Supplementary Figure 1

Spectral data for fluorophores 1–12, 16, 21, and 25.

Normalized absorbance (abs), fluorescence excitation (fl\textsubscript{ex}), and fluorescence emission (fl\textsubscript{em}) spectra for rhodamines (1, 5–12), rhodols (4, 16) carborhodamines (2, 21), and Si-rhodamines (3, 25) in 10 mM HEPES, pH 7.3; the fl\textsubscript{ex} spectra are delineated by a dashed line. Note: the normalized absorbance spectra of fluorophores 3, 21, and 25 exhibit higher noise due to low visible absorption in aqueous buffer.
Supplementary Figure 2

Labeling cells with Janelia Fluor dyes.

(a) Chemical structure of JF525–SNAP-tag (15). (b) Image of COS7 cells expressing SNAP-tag–histone H2B and stained with ligand 15. (c) HaloTag and SNAP-tag ligands have no effect on COS7 cell viability at concentrations used for labeling; HaloTag ligands 13, 14, 17, 23, 26, 27 were incubated with cells for 1 h; SNAP-tag ligands 15, 20, 24, 29 were incubated for 3 h; error bars show ± s.d.; n = 3. (d) Chemical structures of known 488 nm-excited HaloTag ligands 18 and 19. (e) Plot of average cellular fluorescence vs. incubation time for live cells loaded with ligands 17–19. (f) Chemical structure of JF503–SNAP-tag ligand (20). (g) Image of COS7 cells expressing SNAP-tag–histone H2B and stained with ligand 20. (h) Structure of JF585–SNAP-tag ligand (24). (i) Image of COS7 cells expressing histone H2B–SNAP-tag and stained with ligand 24. (j) Multicolor image of U2OS cells expressing Sec61β–HaloTag fusion (stable) and TOMM20–SNAP-tag (transient) labeled with JF503–SNAP-tag ligand 20 (mitochondria, green), JF585–HaloTag ligand 23 (ER, orange), and JF646–Hoechst 33 (nucleus, red). (k) Chemical structure of SiTMR–HaloTag ligand 28. (l) Images of COS7 cells expressing HaloTag–histone H2B fusion and labeled with 250 nM of HaloTag ligand 28 for 1 h and imaged directly without washing. The number indicates mean signal (nuclear) to background (cytosol) ratio (S/B) in three fields of view (n = 152 areas). This image was taken with identical microscope settings to those used with ligands 26 and 27 (Fig. 2m,n). (m) Chemical structure of JF635–SNAP-tag ligand (29). (n) Image of COS7 cells expressing SNAP-tag–histone H2B and stained with ligand 29. (o) Multicolor image of U2OS cells expressing Sec61β–HaloTag fusion (stable) and histone H2B–SNAP-tag (transient) labeled with JF525–SNAP-tag ligand 15 (nucleus, yellow) and JF635–HaloTag ligand 27 (ER, red). Scale bars for all images: 15 μm.
Supplementary Figure 3

*In vivo* imaging using the Janelia Fluor dyes

(a–b) Comparison of Basin cell and pan-neuronal labeling in tissue. (a) SiMView light-sheet microscopy image of the ventral nerve cord region of *Drosophila* larval explant expressing HaloTag protein in Basin neurons and stained with JF₆₃₅–HaloTag ligand (27; same imaging data set as Fig. 3a). Lower panel shows image of the anteroposterior (AP) cross-section of the indicated volume. (b) SiMView light-sheet microscopy image of ventral nerve cord region of *Drosophila* larval explant expressing GCaMP6s protein pan-neuronally (Gal4/UAS system; 57C10-Gal4 driver line). Lower panel shows image of the AP cross-section of the indicated volume. Scale bars for a and b: 50 μm. (c) SiMView light-sheet microscopy image (same as Fig. 3a) with inset showing a single imaging slice from the 3D projection through neuronal cell bodies. (d) Representative images from the labeling time course for JF₅₈₅–HaloTag ligand (23) *in vivo*. Bright field image showing cranial window and epi-fluorescence images of green (GCaMP6s; *t* = 0) and red (JF₅₈₅, *t* = 0 and 6 h. Scale bar: 0.5 mm. (e) Plot of GCaMP6s green fluorescence vs. JF₅₈₅ red fluorescence for 2-photon imaging experiments. Found: Pearson linear correlation coefficient (*ρ*) = 0.768; *n* = 106 regions of interest (ROIs).
SUPPLEMENTARY NOTE: SYNTHESIS AND CHARACTERIZATION OF NEW COMPOUNDS

General Experimental Information for Synthesis

General experimental information. 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 with a silicon oil bath or an aluminum reaction block on top of a stirring hotplate equipped with an electronic contact thermometer to maintain the indicated temperatures.

Reactions were monitored by thin layer chromatography (TLC) on precoated TLC glass plates (silica gel 60 F_{254}, 250 µm thickness) or by LC–MS (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 HPLC analysis was performed with an Agilent Eclipse XDB 4.6 × 150 mm 5 µm C18 column under the indicated conditions. High-resolution mass spectrometry was obtained by the Mass Spectrometry Center in the Department of Medicinal Chemistry at the University of Washington and the High Resolution Mass Spectrometry Facility at the University of Iowa.

NMR spectra were recorded on a 400 MHz spectrometer. ¹H and ¹³C chemical shifts (δ) were referenced to TMS or residual solvent peaks, and ¹⁹F chemical shifts (δ) 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), 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.
Scheme SN1. Synthesis of Janelia Fluor dyes and ligands. (a) Cross-coupling strategy for the synthesis of azetidinyl rhodamines. (b) Synthesis of azetidinyl rhodamines 4 and 16. (c) Synthesis of JF₅₂⁵ ligands 13 and 15 from triflate 33. (d) Synthesis of JF₆₀₃ ligands 17 and 20 from triflate 33. (e) Synthesis of JF₆₀₈ and JF₅₈₅ ligands 22–24 from ketone 42. (f) Synthesis of JF₆₃₅ ligands 27 and 29 from triflate 51.
SYNTHESIS OF DITRIFLATE 45

6-tert-Butoxycarbonylcarbofluorescein bis-(tert-butyldimethylsilyle) (44): A vial was charged with di-tert-butyl 2-bromoterephthalate (43; 1.48 g, 4.14 mmol, 2 eq), sealed, and flushed with nitrogen. After dissolving the bromide in THF (7 mL) and cooling the reaction to -15 °C, i-PrMgCl·LiCl (1.3 M in THF, 3.19 mL, 4.14 mmol, 2 eq) was added. The reaction was warmed to -10 °C and stirred for 4 h. A solution of 3,6-bis((tert-butyldimethylsilyl)oxy)-10,10-dimethylanthracen-9(10H)-one (42; 1.00 g, 2.07 mmol) in THF (4 mL) was then added dropwise. The reaction mixture was warmed to room temperature and stirred for 2 h. It was subsequently quenched with saturated NH₄Cl, diluted with water, and extracted with EtOAc (2×). The combined organics were washed with brine, dried (MgSO₄), filtered, and evaporated. Silica gel chromatography (0–10% Et₂O/hexanes, linear gradient) provided 245 mg (17%) of 44 as a colorless solid. ¹H NMR (CDCl₃, 400 MHz) δ 8.16 (dd, J = 8.0, 1.3 Hz, 1H), 8.02 (dd, J = 8.0, 0.6 Hz, 1H), 7.63–7.59 (m, 1H), 7.09–7.05 (m, 2H), 6.64–6.57 (m, 4H), 1.81 (s, 3H), 1.72 (s, 3H), 1.54 (s, 9H), 0.99 (s, 18H), 0.22 (s, 12H); ¹³C NMR (CDCl₃, 101 MHz) δ 169.9 (C), 164.4 (C), 156.5 (C), 155.5 (C), 147.0 (C), 138.1 (C), 130.3 (CH), 129.7 (C), 129.3 (CH), 125.1 (CH), 125.0 (C), 119.2 (CH), 117.8 (CH), 87.0 (C), 82.5 (C), 38.2 (C), 35.0 (CH₃), 33.2 (CH₃), 28.2 (CH₃), 25.8 (CH₃), 18.4 (C), -4.17 (CH₃), -4.19 (CH₃); HRMS (ESI) calcd for C₄₀H₅₅O₆Si₂ [M+H]⁺ 687.3537, found 687.3533.

6-tert-Butoxycarbonylcarbofluorescein ditriflate (45): To a solution of silyl ether 44 (170 mg, 0.247 mmol) was added TBAF (1.0 M in THF, 990 µL, 0.990 mmol, 4 eq). The reaction was stirred at room temperature for 10 min. It was subsequently diluted with saturated NH₄Cl and extracted with EtOAc (2×). The organic extracts were washed with brine, dried (MgSO₄), filtered, and evaporated to provide an orange residue. The crude intermediate was taken up in CH₂Cl₂ (5 mL) and cooled to 0 °C. Pyridine (160 µL, 1.98 mmol, 8 eq) and trifluoromethanesulfonic anhydride (167 µL, 0.990 µmol, 4 eq) were added, and the ice bath was removed. The reaction was stirred at room temperature for 2 h. It was then diluted with water and extracted with CH₂Cl₂ (2×). The combined organics were washed with brine, dried (MgSO₄), filtered, and concentrated in vacuo. Flash chromatography on silica gel (0–20% EtOAc/hexanes, linear gradient) afforded 159 mg (89%) of 45 as a colorless solid. ¹H NMR (CDCl₃, 400 MHz) δ 8.24 (dd, J = 8.0, 1.3 Hz, 1H), 8.11 (dd, J = 8.0, 0.6 Hz, 1H), 7.63–7.60 (m, 1H), 7.56 (d, J = 2.5 Hz, 2H), 7.10 (dd, J = 8.8, 2.5 Hz, 2H), 6.88 (d, J = 8.8 Hz, 2H), 1.91 (s, 3H), 1.81 (s, 3H), 1.56 (s, 9H); ¹⁹F NMR (CDCl₃, 376 MHz) δ...
C–N CROSS-COUPLED PRODUCTS

General procedure for preparation of rhodamines via C–N cross-coupling of fluorescein ditriflates (Method A): The following procedure for 5 is representative. A vial was charged with fluorescein ditriflate (30; 150 mg, 251 µmol), 3,3-dimethylazetidine hydrochloride (73 mg, 604 µmol, 2.4 eq), Pd2dba3 (23 mg, 25.1 µmol, 0.1 eq), XPhos (36 mg, 75.4 µmol, 0.3 eq), and Cs2CO3 (393 mg, 1.21 mmol, 4.8 eq). The vial was sealed and evacuated/backfilled with nitrogen (3×). Dioxane (2 mL) was added, and the reaction was flushed again with nitrogen (3×). The reaction was then stirred at 100 °C for 4 h. It was subsequently cooled to room temperature, diluted with MeOH, deposited onto Celite, and concentrated to dryness. Purification by silica gel chromatography (0–10% MeOH (2 M NH3/CH2Cl2, linear gradient; dry load with Celite) afforded 5 (101 mg, 86%) as a purple solid.

2-(3,6-Bis(3,3-dimethylazetidin-1-yl)xanthylum-9-yl)benzoate (5): (86%, purple solid) 1H NMR (CD2OD, 400 MHz) δ 8.11–8.06 (m, 1H), 7.67–7.57 (m, 2H), 7.23–7.20 (m, 1H), 7.19 (d, J = 9.2 Hz, 2H), 6.56 (dd, J = 9.1, 2.2 Hz, 2H), 6.49 (d, J = 2.2 Hz, 2H), 3.92 (s, 8H), 1.39 (s, 12H); 13C NMR (CD2OD, 101 MHz) δ 173.1 (C), 160.2 (C), 158.2 (C), 140.8 (C), 134.9 (C), 132.9 (CH), 130.82 (CH), 130.77 (CH), 130.74 (CH), 130.2 (CH), 115.0 (C), 113.1 (CH), 95.5 (CH), 64.4 (CH2), 33.0 (C), 27.1 (CH3); Analytical HPLC: tR = 15.6 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 C30H31N2O3 [M+H]+ 467.2329, found 467.2341.

2-(3,6-Bis(3-(2-methoxy-2-oxoethyl)azetidin-1-yl)xanthylum-9-yl)benzoate (55): Method A was used to prepare the title compound from 30 and methyl 3-azetidineacetate trifluoroacetate (67%, purple solid). 1H NMR (CD2OD, 400 MHz) δ 8.10–8.05 (m, 1H), 7.67–7.57 (m, 2H), 7.21–7.18 (m, 1H), 7.16 (d, J = 9.1 Hz, 2H), 6.54 (dd, J = 9.1, 2.2 Hz, 2H), 6.48 (d, J = 2.2 Hz, 2H), 4.41–4.32 (m, 4H), 3.97–3.88 (m, 4H), 3.69 (s, 6H), 3.26–3.13 (m,
2H), 2.80 (d, J = 7.7 Hz, 4H); 13C NMR (CD3OD, 101 MHz) δ 173.7 (C), 173.0 (C), 158.3 (C), 157.5 (C), 154.9 (C), 140.1 (C), 136.3 (C), 132.7 (CH), 131.1 (CH), 130.8 (CH), 130.4 (CH), 129.8 (CH), 114.5 (C), 112.7 (CH), 95.7 (CH), 57.5 (CH2), 52.2 (CH3), 38.5 (CH2), 27.3 (CH); HRMS (ESI) calcd for C12H12N2O7 [M+H]+ 555.2126, found 555.2132.

2,5-Difluorobenzyl (55): Ester 55 (25 mg, 45.1 µmol) was dissolved in MeOH (2 mL), and 1 M NaOH (180 µL, 180 µmol, 4 eq) was added. After stirring the reaction at room temperature for 18 h, it was acidified with 1 M HCl (200 µL) and directly purified by reverse phase HPLC (10–50% MeCN/H2O, linear gradient, with constant 0.1% v/v TFA additive) to provide 23 mg (80%, TFA salt) of 6 as a red-purple solid. 1H NMR (CD3OD, 400 MHz) δ 8.35 – 8.30 (m, 1H), 7.83 (td, J = 7.5, 1.5 Hz, 1H), 7.78 (td, J = 7.6, 1.5 Hz, 1H), 7.40 – 7.35 (m, 1H), 7.07 (d, J = 9.2 Hz, 2H), 6.62 (dd, J = 9.2, 2.2 Hz, 2H), 6.56 (d, J = 2.2 Hz, 2H), 4.43 (t, J = 9.6 Hz, 4H), 4.05 – 3.96 (m, 4H), 3.28 – 3.16 (m, 2H), 2.78 (d, J = 7.7 Hz, 4H); 13C NMR (CD3OD, 101 MHz) δ 175.1 (C), 167.9 (C), 161.7 (C), 135.4 (C), 133.8 (CH), 132.5 (CH), 132.3 (CH), 131.41 (CH), 131.40 (CH), 115.1 (C), 113.7 (CH), 95.3 (CH), 57.6 (CH2), 38.5 (CH2), 27.3 (CH); Analytical HPLC: tR = 10.5 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 550 nm); HRMS (ESI) calcd for C30H27N2O7 [M+H]+ 527.1813, found 527.1815.

2-(3,6-Bis(3-(carboxymethyl)azetidin-1-yl)xanthyl-9-yl)benzoate (56): Method A was used to prepare the title compound from 30 and methyl azetidine-3-carboxylate hydrochloride (79%, purple solid). 1H NMR (CD3OD, 400 MHz) δ 8.09 – 8.03 (m, 1H), 7.69 – 7.62 (m, 2H), 7.24 – 7.17 (m, 1H), 7.02 (d, J = 8.9 Hz, 2H), 6.48 (dd, J = 8.9, 2.2 Hz, 2H), 6.45 (d, J = 2.1 Hz, 2H), 4.34 (t, J = 9.0 Hz, 4H), 4.25 (dd, J = 9.0, 5.9 Hz, 4H), 3.77 (s, 6H), 3.71 (tt, J = 8.9, 5.9 Hz, 2H); 13C NMR (CD3OD, 101 MHz) δ 174.4 (C), 172.6 (C), 157.0 (C), 156.5 (C), 141.6 (C), 136.7 (C), 135.8 (C), 132.7 (CH), 131.9 (CH), 130.9 (CH), 129.0 (CH), 128.5 (CH), 113.3 (C), 111.7 (CH), 96.8 (CH), 55.2 (CH2), 52.9 (CH3), 34.0 (CH); HRMS (ESI) calcd for C30H27N2O7 [M+H]+ 527.1813, found 527.1823.
2-(3,6-Bis(3-carboxyazetidin-1-yl)xanthylum-9-yl)benzoate (7): Ester 56 (40 mg, 76.0 µmol) was dissolved in MeOH (2.5 mL), and 1 M NaOH (304 µL, 304 µmol, 4 eq) was added. After stirring the reaction at room temperature for 18 h, it was acidified with 1 M HCl (350 µL) and directly purified by reverse phase HPLC (10–50% MeCN/H$_2$O, linear gradient, with constant 0.1% v/v TFA additive) to provide 28 mg (60%, TFA salt) of 7 as a red-purple solid. $^1$H NMR (CD$_3$OD, 400 MHz) δ 8.36 – 8.30 (m, 1H), 7.84 (td, J = 7.5, 1.6 Hz, 1H), 7.79 (td, J = 7.6, 1.5 Hz, 1H), 7.40 – 7.36 (m, 1H), 7.12 (d, J = 9.2 Hz, 2H), 6.66 (dd, J = 9.2, 2.2 Hz, 2H), 6.61 (d, J = 2.2 Hz, 2H), 4.48 (t, J = 9.6 Hz, 4H), 4.39 (dd, $J = 9.9, 5.9$ Hz, 4H), 3.72 (tt, $J = 9.0, 5.1$ Hz, 2H); $^{13}$C NMR (CD$_3$OD, 101 MHz) δ 175.2 (C), 168.0 (C), 162.4 (C), 158.9 (C), 157.9 (C), 157.3 (C), 153.5 (C), 153.0 (C), 135.9 (C), 132.6 (CH), 132.5 (CH), 131.5 (CH), 131.4 (CH), 115.4 (C), 95.6 (CH), 55.3 (CH$_2$), 33.9 (CH); Analytical HPLC: $t_R = 11.2$ min, >99% purity (10–75% 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$_{28}$H$_{23}$N$_2$O$_7$ [M+H]$^+$ 499.1500, found 499.1507.

2-(3,6-Bis(3-(dimethylamino)azetidin-1-yl)xanthylum-9-yl)benzoate (8): Method A was used to prepare the title compound from 30 and 3-(dimethylamino)azetidine dihydrochloride (80%, purple solid). $^1$H NMR (CD$_3$OD, 400 MHz) δ 8.10 – 8.05 (m, 1H), 7.69 – 7.60 (m, 2H), 7.24 – 7.19 (m, 1H), 7.12 (d, J = 9.0 Hz, 2H), 6.56 (dd, J = 9.0, 2.2 Hz, 2H), 6.53 (d, J = 2.2 Hz, 2H), 4.31 – 4.22 (m, 4H), 4.01 (dd, J = 10.5, 5.1 Hz, 4H), 3.39 (tt, J = 7.0, 5.1 Hz, 2H), 2.27 (s, 12H); $^{13}$C NMR (CD$_3$OD, 101 MHz) δ 172.8 (C), 157.9 (C), 157.1 (C), 147.7 (C), 138.7 (C), 138.4 (C), 132.5 (CH), 131.8 (CH), 130.8 (CH), 129.9 (CH), 129.3 (CH), 114.1 (C), 112.4 (CH), 96.3 (CH), 57.0 (CH), 56.6 (CH$_2$), 42.0 (CH$_3$); Analytical HPLC: $t_R = 7.1$ 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$_{30}$H$_{33}$N$_4$O$_3$ [M+H]$^+$ 497.2547, found 497.2561.
2-(3,6-Bis(3-methoxyazetidin-1-yl)xanthylum-9-yl)benzoate (9): Method A was used to prepare the title compound from 30 and 3-methoxyazetidine hydrochloride (83%, purple solid). $^1$H NMR (DMSO-$d_6$, 400 MHz) $\delta$ 8.00 – 7.94 (m, 1H), 7.77 (td, $J = 7.5$, 1.2 Hz, 1H), 7.70 (td, $J = 7.5$, 0.9 Hz, 1H), 7.25 – 7.20 (m, 1H), 6.48 (d, $J = 8.6$ Hz, 2H), 6.26 (d, $J = 2.3$ Hz, 2H), 6.19 (dd, $J = 8.6$, 2.3 Hz, 2H), 4.32 (tt, $J = 6.2$, 4.2 Hz, 2H), 4.07 (dd, $J = 8.0$, 6.6 Hz, 4H), 3.66 (dd, $J = 8.4$, 4.1 Hz, 4H), 3.24 (s, 6H); $^{13}$C NMR (DMSO-$d_6$, 101 MHz) $\delta$ 168.7 (C), 152.1 (C), 129.7 (CH), 129.3 (CH), 127.1 (CH), 124.0 (CH), 119.7 (C), 109.7 (C), 108.1 (CH), 98.7 (CH), 83.9 (C), 83.3 (d, $J = 23.7$ Hz, CH$_2$); Analytical HPLC: $t_r$ = 12.2 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$_{25}$H$_{27}$N$_3$O$_5$ [M+H]$^+$ 471.1914, found 471.1926.

![Image of compound 9](image_url)

2-(3,6-Bis(3-fluoroazetidin-1-yl)xanthylum-9-yl)benzoate (10): Method A was used to prepare the title compound from 30 and 3-fluoroazetidine hydrochloride (89%, pink solid). $^1$H NMR (DMSO-$d_6$, 400 MHz) $\delta$ 8.01 – 7.95 (m, 1H), 7.78 (td, $J = 7.5$, 1.2 Hz, 1H), 7.71 (td, $J = 7.5$, 0.9 Hz, 1H), 7.25 – 7.20 (m, 1H), 6.52 (d, $J = 8.6$ Hz, 2H), 6.33 (d, $J = 2.3$ Hz, 2H), 6.24 (dd, $J = 8.6$, 2.3 Hz, 2H), 5.49 (ddtt, $^2J_{HF} = 57.6$ Hz, $J = 6.0$, 3.1 Hz, 2H), 4.26 – 4.13 (m, 4H), 4.00 – 3.88 (m, 4H); $^{19}$F NMR (DMSO-$d_6$, 376 MHz) $\delta$ -178.95 (dtt, $J_{FH} = 57.4$, 24.2, 20.9 Hz); $^{13}$C NMR (DMSO-$d_6$, 101 MHz) $\delta$ 168.7 (C), 152.54 (d, $^4J_{CF} = 1.3$ Hz, C), 152.47 (C), 151.8 (C), 135.4 (CH), 129.9 (CH), 128.6 (CH), 126.4 (C), 124.5 (CH), 123.9 (CH), 108.6 (CH), 107.8 (C), 98.0 (CH), 83.8 (C), 83.3 (d, $^1J_{CF} = 200.3$ Hz, CFH), 59.2 (d, $^2J_{CF} = 23.7$ Hz, CH$_2$); Analytical HPLC: $t_r$ = 12.1 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$_{26}$H$_{21}$F$_2$N$_3$O$_5$ [M+H]$^+$ 447.1515, found 447.1525.

![Image of compound 10](image_url)

2-(3,6-Bis(3-cyanoazetidin-1-yl)xanthylum-9-yl)benzoate (11): Method A was used to prepare the title compound from 30 and 3-azetidinecarbonitrile hydrochloride (85%, magenta solid). $^1$H NMR (CDCl$_3$, 400 MHz) $\delta$ 8.03 – 7.98 (m, 1H), 7.66 (td, $J = 7.4$, 1.3 Hz, 1H), 7.60 (td, $J = 7.4$, 1.1 Hz, 1H), 7.17 – 7.13 (m, 1H), 6.62 (d, $J = 8.6$ Hz, 2H), 6.25 (d, $J = 2.3$ Hz, 2H), 6.12 (dd, $J = 8.6$, 2.4 Hz, 2H), 4.25 – 4.18 (m, 4H), 4.15 – 4.08 (m, 4H), 3.60 (tt, $J = 8.5$, 6.2 Hz, 2H); $^{13}$C NMR (CDCl$_3$, 101 MHz) $\delta$ 169.6 (C), 153.2 (C), 152.5 (C), 151.9 (C), 135.0 (CH), 129.7 (CH), 129.3 (CH), 127.1 (C), 125.1 (CH), 124.0 (CH), 119.7 (C), 109.7 (C), 108.1 (CH), 98.7 (CH), 83.9 (C), 55.2 (CH$_2$), 18.4 (CH); Analytical HPLC: $t_r$ = 11.0 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_{28}H_{21}N_{4}O_{3} [M+H]^+ 461.1608, found 461.1628.

**JF_{325} (12):** Method A was used to prepare the title compound from 30 and 3,3-difluoroazetidine hydrochloride (91%, pink solid). ^1H NMR (CDCl3, 400 MHz) δ 8.03 – 7.99 (m, 1H), 7.66 (td, J = 7.4, 1.3 Hz, 1H), 7.60 (td, J = 7.4, 1.1 Hz, 1H), 7.17 – 7.14 (m, 1H), 6.64 (d, J = 8.6 Hz, 2H), 6.30 (d, J = 2.4 Hz, 2H), 6.17 (dd, J = 8.6, 2.4 Hz, 2H), 4.25 (t, J_{HF} = 11.7 Hz, 8H); 19F NMR (CDCl3, 376 MHz) δ -100.05 (p, J_{FH} = 11.8 Hz); ^13C NMR (CDCl3, 101 MHz) δ 169.6 (C), 153.3 (C), 152.6 (C), 151.3 (t, J_{CF} = 2.9 Hz, C), 135.0 (CH), 129.7 (CH), 129.3 (CH), 127.2 (C), 125.1 (CH), 124.5 (CH), 112.8 (t, J_{CF} = 274.6 Hz, CF2), 109.7 (C), 108.8 (CH), 99.4 (CH), 83.9 (C), 63.4 (t, J_{CF} = 26.3 Hz, CH2); Analytical HPLC: t_R = 12.7 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 525 nm); HRMS (ESI) calcd for C_{31}H_{27}F_{4}N_{2}O_{5} [M+H]^+ 483.1326, found 483.1336.

**6-tert-Butoxycarbonyl-JF_{325} (35):** Method A was used to prepare the title compound from 6-tert-butoxycarbonylfluorescein ditriflate^2 (33) and 3,3-difluoroazetidine hydrochloride (84%, pink solid). ^1H NMR (CDCl3, 400 MHz) δ 8.21 (dd, J = 8.0, 1.3 Hz, 1H), 8.04 (dd, J = 8.0, 0.8 Hz, 1H), 7.73 (dd, J = 1.2, 0.8 Hz, 1H), 6.61 (d, J = 8.6 Hz, 2H), 6.30 (d, J = 2.4 Hz, 2H), 6.17 (dd, J = 8.6, 2.4 Hz, 2H), 4.25 (t, J_{HF} = 11.7 Hz, 8H), 1.55 (s, 9H); ^19F NMR (CDCl3, 376 MHz) δ -100.06 (p, J_{FH} = 11.7 Hz); ^13C NMR (CDCl3, 101 MHz) δ 168.8 (C), 164.3 (C), 153.3 (C), 152.6 (C), 151.4 (t, J_{CF} = 2.9 Hz, C), 138.4 (C), 130.9 (CH), 130.2 (C), 129.3 (CH), 125.1 (CH), 125.0 (CH), 115.7 (t, J_{CF} = 274.5 Hz, CF2), 109.1 (C), 108.9 (CH), 99.4 (CH), 84.3 (C), 82.7 (C), 63.4 (t, J_{CF} = 26.3 Hz, CH2), 28.2 (CH3); HRMS (ESI) calcd for C_{29}H_{37}F_{4}N_{2}O_{5} [M+H]^+ 583.1851, found 583.1859.
**JF<sub>503</sub> (16):** A vial was charged with fluorescein ditriflate (30; 100 mg, 168 µmol), 3,3-difluoroazetidine hydrochloride (22 mg, 168 µmol, 1 eq), Pd<sub>2</sub>dba<sub>3</sub> (7.7 mg, 8.4 µmol, 0.05 eq), XPhos (12 mg, 25.1 µmol, 0.15 eq), and Cs<sub>2</sub>CO<sub>3</sub> (131 mg, 402 µmol, 2.4 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 then stirred at 100 °C for 1 h. It was subsequently cooled to room temperature, filtered through Celite with CH<sub>2</sub>Cl<sub>2</sub>, and concentrated to dryness. Purification by silica gel chromatography (0–40% EtOAc/hexanes, linear gradient) afforded JF<sub>503</sub> triflate (23 mg, 25%) as a white foam.

The triflate (20 mg, 37.1 µmol) was taken up in 1:1 THF/MeOH (1 mL). After adding 1 M NaOH (74 µL, 74.2 µmol, 2 eq), the reaction was stirred at room temperature for 3 h. It was then neutralized with 1 M HCl (74 µL) and concentrated to dryness. Reverse phase HPLC (10–75% MeCN/H<sub>2</sub>O, linear gradient, with constant 0.1% v/v TFA additive) afforded 16 as an orange solid. <sup>1</sup>H NMR (CD<sub>3</sub>OD, 400 MHz) δ 8.16 (d, <sup>J</sup> = 7.5 Hz, 1H), 7.85–7.79 (m, 1H), 7.79–7.72 (m, 1H), 7.29 (d, <sup>J</sup> = 7.6 Hz, 1H), 6.99–6.83 (m, 3H), 6.75 (d, <sup>J</sup> = 8.6 Hz, 1H), 6.67–6.60 (m, 1H), 6.56 (d, <sup>J</sup> = 8.7 Hz, 1H), 4.49 (t, <sup>3</sup>J<sub>HF</sub> = 11.7 Hz, 4H); <sup>19</sup>F NMR (CD<sub>3</sub>OD, 376 MHz) δ -75.42 (s, 3F), -100.27–100.68 (m, 2F); <sup>13</sup>C NMR (DMSO-d<sub>6</sub>, 101 MHz, 350 K) δ 168.9 (C), 160.3 (C), 152.7 (C), 152.6 (C), 152.2 (C), 152.1 (t, <sup>4</sup>J<sub>CF</sub> = 2.8 Hz, C), 135.7 (CH), 130.4 (CH), 129.3 (CH), 129.1 (CH), 127.0 (C), 125.2 (CH), 124.6 (CH), 116.9 (t, <sup>1</sup>J<sub>CF</sub> = 273.2 Hz, CF<sub>2</sub>), 113.3 (CH), 110.7 (C), 109.8 (C), 102.8 (CH), 99.7 (CH), 63.4 (t, <sup>2</sup>J<sub>CF</sub> = 25.9 Hz, CH<sub>2</sub>); Analytical HPLC: t<sub>R</sub> = 10.5 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 500 nm); HRMS (ESI) calcd for C<sub>23</sub>H<sub>16</sub>F<sub>2</sub>NO<sub>4</sub> [M+H]<sup>+</sup> 408.1042, found 408.1040.

**6-tert-Butoxycarbonyl-JF<sub>503</sub> triflate (39):** A vial was charged with 6-tert-butoxycarbonylfluorescein ditriflate (33; 300 mg, 431 µmol), 3,3-difluoroazetidine hydrochloride (56 mg, 431 µmol, 1 eq), Pd<sub>2</sub>dba<sub>3</sub> (20 mg, 21.5 µmol, 0.05 eq), XPhos (31 mg, 64.6 µmol, 0.15 eq), and Cs<sub>2</sub>CO<sub>3</sub> (337 mg, 1.03 mmol, 2.4 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 then stirred at 100 °C for 1 h. It was subsequently cooled to room temperature, filtered through Celite with CH<sub>2</sub>Cl<sub>2</sub>, and concentrated to dryness. Purification by silica gel chromatography (0–40% EtOAc/hexanes, linear gradient) afforded 39 (63 mg, 23%) as an off-white solid. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz) δ 8.25 (dd, <sup>J</sup> = 8.0, 1.1 Hz, 1H), 8.07 (d, <sup>J</sup> = 8.0 Hz, 1H), 7.74 (bs, 1H), 7.24 (d, <sup>J</sup> = 2.4 Hz, 1H), 6.96 (dd, <sup>J</sup> = 8.8, 2.4 Hz, 1H), 6.86 (d, <sup>J</sup> = 8.8 Hz, 1H), 6.64 (d, <sup>J</sup> = 8.6 Hz, 1H), 6.35 (d, <sup>J</sup> = 2.3 Hz, 1H), 6.22 (dd, <sup>J</sup> = 8.6, 2.3 Hz, 1H), 4.28 (t, <sup>3</sup>J<sub>HF</sub> = 11.7 Hz, 4H), 1.54 (s, 9H); <sup>19</sup>F NMR (CDCl<sub>3</sub>, 376 MHz) δ -73.16 (s, 3F), -100.02 (p, <sup>1</sup>J<sub>HF</sub> = 11.7 Hz, 2F); <sup>13</sup>C NMR (CDCl<sub>3</sub>, 101 MHz) δ 168.3 (C), 164.1 (C), 152.7 (C), 152.2 (C), 152.0 (C), 151.62 (t, <sup>4</sup>J<sub>CF</sub> = 2.9 Hz, C), 150.2 (C), 138.8 (C), 131.4 (CH), 130.2 (CH), 129.6 (C), 129.2 (CH), 125.3 (CH), 125.0 (CH), 119.5 (C), 118.8 (q, Nature Methods: doi:10.1038/nmeth.4403
$J_{CF} = 320.8$ Hz, CF$_3$), 116.9 (CH), 115.6 (t, $J_{CF} = 274.6$ Hz, CF$_3$), 110.7 (CH), 109.7 (CH), 108.0 (C), 99.4 (CH), 83.0 (C), 82.4 (C), 63.4 (t, $J_{CF} = 26.6$ Hz, CH$_2$), 28.2 (CH$_3$); HRMS (ESI) calcd for C$_{29}$H$_{23}$F$_5$NO$_8$S [M+H]$^+$ 640.1059, found 640.1068.

**JF$_{685}$ (21):** Method A was used to prepare the title compound from carbofluorescein ditriflate$^1$ (31) and 3,3-difluoroazetidine hydrochloride (95%, off-white solid). $^1$H NMR (CDCl$_3$, 400 MHz) $\delta$ 8.04 – 7.98 (m, 1H), 7.60 (td, $J = 7.3, 1.5$ Hz, 1H), 7.56 (td, $J = 7.3, 1.3$ Hz, 1H), 7.04 (s, 1H), 6.64 (d, $J = 2.8$ Hz, 2H), 6.63 (d, $J = 8.7$ Hz, 2H), 6.28 (dd, $J = 8.6, 2.5$ Hz, 2H), 4.25 (t, $J_{HF} = 11.8$ Hz, 8H), 1.84 (s, 3H), 1.74 (s, 3H); $^{19}$F NMR (CDCl$_3$, 376 MHz) $\delta$ -99.95 (p, $J_{FH} = 11.8$ Hz); $^{13}$C NMR (CDCl$_3$, 101 MHz) $\delta$ 170.6 (C), 155.2 (C), 150.1 (t, $J_{CF} = 2.7$ Hz, C), 146.9 (C), 134.8 (CH), 129.3 (CH), 129.2 (CH), 127.0 (C), 125.2 (CH), 123.9 (CH), 122.4 (C), 115.9 (t, $J_{CF} = 274.6$ Hz, CF$_2$), 111.6 (CH), 109.2 (CH), 87.2 (C), 63.4 (t, $J_{CF} = 25.9$ Hz, CH$_2$), 38.6 (C), 35.6 (CH$_3$), 32.5 (CH$_3$); Analytical HPLC: $t_R = 14.9$ min, >99% purity (30–95% 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 600 nm); HRMS (ESI) calcd for C$_{29}$H$_{23}$F$_5$NO$_8$S [M+H]$^+$ 509.1847, found 509.1843.

**6-tert-Butoxycarbonyl-JF$_{685}$ (47):** Method A was used to prepare the title compound from ditriflate 45 and azetidine (84%, blue solid). $^1$H NMR (CDCl$_3$, 400 MHz) $\delta$ 8.14 (dd, $J = 8.0, 1.3$ Hz, 1H), 8.00 (dd, $J = 8.0, 0.7$ Hz, 1H), 7.61 (dd, $J = 1.3, 0.8$ Hz, 1H), 6.58 (d, $J = 2.3$ Hz, 2H), 6.54 (d, $J = 8.6$ Hz, 2H), 6.21 (dd, $J = 8.6, 2.4$ Hz, 2H), 3.91 (t, $J = 7.2$ Hz, 8H), 2.38 (p, $J = 7.2$ Hz, 4H), 1.83 (s, 3H), 1.73 (s, 3H), 1.53 (s, 9H); $^{13}$C NMR (CDCl$_3$, 101 MHz) $\delta$ 170.1 (C), 164.6 (C), 155.6 (C), 152.4 (C), 146.8 (C), 137.8 (C), 130.3 (C), 130.1 (CH), 128.9 (CH), 125.1 (CH), 124.8 (CH), 119.9 (C), 110.5 (CH), 108.0 (CH), 88.8 (C), 82.3 (C), 52.3 (CH$_2$), 38.5 (C), 35.5 (CH$_3$), 32.8 (CH$_3$), 28.2 (CH$_3$), 17.0 (CH$_2$); HRMS (ESI) calcd for C$_{34}$H$_{37}$N$_2$O$_4$ [M+H]$^+$ 537.2753, found 537.2768.
6-tert-Butyloxy carbonyl-JF<sub>385</sub> (48): Method A was used to prepare the title compound from ditriflate 45 and 3,3-difluoroazetidine hydrochloride (93%, off-white solid). <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz) δ 8.16 (dd, J = 8.0, 1.3 Hz, 1H), 8.02 (dd, J = 8.0, 0.7 Hz, 1H), 7.60 (dd, J = 1.2, 0.8 Hz, 1H), 6.65 (d, J = 2.4 Hz, 2H), 6.62 (d, J = 8.6 Hz, 2H), 6.29 (dd, J = 8.6, 2.5 Hz, 2H), 4.26 (t, J<sub>HF</sub> = 11.7 Hz, 8H), 1.85 (s, 3H), 1.75 (s, 3H), 1.53 (s, 9H); <sup>19</sup>F NMR (CDCl<sub>3</sub>, 376 MHz) δ -99.96 (p, J<sub>FH</sub> = 11.8 Hz); <sup>13</sup>C NMR (CDCl<sub>3</sub>, 101 MHz) δ 169.9 (C), 164.4 (C), 155.3 (C), 150.1 (t, J<sub>CF</sub> = 2.7 Hz, C), 146.8 (C), 138.1 (C), 130.2 (CH), 129.9 (C), 129.2 (CH), 125.1 (CH), 124.9 (CH), 121.7 (C), 115.9 (t, J<sub>CF</sub> = 194.6 Hz, CF<sub>2</sub>), 111.7 (CH), 109.3 (CH), 87.5 (C), 82.5 (C), 63.4 (t, J<sub>CF</sub> = 26.0 Hz, CH<sub>2</sub>), 38.5 (C), 35.4 (CH<sub>3</sub>), 33.0 (CH<sub>3</sub>), 28.2 (CH<sub>3</sub>); HRMS (ESI) calcd for C<sub>32</sub>H<sub>33</sub>F<sub>2</sub>N<sub>3</sub>O<sub>4</sub> [M+H]<sup>+</sup> 609.2371, found 609.2384.

**JF<sub>385</sub> (25):** Method A was used to prepare the title compound from silafluorescein ditriflate<sup>2</sup> (32) and 3-fluoroazetidine hydrochloride (78%, off-white solid). <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz) δ 7.97 (dt, J = 7.6, 0.9 Hz, 1H), 7.65 (td, J = 7.5, 1.2 Hz, 1H), 7.55 (td, J = 7.5, 0.9 Hz, 1H), 7.29 (dt, J = 7.7, 0.8 Hz, 1H), 6.80 (d, J = 8.7 Hz, 2H), 6.70 (d, J = 2.6 Hz, 2H), 6.30 (dd, J = 8.7, 2.7 Hz, 2H), 5.41 (dtt, J<sub>HF</sub> = 57.0 Hz, J = 5.9, 3.7 Hz, 2H), 4.25 – 4.14 (m, 4H), 4.04 – 3.91 (m, 4H), 0.62 (s, 3H), 0.60 (s, 3H); <sup>19</sup>F NMR (CDCl<sub>3</sub>, 376 MHz) δ -180.48 (dtt, J<sub>FH</sub> = 57.0, 23.9, 18.2 Hz); <sup>13</sup>C NMR (CDCl<sub>3</sub>, 101 MHz) δ 170.6 (C), 154.1 (C), 150.2 (d, J<sub>CF</sub> = 1.0 Hz, C), 137.2 (C), 133.9 (C), 133.86 (CH), 129.0 (CH), 128.1 (CH), 127.0 (C), 126.0 (CH), 124.7 (CH), 116.3 (CH), 112.9 (CH), 91.6 (C), 82.8 (d, J<sub>CF</sub> = 204.8 Hz, CFH), 59.6 (d, J<sub>CF</sub> = 23.8 Hz, CH<sub>2</sub>), 0.5 (CH<sub>3</sub>), -1.4 (CH<sub>3</sub>); Analytical HPLC: t<sub>r</sub> = 14.7 min, 98.7% purity (30–95% 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 650 nm); HRMS (ESI) calcd for C<sub>32</sub>H<sub>33</sub>F<sub>2</sub>N<sub>3</sub>O<sub>4</sub> [M+H]<sup>+</sup> 489.1804, found 489.1810.

6-tert-Butyloxy carbonyl-JF<sub>385</sub> (53): Method A was used to prepare the title compound from 6-tert-butyloxy carbonylsilafluorescein ditriflate<sup>2</sup> (51) and 3-fluoroazetidine hydrochloride (85%, off-white solid). <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz) δ 8.12 (dd, J = 8.0, 1.3 Hz, 1H), 7.97 (dd, J = 7.9, 0.8 Hz, 1H), 7.82 (dd, J = 1.3, 0.8 Hz, 1H), 6.88 (d, J = 8.7 Hz, 2H), 6.70 (d, J = 2.6 Hz, 2H), 6.35 (dd, J = 8.7, 2.7 Hz, 2H), 5.41 (dtt, J = 57.0, 5.9, 3.7 Hz, 2H), 4.26 – 4.15 (m, 4H), 4.05 – 3.93 (m, 4H), 1.55 (s, 9H), 0.67 (s, 3H), 0.60 (s, 3H); <sup>19</sup>F NMR (CDCl<sub>3</sub>, 376 MHz) δ -180.48 (dtt, J<sub>FH</sub> = 57.0, 23.9, 18.2 Hz); <sup>13</sup>C NMR (CDCl<sub>3</sub>, 101 MHz) δ 170.2 (C), 164.4 (C), 155.1 (C), 150.0 (d, J<sub>CF</sub> = 1.2 Hz, C), 137.4 (C), 136.3 (C), 133.5 (C), 130.1 (CH), 129.0 (C), 127.8 (CH), 125.8 (CH), 125.1 (CH), 121.7 (C), 115.9 (t, J<sub>CF</sub> = 131.1 Hz, CF<sub>2</sub>), 111.7 (CH), 109.3 (CH), 87.5 (C), 82.5 (C), 63.4 (t, J<sub>CF</sub> = 26.0 Hz, CH<sub>2</sub>), 38.5 (C), 35.4 (CH<sub>3</sub>), 33.0 (CH<sub>3</sub>), 28.2 (CH<sub>3</sub>); HRMS (ESI) calcd for C<sub>32</sub>H<sub>33</sub>F<sub>2</sub>N<sub>3</sub>O<sub>4</sub>Si [M+H]<sup>+</sup> 529.1856, found 529.1848.
116.3 (CH), 113.3 (CH), 91.4 (C), 82.8 (d, \( J_{CF} = 204.8\), CFH), 82.5 (C), 59.6 (d, \( J_{CF} = 23.8\) Hz, CH2), 28.2 (CH3), 0.2 (CH3), -0.7 (CH3); HRMS (ESI) calcd for \( C_{33}H_{35}F_{2}N_{2}O_{4}Si \) [M+H] + 589.2329, found 589.2335.

**HALOTAG AND SNAP-TAG LIGANDS**

**General procedure for deprotection of tert-butyl esters (Method B):** The following procedure for 36 is representative. Ester 35 (70 mg, 120 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 6 h. Toluene (3 mL) was added; the reaction mixture was concentrated to dryness and then azeotroped with MeOH three times to provide 36 as a dark pink solid (72 mg, 93%, TFA salt). Analytical HPLC and NMR indicated that the material was >95% pure and did not require further purification prior to amide coupling.

**6-Carboxy-JF525 (36):** (93%, dark pink solid, TFA salt) \(^1\)H NMR (CD3OD, 400 MHz) \( \delta \) 8.45 (d, \( J = 8.1\) Hz, 1H), 8.41 (dd, \( J = 8.2, 1.5\) Hz, 1H), 8.01 – 7.97 (m, 1H), 7.24 (d, \( J = 9.1\) Hz, 2H), 6.84 (d, \( J = 2.2\) Hz, 2H), 6.80 (dd, \( J = 9.1, 2.2\) Hz, 2H), 4.72 (t, \( J_{HF} = 11.6\) Hz, 8H); \(^{19}\)F NMR (CD3OD, 376 MHz) \( \delta \) -75.59 (s, 3F), -100.90 (p, \( J_{FH} = 11.6\) Hz, 4F); \(^{13}\)C NMR (CD3OD, 101 MHz) \( \delta \) 167.6 (C), 167.3 (C), 159.1 (C), 157.7 (t, \( J_{CF} = 3.9\) Hz, C), 136.1 (C), 135.8 (C), 134.6 (C), 134.1 (CH), 132.9 (CH), 132.7 (CH), 132.6 (CH), 132.1 (CH), 119.2 (C), 116.5 (t, \( J_{CF} = 271.9\) Hz, CF2), 116.1 (C), 115.2 (CH), 97.4 (CH), 64.2 (t, \( J_{CF} = 29.1\) Hz, CH2); Analytical HPLC: \( t_R = 11.2\) 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 525 nm); HRMS (ESI) calcd for \( C_{27}H_{19}F_{4}N_{2}O_{5}Si \) [M+H] + 527.1225, found 527.1232.

**6-Carboxy-JF503 triflate (40):** Method B was used to convert ester 39 into the title compound (88%, red-orange solid, TFA salt). \(^1\)H NMR (DMSO-d6, 400 MHz) \( \delta \) 13.62 (s, 1H), 8.26 (dd, \( J = 8.0, 1.2\) Hz, 1H), 8.16 (dd, \( J = 8.0, 0.4\) Hz, 1H), 7.79 – 7.75 (m, 1H), 7.66 (d, \( J = 2.5\) Hz, 1H), 7.23 (dd, \( J = 8.8, 2.5\) Hz, 1H), 7.09 (d, \( J = 8.9\) Hz, 1H), 6.71 (d, \( J = 8.6\) Hz, 1H), 6.55 (d, \( J = 2.3\) Hz, 1H), 6.39 (dd, \( J = 8.7, 2.3\) Hz, 1H), 4.36 (t, \( J_{HF} = 12.3\) Hz, 4H); \(^{19}\)F NMR (DMSO-d6, 376 MHz) \( \delta \) -72.20 (s, 3F), -98.42 (p, \( J_{FH} = 12.3\) Hz, 2F); \(^{13}\)C NMR (DMSO-d6, 101 MHz) \( \delta \)
167.6 (C), 166.0 (C), 152.2 (C), 151.8 (t, $J_{CF} = 2.7$ Hz, C), 151.4 (C), 151.1 (C), 149.7 (C), 138.0 (C), 131.3 (CH), 130.7 (CH), 128.9 (CH), 125.5 (CH), 124.5 (CH), 119.4 (C), 118.2 (q, $J_{CF} = 321.0$ Hz, CF), 117.1 (CH), 116.4 (t, $J_{CF} = 273.1$ Hz, CF), 110.6 (CH), 110.3 (CH), 107.3 (C), 99.3 (CH), 81.7 (C), 62.8 (t, $J_{CF} = 25.7$ Hz, CH); HRMS (ESI) calcd for C$_{25}$H$_{15}$F$_{5}$NO$_{8}$S $[M+H]^+$ 584.0433, found 584.0446.

6-Carboxy-JF$_{608}$ (49): Method B was used to convert ester 47 into the title compound (98%, dark blue solid, TFA salt). $^1$H NMR (CD$_3$OD, 400 MHz) $\delta$ 8.34 (dd, $J = 8.2$, 0.5 Hz, 1H), 8.31 (dd, $J = 8.2$, 1.5 Hz, 1H), 7.84 (dd, $J = 1.5$, 0.5 Hz, 1H), 6.93 (d, $J = 9.1$ Hz, 2H), 6.82 (d, $J = 2.2$ Hz, 2H), 6.39 (dd, $J = 9.1$, 2.3 Hz, 2H), 4.33 (t, $J = 7.6$ Hz, 8H), 2.55 (p, $J = 7.6$ Hz, 4H), 1.82 (s, 3H), 1.70 (s, 3H); $^{19}$F NMR (CD$_3$OD, 376 MHz) $\delta$ -75.24 (s); $^{13}$C NMR (CD$_3$OD, 101 MHz) $\delta$ 167.9 (C), 167.5 (C), 165.4 (C), 158.0 (C), 156.8 (C), 139.3 (C), 137.6 (CH), 136.2 (C), 135.5 (C), 132.5 (CH), 132.4 (CH), 131.5 (CH), 121.8 (C), 111.9 (CH), 109.7 (CH), 52.9 (CH$_2$), 42.8 (C), 35.6 (CH$_3$), 32.0 (CH$_3$); Analytical HPLC: $t_R = 9.2$ 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 600 nm); HRMS (ESI) calcd for C$_{30}$H$_{29}$N$_2$O$_4$ $[M+H]^+$ 481.2127, found 481.2120.

6-Carboxy-JF$_{585}$ (50): Method B was used to convert ester 48 into the title compound (99%, dark blue-purple solid, TFA salt). $^1$H NMR (CD$_3$OD, 400 MHz) $\delta$ 8.30 (dd, $J = 8.1$, 1.4 Hz, 1H), 8.23 – 8.15 (m, 1H), 7.69 (s, 1H), 6.92 (d, $J = 2.1$ Hz, 2H), 6.79 (d, $J = 7.6$ Hz, 2H), 6.47 (dd, $J = 8.8$, 2.3 Hz, 2H), 4.45 (t, $J_{HF} = 11.0$ Hz, 8H), 1.88 (s, 3H), 1.76 (s, 3H); $^{19}$F NMR (CD$_3$OD, 376 MHz) $\delta$ -75.81 (s, 3F), -100.32 (m, 4F); $^{13}$C NMR (101 MHz, DMSO-$d_6$, 350 K) $\delta$ 168.3 (C), 165.5 (C), 154.4 (C), 149.9 (t, $J_{CF} = 2.8$ Hz, C), 146.3 (C), 136.8 (C), 129.9 (CH), 128.9 (C), 128.1 (CH), 124.9 (CH), 123.7 (CH), 120.3 (C), 116.2 (t, $J_{CF} = 273.3$ Hz, CF$_2$), 111.8 (CH), 109.5 (CH), 62.5 (t, $J_{CF} = 25.6$ Hz, CH$_2$), 37.8 (C), 34.0 (CH$_3$), 32.8 (CH$_3$); Analytical HPLC: $t_R = 11.9$ min, >99% purity (30–95% 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 600 nm); HRMS (ESI) calcd for C$_{30}$H$_{30}$F$_4$N$_2$O$_4$ $[M+H]^+$ 553.1745, found 553.1741.
6-Carboxy-JF<sub>635</sub> (54): Method B was used to convert ester 53 into the title compound (91%, dark blue solid, TFA salt). <sup>1</sup>H NMR (CD<sub>3</sub>OD, 400 MHz) δ 8.25 (dd, J = 8.0, 1.4 Hz, 1H), 8.11 (d, J = 8.1 Hz, 1H), 7.84 (dd, J = 1.4, 0.7 Hz, 1H), 6.87 (d, J = 2.6 Hz, 2H), 6.83 (d, J = 8.8 Hz, 2H), 6.39 (dd, J = 8.9, 2.6 Hz, 2H), 5.43 (dtt, J = 57.3, 6.1, 3.3 Hz, 2H), 4.41 – 4.20 (m, 4H), 4.16 – 4.01 (m, 4H), 0.65 (s, 3H), 0.56 (s, 3H); <sup>1</sup>9F NMR (376 MHz, CD<sub>3</sub>OD) δ -75.92 (s, 3F), -180.00 – -180.61 (m, 2F); <sup>13</sup>C NMR (101 MHz, DMSO-<sub>d</sub>6, 350 K) δ 168.6 (C), 165.5 (C), 154.6 (C), 149.5 (d, J<sub>CF</sub> = 1.3 Hz, C), 136.3 (C), 135.4 (C), 131.8 (C), 129.6 (CH), 127.8 (C), 127.0 (CH), 125.4 (CH), 123.9 (CH), 115.8 (CH), 113.1 (CH), 83.0 (d, J<sub>CF</sub> = 200.9 Hz, CHF), 58.8 (d, J<sub>CF</sub> = 58.8 Hz, CH<sub>2</sub>), -0.6 (CH<sub>3</sub>), -1.4 (CH<sub>3</sub>); Analytical HPLC: t<sub>R</sub> = 11.8 min, >98% 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 650 nm); HRMS (ESI) calcd for C<sub>29</sub>H<sub>27</sub>F<sub>2</sub>N<sub>2</sub>O<sub>4</sub>Si [M+H]<sup>+</sup> 533.1703, found 533.1710.

General procedure for preparation of HaloTag and SNAP-tag ligands (Method C): The following procedure for 13 is representative. 6-Carboxy-JF<sub>525</sub> (36; 30 mg, 46.8 µmol) was combined with DSC (26 mg, 103 µmol, 2.2 eq) in DMF (2.5 mL). After adding Et<sub>3</sub>N (39 µL, 281 µmol, 6 eq) and DMAP (0.6 mg, 4.7 µmol, 0.1 eq), the reaction was stirred at room temperature for 1 h while shielded from light. HaloTag(O2)amine (HTL-NH<sub>2</sub>, 37; TFA salt; 26 mg, 117 µmol, 2.5 eq, Promega) was then added. The reaction was stirred an additional 2 h at room temperature, then concentrated in vacuo. 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) followed by flash chromatography on silica gel (0–10% MeOH/CH<sub>2</sub>Cl<sub>2</sub>, linear gradient) to provide 21.3 mg (62%) of 13 as a pink solid.

JF<sub>525</sub>=HaloTag ligand (13): (62%, pink solid) <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz) δ 8.05 (dd, J = 8.0, 0.8 Hz, 1H), 7.98 (dd, J = 8.0, 1.4 Hz, 1H), 7.55 (dd, J = 1.5, 0.8 Hz, 1H), 6.75 – 6.70 (m, 1H), 6.62 (d, J = 8.6 Hz, 2H), 6.31 (d, J = 2.4 Hz, 2H), 6.17 (dd, J = 8.6, 2.4 Hz, 2H), 4.26 (t, J<sub>HF</sub> = 11.7 Hz, 8H), 3.69 – 3.57 (m, 6H), 3.56 – 3.48 (m, 4H), 3.40 (t, J = 6.6 Hz, 2H), 1.79 – 1.70 (m, 2H), 1.54 – 1.48 (m, 2H), 1.46 – 1.38 (m, 2H), 1.37 – 1.29 (m, 2H); <sup>19</sup>F NMR (CDCl<sub>3</sub>, 376 MHz) δ -100.04 (p, J<sub>HF</sub> = 11.8 Hz); Analytical HPLC: t<sub>R</sub> = 14.2 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 650 nm).
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 C₃₇H₃₉ClF₄N₃O₆ [M+H]⁺ 732.2458, found 732.2457.

**JF₅₀₃–HaloTag ligand (17):** Acid 40 (20 mg, 28.7 µmol) was combined with TSTU (19 mg, 63.1 µmol, 2.2 eq) in DMF (1.5 mL). After adding DIEA (30 µL, 172 µmol, 6 eq), the reaction was stirred at room temperature for 1 h while shielded from light. HaloTag(O2)amine (HTL-NH₂, 37; TFA salt; 23 mg, 68.8 µmol, 2.4 eq, Promega) was then added, and the reaction was stirred for 1 h at room temperature. After adding MeOH (1 mL) and 1 N NaOH (300 µL) and stirring an additional 3 h at room temperature, the reaction was acidified with 1 N HCl (325 µL) and concentrated to dryness. The crude material was purified by reverse phase HPLC (10–75% MeCN/H₂O, linear gradient, with constant 0.1% v/v TFA additive) to provide 19.4 mg (88%, TFA salt) of 17 as an orange solid.

**JF₆₀₈–HaloTag ligand (22):** Acid 49 was subjected to Method C to prepare the title compound (72%, dark blue solid). ¹H NMR (CDCl₃, 400 MHz) δ 8.02 (dd, J = 8.0, 0.6 Hz, 1H), 7.94 (dd, J = 8.0, 1.4 Hz, 1H), 7.42–7.40 (m, 1H), 6.68–6.62 (m, 1H), 6.57 (d, J = 2.3 Hz, 2H), 6.52 (d, J = 8.6 Hz, 2H), 6.20 (dd, J = 8.6, 2.4 Hz, 2H), 3.91 (t, J = 7.4 Hz, 8H), 3.64–3.56 (m, 6H), 3.55–3.48 (m, 4H), 3.38 (t, J = 6.6 Hz, 2H), 2.37 (p, J = 7.2 Hz, 4H), 1.83 (s, 3H), 1.77–1.68 (m, 2H), 1.72 (s, 3H), 1.56–1.47 (m, 2H), 1.46–1.36 (m, 2H), 1.36–1.28 (m, 2H); Analytical HPLC: tᵣ = 14.2 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 500 nm); HRMS (ESI) calcd for C₃₅H₃₇ClF₂N₂O₇ [M+H]⁺ 657.2174, found 657.2190.
min run; 1 mL/min flow; ESI; positive ion mode; UV detection at 600 nm); HRMS (ESI) calcd for C₄₀H₄₉ClN₃O₅ [M+H]⁺ 686.3361, found 686.3375.

**JF₅₈–HaloTag ligand (23):** Acid 50 was subjected to Method C to prepare the title compound (54%, off-white/bluish solid). ¹H NMR (CDCl₃, 400 MHz) δ 8.04 (dd, J = 8.0, 0.5 Hz, 1H), 7.93 (dd, J = 8.0, 1.4 Hz, 1H), 7.44 – 7.40 (m, 1H), 6.70 – 6.65 (m, 1H), 6.64 (d, J = 2.4 Hz, 2H), 6.60 (d, J = 8.6 Hz, 2H), 6.28 (dd, J = 8.6, 2.4 Hz, 2H), 4.25 (t, J₃HF = 11.7 Hz, 8H), 3.66 – 3.57 (m, 6H), 3.56 – 3.48 (m, 4H), 3.39 (t, J = 6.6 Hz, 2H), 1.85 (s, 3H), 1.79 – 1.70 (m, 5H), 1.57 – 1.48 (m, 2H), 1.46 – 1.37 (m, 2H), 1.37 – 1.26 (m, 2H); ¹⁹F NMR (CDCl₃, 376 MHz) δ -99.94 (p, J₃FH = 11.8 Hz); Analytical HPLC: t_R = 15.4 min, >99% purity (30–95% 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 600 nm); HRMS (ESI) calcd for C₄₀H₄₉ClF₄N₃O₅ [M+H]⁺ 758.2978, found 758.2989.

**JF₆₃₆–HaloTag ligand (27):** Acid 54 was subjected to Method C to prepare the title compound (61%, blue-green solid). ¹H NMR (CDCl₃, 400 MHz) δ 7.99 (dd, J = 7.9, 0.7 Hz, 1H), 7.89 (dd, J = 8.0, 1.4 Hz, 1H), 7.69 (dd, J = 1.4, 0.8 Hz, 1H), 6.81 (d, J = 8.7 Hz, 2H), 6.79 – 6.75 (m, 1H), 6.69 (d, J = 2.6 Hz, 2H), 6.31 (dd, J = 8.7, 2.7 Hz, 2H), 5.52 – 5.30 (m, 2H), 4.26 – 4.14 (m, 4H), 4.04 – 3.92 (m, 4H), 3.68 – 3.58 (m, 6H), 3.58 – 3.53 (m, 2H), 3.50 (t, J = 6.6 Hz, 2H), 3.40 (t, J = 6.7 Hz, 2H), 1.77 – 1.68 (m, 2H), 1.54 – 1.48 (m, 2H), 1.44 – 1.36 (m, 2H), 1.35 – 1.27 (m, 2H), 0.66 (s, 3H), 0.59 (s, 3H); ¹⁹F NMR (CDCl₃, 376 MHz) δ -180.49 (dtt, J₃FH = 56.9, 23.9, 18.2 Hz); Analytical HPLC: t_R = 16.8 min, 98.7% 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 633 nm); HRMS (ESI) calcd for C₃₀H₄₇ClF₂N₃O₅Si [M+H]⁺ 738.2936, found 738.2953.

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**JF<sub>525</sub>-SNAP-tag ligand (15):** Acid 36 and STL-NH<sub>2</sub> (38, NEB) were subjected to Method C to prepare the title compound (72%, red-orange solid). <sup>1</sup>H NMR (CD<sub>3</sub>OD, 400 MHz) δ 8.13 (dd, <i>J</i> = 8.1, 1.4 Hz, 1H), 8.07 (dd, <i>J</i> = 8.0, 0.6 Hz, 1H), 7.80 (s, 1H), 7.65 – 7.60 (m, 1H), 7.42 (d, <i>J</i> = 8.1 Hz, 2H), 7.28 (d, <i>J</i> = 8.1 Hz, 2H), 6.71 (d, <i>J</i> = 8.7 Hz, 2H), 6.46 (d, <i>J</i> = 2.3 Hz, 2H), 6.35 (dd, <i>J</i> = 8.7, 2.4 Hz, 2H), 5.48 (s, 2H), 4.49 (s, 2H), 4.31 (t, <i>J</i><sub>HF</sub> = 11.9 Hz, 8H); <sup>19</sup>F NMR (CD<sub>3</sub>OD, 376 MHz) δ -100.07 – -100.32 (m); Analytical HPLC: <i>t</i><sub>R</sub> = 10.8 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>40</sub>H<sub>31</sub>F<sub>4</sub>N<sub>8</sub>O<sub>5</sub> [M+H]<sup>+</sup> 779.2348, found 779.2355.

**JF<sub>503</sub>-cpSNAP-tag ligand (20):** Acid 40 and cpSTL-NH<sub>2</sub> (41, NEB) were subjected to the same procedure described for 17 to prepare the title compound (71%, orange solid, TFA salt). <sup>1</sup>H NMR (CD<sub>3</sub>OD, 400 MHz) δ 9.20 (t, <i>J</i> = 6.0 Hz, 1H), 8.35 (d, <i>J</i> = 8.2 Hz, 1H), 8.23 (dd, <i>J</i> = 8.2, 1.6 Hz, 1H), 7.80 (d, <i>J</i> = 1.3 Hz, 1H), 7.36 (AB quartet, <i>v</i><sub>A</sub> = 2951.5 Hz, <i>v</i><sub>B</sub> = 2938.7 Hz, <i>J</i><sub>AB</sub> = 8.3 Hz, 4H), 7.19 – 7.10 (m, 2H), 7.07 (d, <i>J</i> = 1.9 Hz, 1H), 6.92 (dd, <i>J</i> = 9.0, 2.0 Hz, 1H), 6.80 (d, <i>J</i> = 1.9 Hz, 1H), 6.77 – 6.70 (m, 1H), 6.07 (s, 1H), 5.32 (s, 2H), 4.67 (t, <i>J</i><sub>HF</sub> = 11.6 Hz, 4H), 4.60 – 4.53 (m, 2H); <sup>19</sup>F NMR (CD<sub>3</sub>OD, 376 MHz) δ -100.75 – -75.60 (s, 3F), -100.77 (p, <i>J</i><sub>HF</sub> = 11.7 Hz, 2F); Analytical HPLC: <i>t</i><sub>R</sub> = 12.4 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 500 nm); HRMS (ESI) calcd for C<sub>46</sub>H<sub>31</sub>F<sub>4</sub>N<sub>5</sub>O<sub>5</sub> [M+H]<sup>+</sup> 698.1612, found 698.1627.
**JF₅₈₅–SNAP-tag ligand (24):** Acid 50 and STL-NH₂ (38, NEB) were subjected to Method C to prepare the title compound (71%, off-white solid). $^1$H NMR (CD₃OD, 400 MHz) δ 8.07 (dd, $J = 8.0, 1.3$ Hz, 1H), 8.04 (dd, $J = 8.0, 0.7$ Hz, 1H), 7.81 (s, 1H), 7.48 – 7.44 (m, 1H), 7.42 (d, $J = 8.2$ Hz, 2H), 7.26 (d, $J = 8.2$ Hz, 2H), 6.81 (d, $J = 2.4$ Hz, 2H), 6.58 (d, $J = 8.6$ Hz, 2H), 6.39 (dd, $J = 8.6, 2.4$ Hz, 2H), 5.48 (s, 2H), 4.47 (s, 2H), 4.24 (t, $f_{JHF} = 12.0$ Hz, 8H), 1.85 (s, 3H), 1.74 (s, 3H); $^{19}$F NMR (CD₃OD, 376 MHz) δ -99.82 (p, $f_{JFH} = 11.9$ Hz); Analytical HPLC: $t_R = 13.3$ 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 600 nm); HRMS (ESI) cale for C₄₃H₃₇F₄N₈O₄ [M+H]$^+$ 805.2868, found 805.2876.

**JF₆₃₅–SNAP-tag ligand (29):** Acid 54 and STL-NH₂ (38, NEB) were subjected to Method C to prepare the title compound (48%, off-white solid). $^1$H NMR (CD₃OD, 400 MHz) δ 8.02 (dd, $J = 8.0, 1.3$ Hz, 1H), 8.00 (dd, $J = 8.0, 0.8$ Hz, 1H), 7.81 (s, 1H), 7.68 – 7.63 (m, 1H), 7.47 (d, $J = 8.2$ Hz, 2H), 7.32 (d, $J = 8.2$ Hz, 2H), 6.79 (d, $J = 2.6$ Hz, 2H), 6.75 (d, $J = 8.7$ Hz, 2H), 6.39 (dd, $J = 8.7, 2.7$ Hz, 2H), 5.52 (s, 2H), 5.50 – 5.31 (m, 2H), 4.52 (s, 2H), 4.25 – 4.12 (m, 4H), 3.98 – 3.86 (m, 4H), 0.60 (s, 3H), 0.54 (s, 3H); $^{19}$F NMR (CD₃OD, 376 MHz) δ -179.91 (dtt, $f_{JFH} = 57.5, 23.8, 19.3$ Hz); Analytical HPLC: $t_R = 12.6$ 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 633 nm); HRMS (ESI) cale for C₄₂H₃₉F₂N₆O₄Si [M+H]$^+$ 785.2826, found 785.2828.

**REFERENCES**

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CO

56

Nature Methods: doi:10.1038/nmeth.4403
Nature Methods: doi:10.1038/nmeth.4403
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Nature Methods: doi:10.1038/nmeth.4403
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Nature Methods: doi:10.1038/nmeth.4403
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<tr>
<td>Spectral Width</td>
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<tr>
<td>Lowest Frequency</td>
<td>-1646.8</td>
</tr>
<tr>
<td>Nucleus</td>
<td>1H</td>
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<tr>
<td>Acquired Size</td>
<td>32768</td>
</tr>
<tr>
<td>Spectral Size</td>
<td>65536</td>
</tr>
</tbody>
</table>

**Chemical Structures**

![Chemical Structure Image]

Chemical formula: `NNCO₂–t-BuO₂C₄`

**Spectroscopic Data**

- **1H NMR Spectrum**: [View Spectrum]
- **13C NMR Spectrum**: [View Spectrum]
Data File C:\CHEM32\1\DATA\2014_11\DAILYSQUENCE_LC 2014-12-02 11-37-40\2014_11000002.D
Sample Name: JBG16039
Instrument 1 12/2/2014 4:50:22 PM

**MSD1 SPC, time=14.701:14.886 of C:\CHEM32\1\DATA\2014_11\DAILYSQUENCE_LC 2014-12-02 11-37-40\2014_11000002.D** ES-API
Max: 1.0495e+006

**DAD1 E, Sig=650.16 Ref=off (2014_11\DAILYSQUENCE_LC 2014-12-02 11-37-40\2014_11000002.D)**

Spectra averaged over upper half of peaks.
Noise Cutoff: 1000 counts.
Reportable Ion Abundance: > 10%.

Retention                    Mol. Weight
Time (MS)      MS Area      or Ion
14.771      28159928       491.10 I
490.10 I
489.10 I

*** End of Report ***

Nature Methods: doi:10.1038/nmeth.4403
### Spectrum 1

- **Origin**: Bruker BioSpin GmbH
- **Solvent**: MeOD
- **Temperature**: 300.0
- **Pulse Sequence**: zg30
- **Experiment**: 1D
- **Number of Scans**: 16
- **Acquisition Date**: 2015-09-28T15:41:00
- **Modification Date**: 2015-09-28T15:41:08
- **Spectrometer Frequency**: 400.13
- **Spectral Width**: 8012.8
- **Lowest Frequency**: -1560.6
- **Nucleus**: 1H
- **Acquired Size**: 32768
- **Spectral Size**: 65536

![Spectrum 1 Image]

### Spectrum 2

- **Origin**: Bruker BioSpin GmbH
- **Solvent**: MeOD
- **Temperature**: 300.0
- **Pulse Sequence**: zg30
- **Experiment**: 1D
- **Number of Scans**: 1024
- **Acquisition Date**: 2015-03-16T16:09:00
- **Modification Date**: 2015-03-16T16:09:30
- **Spectrometer Frequency**: 100.62
- **Spectral Width**: 24038.5
- **Lowest Frequency**: -1815.8
- **Nucleus**: 13C
- **Acquired Size**: 32768
- **Spectral Size**: 65536

![Spectrum 2 Image]
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<tr>
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<tbody>
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<tr>
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<td>Lowest Frequency</td>
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<tr>
<td>Nucleus</td>
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<td>Acquired Size</td>
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<td>Spectral Size</td>
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</table>

![Chemical Structure](image1.png)

<table>
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<th>Origin</th>
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<tbody>
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</tr>
<tr>
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<tr>
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<tr>
<td>Nucleus</td>
<td>13C</td>
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<td>Acquired Size</td>
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<tr>
<td>Spectral Size</td>
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</tbody>
</table>

![Chemical Structure](image2.png)
Origin: Bruker BioSpin GmbH
Solvent: MeOD
Temperature: 300.0
Pulse Sequence: zg30
Experiment: 1D
Number of Scans: 16
Acquisition Date: 2013-08-13T16:58:00
Spectrometer Frequency: 600.13
Spectral Width: 2223.7
Lowest Frequency: -1664.1
Nucleus: 1H
Acquired Size: 32768
Spectral Size: 65536

Origin: Bruker BioSpin GmbH
Solvent: MeOD
Temperature: 300.0
Pulse Sequence: zg30
Experiment: 1D
Number of Scans: 8192
Acquisition Date: 2013-08-15T04:51:00
Spectrometer Frequency: 100.62
Spectral Width: 24038.5
Lowest Frequency: -1816.0
Nucleus: 13C
Acquired Size: 32768
Spectral Size: 65536
**Nature Methods**: doi:10.1038/nmeth.4403
Origin: Bruker BioSpin GmbH
Solvent: MeOD
Temperature: 300.0
Pulse Sequence: zg30
Experiment: 1D
Number of Scans: 16
Acquisition Date: 2016-10-03 13:00
Modification Date: 2016-10-03 13:36
Spectrometer Frequency: 400.13
Spectral Width: 8012.8
Lowest Frequency: -1543.3
Number: 1H
Acquired Size: 32768
Spectral Size: 65536

Nature Methods: doi:10.1038/nmeth.4403
Origin: Bruker BioSpin GmbH
Solvent: CDC13
Temperature: 300.0
Pulse Sequence: zg30
Experiment: 1D
Number of Scans: 16
Acquisition Date: 2014-01-31T08:45:00
Spectrometer Frequency: 8223.7
Lowest Frequency: -1645.1
Nucleus: 1H
Acquired Size: 32768
Spectral Size: 65536


Max: 268235


Max: 268235
Data File C:\CHEM32\1\DATA\2015_03\DAILYSQUENCE_LC 2015-03-16 13-12-15\2015_03000010.D
Sample Name: AKM-1-193-conc2

**Origin**
Bruker BioSpin GmbH

**Solvent**
CDCl3

**Temperature**
300.0

**Pulse Sequence**
zg30

**Experiment**
1D

**Number of Scans**
16

**Acquisition Date**
2015-03-17T12:06:00

**Modification Date**
2015-03-17T12:06:00

**Spectrometer Frequency**
400.13

**Spectral Width**
8223.7

**Lowest Frequency**
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**Nucleus**
1H

**Acquired Size**
32768

**Spectral Size**
65536

**Retention Time**
16.784

**Mol. Weight**
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**MS Area**
1499.40 I

**Ion**
1498.35 I

**m/z**
741.20 I

**DAD1 A, Sig=254.8 Ref=off (2015_03\DAILYSQUENCE_LC 2015-03-16 13-12-15\2015_03000010.D)**

**Max:** 185315

**Nature Methods:** doi:10.1038/nmeth.4403