8\) Hz, 8H), 3.36 (t, \(J = 7.2\) Hz, 2H), 2.90 (t, \(J = 7.6\) Hz, 2H), 1.63 (m, 4H), 1.40 (m, 4H). \textsuperscript{19}F NMR (CD\textsubscript{3}OD, 376 MHz) \(\delta\) –75.35 (s, 6F), –100.77 (p, \(J_{HF} = 12.2\) Hz, 4F), –133.10 – –135.26 (m, 2F). Analytical LC–MS: \(t_r = 9.86\) min, >99% purity (10–95% MeCN/H\textsubscript{2}O, linear gradient, with constant 0.1% v/v TFA additive; 20 min run; 1 mL/min flow; ESI; positive ion mode; detection at 550 nm). HRMS (ESI) calcd for C\textsubscript{33}H\textsubscript{31}F\textsubscript{6}N\textsubscript{4}O\textsubscript{4} [M+H]\textsuperscript{+} 661.2244, found 661.2241.
**JF<sub>526</sub>–Pepstatin A (10<sub>PEP</sub>).** Pepstatin A (S8, 8.3 mg, 12 µmol, 2 equiv) was combined with TSTU (4 mg, 15 µmol, 2.4 equiv) in DMF (0.3 mL). After adding DIEA (53 µL, 305 µmol, 50 equiv), the reaction was stirred at room temperature for 5 min while shielded from light. S7 (4 mg, 6 µmol, 1 equiv) was then added. The reaction was stirred for 2 days at room temperature, then concentrated under reduced pressure. The crude material was purified by reverse phase HPLC (10–90% MeCN/H<sub>2</sub>O, linear gradient, with constant 0.1% v/v TFA additive) to provide 1.7 mg of 10<sub>PEP</sub> (19%, TFA salt) as an off-white solid. <sup>1</sup>H NMR (CD<sub>3</sub>OD, 400 MHz) δ 8.67 (s, 1H), 8.30 – 8.07 (m, 2H), 7.98 (d, <i>J</i> = 8.0 Hz, 1H), 7.95 (d, <i>J</i> = 8.3 Hz, 1H), 7.66 (m, 2H), 7.42 (d, <i>J</i> = 9.4 Hz, 1H), 6.66 (d, <i>J</i><sub>HF</sub> = 7.5 Hz, 2H), 6.55 (d, <i>J</i><sub>HF</sub> = 12.3 Hz, 2H), 4.51 (t, <i>J</i> = 11.9 Hz, 8H), 4.25 (q, <i>J</i> = 7.1 Hz, 1H), 4.19 – 4.08 (m, 2H), 4.05 – 3.88 (m, 2H), 3.21 – 3.06 (m, 2H), 2.36 (m, 2H), 2.25 (d, <i>J</i> = 6.6 Hz, 2H), 2.18 – 1.93 (m, 5H), 1.69 – 1.45 (m, 7H), 1.43 – 1.23 (m, 8H), 1.09 – 0.79 (m, 30H). <sup>19</sup>F NMR (CD<sub>3</sub>OD, 376 MHz) δ −77.03 (s, 18F), −102.47 (p, <sup>3</sup> <i>J</i><sub>FH</sub> = 11.9 Hz, 4F), −135.83 – −137.96 (m, 2F). Analytical LC–MS: <i>t</i><sub>r</sub> = 13.72 min, >99% purity (10–95% MeCN/H<sub>2</sub>O, linear gradient, with constant 0.1% v/v TFA additive; 20 min run; 1 mL/min flow; ESI; positive ion mode; detection at 550 nm). HRMS (ESI) calcd for C<sub>67</sub>H<sub>92</sub>F<sub>6</sub>N<sub>3</sub>O<sub>12</sub> [M+H]<sup>+</sup> 1328.6770, found 1328.6783.

![Image](https://via.placeholder.com/150)

**JF<sub>525</sub>–Taxol (7<sub>TXL</sub>).** 6-Carboxy-JF<sub>525</sub> (S9, 5 mg, 8 µmol) was combined with TSTU (2.8 mg, 9 µmol, 1.2 equiv) in DMF (1 mL). After adding DIEA (28 µL, 160 µmol, 20 equiv), the reaction was stirred at room temperature for 1 h. S3 (1.5 mg, 9 µmol, 1.1 equiv, Scheme S1) was then added and the reaction was stirred for 2 days at room temperature while shielded from light. TSTU (2.8 mg, 9 µmol, 1.2 equiv) and S5 (13 mg, 22 µmol, 2 equiv, Scheme S1) was then added and the reaction was stirred for 4 days at room temperature while shielded from light, then concentrated under reduced pressure. The crude material was purified by reverse phase HPLC (10–90% MeCN/H<sub>2</sub>O, linear gradient, with constant 0.1% v/v TFA additive) to provide 1.0 mg of 7<sub>TXL</sub> (9%, TFA salt) as a pink solid. <sup>1</sup>H NMR (CD<sub>3</sub>OD, 400 MHz) δ 8.38 (d, <i>J</i> = 8.2 Hz, 1H), 8.18 (dd, <i>J</i> = 8.2, 1.8 Hz, 1H), 8.12 – 8.02 (m, 2H), 7.79 (d, <i>J</i> = 1.7 Hz, 1H), 7.63 (t, <i>J</i> = 7.4 Hz, 1H), 7.53 (t, <i>J</i> = 7.6 Hz, 2H), 7.44 – 7.32 (m, 4H), 7.25 (t, <i>J</i> = 6.9 Hz, 1H), 7.16 (dd, <i>J</i> = 9.2, 3.0 Hz, 2H), 6.81 (s, 2H), 6.77 – 6.70 (m, 2H), 6.13 (t, <i>J</i> = 9.0 Hz, 1H), 5.61 (d, <i>J</i> = 7.1 Hz, 1H), 5.46 (d, <i>J</i> = 4.2 Hz, 1H), 5.25 (s, 1H), 4.68 (t, <i>J</i><sub>HF</sub> = 11.6 Hz, 8H), 4.58 (d, <i>J</i> = 4.3 Hz, 1H), 4.26 – 4.09 (m, 3H), 3.85 (d, <i>J</i> = 7.2 Hz, 1H), 3.35 (m, 3H), 2.52 – 2.38 (m, 1H), 2.34 (s, 3H), 2.32 – 2.16 (m, 2H), 2.08 – 1.98 (m, 2H), 1.82 (d, <i>J</i> = 1.4 Hz, 2H), 1.67 (s, 3H), 1.64 – 1.50 (m, 4H), 1.31 (m, 6H), 1.15 (s, 6H), 1.08 (s, 6H). <sup>19</sup>F NMR (CD<sub>3</sub>OD, 376 MHz) δ −75.39 (s, 9F), −100.74 (p, <sup>3</sup> <i>J</i><sub>FH</sub> = 11.6 Hz, 4F). Analytical LC: <i>t</i><sub>r</sub> = 12.52 min, >99% purity (10–95% MeCN/H<sub>2</sub>O, linear gradient, with constant 0.1% v/v TFA additive; 20 min run; 1 mL/min flow; ESI; positive ion mode; detection at 550 nm). Analytical LC–MS: <i>t</i><sub>r</sub> = 3.44 min, >99% purity (Phenomenex Kinetex 2.1 mm × 30 mm 2.6 µm C18 column; 5 µL injection; 5–98% MeCN/H<sub>2</sub>O, linear gradient, with constant 0.1% v/v HCO<sub>2</sub>H.
additive; 6 min run; 0.5 mL/min flow; ESI; positive ion mode; detection at 550 nm). HRMS (ESI) calcd for C_{73}H_{77}F_{4}N_{4}O_{17} [M+H]⁺ 1357.5220, found 1357.5232.

3',6'-dibromo-2',7'-difluoro-3H-spiro[isobenzofuran-1,9'-xanthene]-6-carboxylic acid (33). 2',7'-Fluoro-6-carboxyfluorescein dibromide (22, 420 mg, 0.78 mmol) was taken in 60 mL 5:1 isopropanol/THF mixture and cooled to 0 °C, and LiBH₄ (4.9 mL of 2.0 M solution in THF, 9.8 mmol, 12.5 equiv) was added dropwise. The reaction was stirred at room temperature for 8 h, before being diluted with saturated NH₄Cl(aq) and extracted with CH₂Cl₂ (2×). The combined organic extracts were dried over MgSO₄(s), filtered, and concentrated under reduced pressure. Purification by silica gel chromatography (0–3% MeOH/CH₂Cl₂, linear gradient) afforded 33 (222 mg, 54%) as a white solid. ¹H NMR (CD₂OD, 400 MHz) δ 8.94 (d, J = 1.8 Hz, 1H), 8.09 (dd, J = 8.1, 1.8 Hz, 1H), 7.71 (d, J = 8.0 Hz, 1H), 7.57 (d, J = 5.8 Hz, 2H), 6.75 (d, J = 8.8 Hz, 2H), 3.78 (s, 2H). ¹³C NMR (101 MHz, CD₂OD) δ 170.0 (C), 156.6 (d, J = 242.5 Hz, CF), 147.5 (d, J = 25.0 Hz, C), 145.1 (C), 142.9 (C), 130.8 (CH), 130.3 (C), 128.4 (CH), 127.3 (d, J = 6.0 Hz, CH), 122.3 (CH), 116.4 (d, J = 25.0 Hz, CH), 110.6 (d, J = 23.5 Hz, C), 69.3 (C), 61.6 (CH₂). ¹⁹F NMR (CD₂OD, 376 MHz) δ −115.79 (dd, J = 8.9, 6.1 Hz). HRMS (ESI) calcd for C_{21}H_{11}Br₂F₂O₄, [M+H]⁺ 522.8992, found 522.8994.

**tert-Butyl 3',6'-dibromo-2',7'-difluoro-3H-spiro[isobenzofuran-1,9'-xanthene]-6-carboxylate (34).** A suspension of 3',6'-dibromo-2',7'-difluoro-3H-spiro[isobenzofuran-1,9'-xanthene]-6-carboxylic acid (33, 125 mg, 0.24 mmol) in toluene was heated to 80 °C and N,N-dimethylformamide di-tert-butyl acetal (23, 480 µL, 2.4 mmol, 10 equiv) was added dropwise. The reaction was stirred at 80 °C for 15 min. After cooling the mixture to room temperature, it was diluted with saturated NaHCO₃(aq) and extracted with CH₂Cl₂ (2×). The combined organic extracts were dried (MgSO₄), filtered, and concentrated under reduced pressure. Flash chromatography (0–50% CH₂Cl₂/hexanes, linear gradient) provided 34 as a white solid (87 mg, 63%). ¹H NMR (CDCl₃, 400 MHz) δ 8.05 (dd, J = 7.9, 1.5 Hz, 1H), 7.47 (d, J = 5.7 Hz, 2H), 7.45 – 7.41 (m, 2H), 6.72 (d, J = 8.5 Hz, 2H), 5.41 (s, 2H), 1.54 (s, 9H). ¹³C NMR (CDCl₃, 101 MHz) δ 164.9 (C), 155.5 (d, J = 243.7 Hz, CF), 146.4 (C), 144.2 (C), 142.4 (C), 133.5 (C), 130.7 (CH), 124.7 (CH), 124.2 (d, J = 6.1 Hz, C), 121.7 (CH), 121.3 (CH), 115.0 (d, J = 24.7 Hz, CH), 110.5 (d, J = 23.3 Hz, C), 81.9 (CH₂), 73.2 (C), 28.3 (CH₃). ¹⁹F NMR (CDCl₃, 376 MHz) δ −113.82 (dd, J = 8.5 Hz, 5.8 Hz). HRMS (ESI) calcd for C_{25}H_{19}Br₂F₂O₄ [M+H]⁺ 578.9618, found 578.9619.
tert-Butyl 3',6'-bis(3,3-difluoroazetidin-1-yl)-2',7'-difluoro-3H-spiro[isobenzofuran-1,9'-xanthene]-6-carboxylate (35). The title compound was prepared from dibromide 34 and 3,3-difluoroazetidine hydrochloride (29) following the C–N cross-coupling method described for 9 (64%, white solid). 1H NMR (CDCl3, 400 MHz) δ 8.02 (dd, J = 7.9, 1.5 Hz, 1H), 7.48 (d, J = 1.3 Hz, 1H), 7.04 (d, J = 7.9 Hz, 1H), 6.48 (d, 3J_HF = 12.4 Hz, 2H), 6.26 (d, 4J_HF = 7.7 Hz, 2H), 5.30 (s, 2H), 4.32 (td, 3J_HF = 11.9, 5J_HF = 2.1 Hz, 8H), 1.54 (s, 9H). 13C NMR (CDCl3, 101 MHz) δ 165.3 (C), 147.7 (d, 1J_CF = 238.6 Hz, CF), 147.1 (d, 4J_CF = 1.1 Hz, C), 144.7 (C), 143.4 (C), 133.2 (C), 130.1 (CH), 125.2 (CH), 121.0 (CH), 115.0 (d, 2J_CF = 21.1 Hz, CH), 114.4 (d, 3J_CF = 6.1 Hz, C), 101.9 (d, 3J_CF = 3.7 Hz, CH), 83.7 (C), 81.6 (C), 72.0 (CH2), 64.5 (td, 2J_CF = 26.0, 4J_CF = 2.1 Hz, CH), 28.3 (CH3). 19F NMR (CDCl3, 376 MHz) δ −100.67 (p, 3J_FH = 12.0 Hz, 4F), −137.90 (ddd, 3J_FH = 12.6, 4J_FH = 7.7, 5J_FH = 2.1 Hz, 2F). HRMS (ESI) calcld for C31H27F6N2O4, [M+H]+ 605.1870, found 605.1875.

6-Carboxy-hydroxymethyl-JF326 (6-carboxy-HM-JF326, 36). Ester 35 (25 mg, 33 µmol) was dissolved in CH2Cl2 (2.5 mL), and trifluoroacetic acid (0.5 mL) was added. The reaction was stirred at room temperature for 1 h. Toluene (3 mL) was added and the reaction mixture was concentrated under reduced pressure. The residue was dissolved in MeOH after which Na2CO3 (50 mg) was added, and the reaction was stirred at room temperature for 1 h, filtered, and concentrated under reduced pressure. Flash chromatography on silica gel (2–70% EtOAc/hexanes, linear gradient) afforded 36 as a light pink solid (13 mg, 72%). 1H NMR (DMSO-d6, 400 MHz) δ 8.08 (dd, J = 8.0, 1.4 Hz, 1H), 7.61 (d, J = 7.9 Hz, 1H), 7.45 (s, 1H), 6.68 (d, 3J_HF = 12.6 Hz, 2H), 6.46 (d, 4J_HF = 7.8 Hz, 2H), 5.46 (s, 2H), 4.42 (td, 3J_HF = 12.1, 5J_HF = 2.1 Hz, 8H). 13C NMR (DMSO-d6, 101 MHz) δ 166.1 (C), 148.8 (d, 1J_CF = 237.2 Hz, CF), 146.7 (d, 4J_CF = 1.6 Hz, C), 145.9 (C), 143.8 (C), 139.0 (dt, 2J_CF = 13.4, 4J_CF = 2.7 Hz, C), 131.3 (C), 129.9 (CH), 124.4 (CH), 121.7 (CH), 119.5 (t, 1J_CF = 270.4 Hz, CF2), 115.1 (d, 3J_CF = 5.9 Hz, C), 114.4 (d, 2J_CF = 21.3 Hz, CH), 102.1 (d, 3J_CF = 3.7 Hz, CH), 83.0 (C), 72.2 (CH2), 64.0 (td, 2J_CF = 26.0, 4J_CF = 2.5 Hz, CH2). 19F NMR (DMSO-d6, 376 MHz) δ −100.37 (p, 3J_FH = 12.1 Hz, 4F), −137.16 − −137.65 (m, 2F). HRMS (ESI) calcld for C27H19F6N2O4 [M+H]+ 549.1244, found 549.1249.
**HM-JF$_{52e}$-NHS (37).** Acid 36 (13 mg, 23.7 µmol) was combined with TSTU (14 mg, 47 µmol, 2 eq) in DMF (1 mL), and DIEA (35 µL, 295 µmol, 9 eq) was added. After stirring the reaction at room temperature for 30 min, it was concentrated under reduced pressure. Flash chromatography on silica gel (5–60% EtOAc/hexanes, linear gradient) afforded 36 as a yellow solid (11.6 mg, 76%). $^1$H NMR (CDCl$_3$, 400 MHz) $\delta$ 8.17 (dd, $J = 8.0$, 1.6 Hz, 1H), 7.60 (d, $J = 1.4$ Hz, 1H), 7.52 (dd, $J = 8.0$, 0.8 Hz, 1H), 6.49 (d, $^3J_{HF} = 12.2$ Hz, 2H), 6.26 (d, $^4J_{HF} = 7.7$ Hz, 2H), 5.37 (s, 2H), 4.34 (td, $^3J_{HF} = 11.8$, $^5J_{HF} = 2.0$ Hz, 8H), 2.87 (d, $J = 6.5$ Hz, 4H). $^{19}$F NMR (CDCl$_3$, 376 MHz) $\delta$ −100.38 (p, $^3J_{FH} = 12.1$ Hz, 4F), −137.14 – −137.31 (m, 2F). Analytical LC–MS: $t_R = 12.99$ min, 98% purity; (10–95% MeCN/H$_2$O, linear gradient, with constant 0.1% v/v TFA additive; 20 min run; 1 mL/min flow; ESI; positive ion mode; detection at 550 nm). HRMS (ESI) calcd for C$_{31}$H$_{22}$F$_6$N$_3$O$_6$ [M+H]$^+$ 646.1407, found 646.1419.
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NMR and LC-MS

**Parameters**
- Origin: Bruker Biospin GmbH
- Solvent: DMSO
- Temperature: 300 K
- Pulse Sequence: zg40
- Experiment: 1D
- Number of Scans: 16
- Spectrometer Frequency: 400.13 MHz
- Spectral Width: 10240.0 Hz
- Lowest Frequency: -1946.8 Hz
- Nucleus: 1H
- Acquired Size: 52708
- Spectral Size: 52708

**Spectra**
| Origin          | Draken-BoSeim GmbH       |
|----------------|--------------------------|
| Solvent        | MeOD                     |
| Temperature    | 300°C                    |
| Pulse Sequence | zg39                     |
| Experiment     | 1C                       |
| Number of Scan | 16                       |
| Spectrometer Frequency | 400.13                  |
| Spectral Width | 5012.8                   |
| Lowest Frequency | t3453.1                 |
| Number         | 512                      |
| Acquired Size  | 30758                    |
| Spectral Freqs | 00536                    |

**DAD1 D, Sig=600.4 Ref=off (2018_09/DAILY_SEQUENCE_LC_2018-09-19 14-27-48/2018_0900000002.D)***

**MSD2 SPC, time=10.698:1:771 of C:\CHEM321\DATA\2018_09/DAILY_SEQUENCE_LC_2018-09-19 14-27-48/2018_0900000002.D ES-***

Max: 349210
**Graphical Data**

- **DAD1 C, Sig=550.4 Ref=off (2017_03/DAILY_SEQUENCE LC 2017-03-17 12:00-17:2017_03000002.D)**

- **MSD2 SPC, time=9.20:10.011 of C:\CHEM321\DATA/2017_03/DAILY_SEQUENCE LC 2017-03-17 12:00-17:2017_03000002.D ES-4**

**Chemical Structures**

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**Origin**: Bruker BioSpin GmbH

**Solvent**: MeOH

**Temperature**: 300 K

**Pulse Sequence**: zg180

**Experiment**: 1D

**Number of Scans**: 128

**Spectrometer Frequency**: 400.13 MHz

**Spectral Width**: 8012.0 Hz

**Lowest Frequency**: 1463.2 Hz

**Full Scan Data**: 52788

**Spectral Size**: 60536
