An expanded palette of fluorogenic HaloTag probes with enhanced contrast for targeted cellular imaging

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Supplementary Information

I. Materials and methods

Materials. Chemical reagents and solvents were purchased from Sigma-Aldrich or TCI and were used as received. Purified GST-Halotag protein and Halo-TMR were purchased from Promega Corp. (Madison, Wi, USA). Mitotracker Deep Red FM was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Hela cells (CCL-2) were obtained from the ATCC.

Chemical analysis. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Advance 300 MHz spectrometer. All chemical shifts (δ) for ^{1}H and ^{13}C NMR spectra are reported in parts per million (ppm) relative to the internal residual solvent signals. The abbreviations used are: singlet (s), doublet (d), doublet of doublet (dd), triplet (t), doublet of triplet (dt), multiplet (m) and coupling constants are reported in hertz (Hz). ESI-MS experiments were carried out using a LTQ-Orbitrap XL from Thermo Scientific (Thermo Fisher Scientific, Courtaboeuf, France) and operated in positive or negative ionization mode, with a spray voltage at 3.6 kV. Applied voltages were 40 and 100 V for the ion transfer capillary and the tube lens, respectively. The ion transfer capillary was held at 275°C. Detection was achieved in the Orbitrap with a resolution set to 100,000 (at m/z 400) and a m/z range between 200-2000 in profile mode. Spectrum was analyzed using the acquisition software XCalibur 2.1 (Thermo Fisher Scientific, Courtaboeuf, France). The automatic gain control (AGC) allowed accumulation of up to 2.105 ions for FTMS scans, Maximum injection time was set to 300 ms and 1 µscan was acquired. 10 µL was injected using a Thermo Finnigan Surveyor HPLC system (Thermo Fisher Scientific, Courtaboeuf, France) with a continuous infusion of methanol at 100 µL.min-1. Thin layer chromatography (TLC) analysis was run on silica gel (Merck 60F – 254) with visualization at 254 nm.

Absorption and fluorescence spectroscopy. UV spectra were recorded on a Cary 300 spectrophotometer (Agilent technologies, Santa Clara, CA, USA). Scanning was set at 600 nm/min with a step of 1 nm. Fluorescence emission spectra were recorded on a Jasco FP8300 spectrofluorometer (Jasco Inc., Easton, MD, USA). Scanning speed was set to 500 nm/min, excitation and emission slits were set to 5 nm and the PMT to medium. Measurements in water and with HaloTag were performed in quartz cuvettes (1 cm pathlength, Hellma Analytics). Measurements in glycerol were performed using 3 mL disposable cuvettes (1 cm pathlength, Sigma-Aldrich).

Fluorescence quantum yields measurements in glycerol. Fluorescence quantum yield were calculated by relative measurement using Rhodamine 6G as reference (ϕ_F = 0.94 in ethanol) except for CCVJ-Halo for which Fluorescein (ϕ_F = 0.91 in 0.1 M NaOH) was used. The absorption and fluorescence spectra of the references and samples solutions were measured at 3 to 5 different concentrations while keeping the absorption < 0.1. For each sample and reference, the integrated fluorescence intensity was plotted against the absorbance at the excitation wavelength to give a linear curve. Using the slope value (*grad*), the quantum yield was calculated according to the following equation:

$$\varphi_f^i = \varphi_f^s \frac{grad^i n_i^2}{grad^s n_s^2}$$

Where *i* and *s* refer to the sample and reference respectively and *n* is the refractive index of the solvent.

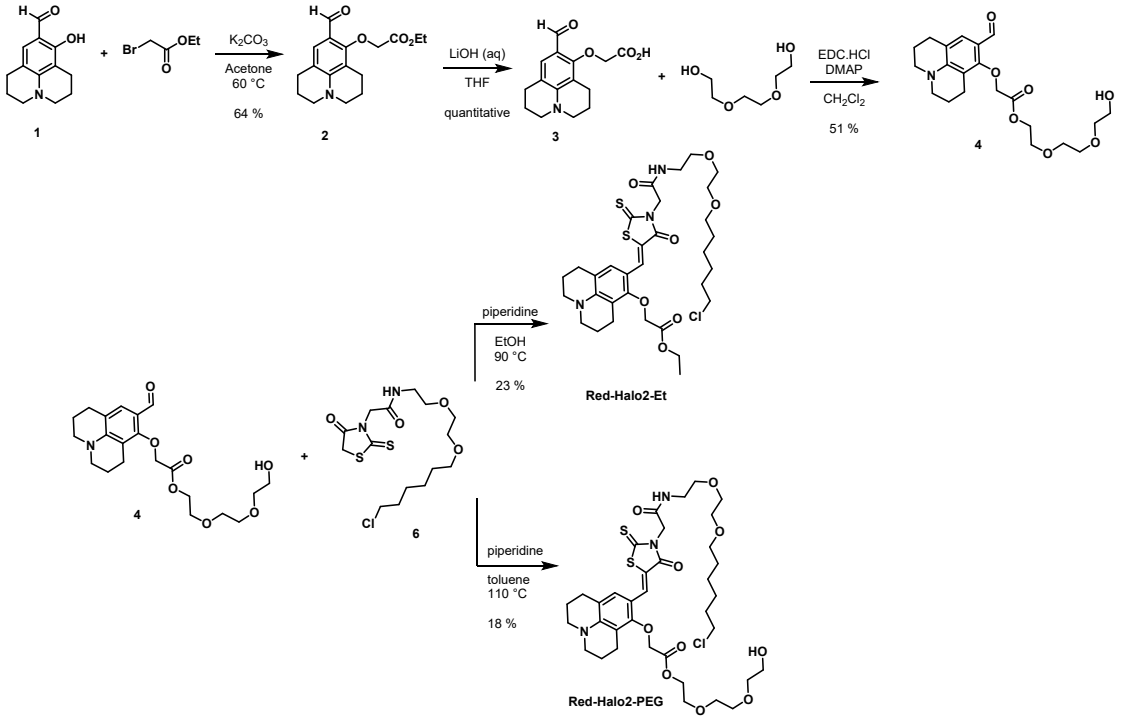
Fluorescence quantum yields measurements in presence of HaloTag. Fluorescence quantum yield were calculated using the same references as in glycerol. After complete reaction with HaloTag, the absorption spectra of the probes were recorded. The fluorescence was next recorded at three different excitation wavelength to check for potential baseline error during the absorption spectra measurements. The fluorescence quantum yield was then calculated at each wavelength using the following equation and averaged:

$$\varphi_f^i = \varphi_f^s \frac{F^i(1-10^{-A^s})n_i^2}{F^s(1-10^{-A^i})n_s^2}$$

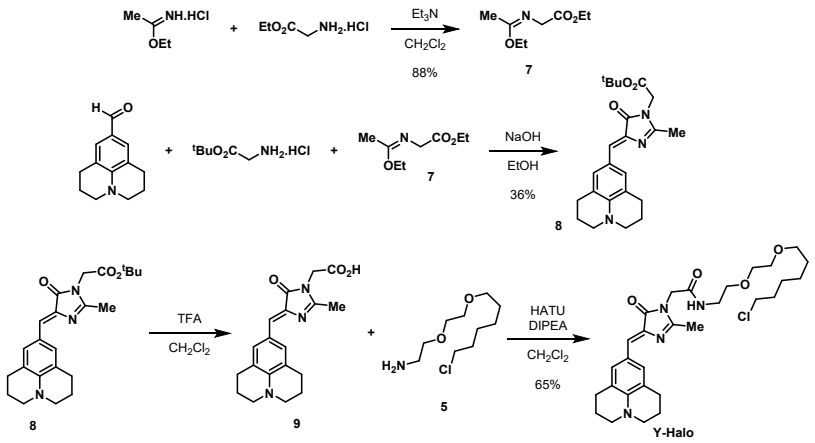
Where ϕ_F is the fluorescence quantum yield, F the integrated fluorescence intensity, A the absorbance at the excitation wavelength, n the refractive index of the solvent and the superscript *s* and *i* refer to the standard and the sample respectively.

Molecular Biology. The HaloTag-NLS and HaloTag-Lifeact plasmids were previously reported.[1] Synthetic oligonucleotides used for cloning were purchased from Integrated DNA Technology. PCR reactions were performed with Q5 polymerase (New England Biolabs) in the buffer provided. PCR products were purified using QIAquick PCR purification kit (Qiagen). Isothermal assemblies (Gibson assembly) were performed using homemade mix prepared according to previously described protocols (modified from the original described protocol).[2] Gibson products were purified using MinElute PCR purification kit (Qiagen). Gibson products were transformed in DH10 E. coli. Small-scale isolation of plasmid DNA was done using QIAprep miniprep kit (Qiagen) from 3 mL of overnight culture supplemented with appropriate antibiotics. Large-scale isolation of plasmid DNA was done using the QIAprep maxiprep kit (Qiagen) from 150 mL of overnight culture supplemented with appropriate antibiotics. All plasmid sequences were confirmed by Sanger sequencing with appropriate sequencing primers (GATC Biotech).

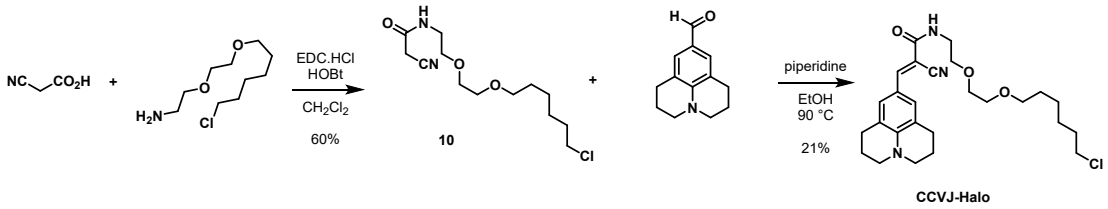
II. Supplementary schemes and figures



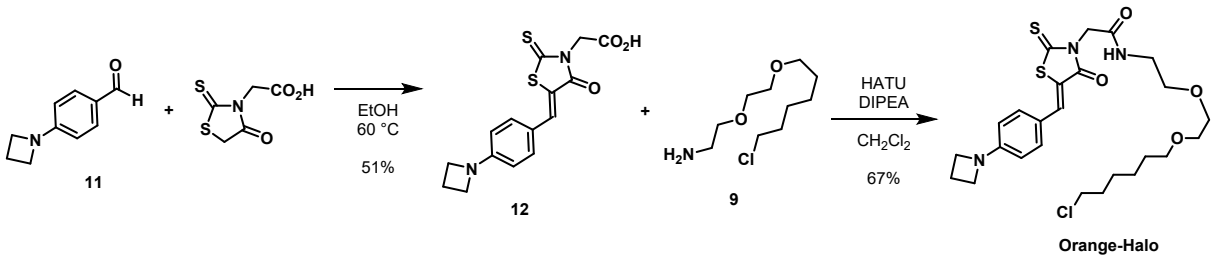
Scheme S1. Synthesis of **Red-Halo2-Et** and **Red-Halo2-PEG**



Scheme S2. Synthesis of **Y-Halo**



Scheme S3. Synthesis of **CCVJ-Halo**



Scheme S4. Synthesis of **Orange-Halo**.

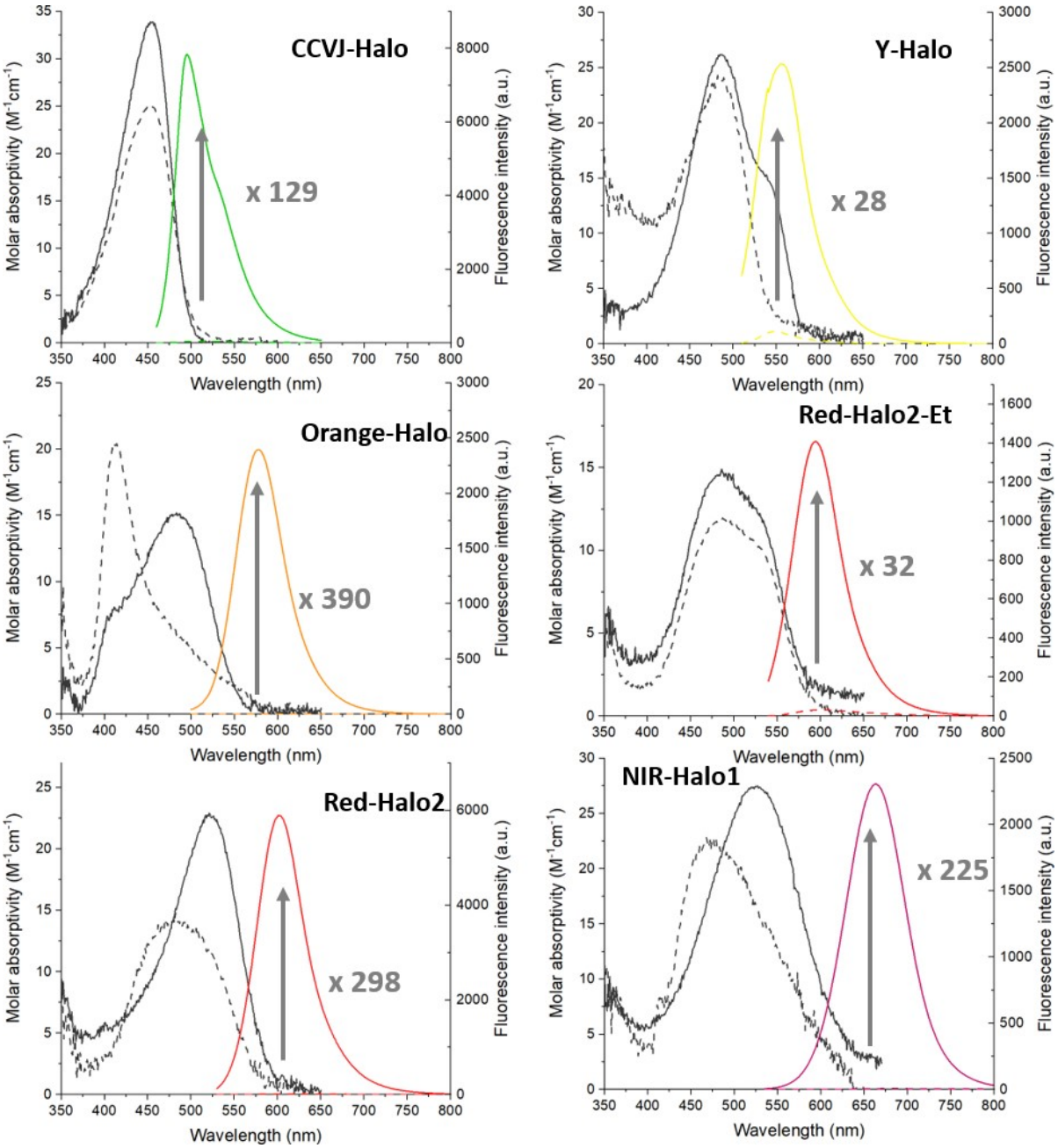


Figure S1. Viscosity-dependent emission of the molecular rotor probes.
Absorption (black lines) and fluorescence emission (colored lines) in water (dashed lines) and in glycerol (full lines) of the Halo probes. On each panel is also indicated the fluorescence enhancement factor F/F_0 where F is the fluorescence intensity in glycerol and F_0 the fluorescence in water.

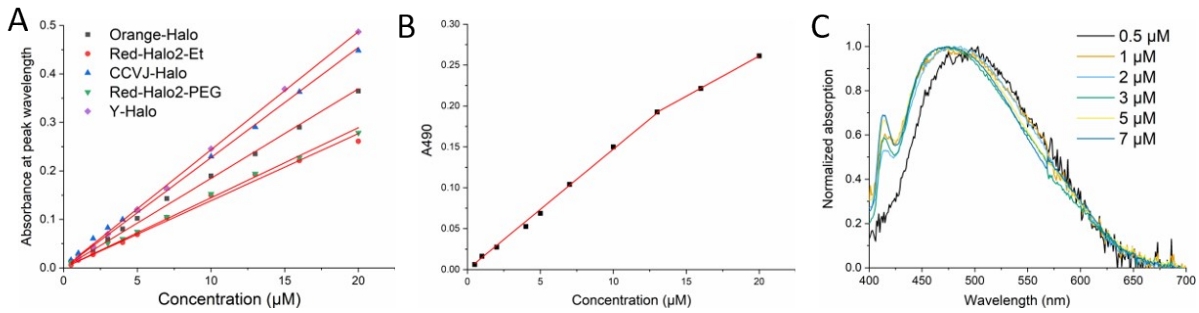


Figure S2. Solubility of the fluorophores.
(A) Absorbance at peak wavelength versus the concentration between 0 and 20 µM. Curves were fitted to a linear model following Lambert-Beer's law. (B) Absorbance at peak wavelength versus the concentration for **Red-Halo2-Et**. Data was fitted to two linear models, showing the solubility limit around 13µM. (C) Normalized absorption spectra of **NIR-Halo1** between 0.5 and 7 µM.

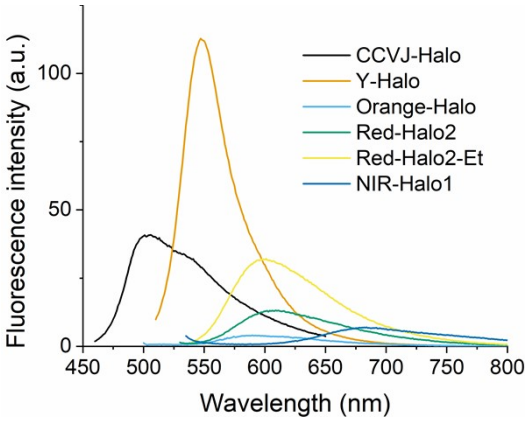
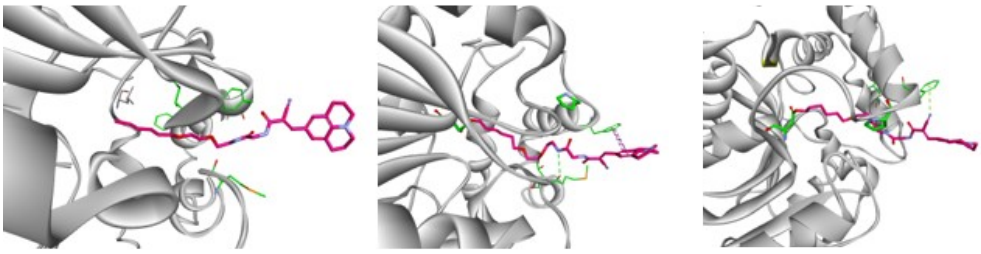


Figure S3. Fluorescence spectra of 1 µM solutions of HaloTag fluorogens in PBS.

Fluorogen	Mean	Median
CCVJ-Halo	10.79	7.75
Y-Halo	24.46	19.27
Orange-Halo	9.93	8.42
Red-Halo2-Et	22.75	19.13
Red-Halo2-PEG	5.21	4.63
Red-Halo2	5.94	5.39
NIR-Halo1	4.66	3.84

Table S1. Mean and median values of the S/NS contrast values from figure 3B.

CCVJ

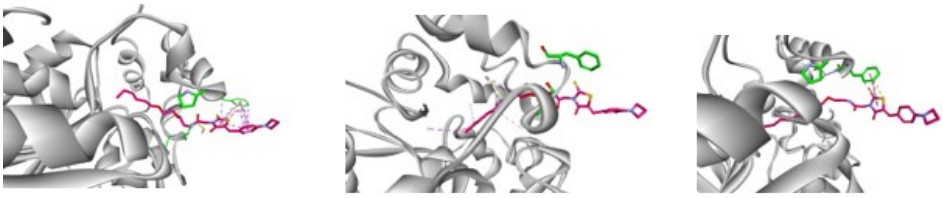


58 % of poses | 26 % of poses | 16 % of poses

Y-Halo

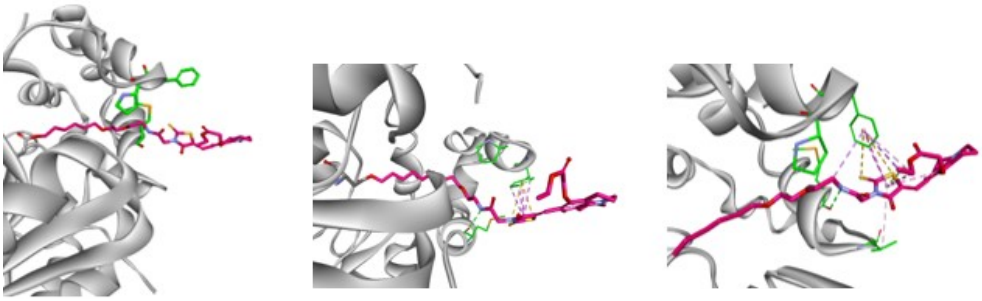
70 % of poses | 22 % of poses | 8 % of poses

Orange-Halo



53 % of poses | 36 % of poses | 11 % of poses

Red-Halo2-Et



60 % of poses | 33 % of poses | 7 % of poses

Figure S4. Representative structures for each cluster of conformations of the fluorogen/HaloTag complexes obtained by molecular dynamics calculations.

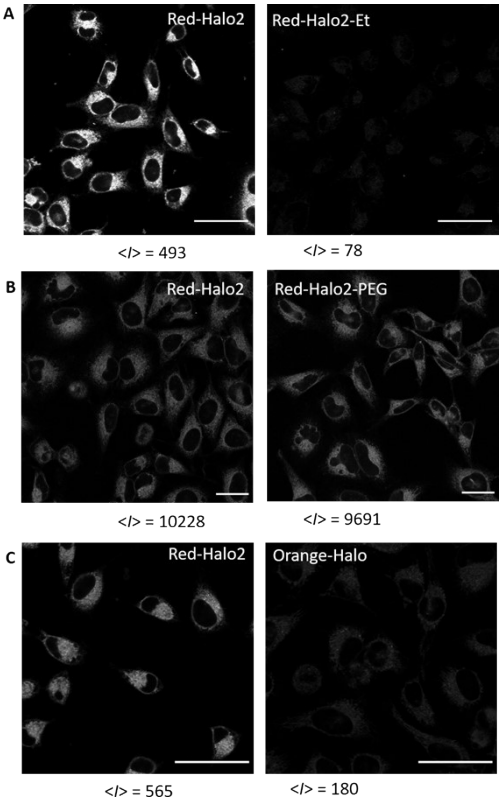


Figure S5. Non-specific signal of Red-Halo2-CO_2Et (A) Red-Halo2-PEG (B) and Orange-Halo (C) compared to Red-Halo2. Live confocal imaging of wild-type Hela cells incubated with 0.5 µM of **Red-Halo2**, **Red-Halo2-CO_2Et, Red-Halo2-PEG** or **Orange-Halo** and imaged without washing or changing the medium. Each pair of images was acquired with the exact same microscope settings and is presented with identical brightness and contrast parameters. Note that intensities in panel A, B and C cannot be compared with each other. </> = average intensity above the threshold. A threshold was set to exclude the background and then the average intensity was measured using imageJ.

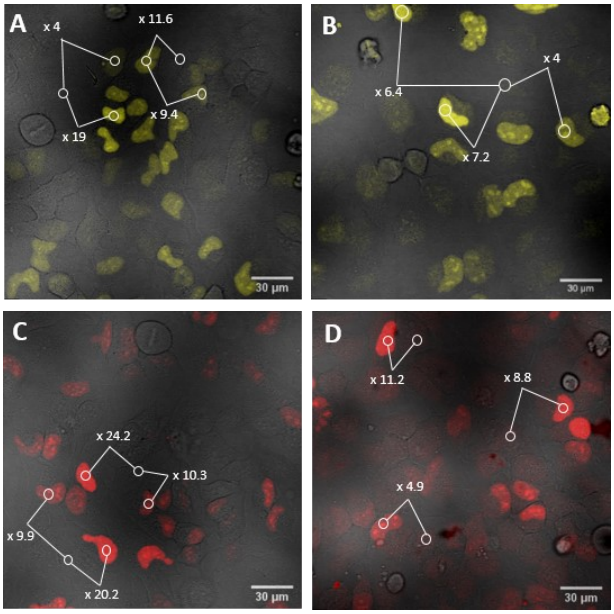


Figure S6. Effect of fluorogen concentration on the imaging contrast.
Wash-free imaging of Hela cells transiently expressing a HaloTag-NLS protein and incubated with **Y-Halo** at 1 µM (A) and 5 µM (B) or with **Red-Halo2-Et** at (1 µM) and (5 µM). Composite of transmission and fluorescence images. Contrast ratios were calculated for a sample of cells displaying various expression levels. Some cells are purposely saturated to make cells with lower expression levels visible.

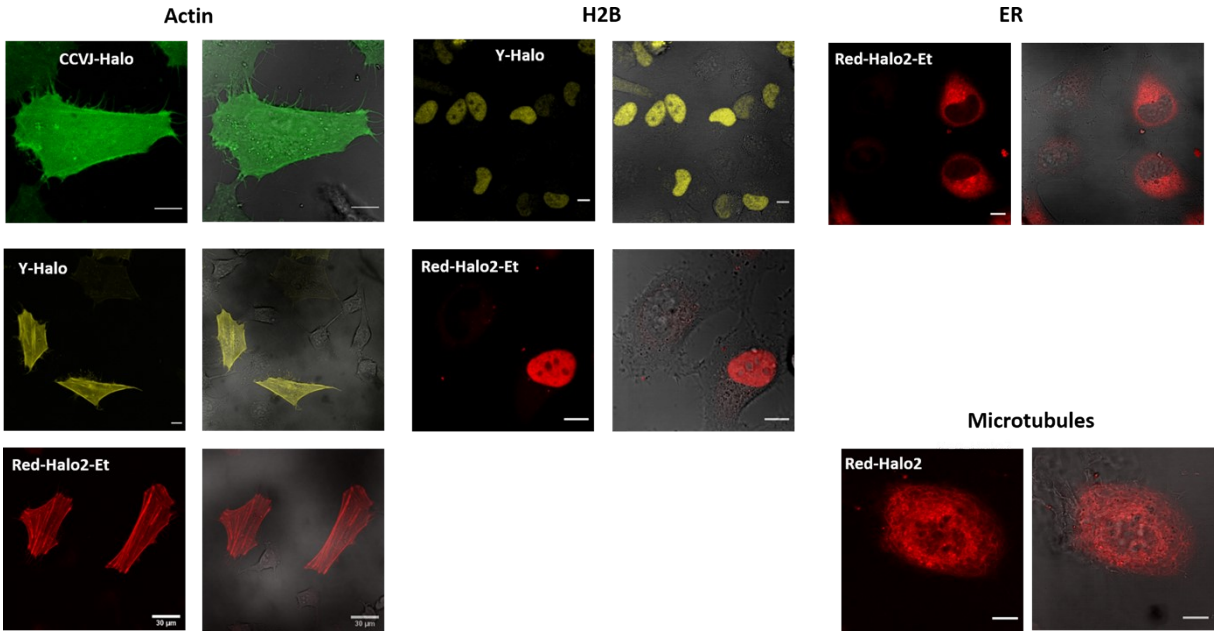
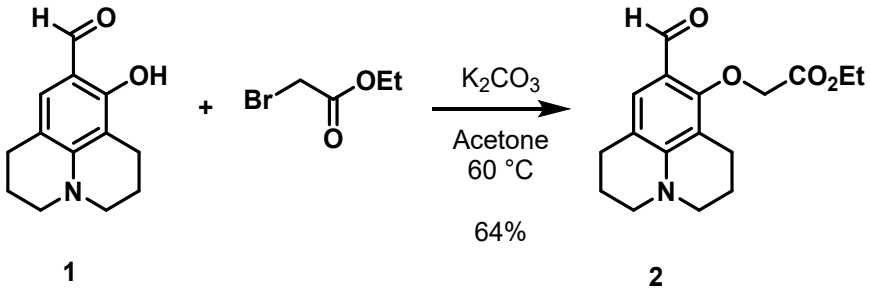


Figure S7. Additional microscopy images.

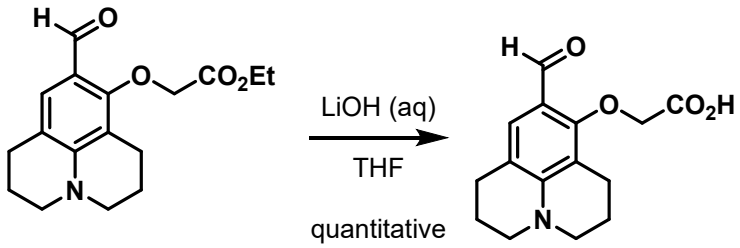
III. Synthetic procedures

Intermediates **1**,[3] **5**[4] and **11**[5] were synthesized according to previously reported procedures.



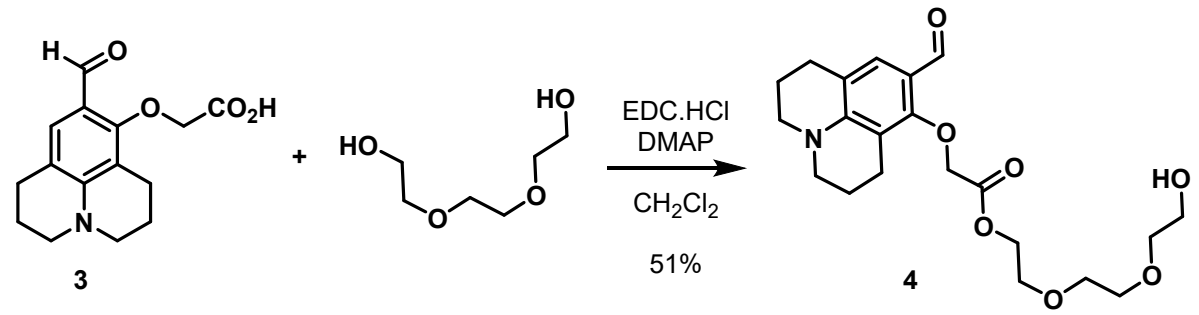
1 **2**

To a stirred suspension of **1** (500 mg, 2.30 mmol, 1.0 eq) and potassium carbonate (636 mg, 4.60 mmol, 2.0 eq) in acetone (1.0 mL) was added ethyl bromoacetate (0.38 mL, 3.45 mmol, 1.5 eq) and the reaction was stirred at reflux for 5 hours. The solution was evaporated to dryness and the crude product was extracted with ethyl acetate and washed with water and brine. The organic layer was dried over $MgSO_4$, filtered and evaporated to dryness and the crude product was purified by column chromatography (EtOAc:cyclohexane, 0:1 to 2:3) to give **2** (444 mg, 64%) as a yellow solid. **^{1}H NMR** (300 MHz, $CDCl_3$): δ 9.98 (s, 1H), 7.29 (s, 1H), 4.51 (s, 2H), 4.28 (q, J = 7.0 Hz, 2H), 3.27 (dd, J = 6.5, 5.0 Hz, 4H), 2.79 (t, J = 6.5 Hz, 2H), 2.71 (t, J = 6.5 Hz, 2H), 1.98-1.88 (m, 4H), 1.32 (t, J = 7.0 Hz, 3H). **^{13}C NMR** (75 MHz, $CDCl_3$): δ 187.7, 169.0, 158.3, 149.0, 128.5, 117.6, 116.7, 112.6, 71.6, 61.4, 50.2, 49.9, 27.4, 21.4, 21.2, 20.8, 14.3. **HRMS** calcd for $C_{17}H_{22}NO_4$: 304.1543. Found: 304.1544.



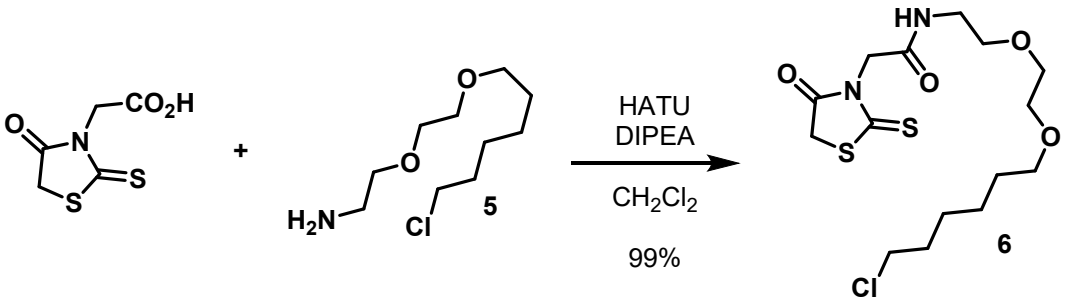
2 **3**

To a stirred solution of **2** (429 mg, 1.41 mmol, 1.0 eq) in THF (14.0 mL) was added lithium hydroxide (1.0 M, 14.0 mL, 14.0 mmol, 10 eq) and the reaction was stirred at room temperature overnight. The solution was extracted with ethyl acetate, then acidified with HCl until pH 1 and extracted with ethyl acetate. This organic layer was dried over $MgSO_4$, filtered and evaporated to dryness to give **3** (479 mg, quantitative yield) as a yellow solid. **^{1}H NMR** (300 MHz, $CDCl_3$): δ 9.40 (s, 1H), 7.05 (s, 1H), 4.47 (s, 2H), 3.32 (dd, J = 6.5, 5.0 Hz, 4H), 2.73-2.67 (m, 4H), 2.00-1.86 (m, 4H). **^{13}C NMR** (75 MHz, $CDCl_3$): δ 189.7, 170.8, 156.7, 149.8, 134.9, 117.2, 114.7, 113.3, 71.4, 50.3, 49.8, 27.3, 21.9, 21.1, 20.5. **HRMS** calcd for $C_{15}H_{16}NO_4$: 274.1085. Found: 274.1086.



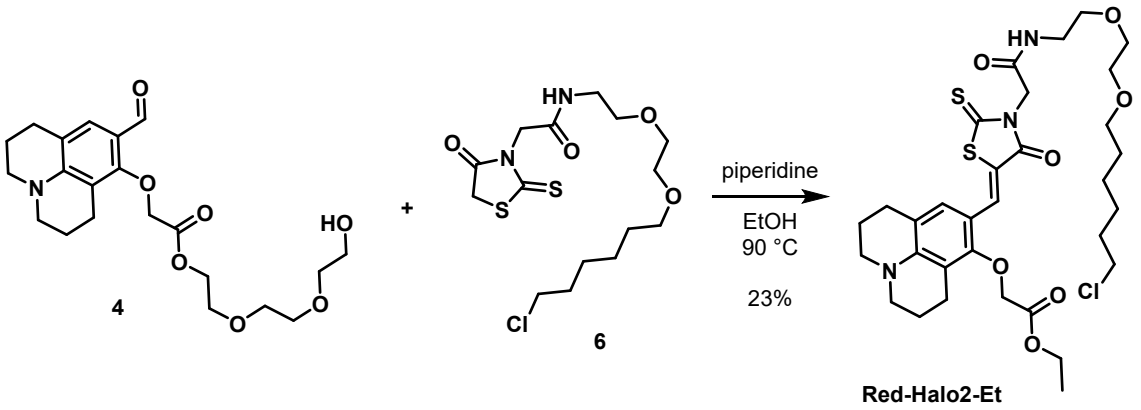
3 4

To a stirred solution of **3** (49 mg, 0.18 mmol, 1.0 eq) and triethylene glycol (0.24 mL, 1.78 mmol, 10.0 eq) in dichloromethane (18 mL) was added DMAP (24 mg, 0.20 mmol, 1.1 eq) and EDC hydrochloride (38 mg, 0.20 mmol, 1.1 eq) and the reaction was stirred at room temperature overnight. The mixture was extracted with dichloromethane and washed with HCl (1M) and brine. The organic layer was dried over $MgSO_4$, filtered and evaporated to dryness and the crude product was purified by column chromatography (EtOAc:cyclohexane, 7:3 to 1:0) to give **4** (37 mg, 51%) as a colorless oil. **^{1}H NMR** (300 MHz, $CDCl_3$): δ 9.94 (s, 1H), 7.25 (s, 1H), 4.54 (s, 2H), 4.38-4.32 (m, 2H), 3.75-3.67 (m, 4H), 3.65 (s, 4H), 3.60-3.55 (m, 2H), 3.29-3.21 (m, 4H), 2.75 (t, J = 6.5 Hz, 2H), 2.68 (t, J = 6.5 Hz, 2H), 2.41 (brs, 2H), 1.95-1.85 (m, 4H). **^{13}C NMR** (75 MHz, $CDCl_3$): δ 187.8, 169.0, 158.1, 149.0, 128.4, 117.5, 116.5, 112.4, 72.6, 71.2, 70.7, 70.4, 69.0, 64.2, 61.8, 50.1, 49.8, 27.4, 21.4, 21.2, 20.8. **HRMS** calcd for $C_{21}H_{29}NO_7Na$: 430.1836. Found: 430.1829.

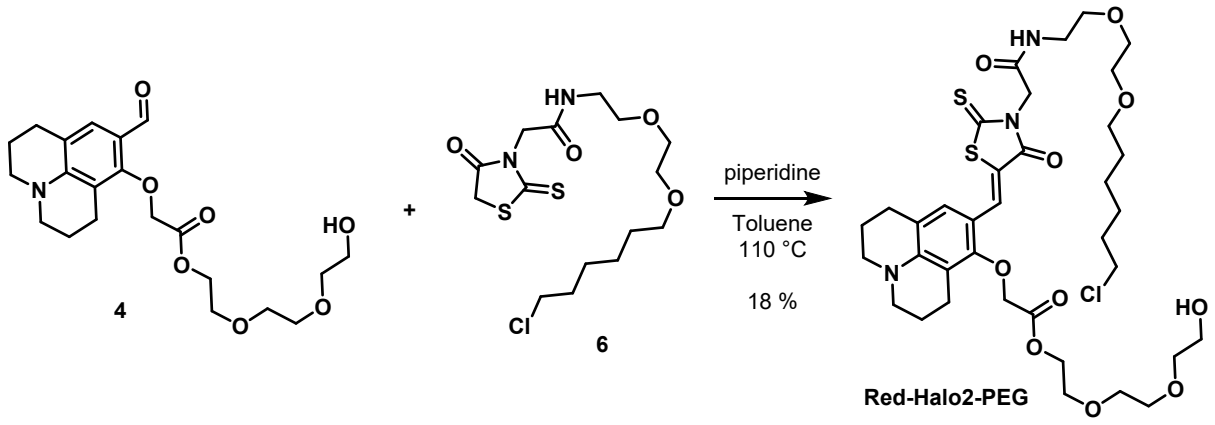


5 6

To a stirred solution of 2-(4-oxo-2-thioxothiazolidin-3-yl)acetic acid (291 mg, 1.52 mmol, 1.0 eq), **5** (408 mg, 1.82 mmol, 1.2 eq) and DIPEA (0.52 mL, 3.04 mmol, 2.0 eq) in CH_2Cl_2 (30.0 mL) was added HATU (578 mg, 1.52 mmol, 1.0 eq) and the reaction was stirred at room temperature overnight in the dark. The mixture was washed with brine and extracted with dichloromethane. The organic layer was dried over $MgSO_4$, filtered and evaporated to dryness and the crude product was purified by column chromatography (EtOAc: CH_2Cl_2, 2:3 to 7:3) to give **6** (598 mg, 99%) as a light yellow oil. **^{1}H NMR** (300 MHz, $CDCl_3$) δ 6.52-6.43 (m, 1H), 4.62 (s, 2H), 4.07 (s, 2H), 3.70-3.39 (m, 12H), 1.82-1.71 (m, 2H), 1.66-1.55 (m, 2H), 1.53-1.30 (m, 4H). **^{13}C NMR** (75 MHz, $CDCl_3$) δ 201.0, 173.4, 164.6, 71.4, 70.5, 70.2, 69.7, 46.5, 45.2, 39.7, 35.8, 32.7, 29.6, 26.8, 25.6. **HRMS** calcd for $C_{15}H_{25}ClN_2O_4S_2Na$: 419.0836. Found: 419.0835.

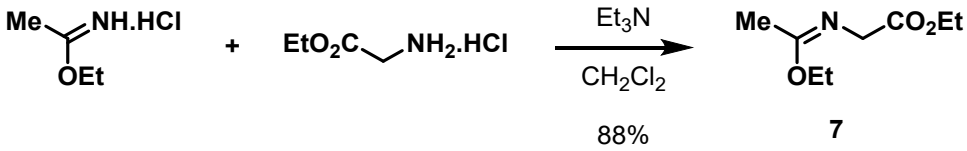


To a stirred solution of **4** (23 mg, 0.06 mmol, 1.0 eq) and **6** (22 mg, 0.06 mmol, 1.0 eq) in ethanol (1.0 mL) was added a catalytic amount of piperidine (one drop) and the reaction was stirred at reflux overnight. The solution was evaporated to dryness and the crude product was purified by column chromatography (EtOAc:dichloromethane, 0:1 to 3:7) to give **Red-Halo2-CO_2Et** (10 mg, 23%) as a red solid. **^{1}H NMR** (300 MHz, DMSO-d_6): δ 8.31 (t, J = 5.5 Hz, 1H), 7.96 (s, 1H), 6.89 (s, 1H), 4.63 (s, 2H), 4.47 (s, 2H), 4.21 (q, J = 7.0 Hz, 2H), 3.61 (t, J = 6.5 Hz, 2H), 3.53-3.48 (m, 4H), 3.45-3.34 (m, 6H), 3.33-3.16 (m, 8H), 2.76-2.65 (m, 4H), 1.92-1.79 (m, 4H), 1.76-1.65 (m, 2H), 1.56-1.45 (m, 2H), 1.43-1.29 (m, 4H), 1.28-1.21 (m, 6H). **^{13}C NMR** (75 MHz, DMSO-d_6): δ 192.7, 168.2, 166.6, 164.7, 156.1, 147.0, 129.5, 127.2, 118.3, 113.2, 112.9, 112.0, 70.6, 70.2, 69.6, 69.4, 68.9, 60.7, 49.4, 48.9, 45.9, 45.3, 32.0, 29.0 26.9, 26.1, 24.9, 21.0, 20.7, 20.0, 14.0. **HRMS** calcd for $C_{32}H_{44}ClN_3O_7S_2Na$: 704.2201 Found: 704.2196

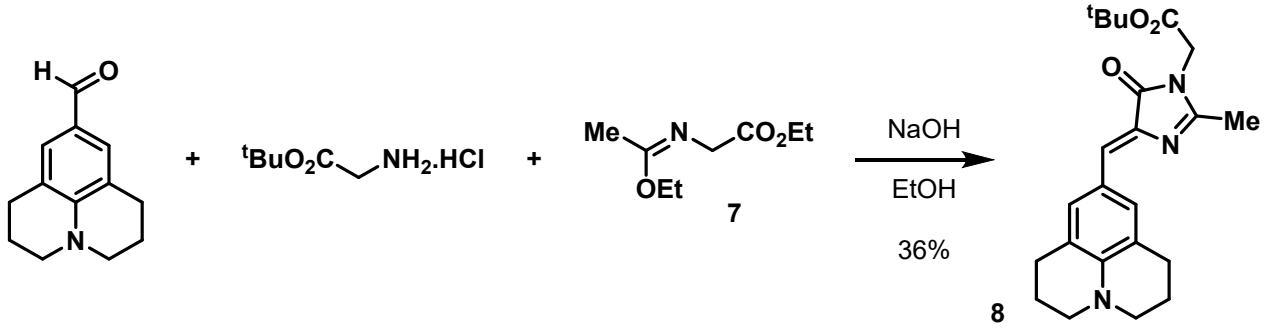


To a stirred solution of **4** (27 mg, 0.06 mmol, 1.0 eq) and **6** (50 mg, 0.13 mmol, 2.0 eq) in toluene (3 mL) was added a catalytic amount of piperidine (three drop). The mixture rapidly turns red and was stirred at 110°C overnight. A red solid precipitates. The suspension was evaporated to dryness and the crude product was purified by flash chromatography (dichloromethane:AcOEt:acetonitrile, 1:9:0 to 0:1:0 to 0:9:1) to give **Red-Halo2-PEG** (9 mg, 18 %) as a red solid. **^{1}H NMR** (300 MHz, $CDCl_3$) δ 8.07 (s, 1H), 6.93 (s, 1H), 6.53 – 6.38 (m, 1H), 4.76 (s, 2H), 4.52 – 4.28 (m, 4H), 3.86 – 3.40 (m, 24H), 3.36 – 3.19 (m, 4H), 2.75 (p, *J* = 5.9 Hz, 4H), 2.03 – 1.87 (m, 4H), 1.76 (p, J = 6.9 Hz, 2H), 1.60 (p, *J* = 6.8 Hz, 2H), 1.52 – 1.31 (m, 4H). **^{13}C NMR** (75 MHz, $CDCl_3$) δ 193.51, 168.63, 167.50, 165.33, 156.61, 147.05, 130.48, 128.14, 118.67, 114.77, 113.86, 113.41, 72.60, 71.44, 71.11,

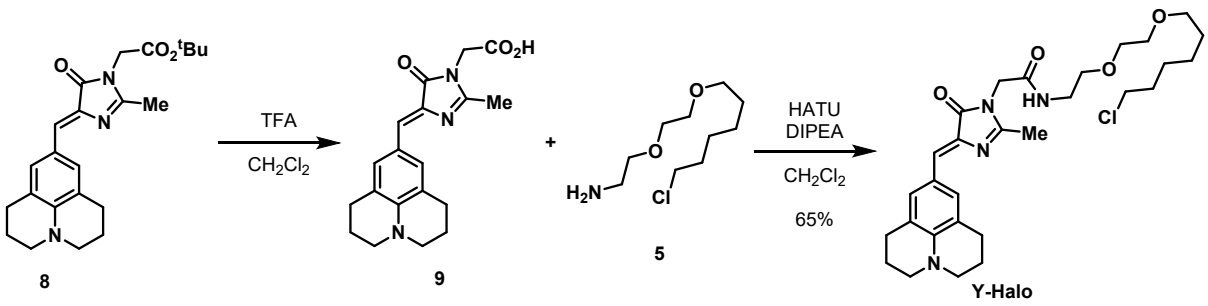
70.74, 70.53, 70.43, 70.22, 69.82, 68.99, 64.40, 61.87, 50.24, 49.85, 46.77, 45.19, 39.67, 32.67, 29.58, 27.74, 26.82, 25.54, 21.61, 21.41, 20.80. **HRMS** calcd for $C_{36}H_{52}ClN_3O_{10}S_2Na$: 808.2675 Found: 808.2671.



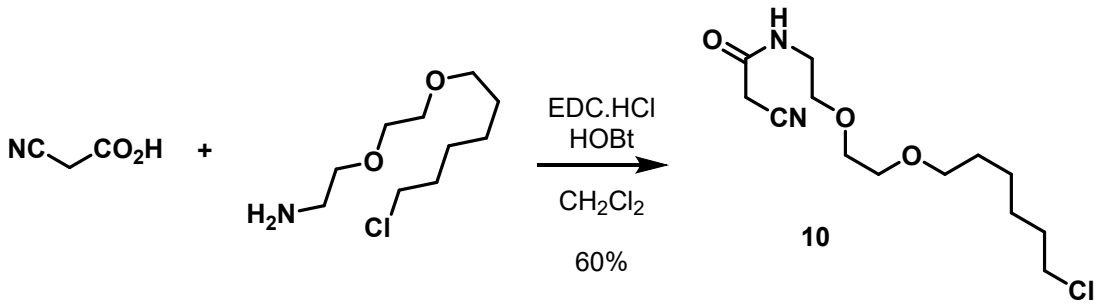
To a stirred suspension of ethyl acetimidate hydrochloride (885 mg, 7.16 mmol, 1.0 eq) and glycine ethyl ester hydrochloride (1.00 g, 7.16 mmol, 1.0 eq) in dichloromethane (30 mL) was added triethylamine (1.00 mL, 7.16 mmol, 1.0 eq) and the reaction was stirred at room temperature for 3 hours. The solution was extracted with dichloromethane and washed with water and brine. The organic layer was dried over $MgSO_4$, filtered and evaporated to dryness to give **7** (1.09 g, 88%) as a colorless oil. **^{1}H NMR** (300 MHz, $CDCl_3$): δ 4.19 (q, J = 7.0 Hz, 2H), 4.11 (q, J = 7.0 Hz, 2H), 4.03 (s, 2H), 1.87 (s, 3H), 1.27 (t, J = 7.0 Hz, 3H), 1.26 (t, J = 7.0 Hz, 3H). **^{13}C NMR** (75 MHz, $CDCl_3$): δ 171.3, 164.9, 61.1, 60.9, 51.4, 15.4, 14.4, 14.3. **HRMS** calcd for $C_8H_{16}NO_3$: 174.1125. Found: 174.1124.



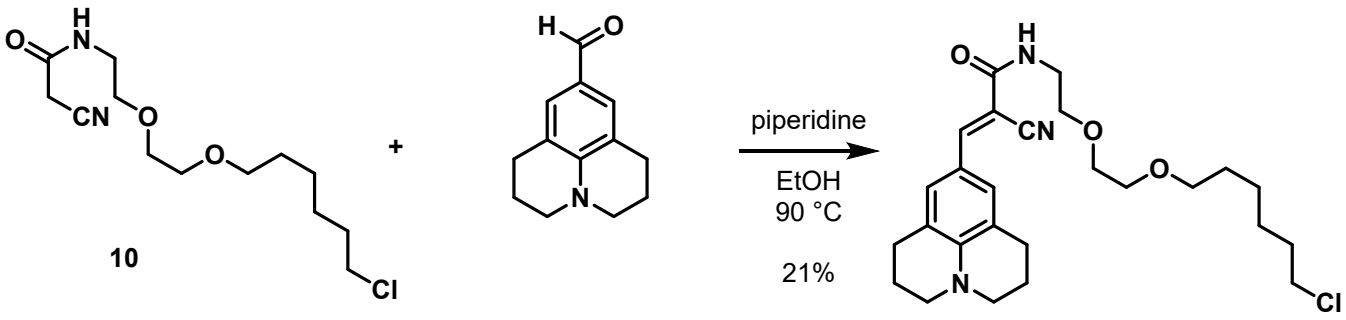
To a stirred solution of **7** (172 mg, 0.99 mmol, 1.0 eq) in ethanol (1.0 mL) was added 9-julolidinecarboxaldehyde (200 mg, 0.99 mmol, 1.0 eq) and the reaction was stirred overnight. Glycine tert-butyl ester hydrochloride (183 mg, 1.09 mmol, 1.1 eq) and sodium hydroxide (40 mg, 0.99 mmol, 1.0 eq) were added and the reaction was stirred for another night. The mixture was extracted with dichloromethane and washed with water and brine. The organic layer was dried over $MgSO_4$, filtered and evaporated to dryness and the crude product was purified by column chromatography (EtOAc:cyclohexane, 0:1 to 1:1) to give **8** (142 mg, 36%) as an deep orange oil. **^{1}H NMR** (300 MHz, $CDCl_3$): δ 7.61 (s, 2H), 7.00 (s, 1H), 4.27 (s, 2H), 3.31-3.21 (m, 4H), 2.79-2.73 (m, 4H), 2.30 (s, 3H), 2.02-1.90 (m, 4H), 1.46 (s, 9H). **^{13}C NMR** (75 MHz, $CDCl_3$): δ 167.1, 157.2, 145.2, 132.2 (x2), 130.3, 121.3, 121.1, 82.9, 50.2, 42.3, 28.1, 27.9, 21.7, 15.5, 14.3. **HRMS** calcd for $C_{23}H_{30}N_3O_4$: 396.2282. Found: 396.2281.



To a stirred solution of **8** (50 mg, 0.13 mmol, 1.0 eq) in dichloromethane (0.5 mL) was added trifluoroacetic acid (0.5 mL) and the reaction was stirred at rt for 5 hours. The mixture was evaporated to dryness and used without further purification. The residue was dissolved in dichloromethane (1.5 mL), diisopropylethylamine (0.11 mL, 0.63 mmol, 5.0 eq), **5** (34 mg, 0.15 mmol, 1.2 eq) and HATU (48 mg, 0.13 mmol, 4.0 eq) were added successively and the reaction was stirred at rt overnight. The mixture was extracted with dichloromethane and washed with water and brine. The organic layer was dried over $MgSO_4$, filtered and evaporated to dryness and the crude product was purified by column chromatography (EtOAc:dichloromethane, 0:1 to 1:0) to give **Y-Halo** (45 mg, 65% over two steps) as a deep orange oil. **^{1}H NMR** (300 MHz, $CDCl_3$): δ 7.61 (s, 2H), 7.00 (s, 1H), 6.52 (t, J = 5.5 Hz, 1H), 4.24 (s, 2H), 3.59-3.38 (m, 12H), 3.29-3.22 (m, 4H), 2.80-2.71 (m, 4H), 2.36 (s, 3H), 2.00-1.90 (m, 4H), 1.80-1.69 (m, 2H), 1.62-1.50 (m, 2H), 1.47-1.29 (m, 4H). **^{13}C NMR** (75 MHz, $CDCl_3$): δ 170.3, 167.3, 156.8, 145.5, 132.7, 132.3, 131.0, 121.1, 121.0, 71.4, 70.5, 70.2, 69.6, 50.2, 45.2, 44.3, 39.6, 32.7, 29.5, 27.8, 26.8, 25.5, 21.6, 15.6. **HRMS** calcd for $C_{29}H_{42}ClN_4O_4$: 545.2889. Found: 545.2884.



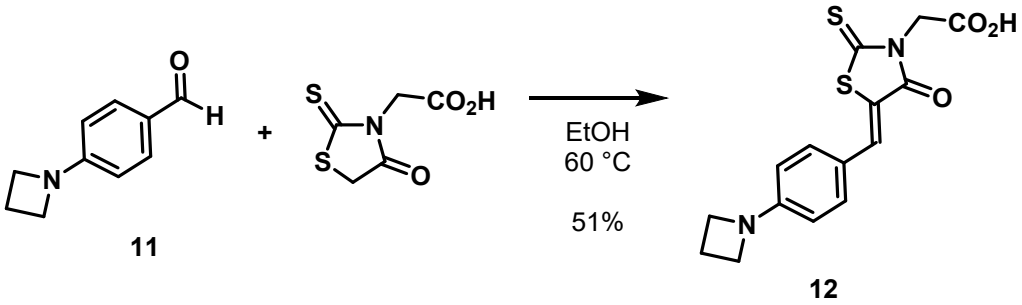
To a stirred solution of 2-cyanoacetic acid (29 mg, 0.34 mmol, 1.5 eq), 2-(2-((6-chlorohexyl)oxy)ethoxy)ethan-1-amine (50 mg, 0.22 mmol, 1.0 eq) and HOBt (46 mg, 0.34 mmol, 1.5 eq) in CH_2Cl_2 (2.5 mL) was added EDC.HCl (65 mg, 0.34 mmol, 1.5 eq) and the reaction was stirred at room temperature overnight. The mixture was washed with brine and extracted with dichloromethane. The organic layer was dried over $MgSO_4$, filtered and evaporated to dryness and the crude product was purified by column chromatography (EtOAc: cyclohexane, 3:7 to 7:3) to give **10** (59 mg, 60%) as a colorless oil. **^{1}H NMR** (300 MHz, $CDCl_3$): δ 6.68 (brs, 1H), 3.67-3.42 (m, 12H), 3.36 (s, 2H), 1.84-1.70 (m, 2H), 1.69-1.54 (m, 2H), 1.54-1.29 (m, 4H). **^{13}C NMR** (75 MHz, $CDCl_3$): δ 161.0, 114.5, 71.3, 70.4, 70.1, 69.2, 45.0, 40.1, 32.5, 29.4, 26.7, 25.9, 25.4. **HRMS** calcd for $C_{13}H_{24}ClN_2O_3$: 291.1470. Found: 291.1471.



10

CCVJ-Halo

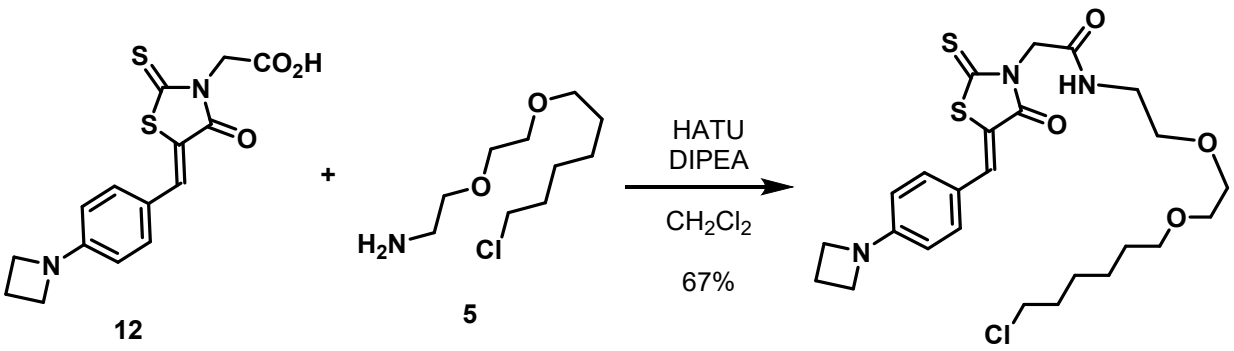
To a stirred solution of 9-julolidinecarboxaldehyde (30 mg, 0.15 mmol, 1.0 eq) and **10** (44 mg, 0.15 mmol, 1.0 eq) in ethanol (1.5 mL) was added a catalytic amount of piperidine (one drop) and the reaction was stirred at reflux overnight. The solution was evaporated to dryness and the crude product was purified by column chromatography (EtOAc:dichloromethane, 0:1 to 3:7) to give **CCVJ-Halo** (15 mg, 21%) as a red solid. **^{1}H NMR** (300 MHz, $CDCl_3$): δ 8.01 (s, 1H), 7.45 (s, 2H), 6.65-6.57 (m, 1H), 3.66-3.56 (m, 8H), 3.54-3.44 (m, 4H), 3.34-3.27 (m, 4H), 2.79-2.70 (m, 4H), 2.01-1.90 (m, 4H), 1.81-1.71 (m, 2H), 1.67 -1.57 (m, 2H), 1.48-1.35 (m, 4H). **^{13}C NMR** (75 MHz, $CDCl_3$): δ 162.8, 152.5, 147.1, 131.2, 120.9, 119.9, 118.9, 93.8, 71.5, 70.7, 70.3, 69.9, 50.3, 45.2, 40.2, 32.7, 29.6, 27.8, 26.9, 25.6, 21.4. **HRMS** calcd for $C_{26}H_{36}ClN_3O_3Na$: 496.2337. Found: 496.2333.



11

12

11 (100 mg, 0.62 mmol, 1.0 eq) and 3-rhodanine acetic acid (130 mg, 0.68 mmol, 1.1 eq) were dissolved in 5 mL of ethanol in a sealed tube and heated with stirring at 80°C overnight. The reaction mixture was then cooled in an ice bath and the bright red precipitate filtered and washed with cold ethanol to give **12** (106 mg, 51%) as a red powder. **^{1}H NMR** (300 MHz, DMSO-d_6): δ 13.37 (brs, 1H), 7.74 (s, 1H), 7.49 (d, J = 8.5 Hz, 2H), 6.51 (d, J = 8.5 Hz, 2H), 4.71 (s, 2H), 4.00 (t, J = 7.5 Hz, 4H), 2.38 (p, J = 7.5 Hz, 2H). **^{13}C NMR** (75 MHz, DMSO-d_6): δ 192.6, 167.4, 166.4, 152.8, 135.5, 133.3, 120.3, 113.5, 111.0, 51.2, 45.0, 15.9. **HRMS** calcd for $C_{15}H_{13}N_2O_3S_2$: 333.0373. Found: 333.0375.



To a stirred solution of **12** (50 mg, 0.15 mmol, 1.0 eq), **5** (37 mg, 0.17 mmol, 1.1 eq) and DIPEA (0.05 mL, 0.30 mmol, 2.0 eq) in CH_2Cl_2 (1.5 mL) was added HATU (57 mg, 0.15 mmol, 1.0 eq) and the reaction was stirred at room temperature overnight in the dark. The mixture was washed with brine and extracted with dichloromethane. The organic layer was dried over $MgSO_4$, filtered and evaporated to dryness and the crude product was purified by column chromatography (EtOAc:CH_2Cl_2, 3:7) to give **Orange-Halo** (54 mg, 67%) as a red solid. **^{1}H NMR** (300 MHz, $CDCl_3$) δ 7.69 (s, 1H), 7.37 (d, J = 8.5 Hz, 2H), 6.42 (d, J = 8.5 Hz, 2H), 6.32 (t, J = 4.5 Hz, 1H), 4.77 (s, 2H), 4.04 (t, J = 7.5 Hz, 4H), 3.67-3.43 (m, 12H), 2.54-2.38 (m, 2H), 1.83-1.71 (m, 2H), 1.66-1.53 (m, 2H), 1.53-1.31 (m, 4H). **^{13}C NMR** (75 MHz, $CDCl_3$) δ 193.3, 167.7, 165.1, 152.6, 135.7, 133.3, 121.8, 115.7, 111.2, 77.6, 77.2, 76.7, 71.5, 70.6, 70.2, 69.8, 51.8, 46.8, 45.2, 39.7, 32.7, 29.6, 26.8, 25.6, 16.6. **HRMS** calcd for $C_{25}H_{35}ClN_3O_4S_2$: 540.1752. Found: 540.1753.

IV. Plasmids construction

The plasmid pAG842, encoding HaloTag fused to the platelet derived growth factor receptor (PDGFR) transmembrane domain (HaloTag-PDGFR), was constructed by Gibson assembly from the pDisplay vector (Life Technology). The plasmid backbone was amplified by PCR using primers ag571/ag313 and ag853/ag314. The insert coding for HaloTag was amplified by PCR using the primers ag1301/ag1303. The three fragments were then assembled by Gibson assembly.

The plasmid pAG846, encoding HaloTag fused to an endoplasmic reticulum (ER) targeting sequence (ER-HaloTag), was constructed by Gibson assembly from the pIRES vector. The plasmid backbone was amplified by PCR using primers ag1322/ag314 and ag313/ag1306. The insert coding for HaloTag was amplified by PCR using the primers ag1305/ag1321 including the endoplasmic reticulum (ER) targeting sequence. The three fragments were then assembled by Gibson assembly.

The plasmid pAG847, encoding HaloTag fused to the N-terminal 81 amino acids of the human beta-1,4-galactosyltransferase for Golgi targeting (Golgi-HaloTag), was obtained by inserting the sequence coding for HaloTag using the BglII and HindIII restriction sites in the previously described plasmid pAG157 Golgi-FAST[6] with BglII and HindIII restriction enzymes.

The plasmid pAG848, encoding Halotag fused to zebrafish H2B (H2B-HaloTag) was constructed by Gibson assembly from the previously described plasmid pAG109 H2B-FAST (Addgene #130722, Plamont, PNAS 2016). The plasmid backbone was amplified by PCR using primers ag313/ag492 and ag311/ag314. The insert coding for HaloTag was amplified by PCR using the primers ag1295/ag1291. The three fragments were then assembled by Gibson assembly.

The plasmid pAG849, encoding Halotag fused to microtubule-associated protein (MAP) 4 (MAP4-HaloTag) was constructed by Gibson assembly from the previouly described plasmid pAG498 MAP4-iFAST (Addgene #130820). The plasmid backbone was amplified by PCR using primers ag313/ag979 and ag311/ag314. The insert coding for HaloTag was amplified by PCR using the primers ag1297/ag1291. The three fragments were then assembled by Gibson assembly.

The plasmid pAG850, encoding HaloTag fused to the actin binding peptide LifeAct (LifeAct-Halotag) was constructed by Gibson from the previously described plasmid pAG470 LifeAct-iFAST (Addgene #130821). The plasmid backbone was amplified by PCR using primers ag313/ag1298 and ag311/ag314. The insert coding for HaloTag was amplified by PCR using the primers ag1299/ag1291. The three fragments were then assembled by Gibson assembly.

Primers used in this study

primer	sequence
ag311	aaagcttatttctgaagaggacttgtaataggcggccgcgactctagatcataatc
ag313	ctcaccttgctcctgccgagaaagtatcca
ag314	tggatactttctcggcaggagcaaggtgag
ag358	ggtggcagatctgagtccggtag
ag492	catggtggcagatccgcctcc
ag555	gagtccttgcccttggacttgatg
ag571	gtcgacgaacaaaaactcatctcag
ag572	agatctggccggctgggcc
ag853	gatgagtttttgttcgtcgacggaaagggctttcttcatgtgc
ag979	gtctggtttaatcacactcatggtggcgacc
ag1290	catcaagtccaagggcaaggactccgccggcggcggctccatggcagaaatcggtactggct
ag1291	gtcctcttcagaaataagcttttgttcggatccgccggaaatctcgagcgtcg
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ag1297	caccatgagtgtgattaaaccagacatggcagaaatcggtactggct
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ag1300	ccggactcagatctgccaccatggcagaaatcggtactggct
ag1301	gatgagtttttgttcgtcgacgccggaaatctcgagcgtcg

ag1302	ggggcccagccggccagatctatggcagaaatcggtactggct
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ag1305	ctgctgctgggcctgctgggcgccgccgccgacgcagaaatcggtactggctttc
ag1306	cagcaggcccagcagcaggggacgggcagcagcatggtggcagatctgagtcc
ag1321	agaaataagcttttgttcggatcccagctcgtccttgccggaaatctcgagcgt
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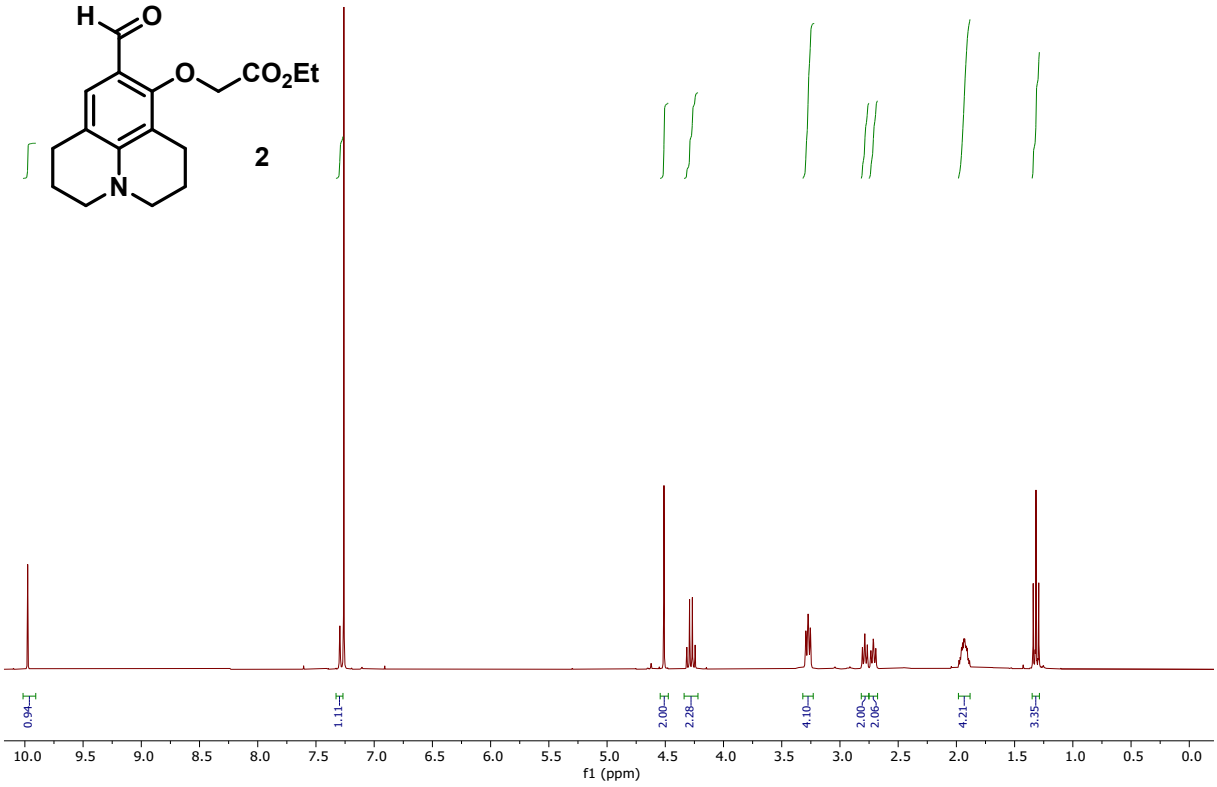
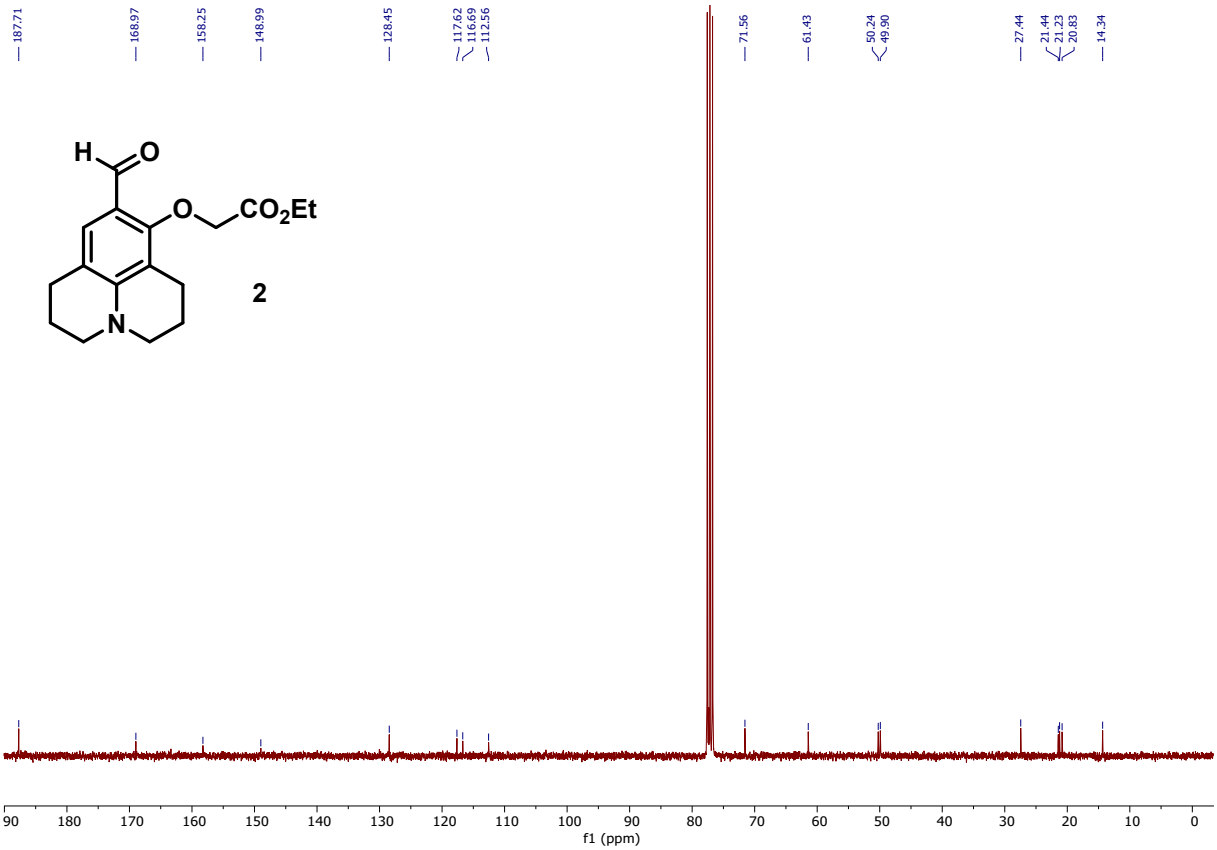
V. NMR spectra

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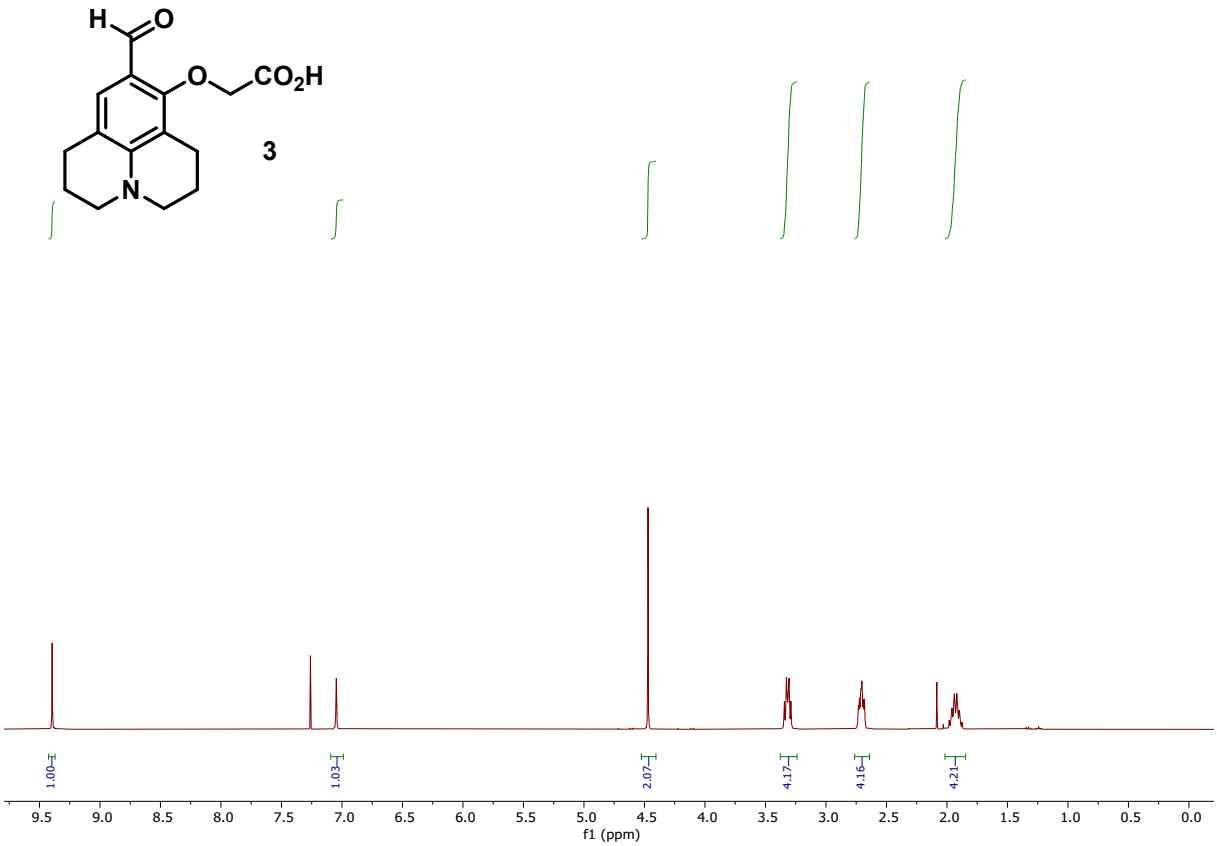
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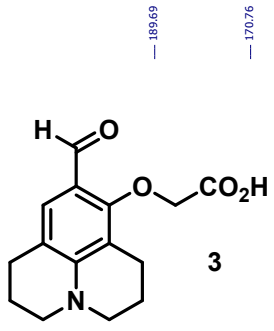
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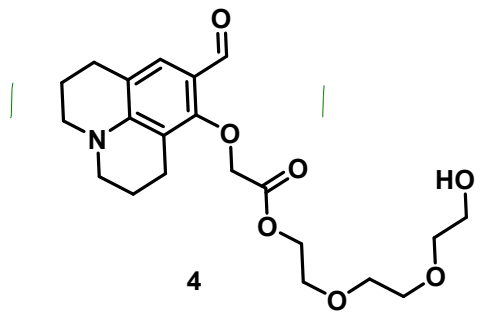
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1.33
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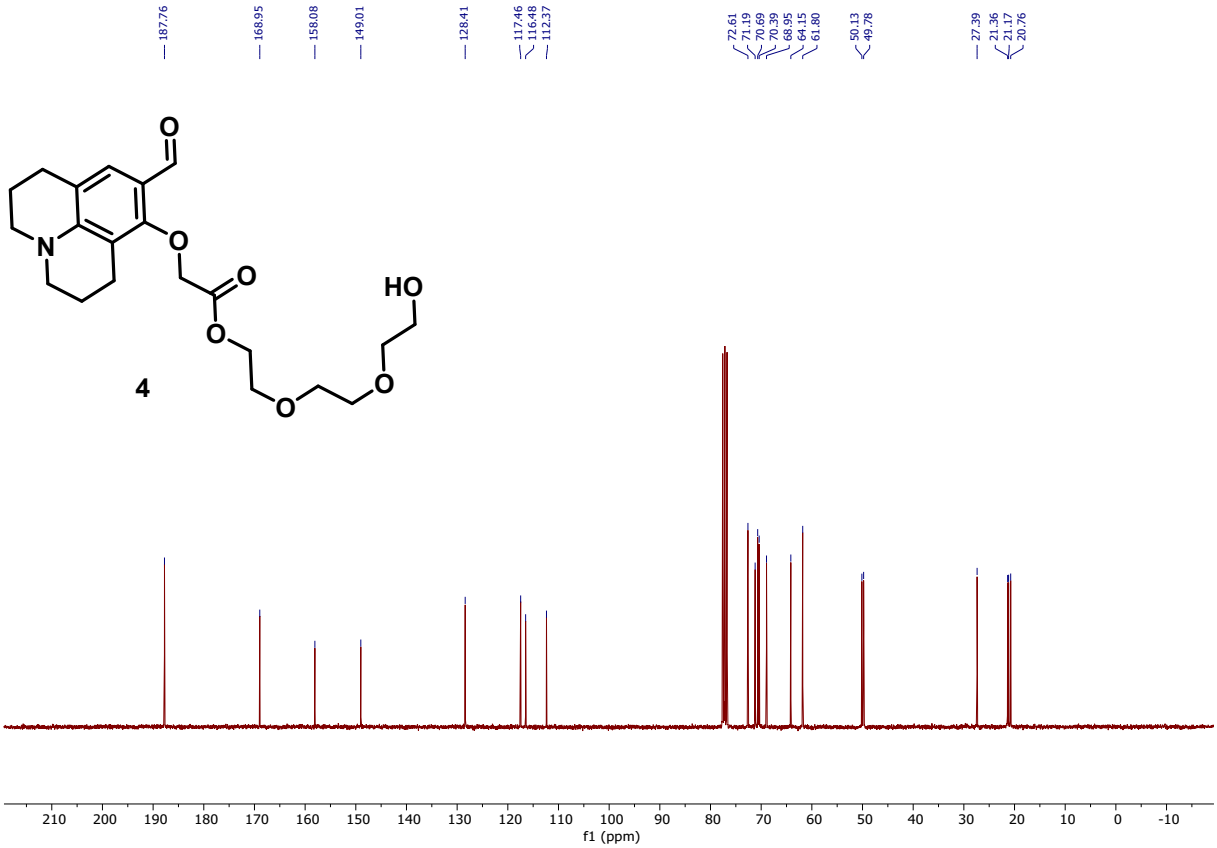
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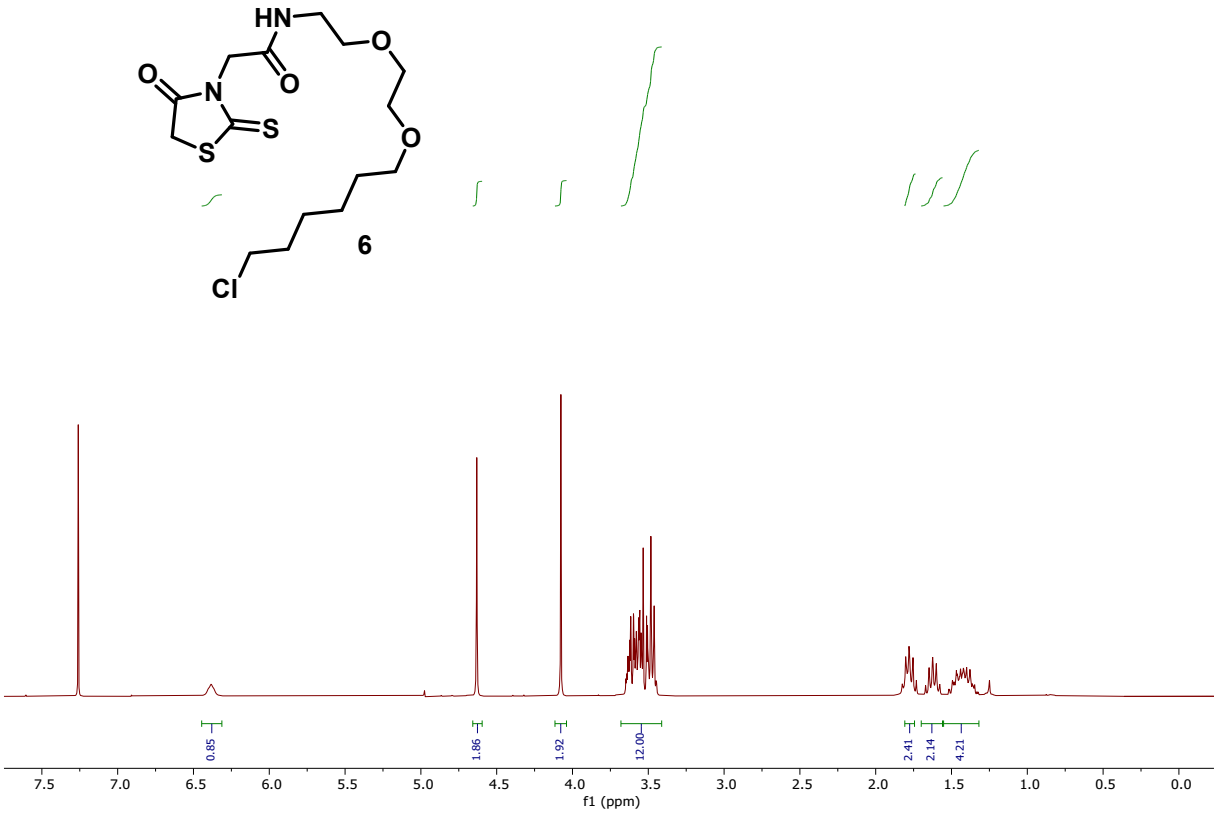
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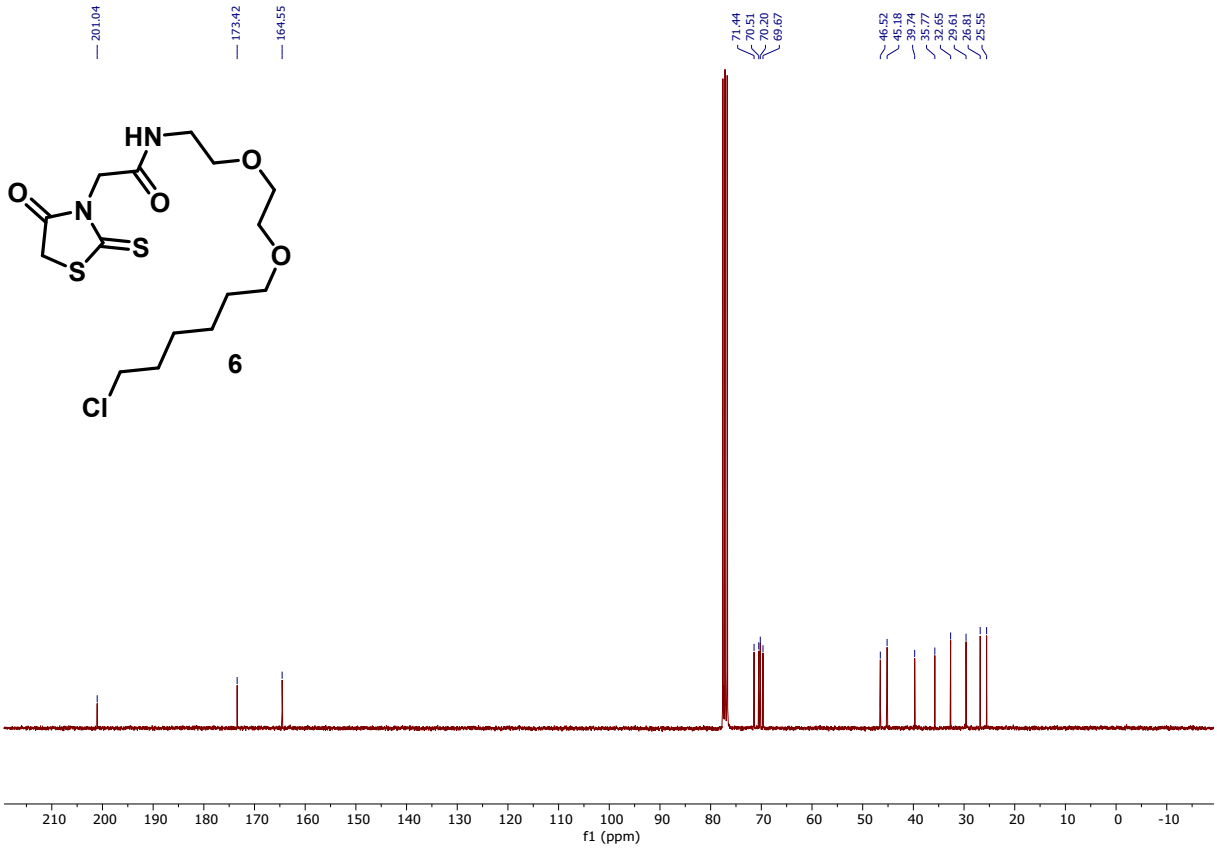
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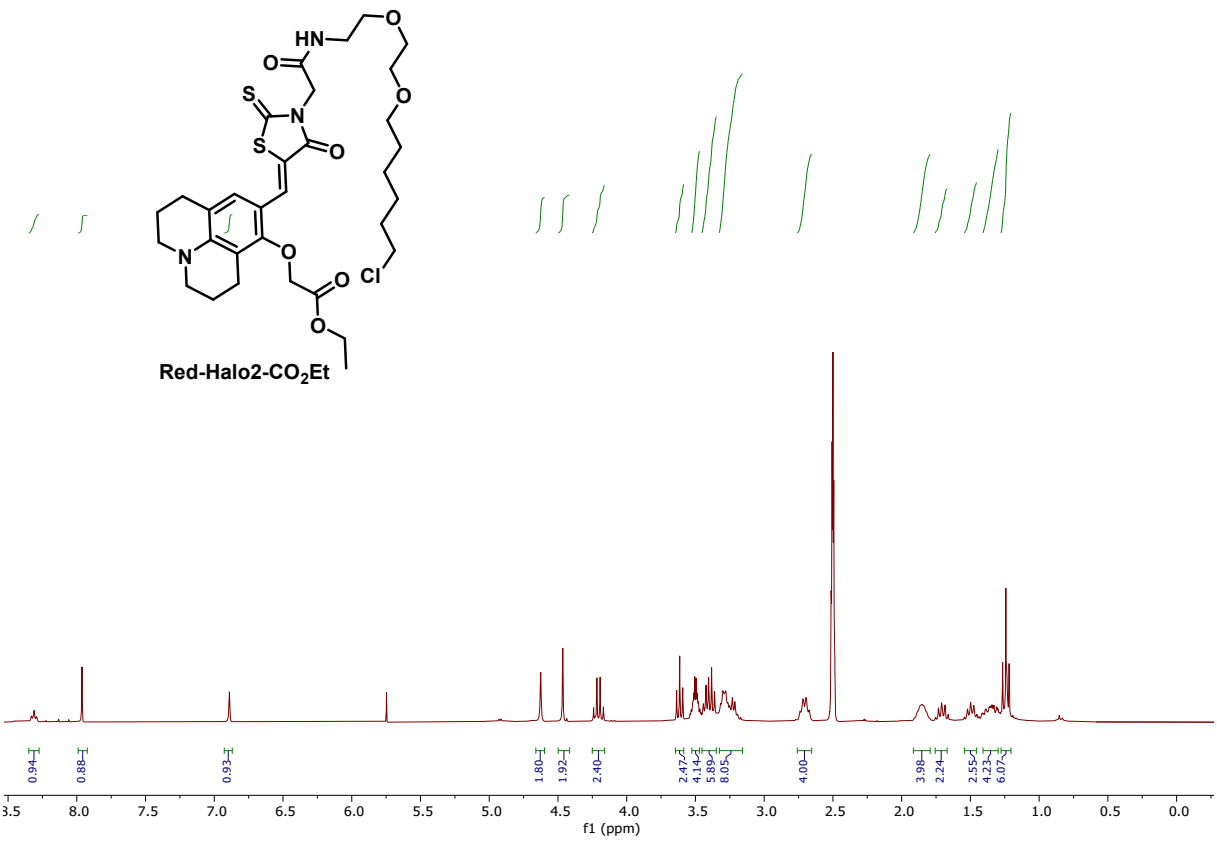
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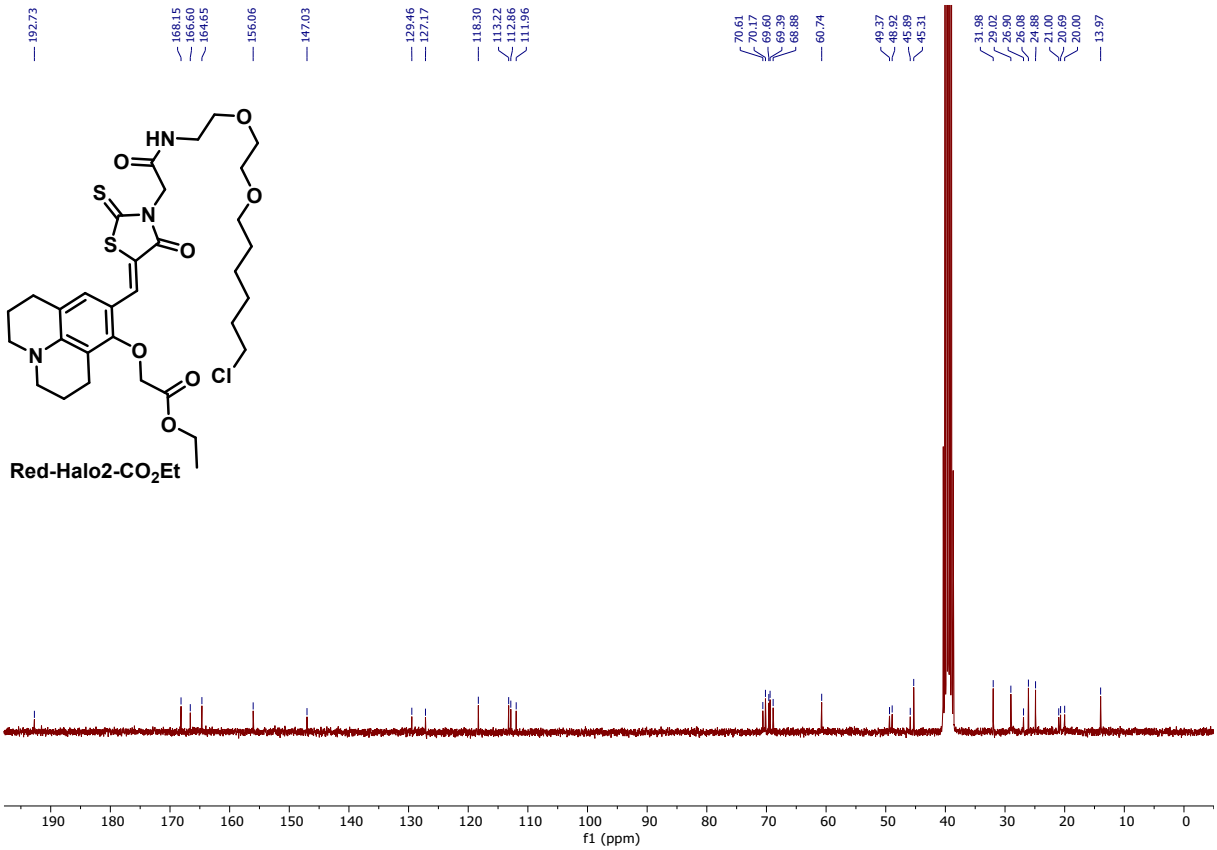
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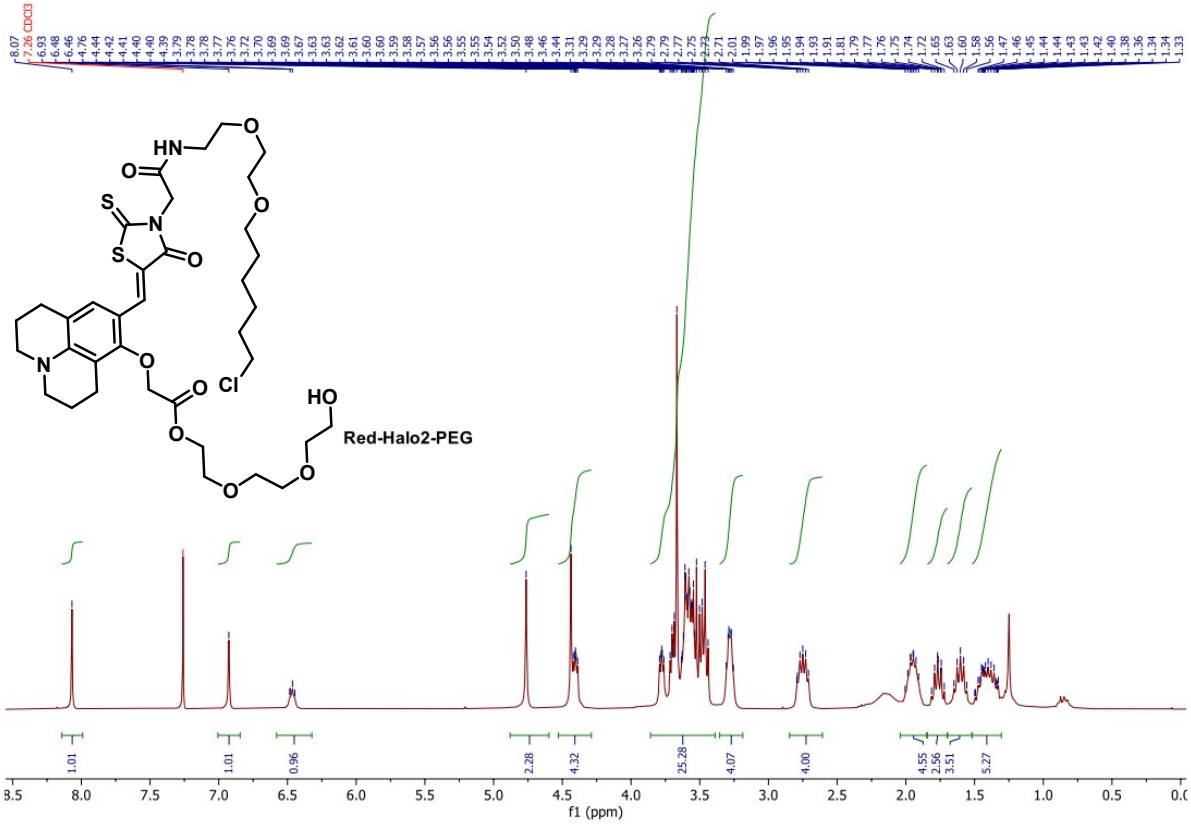
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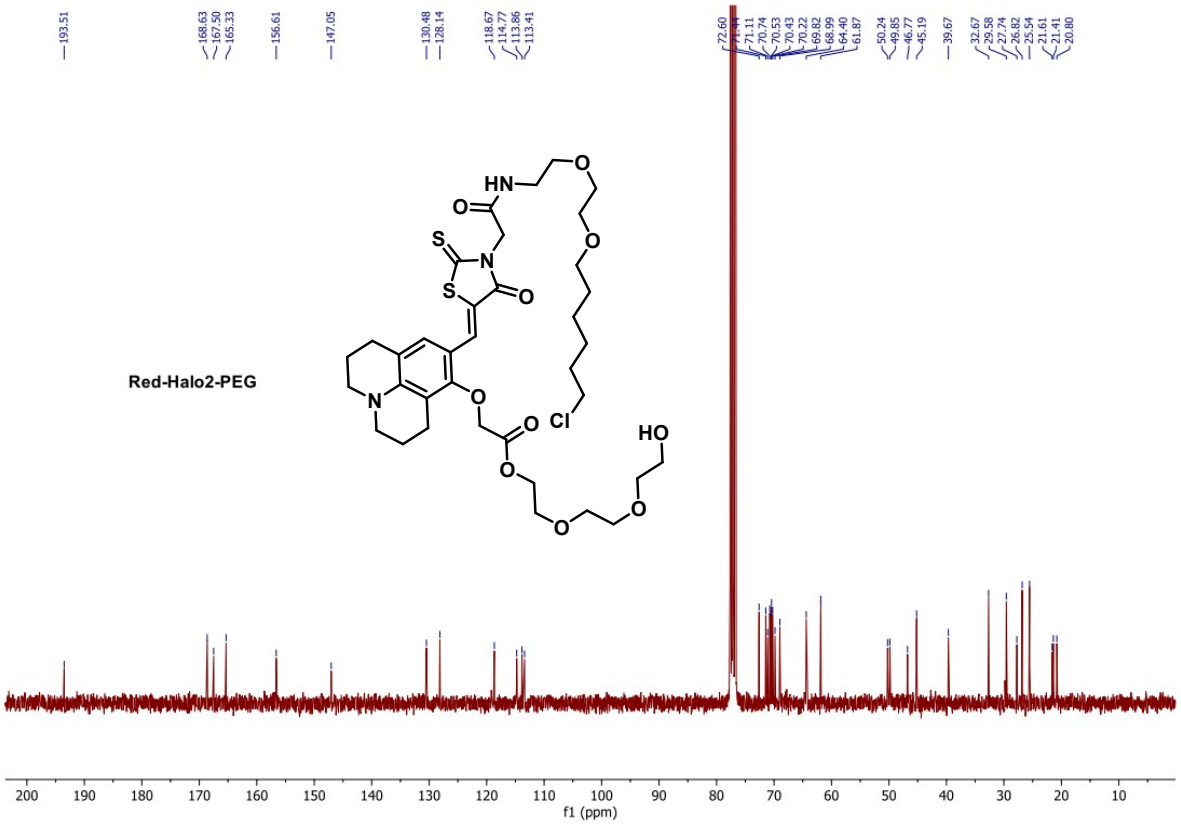
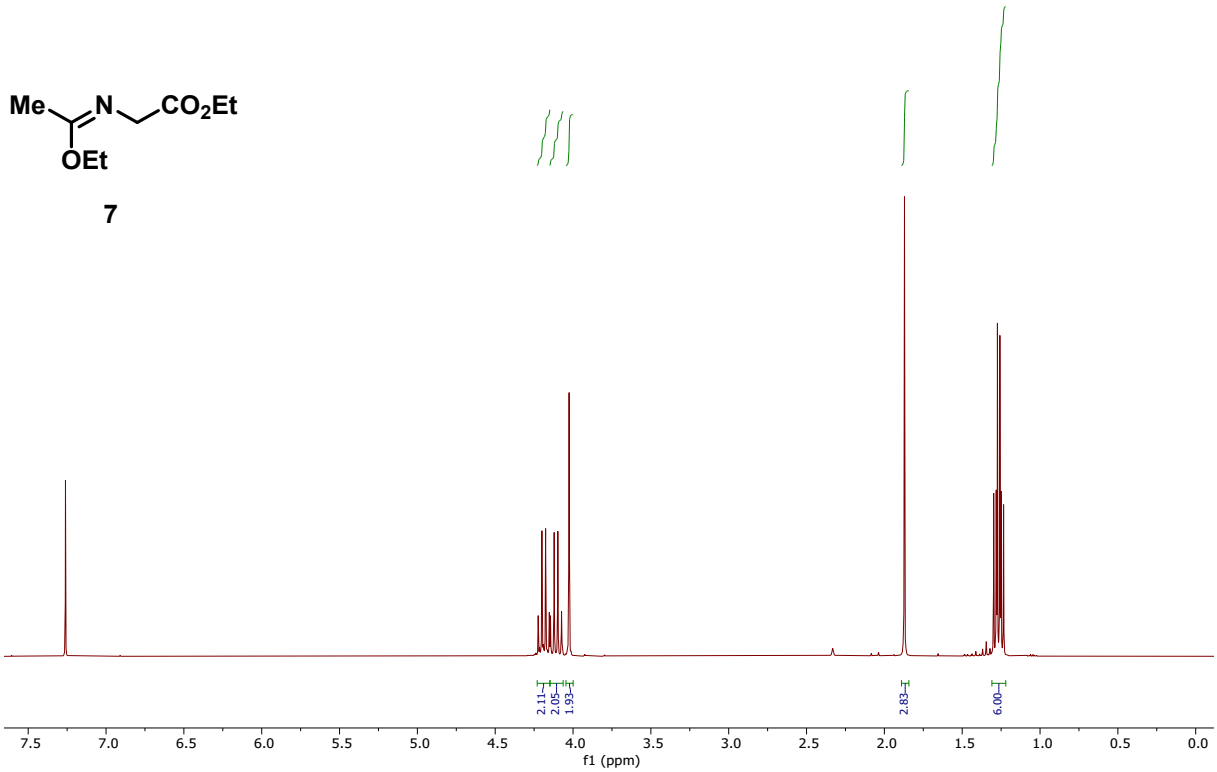
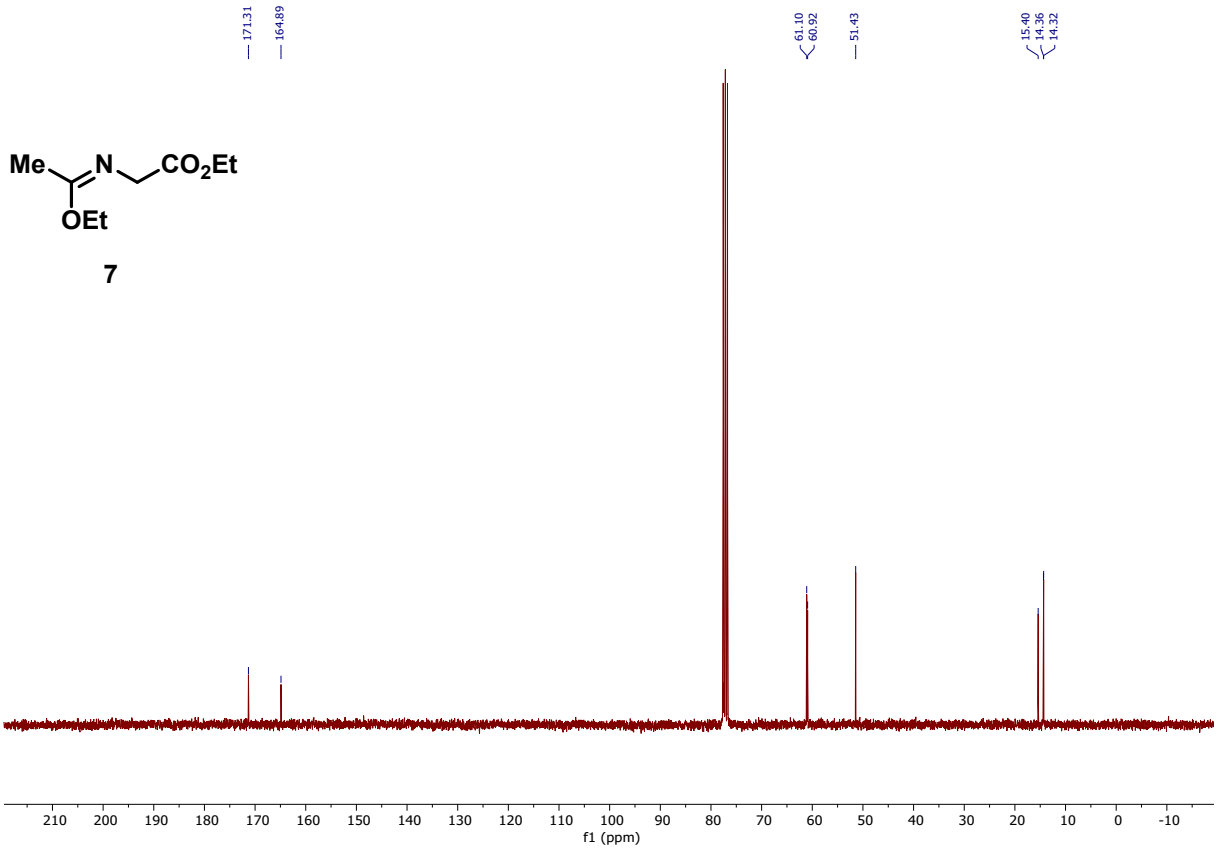
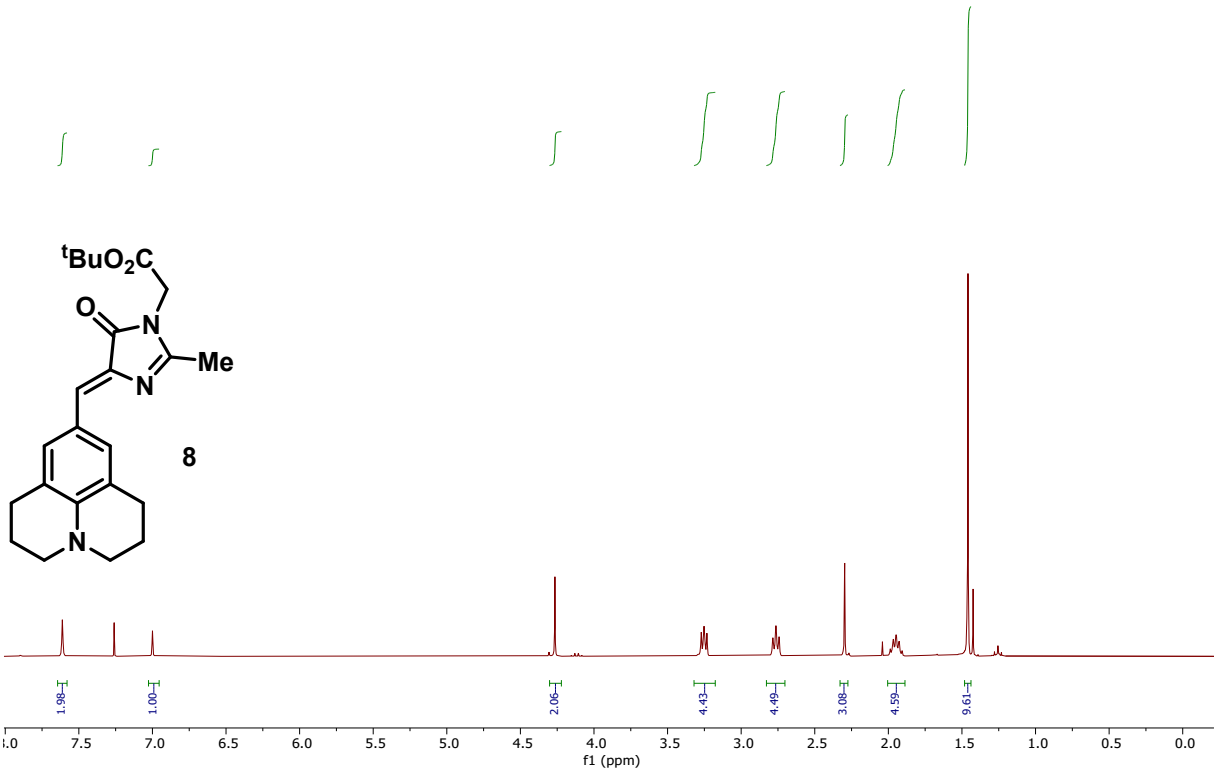

Red-Halo2-PEG
f1 (ppm)

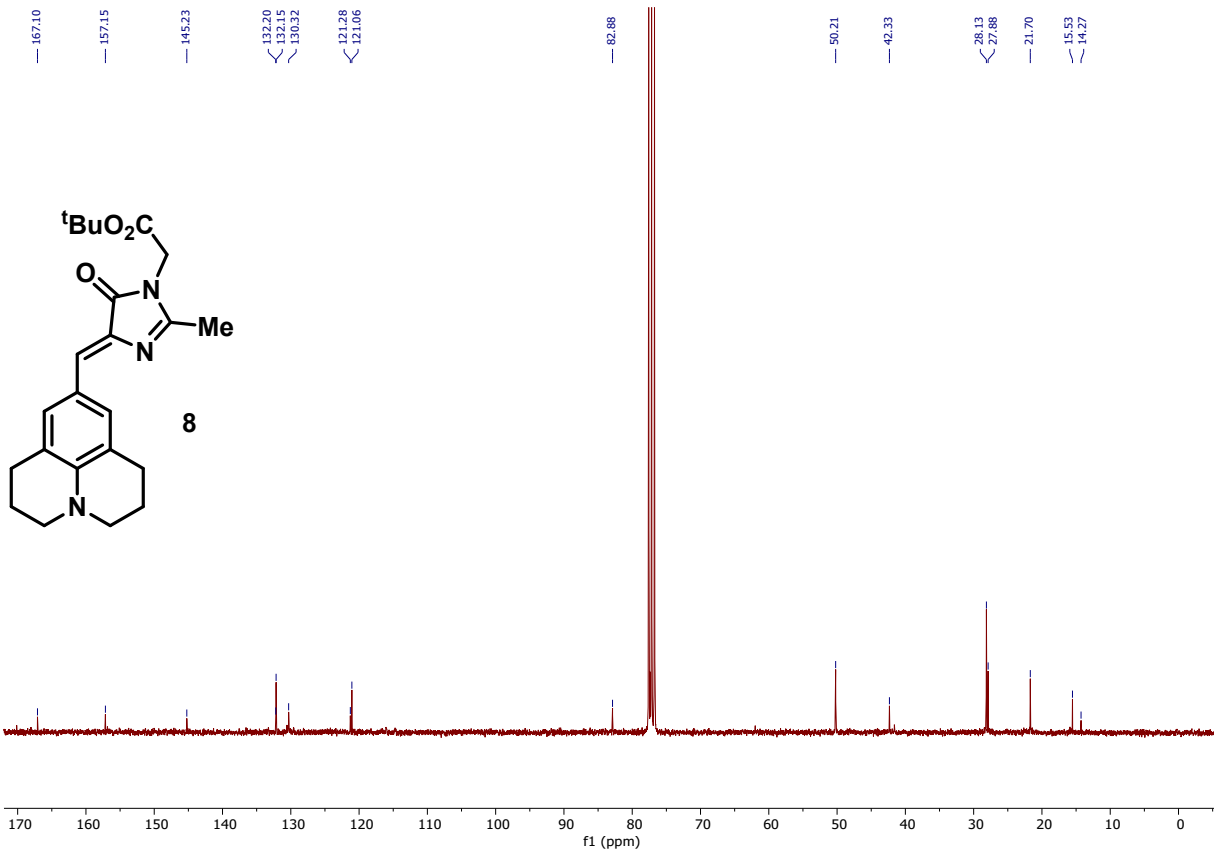

Me
N
CO2Et
OEt
2.11
2.05
1.93
2.83
6.00
7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
f1 (ppm)

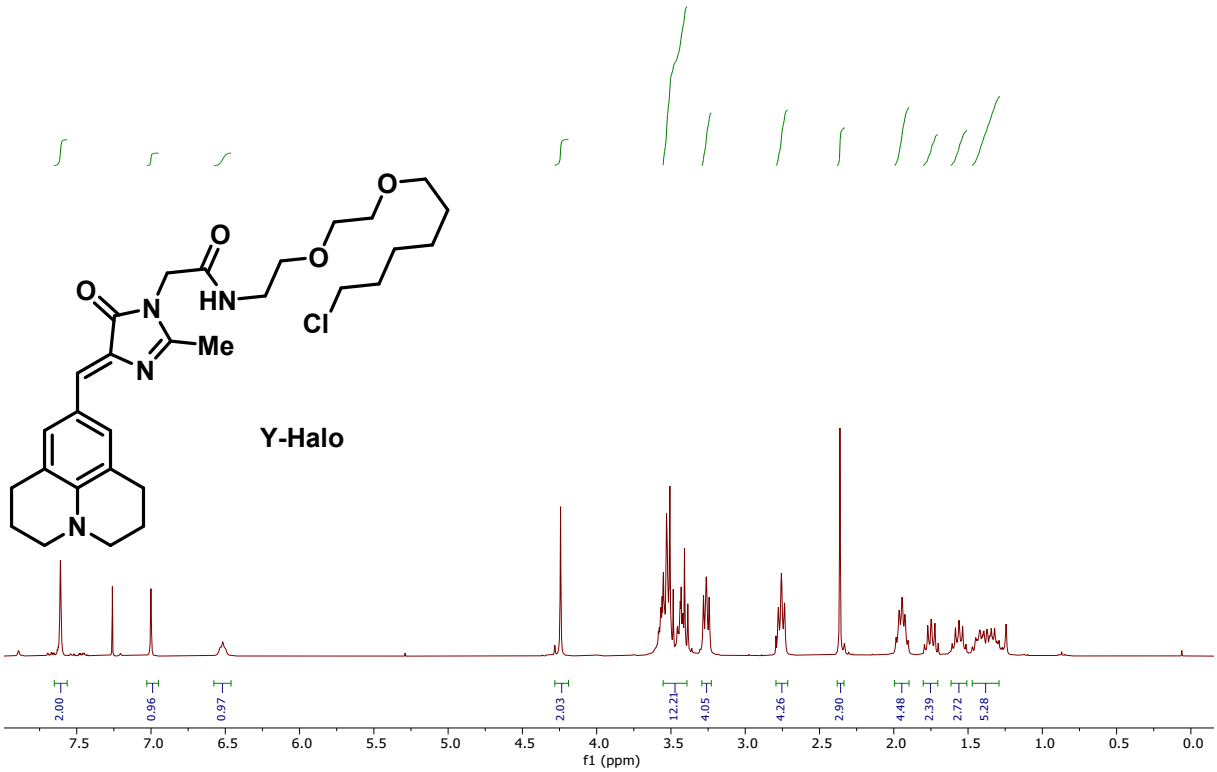
7

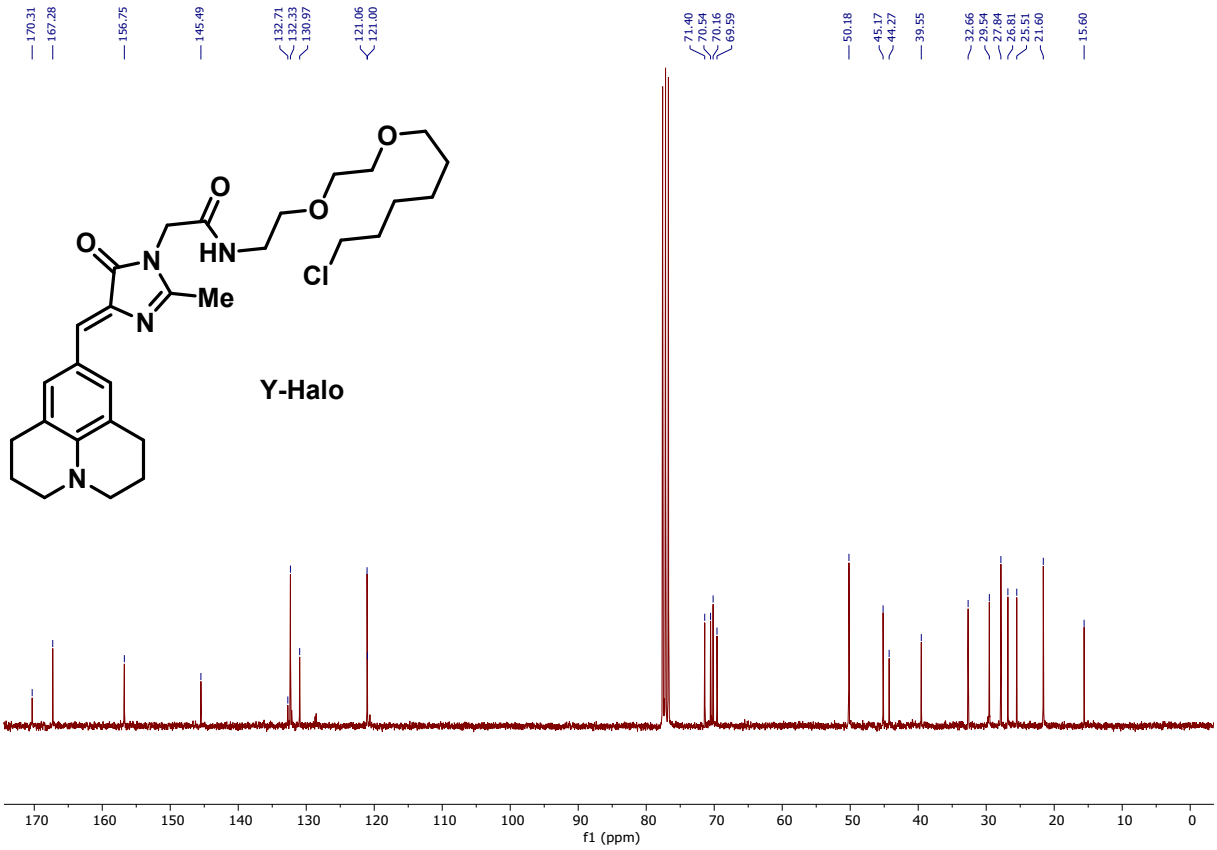

171.31
164.89
61.10
60.92
51.43
15.40
14.36
14.32
Me
N
CO2Et
OEt
210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10
f1 (ppm)

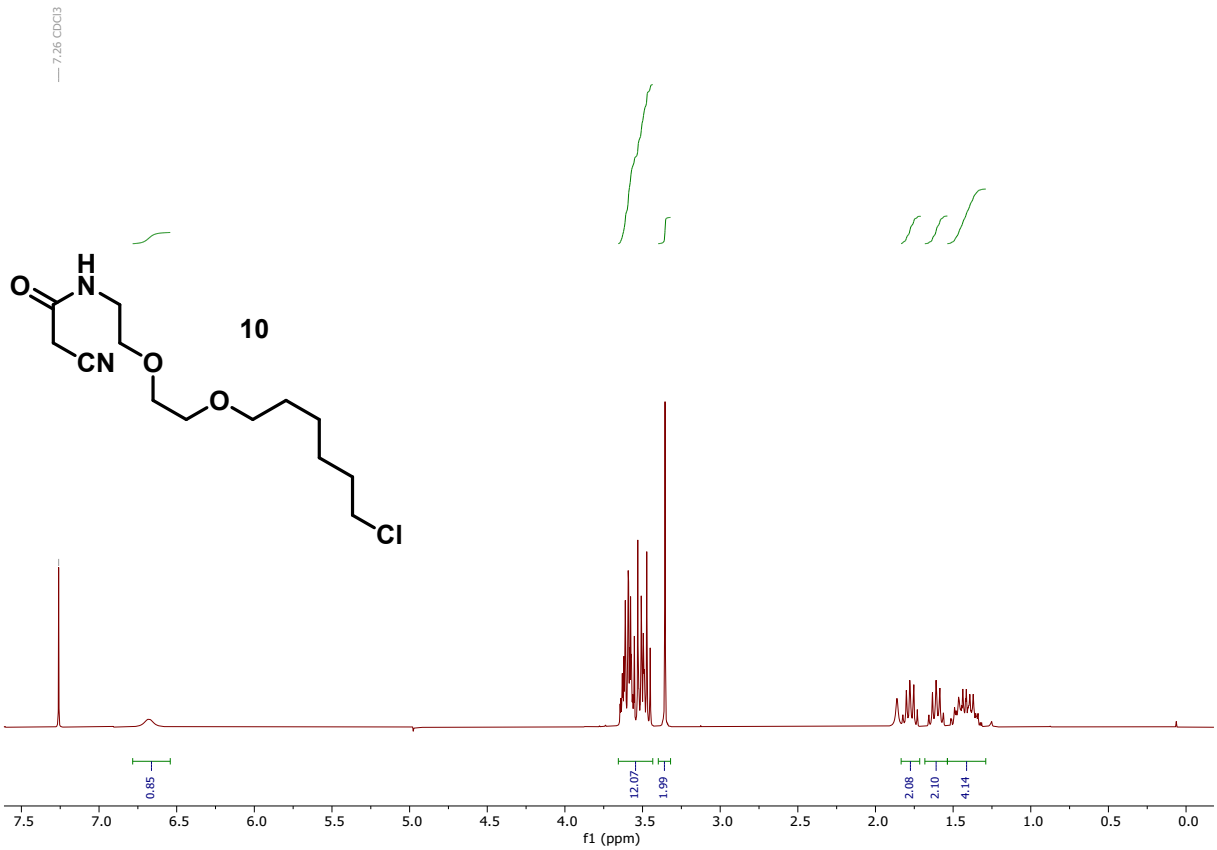
7

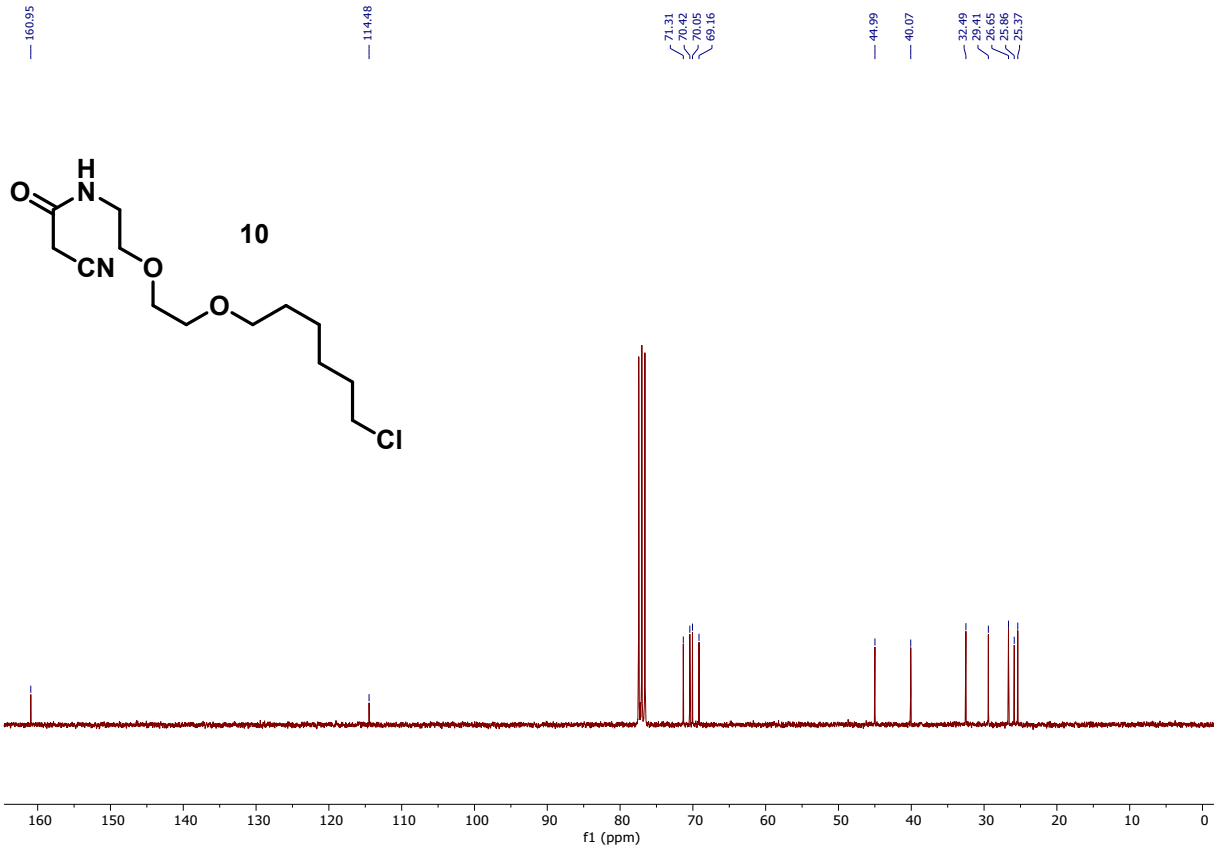

tBuO2C
O
N
Me
N
8
N
1.98
1.00
2.06
4.43
4.49
3.08
4.59
9.61
f1 (ppm)

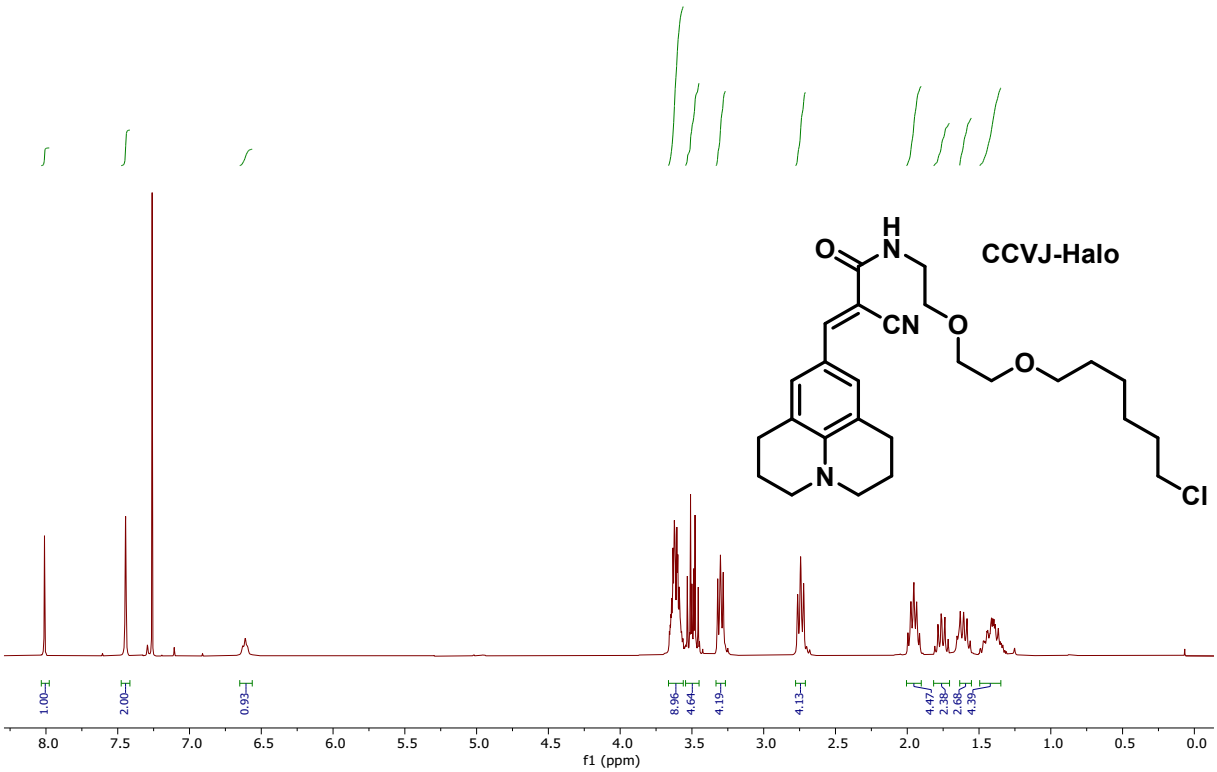

167.10
157.15
145.23
132.20
132.15
130.32
121.28
121.06
82.88
50.21
42.33
28.13
27.88
21.70
15.53
14.27
tBuO2C
O
N
Me
N
8
N
f1 (ppm)

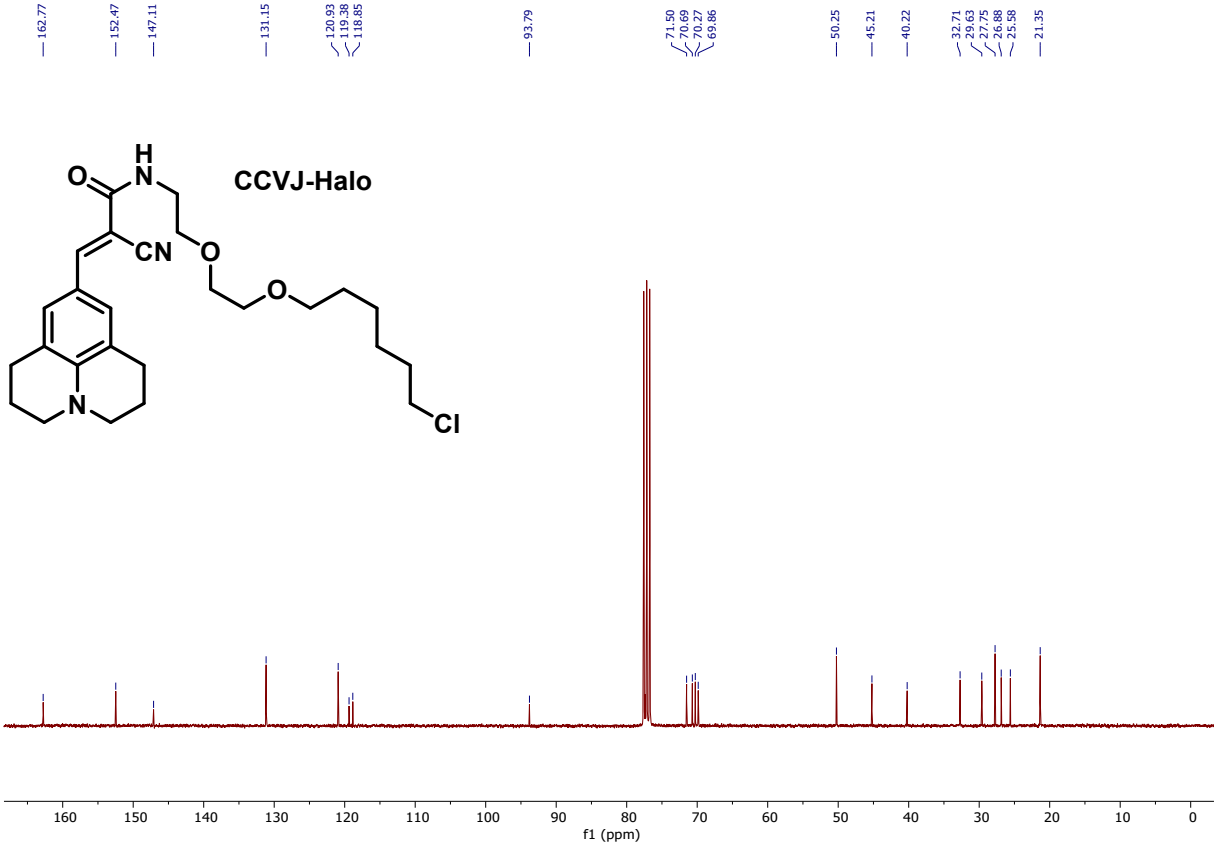

Y-Halo
2.00
0.96
0.97
2.03
12.21
4.05
4.26
2.90
4.48
2.39
2.72
5.28
f1 (ppm)

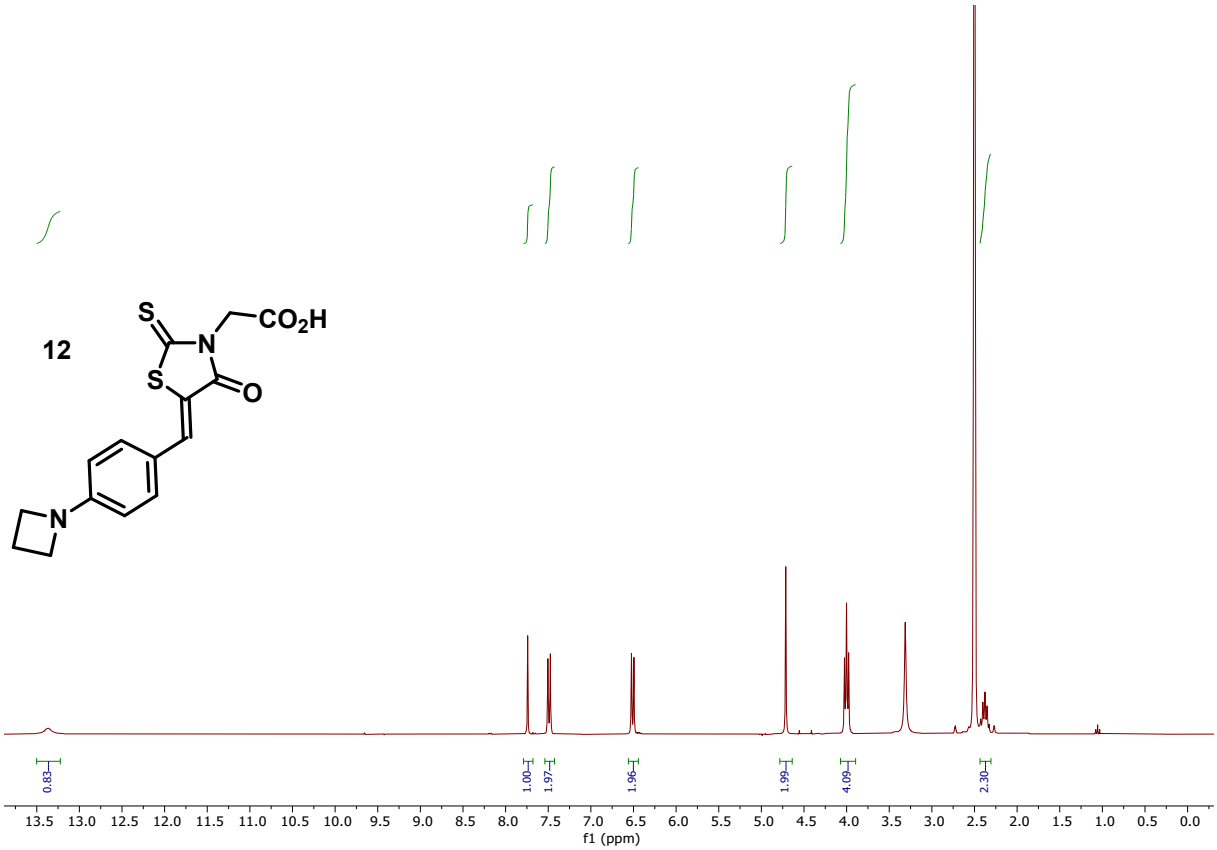

170.31
167.28
156.75
145.49
132.71
132.33
130.97
121.06
121.00
71.40
70.54
70.16
69.59
50.18
45.17
44.27
39.55
32.66
29.54
27.84
26.81
25.51
21.60
15.60
Y-Halo
f1 (ppm)

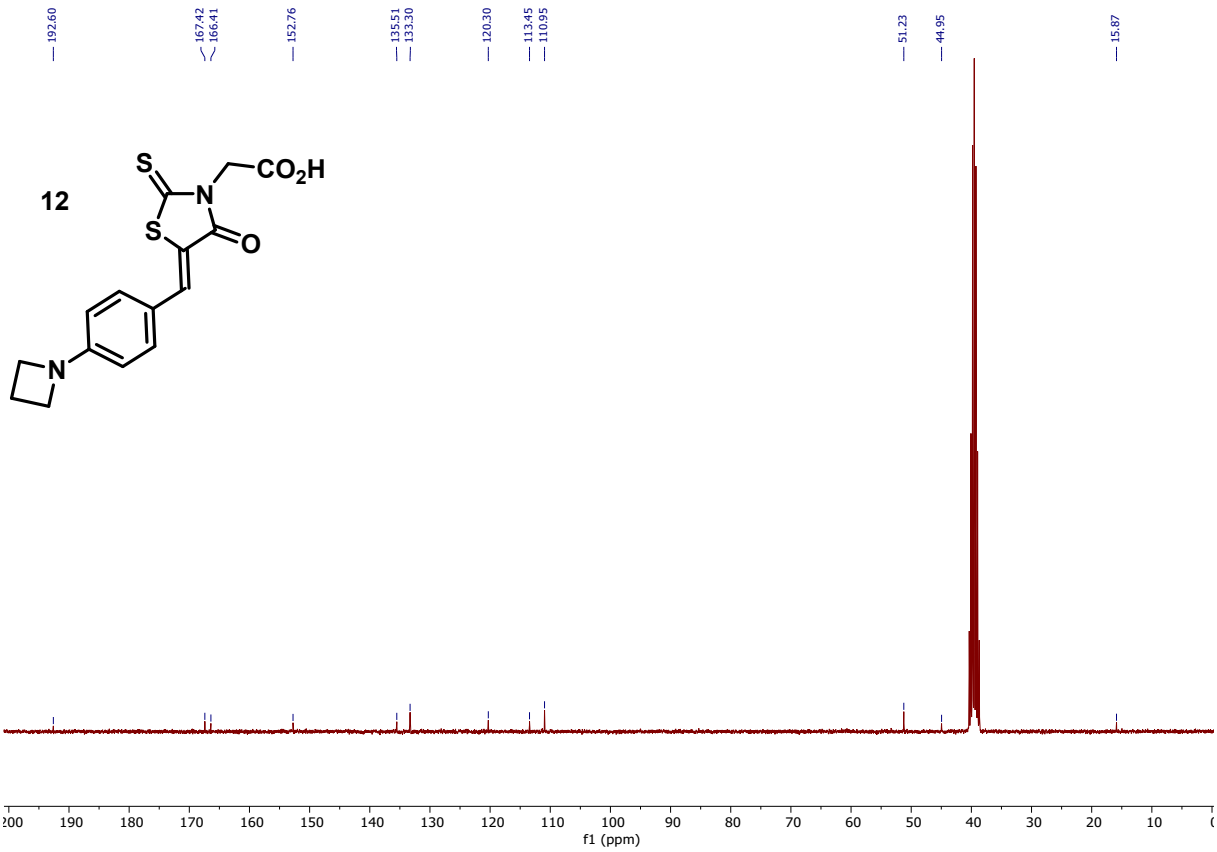

7.26 CDCl3
10
0.85
12.07
1.99
2.08
2.10
4.14
f1 (ppm)

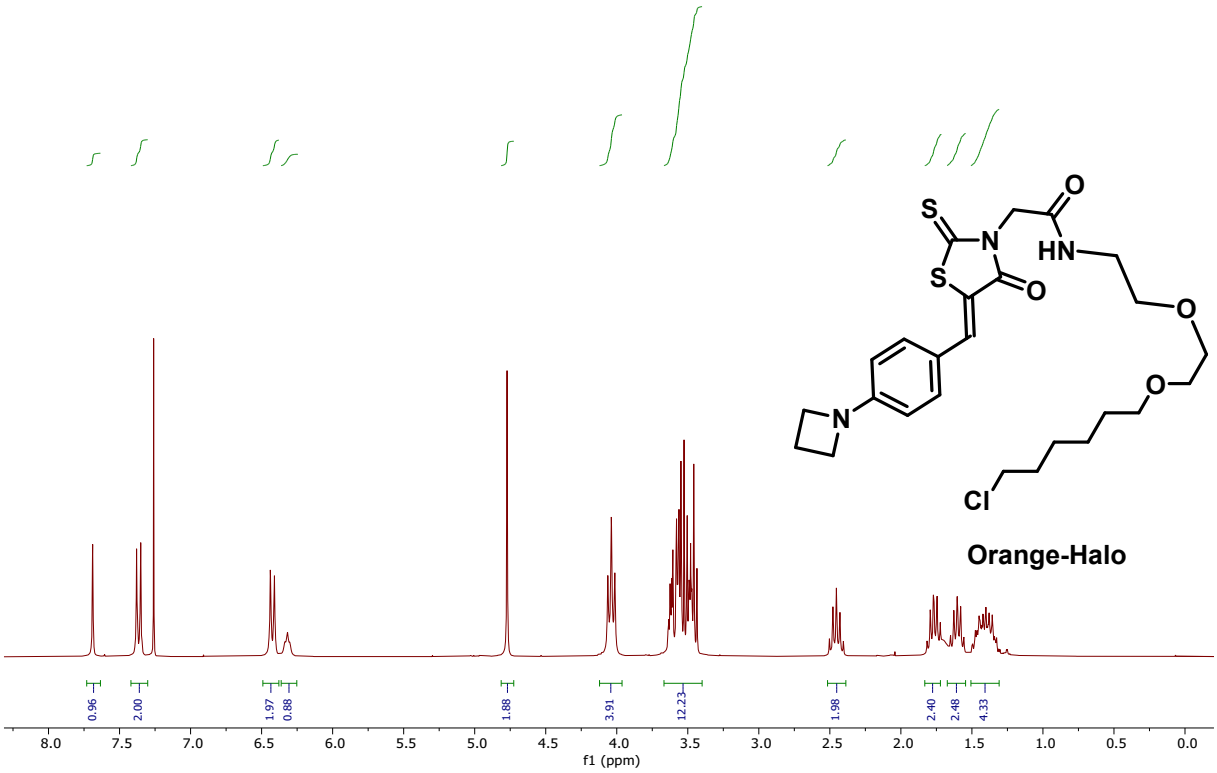

160.95
114.48
71.31
70.42
70.05
69.16
44.99
40.07
32.49
29.41
26.65
25.86
25.37
10
f1 (ppm)

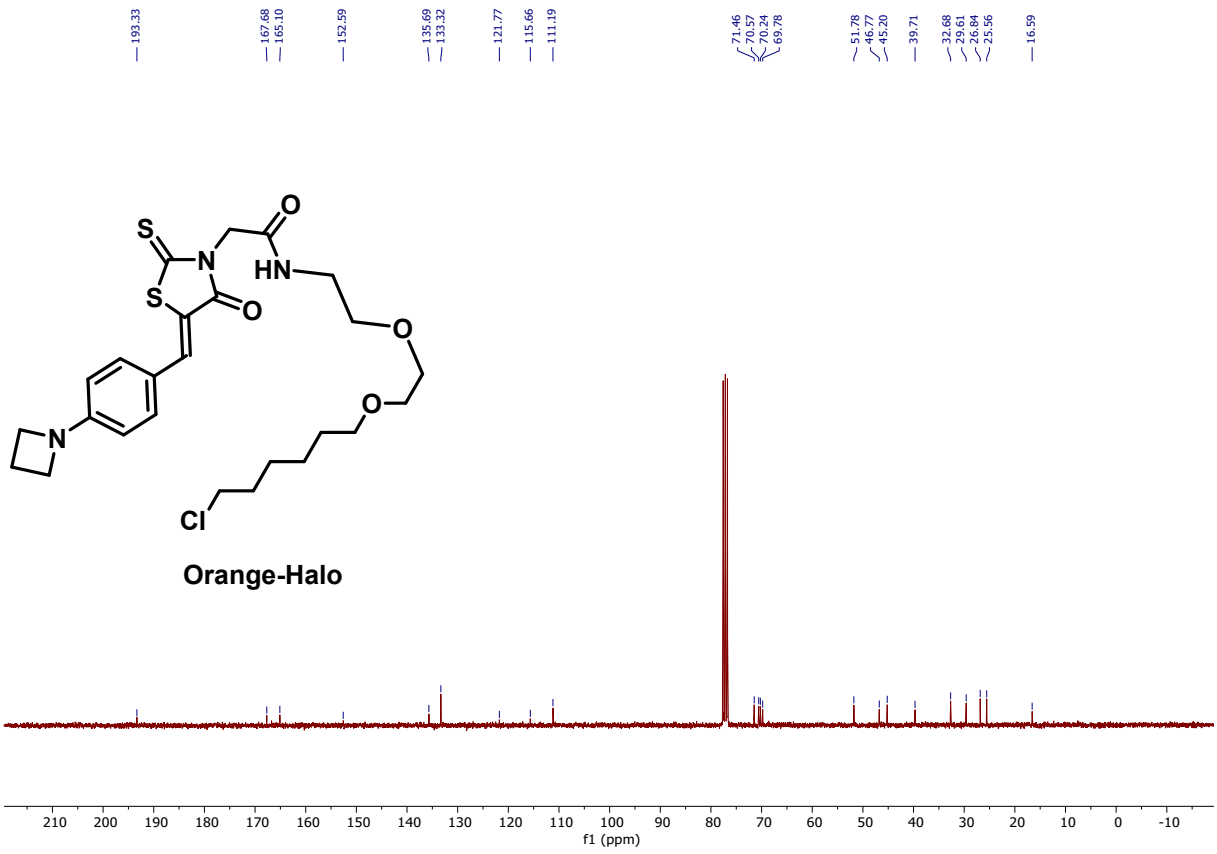

CCVJ-Halo
1.00
2.00
0.93
8.96
4.64
4.19
4.13
4.47
2.38
2.68
4.39
f1 (ppm)


162.77
152.47
147.11
131.15
120.93
119.38
118.85
93.79
71.50
70.69
70.27
69.86
50.25
45.21
40.22
32.71
29.63
27.75
26.88
25.58
21.35
CCVJ-Halo
f1 (ppm)


12
S
N
CO2H
S
O
N
0.83
1.00
1.97
1.96
1.99
4.09
2.30
13.5 13.0 12.5 12.0 11.5 11.0 10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
f1 (ppm)


192.60
167.42
166.41
152.76
135.51
133.30
120.30
113.45
110.95
51.23
44.95
15.87
12
S
N
CO2H
S
O
N
200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10
f1 (ppm)


Orange-Halo
0.96
2.00
1.97
0.88
1.88
3.91
12.23
1.98
2.40
2.48
4.33
f1 (ppm)


193.33
167.68
165.10
152.59
135.69
133.32
121.77
115.66
111.19
71.46
70.57
70.24
69.78
51.78
46.77
45.20
39.71
32.68
29.61
26.84
25.56
16.59
Orange-Halo
f1 (ppm)

VI. References

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