Angewandte Chemie
Eine Zeitschrift der Gesellschaft Deutscher Chemiker

Supporting Information

69451 Weinheim, Germany

Bioorthogonal Imaging of Aurora Kinase A in Live Cells**

*Katherine S. Yang, Ghyslain Budin, Thomas Reiner, Claudio Vinegoni, and Ralph Weissleder**

anie_201200994_sm_miscellaneous_information.pdf

Supporting Information

Table of Contents

General Experimental Procedures..... 1
Materials..... 1
Instrumentation..... 1
Chemical synthesis..... 2
Methods.....9
Supplemental figures.....12
Spectra..... 12

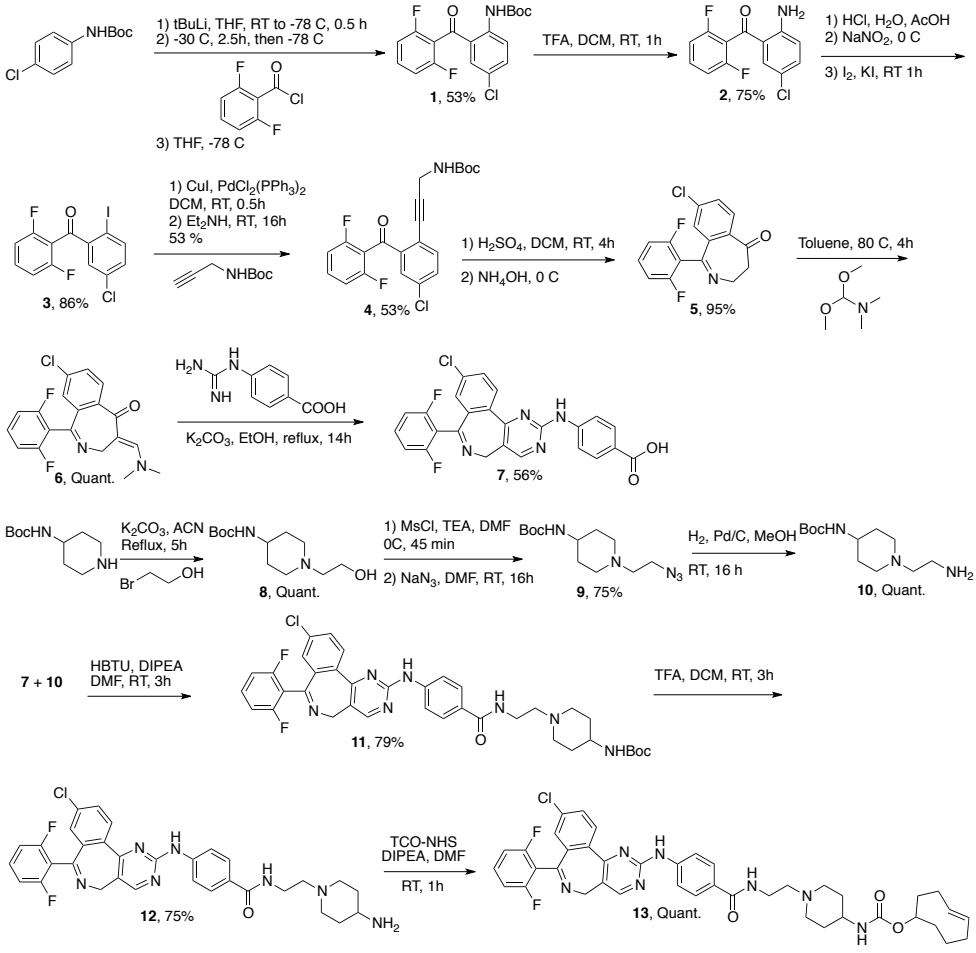
General experimental procedures. Unless otherwise noted, reactions were carried out under an atmosphere of nitrogen or argon in air-dried glassware with magnetic stirring. Air- and/or moisture-sensitive liquids were transferred *via* syringe. Organic solutions were concentrated by rotary evaporation at 25 - 60 °C at 15-30 torr. Analytical thin layer chromatography (TLC) were performed using plates cut from glass sheets (silica gel 60 F-254 from Silicycle). Visualization was achieved under a 254 or 365 nm UV light and by immersion in an ethanolic solution of cerium sulfate, followed by treatment with a heat gun. Column chromatography was carried out as "Flash Chromatography" using silica gel G-25 (40-63 μM).

Materials. All reagents were obtained from commercial sources and used without further purifications. Dry MeOH, DCM and DMF were obtained from Aldrich. Texas Red-Tz[1-3] and (*E*)-cyclooct-4-enyl 2,5-dioxopyrrolidin-1-yl carbonate[2] were synthesized as described earlier. Histidine-tagged recombinant human Aurora Kinase A, the Z´-LYTE Serine/Threonine-1 peptide assay kit, and Hoechst 33258 pentahydrate were purchased from Invitrogen. The anti-AKA antibody was from abcam (ab1287), the paraformaldehyde was from Electron Microscopy Sciences, and the Odyssey Blocking Buffer was purchased from Licor. DRAQ5 live cell DNA stain was purchased from Cell Signaling Technology.

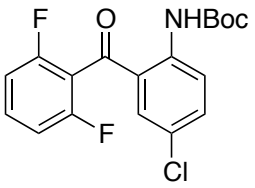
Instrumentation. ^{1}H and ^{13}C NMR spectra were recorded at 23°C on a Varian 400 MHz spectrometers. Recorded shifts are reported in parts per million (δ) and calibrated using residual undeuterated solvent. Data are represented as follows: Chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, m = multiplet, br = broad), coupling constant (*J*, Hz) and integration. LC-ESI-MS analysis and HPLC-purifications were performed on a Waters (Milford, MA) LC-MS system. For LC-ESI-MS analyses, a Waters XTerra® C18 5 μm column was used. For preparative runs, an Atlantis® Prep T3 OBDTM 5 μM column was used (eluents 0.1% TFA (v/ v) in water and MeCN; gradient: 0-1.5 min, 5-100% B; 1.5-2.0 min 100% B). High-resolution electrospray ionization (ESI) mass spectra were obtained on a Bruker Daltonics APEXIV 4.7 Tesla Fourier Transform mass spectrometer (FT-ICR-MS) in the Department of Chemistry Instrumentation Facility at the Massachusetts Institute of Technology. Melting points

(m.p.) were recorded on a Mel-Temp apparatus from Electrothermal and are uncorrected. IC_{50} assays and Fluorescein Diacetate-Tz activation assays were analyzed using a Tecan Safire^2 microplate system (Männedorf, Switzerland). Data were analyzed using Prism 5 for Mac (GraphPad, La Jolla, CA). Imaging was done at 40X, 1.6 zoom using a DeltaVision deconvolution microscope equipped with a live cell imaging chamber (Applied Precision Instruments). Cancer cell lines were screened and quantified for AKA expression levels using a cellWoRx screening system (Applied Precision Instruments) with a 10X objective.

Chemical Synthesis



tert-butyl (4-chloro-2-(2,6-difluorobenzoyl)phenyl)carbamate **1**



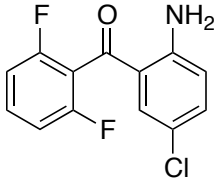
4-Chloro-N-Boc-aniline (3.0 g, 13.17 mmol) was dissolved in dry THF (36 mL) under argon and cooled to -78 ºC. t-BuLi (1.7 M in pentane, 17.82 mL, 30.3 mmol) was cooled in a dry ice/acetone bath and added to the Boc-aniline solution, via a cannula, over 20 min. The yellow solution was stirred at -78 ºC. for 30 min, warmed up to -30 ºC for an additional 2.5 h, and then cooled to -78 ºC. 2,6-Difluorobenzoyl chloride (2.56 g, 14.49 mmol) was dissolved in dry THF (27 mL) and cooled to -78 ºC. under argon. The *o*-lithiated aniline was added, via a cannula, to the acid chloride solution over 30 min. The solution was stirred for an additional 20 min before quenching with 1N HCl (45 mL). The solution was diluted with EtOAc and the organic portion was separated, dried over $MgSO_4$ and concentrated to dryness in vacuo. The resulting orange oil was purified by column chromatography (silica gel, Hexanes:EtOAc 100/0 to 98/2) to provide the Boc protected amino-benzophenone **1** (2.54 g, 53%) as a yellow oil.

^{1}H NMR (400 MHz, cdcl$_3$) δ 10.70 (s, 1H), 8.57 (dd, *J* = 9.2, 0.8 Hz, 1H), 7.57 – 7.41 (m, 2H), 7.38 (d, *J* = 1.1 Hz, 1H), 7.03 (dd, *J* = 11.6, 4.2 Hz, 2H), 1.54 (s, 9H).

^{13}C NMR (101 MHz, cdcl$_3$) δ 191.35, 160.56 (d, *J* = 7.1 Hz), 158.05 (d, *J* = 7.2 Hz), 152.81, 141.48, 136.08, 132.99, 132.51 (t, *J* = 9.8 Hz), 126.13, 122.42, 120.64, 116.82 (t, *J* = 21.5 Hz), 112.32, 112.07, 81.39, 28.33.

HRMS : $[M+Na]^+$ m/z calcd 390.0679 for $C_{18}H16ClF_2NO3$, found 390.0675

(2-amino-5-chlorophenyl)(2,6-difluorophenyl)methanone **2**



The N-Boc-aminobenzophenone **1** (2.54 g, 6.92 mmol) was dissolved in dry DCM (38 mL) and trifluoroacetic acid (38 mL) was added at 0 ºC. After stirring for 1 h at RT, volatiles were evaporated *in vacuo*. The resulting residue was dissolved in EtOAc (100 mL) and washed with a saturated aqueous solution of $NaHCO_3$ then with H_2O, dried over $MgSO_4$, and concentrated to dryness *in vacuo* to provide compound **2** (1.39 g, 75%) as a yellow powder.

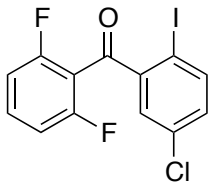
Mp : 95 °C

^{1}H NMR (400 MHz, cdcl$_3$) δ 7.42 (tt, *J* = 8.4, 6.4 Hz, 1H), 7.24 (dd, *J* = 8.8, 2.5 Hz, 1H), 7.20 (s, 1H), 7.04 – 6.94 (m, 2H), 6.66 (d, *J* = 8.8 Hz, 1H), 6.47 (s, 2H)

^{13}C NMR (101 MHz, cdcl$_3$) δ 189.71, 160.53 (d, *J* = 7.8 Hz), 158.03 (d, *J* = 7.7 Hz), 149.87, 135.82, 132.61, 131.72 (t, *J* = 9.8 Hz), 120.39, 118.74, 118.68, 112.14 (d, *J* = 3.9 Hz), 112.05 (d, *J* = 25.0 Hz), 111.96 (d, *J* = 3.9 Hz).

HRMS : $[M+H]^+$ m/z calcd 268.0335 for $C_{13}H8ClF_2NO$, found 268.0332

(5-chloro-2-iodophenyl)(2,6-difluorophenyl)methanone **3**



Compound **2** (800 mg, 3.0 mmol) was dissolved in glacial acetic acid (3.1 mL) and concentrated HCl (1.2 mL) and the solution was cooled to 0 ºC. A solution of $NaNO_2$ (228 mg, 3.3 mmol) in H_2O (1.9 mL) was added dropwise so as to maintain a temperature of between 0-5 ºC. Following this addition, the reaction mixture was stirred at 0 ºC for 30 min. Cold EtOAc (6.5 mL) was added dropwise and the solution was stirred for 20 min at 0 ºC. Iodine (457 mg, 1.8 mmol) and potassium iodide (598 mg, 3.6 mmol) in H_2O (3.2 mL) were added dropwise and the mixture was warmed to room temperature and stirred for 1 h. The reaction mixture was diluted with EtOAc and washed four times with saturated aqueous sodium thiosulfate. The combined aqueous portions were extracted three times with EtOAc. The combined organic portions were then washed three times with a saturated aqueous $NaHCO_3$ solution, twice with H_2O, dried over $MgSO_4$, filtered and evaporated in vacuo to afford **3** (978 mg, 86%) as an orange powder.

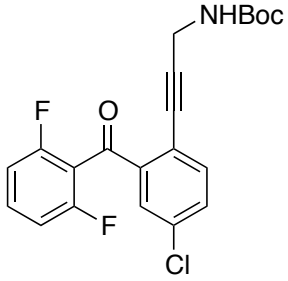
Mp : 92 °C

^{1}H NMR (400 MHz, cdcl$_3$) δ 7.88 (d, *J* = 8.4 Hz, 1H), 7.51 (tt, *J* = 8.5, 6.2 Hz, 1H), 7.43 (d, *J* = 2.5 Hz, 1H), 7.18 (dd, *J* = 8.4, 2.5 Hz, 1H), 7.03 – 6.94 (m, 2H)

^{13}C NMR (101 MHz, cdcl$_3$) δ 189.01, 162.15 (d, *J* = 6.2 Hz), 159.60 (d, *J* = 6.2 Hz), 144.25, 142.23, 134.96, 134.07 (t, *J* = 10.5 Hz), 132.98, 130.51, 112.50 (d, *J* = 2.9 Hz), 112.41 (d, *J* = 25.3 Hz), 112.32 (d, *J* = 2.9 Hz), 89.42.

HRMS : $[M+Na]^+$ m/z calcd 400.9012 for $C_{37}H38ClF_2N_7O3$, found 400.9013

tert-butyl (3-(4-chloro-2-(2,6-difluorobenzoyl)phenyl)prop-2-yn-1-yl)carbamate **4**



Compound **3** (1.33 g, 3.53 mmol), prop-2-ynyl-carbamic acid tert-butyl ester (656 mg, 4.23 mmol) which was prepared following described procedure,[4] $PdCl_2(PPh_3)_2$ (147 mg, 0.21 mmol) and Cu(I)I (41 mg, 0.21 mmol) were suspended in anhydrous DCM (11.8 mL) and the mixture was degassed with nitrogen for 30 min. Diethylamine (0.6 mL) was added and the solution was stirred at room temperature overnight. The solution was concentrated in vacuo and the resulting residue purified by column chromatography (0 to 5% EtOAc/hexanes). Desired compound was recrystalized in Et_2O to afford **4** (757 mg, 53%) as a white powder.

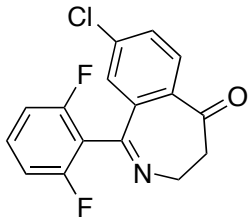
Mp : 103 °C

^{1}H NMR (400 MHz, cdcl$_3$) δ 7.70 (d, *J* = 2.1 Hz, 1H), 7.53 – 7.38 (m, 2H), 6.99 (m, 1H), 4.40 (s, 1H), 3.86 (d, *J* = 5.4 Hz, 1H), 1.46 (s, 5H).

^{13}C NMR (101 MHz, cdcl$_3$) δ 187.93, 161.93 (d, *J* = 6.6 Hz), 159.40 (d, *J* = 6.9 Hz), 155.11, 141.48, 135.25, 134.97, 133.15 (t, *J* = 10.3 Hz), 132.46, 129.61, 120.59, 112.19 (d, *J* = 5.3 Hz), 111.99 (d, *J* = 5.5 Hz), 93.03, 80.27, 79.66, 31.10, 28.50.

HRMS : $[M+Na]^+$ m/z calcd 428.0835 for $C_{21}H18ClF_2NO3$, found 428.0838

8-chloro-1-(2,6-difluorophenyl)-3*H*-benzo[*c*]azepin-5(4*H*)-one **5**



A solution of **4** (364 mg, 0.9 mmol) in DCM (1.3 mL) was added dropwise to a 0.7 mL of concentrated sulfuric acid which was cooled to 0 ºC. The mixture was stirred at 0 ºC for 4 h, poured over crushed ice, and neutralized with ammonium hydroxide. The organic portion was separated and the aqueous portion was extracted with DCM. The combined organic portions were washed three times with H_2O, dried over $MgSO_4$, filtered and evaporated in vacuo. The resulting residue was purified by column chromatography (10 to 20% EtOAc/hexanes) to provide **5** (261 mg, 95%) as a white powder.

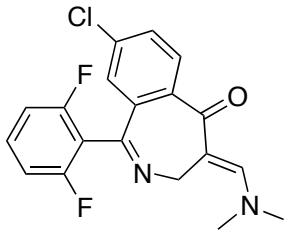
Mp : 141 °C

^{1}H NMR (400 MHz, cdcl$_3$) δ 7.88 (d, *J* = 8.4 Hz, 1H), 7.53 (dd, *J* = 8.4, 1.6 Hz, 1H), 7.43 – 7.34 (m, 1H), 7.14 (d, *J* = 1.4 Hz, 1H), 6.97 (t, *J* = 8.1 Hz, 2H), 4.05 – 3.99 (m, 2H), 3.12 – 3.05 (m, 2H)

^{13}C NMR (101 MHz, cdcl$_3$) δ 200.73 (s), 160.90 (s), 159.18 (d, *J* = 6.5 Hz), 139.62 (s), 135.27 (s), 134.53 (s), 131.49 (t, *J* = 10.5 Hz), 131.29 (s), 130.97 (s), 128.77 (s), 118.10 (s), 112.28 (s), 112.15 (d, *J* = 17.7 Hz), 112.03 (s), 47.13 (s), 45.89 (s).

HRMS : $[M+H]^+$ m/z calcd 306.0492 for $C_{16}H10ClF_2NO$, found 306.0494

(*E*)-8-chloro-1-(2,6-difluorophenyl)-4-((dimethylamino)methylene)-3*H*-benzo[*c*]azepin-5(4*H*)-one **6**



Compound **5** (35 mg, 0.11 mmol) was dissolved in toluene (733 µL) and N,N-dimethylformamide dimethyl acetal (145 µL) and heated at 80 ºC for 4 h. The solution was evaporated in vacuo and the resulting residue was purified by column chromatography (30 to 75% EtOAc/ hexanes) to afford **6** (40 mg, Quant.) as a yellow powder.

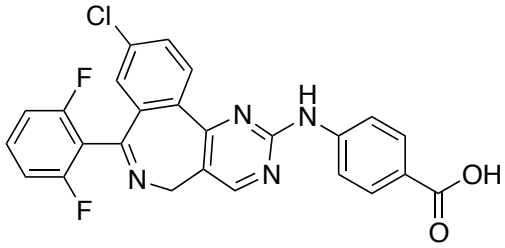
Mp : 231 °C

^{1}H NMR (400 MHz, cdcl$_3$) δ 8.03 (d, *J* = 8.4 Hz, 1H), 7.79 (s, 1H), 7.48 (dd, *J* = 8.4, 2.0 Hz, 1H), 7.34 (ddd, *J* = 14.8, 8.4, 6.4 Hz, 1H), 7.08 (d, *J* = 1.6 Hz, 1H), 6.93 (t, *J* = 8.4 Hz, 2H), 4.95 (d, *J* = 12.5 Hz, 1H), 3.71 (d, *J* = 12.4 Hz, 1H), 3.27 (s, 6H).

^{13}C NMR (101 MHz, cdcl$_3$) δ 189.66, 161.72 (d, *J* = 6.9 Hz), 159.23 (d, *J* = 6.8 Hz), 158.69, 151.39, 139.50, 136.48, 135.02, 131.15, 130.91 (t, *J* = 10.1 Hz), 130.52, 127.63, 118.19, 112.06 (d, *J* = 3.6 Hz), 111.85 (d, *J* = 3.5 Hz), 106.69, 48.44, 44.32.

HRMS : $[M+H]^+$ m/z calcd 361.0914 for $C_{19}H15ClF_2N_2O$, found 361.0905

4-((9-chloro-7-(2,6-difluorophenyl)-5*H*-benzo[*c*]pyrimido[4,5-*e*]azepin-2-yl)amino)benzoic acid **7**



Compound **6** (161 mg, 0.45 mmol), 4-guanidino-benzoic acid hydrochloride (125 mg, 0.58 mmol) and K_2CO_3 (142 mg, 1.03 mmol) in EtOH (3 mL) were refluxed for 14 h. The mixture was cooled and then poured into H_2O (30 mL) and acidified with a 1N HCl solution to pH=3. The aqueous layer was extracted twice with EtOAc and the combined organic portions were washed twice with H_2O, dried over MgSO4 and concentrated to dryness in vacuo. The residue was suspended in DCM and filtered affording **7** as a light pink powder (121 mg, 56%).

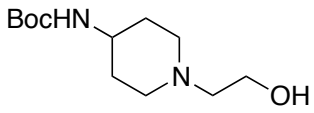
Mp : 283 °C

^{1}H NMR (400 MHz, dmso) δ 10.28 (s, 1H), 8.73 (s, 1H), 8.30 (d, *J* = 8.5 Hz, 1H), 7.95 (d, *J* = 8.9 Hz, 1H), 7.90 (d, *J* = 8.9 Hz, 1H), 7.86 (dd, *J* = 8.5, 2.0 Hz, 1H), 7.60 – 7.47 (m, 1H), 7.33 (d, *J* = 1.9 Hz, 1H), 7.16 (t, *J* = 8.3 Hz, 1H), 4.94 (s, 1H), 3.90 (s, 2H).

^{13}C NMR (101 MHz, dmso) δ 165.30, 159.00, 158.96, 157.60, 156.53 (d, *J* = 6.7 Hz), 156.19, 155.47, 142.87, 135.35, 133.64, 133.53, 130.79 – 130.38 (m), 129.87, 129.54, 128.96, 126.41, 122.20, 122.11, 116.55, 116.34, 111.17, 110.95, 49.51.

HRMS : $[M+H]^+$ m/z calcd 477.0924 for $C_{25}H15ClF_2N_4O2$, found 477.0916

tert-butyl (1-(2-hydroxyethyl)piperidin-4-yl)carbamate **8**



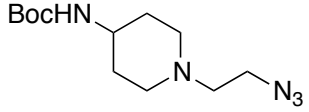
tert-butyl 4-piperidinylcarbamate (600 mg, 2.99 mmol), K_2CO_3 (3.32 g, 24 mmol) and bromoethanol (255 µL, 3.6 mmol) were suspended in ACN (12 mL) and refluxed for 5 h. The reaction mixture was cooled to room temperature and diluted with water. The aqueous layer was extracted three times with DCM. Organics were combined, dried over $MgSO_4$ and evaporated in vacuo to afford **8** (788 mg, Quant.) as a colorless oil.

^{1}H NMR (400 MHz, cdcl$_3$) δ 8.46 (s, 1H), 4.65 (d, *J* = 7.5 Hz, 1H), 3.88 – 3.81 (m, 2H), 3.62 (s, 1H), 3.40 (d, *J* = 8.7 Hz, 2H), 2.96 – 2.85 (m, 2H), 2.62 (t, *J* = 11.3 Hz, 2H), 2.07 (d, *J* = 12.0 Hz, 2H), 1.82 (dd, *J* = 22.3, 10.7 Hz, 2H), 1.43 (s, 9H).

^{13}C NMR (101 MHz, cdcl$_3$) δ 155.51, 79.37, 58.73, 56.18, 51.65, 45.40, 29.20, 28.37.

HRMS : $[M+H]^+$ m/z calcd 245.1860 for $C_{12}H24N_2O3$, found 245.1857

tert-butyl (1-(2-azidoethyl)piperidin-4-yl)carbamate **9**



Compound **8** (50 mg, 0.20 mmol) and TEA (57 µL, 0.41 mmol) were dissolved in 0.7 mL of THF at 0 ºC. Methanesulfonyl chloride (18 µL, 0.23 mmol) was added and the solution was stirred for 45 minutes at 0 ºC. TLC (DCM/MeOH 90/10) showed clean conversion to a new

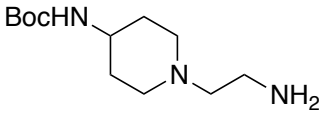
product spot. Then, 1.3 mL of DMF and sodium azide (16 mg, 0.24 mmol) were added, and the solution was stirred overnight at room temperature. The reaction was quenched with water, and extracted three times with EtOAc. The combined organic portions were washed twice with water, once with a saturated solution of NaCl, dried over $MgSO_4$ and evaporated in vacuo. The resulting residue was purified by column chromatography (2 % MeOH/DCM) to afford **9** (40 mg, 75%) as a light yellow oil.

^{1}H NMR (400 MHz, cdcl$_3$) δ 4.84 (s, 1H), 3.45 (t, *J* = 5.9 Hz, 2H), 3.45 (s, 1H), 3.05 (d, *J* = 9.8 Hz, 2H), 2.71 (t, *J* = 6.0 Hz, 2H), 2.36 (t, *J* = 11.1 Hz, 2H), 1.91 (d, *J* = 11.5 Hz, 2H), 1.56 (td, *J* = 14.1, 3.4 Hz, 2H), 1.35 (s, 9H).

^{13}C NMR (101 MHz, cdcl$_3$) δ 155.37, 79.45, 57.33, 52.67, 48.59, 47.84, 32.73, 28.61.

HRMS : $[M+H]^+$ m/z calcd 270.1925 for $C_{12}H23N_5O_2$, found 270.1926

tert-butyl (1-(2-aminoethyl)piperidin-4-yl)carbamate **10**



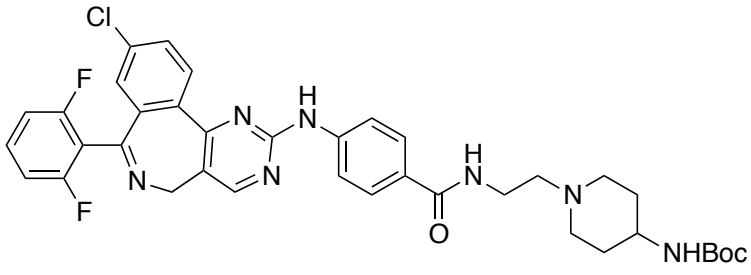
Compound **9** (40 mg, 0.15 mmol) was dissolved in MeOH. Pd/C 10% (8 mg) was added and the mixture was degassed under vacuum and stirred under H_2 overnight. Pd/C was then filtrated through Cellite and the filtrate was evaporated in vacuo affording compound **10** (34 mg, 94%) as a colorless sticky solid.

^{1}H NMR (400 MHz, cd$_3$od) δ 8.47 (s, 2H), 3.53 – 3.43 (m, 1H), 3.07 – 2.96 (m, 2H), 2.87 (d, *J* = 12.1 Hz, 2H), 2.59 (t, *J* = 6.0 Hz, 2H), 2.18 (t, *J* = 11.3 Hz, 2H), 1.85 (d, *J* = 11.2 Hz, 2H), 1.56 – 1.44 (m, 2H), 1.42 (s, 9H).

^{13}C NMR (101 MHz, cd$_3$od) δ 162.66, 79.51, 55.55, 53.33, 37.80, 33.16, 29.08, 29.05.

HRMS : $[M+H]^+$ m/z calcd 244.2020 for $C_{12}H25N_3O2$, found 244.2017

tert-butyl (1-(2-(4-((9-chloro-7-(2,6-difluorophenyl)-5*H*-benzo[*c*]pyrimido[4,5-*e*]azepin-2-yl) amino)benzamido)ethyl)piperidin-4-yl)carbamate **11**



Compound **7** (30 mg, 0.063 mmol), DIPEA (22 µL, 0.12 mmol) and HBTU (26 mg, 0.069 mmol) were dissolved in 0.9 mL of DMF and stirred at room temperature for 0.5 h. Compound **10** (17 mg, 0.069 mmol) was then added and the reaction was stirred at room temperature for 3 h. The reaction mixture was purified on HPLC affording **11** (35 mg, 79%) as a light yellow powder.

Mp : 192 °C

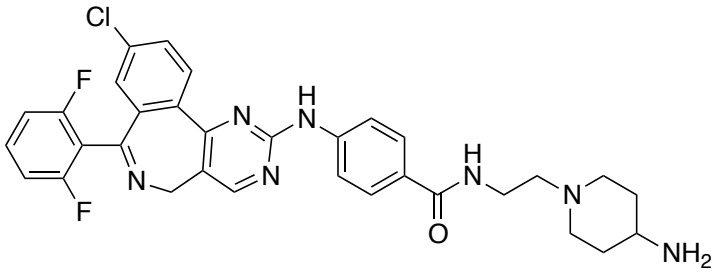
^{1}H NMR (400 MHz, cd$_3$od) δ 8.59 (s, 1H), 8.41 (s, 1H), 8.33 (d, *J* = 8.5 Hz, 1H), 7.93 (d, *J* = 8.5 Hz, 2H), 7.85 (d, *J* = 8.5 Hz, 2H), 7.74 (dd, *J* = 8.5, 1.6 Hz, 1H), 7.51 (dt, *J* = 14.8, 7.5 Hz, 1H), 7.30 (d, *J* = 1.6 Hz, 1H), 7.07 (t, *J* = 7.1 Hz, 2H), 3.97 (brs, 2H), 3.80 – 3.69 (m, 2H), 3.67 –

3.49 (m, 3H), 3.28 – 3.20 (m, 2H), 3.13 – 2.98 (m, 2H), 2.10 (d, *J* = 11.9 Hz, 2H), 1.77 (dd, *J* = 21.2, 10.1 Hz, 2H), 1.44 (s, 9H).

^{13}C NMR (101 MHz, cd$_3$od) δ 170.69, 162.67, 161.55, 161.27, 160.30 (d, *J* = 6.2 Hz), 158.20, 145.43, 138.07, 137.29, 136.93, 133.33, 132.39, 132.24, 129.57, 129.42, 127.24, 124.37, 119.14, 113.15 (d, *J* = 3.7 Hz), 112.92 (d, *J* = 4.8 Hz), 106.18, 80.34, 57.88, 52.74, 51.00, 36.38, 30.47, 28.75, 28.71.

HRMS : $[M+H]^+$ m/z calcd 702.2765 for $C_{37}H38ClF_2N_7O3$, found 702.2765

N-(2-(4-aminopiperidin-1-yl)ethyl)-4-((9-chloro-7-(2,6-difluorophenyl)-5*H*-benzo[*c*]pyrimido [4,5-*e*]azepin-2-yl)amino)benzamide **12**

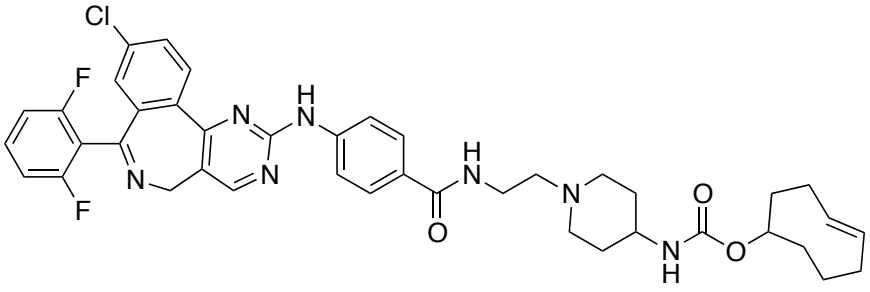


Compound **11** (7 mg, 0.01 mmol) was dissolved in 0.25 mL of DCM and trifluoroacetic acid (0.15 mL) was added at 0 ºC. The solution was stirred at room temperature for 3 h then evaporated in vacuo. The resulting residue was purified by HPLC affording **12** (4.5 mg, 75%) as a light yellow solid.

^{1}H NMR (400 MHz, cd$_3$od) δ 8.59 (s, 1H), 8.34 (d, *J* = 8.5 Hz, 1H), 8.31 (s, 1H), 7.92 (d, *J* = 8.3 Hz, 2H), 7.83 (d, *J* = 8.4 Hz, 2H), 7.74 (d, *J* = 8.4 Hz, 1H), 7.51 (dt, *J* = 14.5, 7.3 Hz, 1H), 7.31 (s, 1H), 7.12 – 7.00 (m, 2H), 5.02 – 4.95 (m, 2H), 3.97 (s, 2H), 3.71 – 3.61 (m, 2H), 3.46 (d, *J* = 11.4 Hz, 2H), 3.37 – 3.32 (m, 1H), 3.06 – 2.95 (m, 2H), 2.67 (t, *J* = 11.3 Hz, 2H), 2.14 (d, *J* = 10.9 Hz, 2H), 1.91 – 1.75 (m, 2H).

HRMS : $[M+H]^+$ m/z calcd 602.2241 for $C_{32}H30ClF_2N_7O$, found 602.2212

(*E*)-cyclooct-4-en-1-yl (1-(2-(4-((9-chloro-7-(2,6-difluorophenyl)-5*H*-benzo[*c*]pyrimido[4,5-*e*] azepin-2-yl)amino)benzamido)ethyl)piperidin-4-yl)carbamate **13**



Compound **12** (4.5 mg, 7.5 µmol), TCO-NHS (1 mg, 3.7 µmol) and DIPEA (1.3 µL, 7.5 µmol) were dissolved in 550 µL DMF and stirred for 1 h at room temperature. The mixture was purified by HPLC affording **13** (2.8 mg, Quant.) as a colorless solid.

^{1}H NMR (400 MHz, cd$_3$od) δ 8.62 (s, 1H), 8.45 (s, 1H), 8.37 (d, *J* = 8.5 Hz, 1H), 7.94 (d, *J* = 8.5 Hz, 2H), 7.85 (d, *J* = 8.3 Hz, 2H), 7.77 (dd, *J* = 8.5, 1.9 Hz, 1H), 7.52 (ddd, *J* = 15.0, 8.4, 6.9 Hz, 1H), 7.32 (d, *J* = 1.7 Hz, 1H), 7.08 (t, *J* = 8.3 Hz, 2H), 5.67 – 5.55 (m, 1H), 5.53 – 5.42 (m,

1H), 4.41 – 4.26 (m, 2H), 4.01 (s, 2H), 3.72 – 3.62 (m, 2H), 3.61 – 3.50 (m, 1H), 3.05 – 2.96 (m, 2H), 2.82 – 2.68 (m, 2H), 2.40 – 2.27 (m, 3H), 2.07 – 1.83 (m, 6H), 1.79 – 1.51 (m, 6H).
HRMS : $[M+H]^+$ m/z calcd 754.3078 for $C_{41}H42ClF_2N_7O3$, found 754.3087

General procedure for the synthesis of the tetrazine fluorophore conjugates.
To a solution of 3-(4-benzylamino)-1,2,4,5-tetrazine (10 μmol) in anhydrous DMF (0.5 mL) was added the succinimidyl ester of the appropriate fluorophore (2.5 μmol) and triethylamine (10 μmol). The resulting solution was allowed to shake overnight in the dark. The crude reaction mixture was then purified by preparative reverse phase HPLC. The identity and purity of the conjugates were confirmed by electrospray mass spectrometry and analytical HPLC, respectively.

Fluorophore	Formula	Calcd. mass	Found mass	Yield (%)
Fluorescein diacetate-Tz	$C_{34}H_{24}N_5O_8^+$	$[M+H]^+$ 630.16	$[M+H]^+$ 630.31	52
Bodipy-FL-Tz	$C_{23}H_{23}BF_2N_7NaO^+$	$[M+Na]^+$ 462.2	$[M+Na]^+$ 484.32	43
NBD-Tz	$C_{15}H_{11}N_8O_3^+$	$[M+H]^+$ 351.09	$[M+H]^+$ 351.14	40
Dansyl-Tz	$C_{21}H_{21}N_6O_2S^+$	$[M+H]^+$ 421.14	$[M+H]^+$ 421.54	11
ATTO495-Tz	$C_{30}H_{33}N_8O^+$	$[M+H]^+$ 521.28	$[M+H]^+$ 521.40	42
Oregon green-Tz	$C_{30}H_{18}F_2N_5O_6^+$	$[M+H]^+$ 582.12	$[M+H]^+$582.29	38
Texas Red-Tz	$C_{46}H_{49}N_8O_7S_2^+$	$[M+H]^+$ 889.31	$[M+H]^+$ 889.29	34
TAMRA-Tz	$C_{34}H_{30}N_7O_4^+$	$[M+H]^+$ 600.23	$[M+H]^+$ 600.33	29
ATTO 610	$C_{34}H_{38}N_7O^+$	$[M+H]^+$ 560.31	$[M+H]^+$ 560.47	30
Rhodamine 101	$C_{41}H_{38}N_7O_2^+$	$[M+H]^+$ 660.31	$[M+H]^+$ 660.53	27

Methods
In vitro kinase assays. The IC_{50} of MLN8054 **7**, MLN8054-TCO **13**, MLN8054-TCO **13**/Texas Red-Tz, and Texas Red-Tz for AKA were determined using the Z´-LYTE Ser/Thr-1 peptide kinase assay kit according to Invitrogen's instructions with minor modifications. The kinase reaction buffer was supplemented with a final concentration of 0.936 mM DTT and 0.67% DMSO. Inhibitors were prepared by 3-fold serial dilution at 75X the final concentration in 100% DMSO (75 μM to 4 nM). This stock was then diluted to 3X the final concentration in kinase reaction buffer. 5 μl of the 3X stock was added to the final 15 μl reaction volume. The final concentration of recombinant AKA was 625 ng/ml. Both the Z´-LYTE control phosho-peptide and the Z´-LYTE peptide were used at a final concentration of 2 μM. ATP was added at a final concentration of 10 μM to initiate the reaction, which proceeded for 1 hour at room temperature. Development reagent was then added and the reaction was incubated for 1 hour at room

temperature. After terminating the reaction with stop reagent, the coumarin and fluorescein emission was measured on a TECAN Saffire^2 plate reader (ex: 400 nm, em: 445 and 520 nm). IC_{50} values were obtained by fitting the dose-response curve using Prism (GraphPad).

DNA constructs. The pTRE-Tight-GFP and pTRE-Tight-mCherry constructs were made by ligating AcGFP1 or mCherry from pAcGFP1-Hyg-C1 or pmCherry-Hyg-C1 (Clontech) into pTRE-Tight (Clontech), using the NheI and AccI restriction enzyme sites. pTRE-Tight-GFP-AKA or pTRE-Tight-mCherry-AKA were cloned using the In-Fusion HD cloning kit (Clontech). pTRE-Tight-GFP or pTRE-Tight-mCherry were digested with BglII and EcoRV restriction enzyme sites, while AKA was PCR amplified from pCMV-SPORT6 (Open Biosystems) using Advantage HF2 polymerase (Clontech) with the following primer pairs 5′-GTCCGGACTCAGATCTATGGACCGATCTAAGGAGAACTGCATATCA-3′ and 5′-TGATCCTCTAGAGATATCCTAAGACTGCTTGCTAGCTGATTCCTTGTT-3′. The in-fusion reaction was used to fuse AKA in-frame to the C-terminus of AcGFP1 or mCherry in the pTRE-Tight vector. pmApple-N1 was made by ligating mApple from Myo1E-pmAppleC1 (Addgene, Prof. Christien Merrifield[5]) into pmCherry-N1 (Clontech) using AfeI and BsrG1 restriction enzymes. The pTag-H2B-Apple construct was made by ligating mApple (pmApple-N1) into pTag-H2B-BFP (Evrogen) using the AgeI and NotI restriction enzyme sites. All plasmids were confirmed by sequencing the inserts in their entirety.

Cell lines. IMR-90 and HT1080 cells were maintained in Minimum Essential Media supplemented with 10% FBS, 100 I.U. penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, non-essential amino acids, and 1 mM sodium pyruvate. MDA-MB-231 cells were maintained in RPMI 1640 supplemented with 10% FBS, 100 I.U. penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. HeLa, A549, SKOV-3, PANC-1, MIA PaCa-2, U-87 MG, SW480, and HEK-293 cells were maintained in Dulbecco's Modified Eagle Medium supplemented identical to RPMI. PANC-1 cells were stably transfected with pTet-On Advanced (Clontech) using Xfect transfection reagent (Clontech) to develop a Tet-On Advanced cell line. Cells were selected in 2 mg/ml G418 (InvivoGen). Single clones were isolated and tested for doxycycline response. Cells were maintained in DMEM supplemented with tet-system approved FBS (Clontech) and 100 μg/ml G418. A clone with high doxycycline responsiveness was then stably transfected with pTRE-Tight-GFP-AKA or pTRE-Tight-mCherry-AKA using FuGENE HD transfection reagent (Roche). Single clones were selected in 200 μg/ml hygromycin (InvivoGen) and were screened for GFP-AKA or mCherry-AKA expression by fluorescence microscopy after addition of 1 μg/ml doxycycline. Stable cell lines were maintained in DMEM containing 50 μg/ml G418 and 20 μg/ml hygromycin. HT1080 cells were transfected with pTag-H2B-Apple using X-tremeGENE HP transfection reagent (Roche). Single clones were selected in 500 μg/ml G418. Clones were screened for H2B-Apple expression by fluorescence microscopy and were maintained in MEM containing 100 μg/ml G418.

Fixed cell fluorescence microscopy. PANC-1 Tet-On cells expressing GFP-AKA were plated at 5500 cells per well in 96-well black μ-clear bottom plates (Grenier Bio-One) and were grown for 48 hrs in the presence of 1 μg/ml doxycycline. On the day of imaging, cells were incubated with

a final concentration of 1.5 μM MLN8054-TCO **13** (0.1% DMSO in growth media) for 20 min at 37°C. Cells were washed once in media and were then incubated with 1 μM TR-Tz (0.1% DMSO in growth media) for 20 min at 37°C. Cells were washed twice with media, fixed in 2% paraformaldehyde, and washed overnight in PBST (PBS containing 0.1% Tween-20). DNA was stained with Hoechst 33258. Imaging was done on a DeltaVision microscope (Applied Precision Instruments) at 40X, with 1.6 zoom. Fluorescence images were processed for noise reduction.

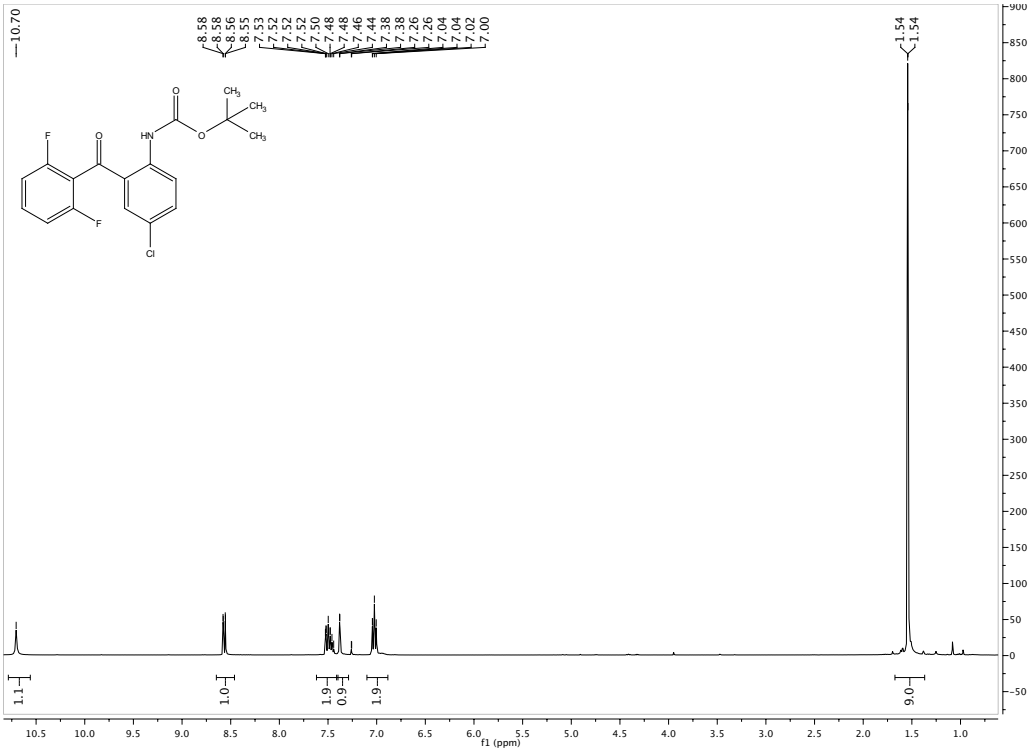
Live cell fluorescence microscopy. PANC-1 Tet-On cells expressing mCherry-AKA were plated as above. Cells were incubated with 125 nM MLN8054-TCO **13** (0.1% DMSO in growth media) for 30 min at 37°C. Cells were washed twice with media and were then incubated with 187.5 nM FDA-Tz (0.1% DMSO in growth media) for 30 min at 37°C. Following several wash steps over two hours, cells were incubated for 10 min with 5 μM DRAQ5 to label nuclei. Live cells were imaged in a humidified environmental chamber of a DeltaVision microscope at 40X, with 1.6 zoom. Fluorescence images were processed for noise reduction. Imaging of HT1080 H2B-Apple live cells was identical to the procedure described above with minor modifications. Cells were plated at 8000 cells/well and grown for 24 hrs and 1 μM FDA-Tz was used for the second step of the bioorthogonal reaction.

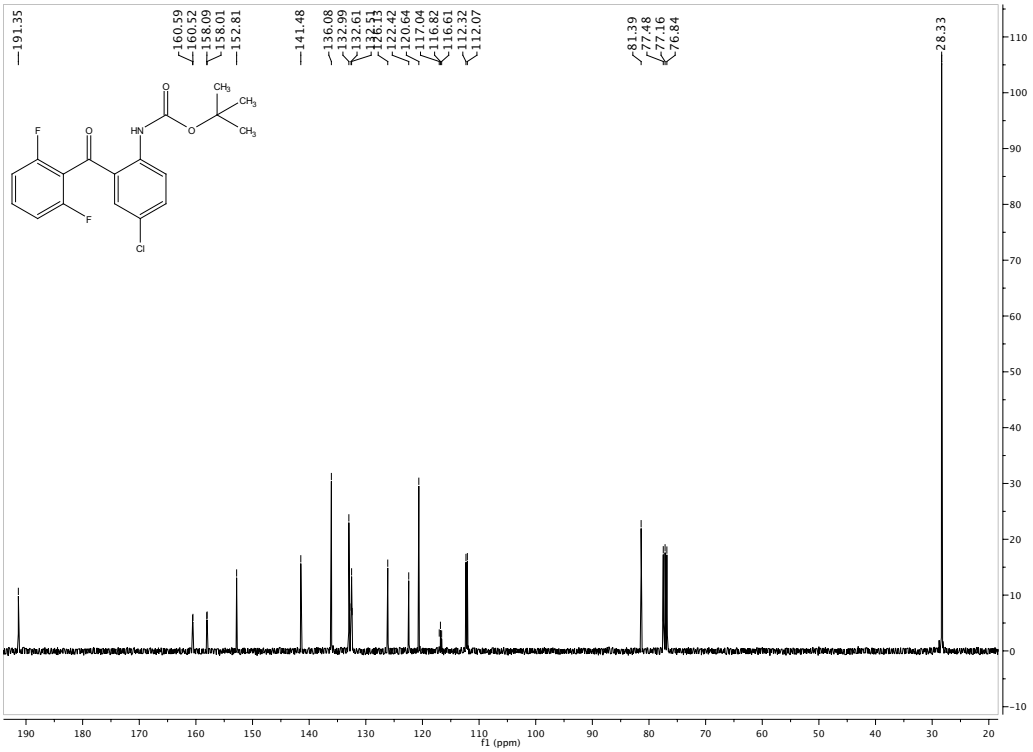
Cell line quantification. HeLa, IMR-90, PANC-1, A549, SKOV-3, MDA-MB-231, U-87 MG, SW480, MIA PaCa-2, and HEK-293 cells were plated in duplicate in a 96-well plate 48 hrs prior to assay. Cells were incubated first with 1.5 μM MLN8054-TCO **13** for 20 min (0.1% DMSO in media). Cells were then washed twice with media before incubation with 1 μM TR-Tz (0.1% DMSO in media). Cells were washed several times in media, fixed with 2% paraformaldehyde, washed with PBST (PBS containing 0.1% Tween-20), permeabilized in 100% methanol at room temperature, and washed again with PBST. Cells were then blocked 1 hr in Odyssey blocking buffer, followed by AKA antibody (1:500, abcam) staining overnight at 4°C. Cells were washed in PBST and then incubated 1 hr in Alexa 488 donkey α-rabbit secondary antibody (1:500, Invitrogen). Cells were washed in PBST, followed by PBS and nuclei were stained with Hoechst 33258, while the whole cell was stained with Cellomics blue whole cell stain (ThermoScientific). Imaging was done using a CellWoRX microscope (Applied Precision Instrumnets) at 10X magnification. Images were analyzed for nuclear integrated fluorescence intensity using ImageRail software.[6]

Fluorescein diacetate-Tz activation. HT1080 cells were washed twice with ice-cold 1X PBS and were then scraped into RIPA buffer (150 mM NaCl, 10mM Tris pH 7.2, 0.1% sodium dodecylsulfate, 1% Triton X-100, 17 mM deoxycholate, and 2.7 mM EDTA). Soluble proteins were isolated by centrifugation at 14,000 x g for 15 min at 4°C. Cell lysate (0, 1, 2.5, 5, 10 and 20 μg of total protein, determined by the BCA assay, Pierce) was incubated with FDA-Tz (20 μM in PBS). Activation kinetics were followed by measuring, in duplicate, the fluorescence emission on a TECAN Saffire^2 plate reader (ex: 488 nm, em: 523 nm) after 10, 30, 60, 150, 240 and 960 min. The fluorescence emission spectra before and after activation was also measured (ex: 488 nm, em: 507-600 nm).

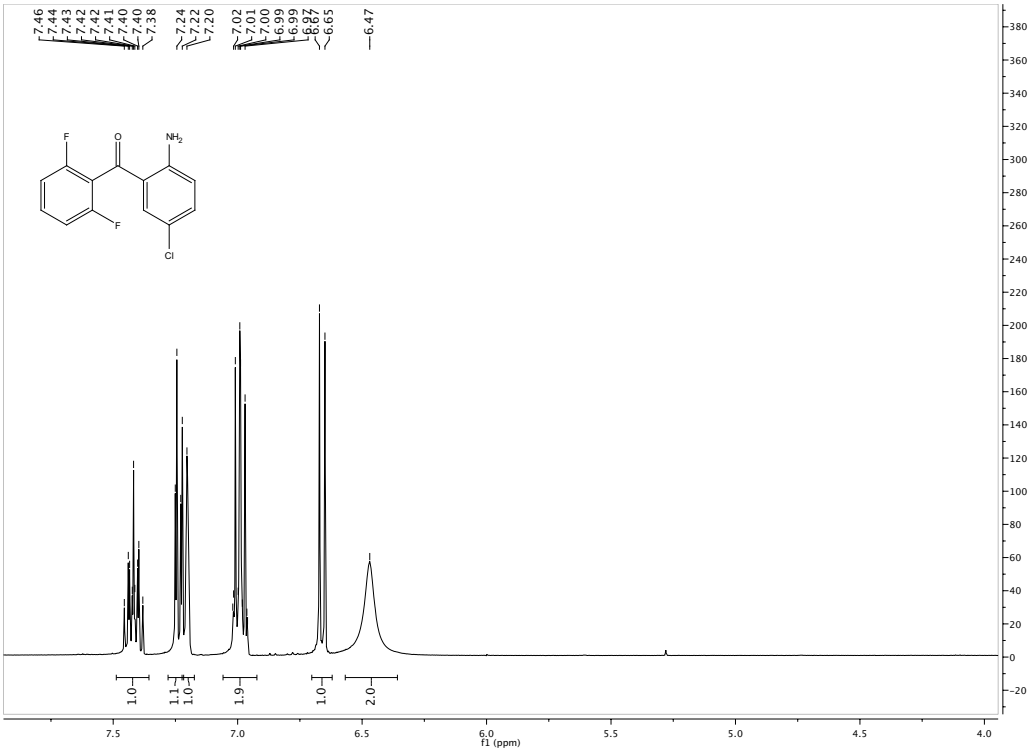
Supplemental Figures

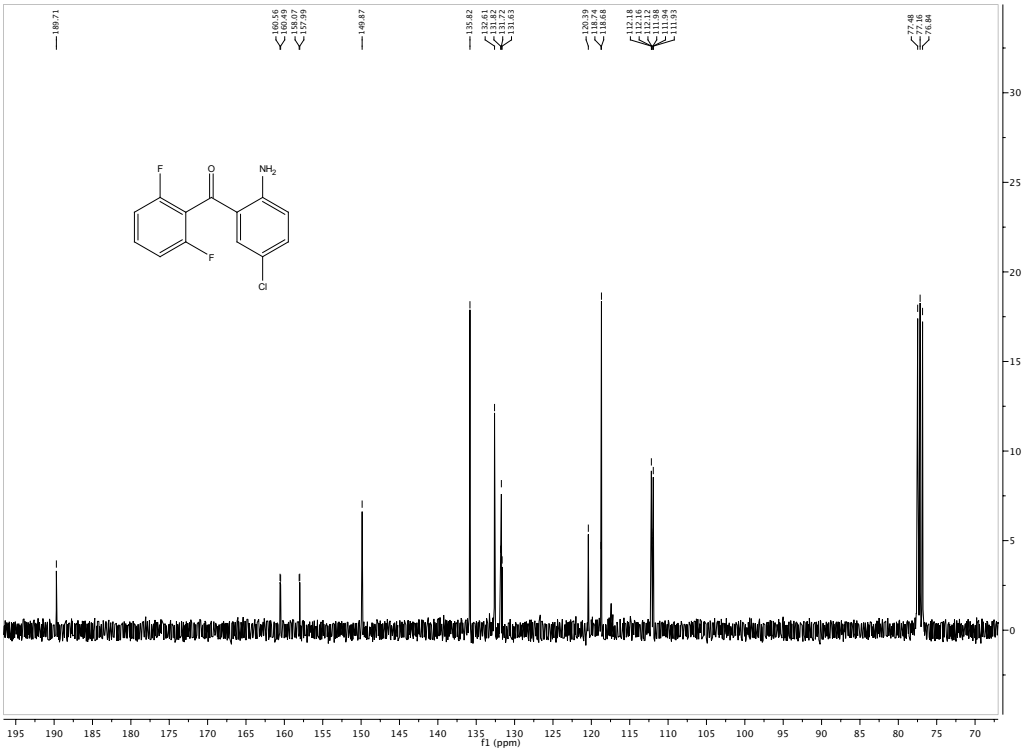
Spectra ^{1}H and ^{13}C of compound 1



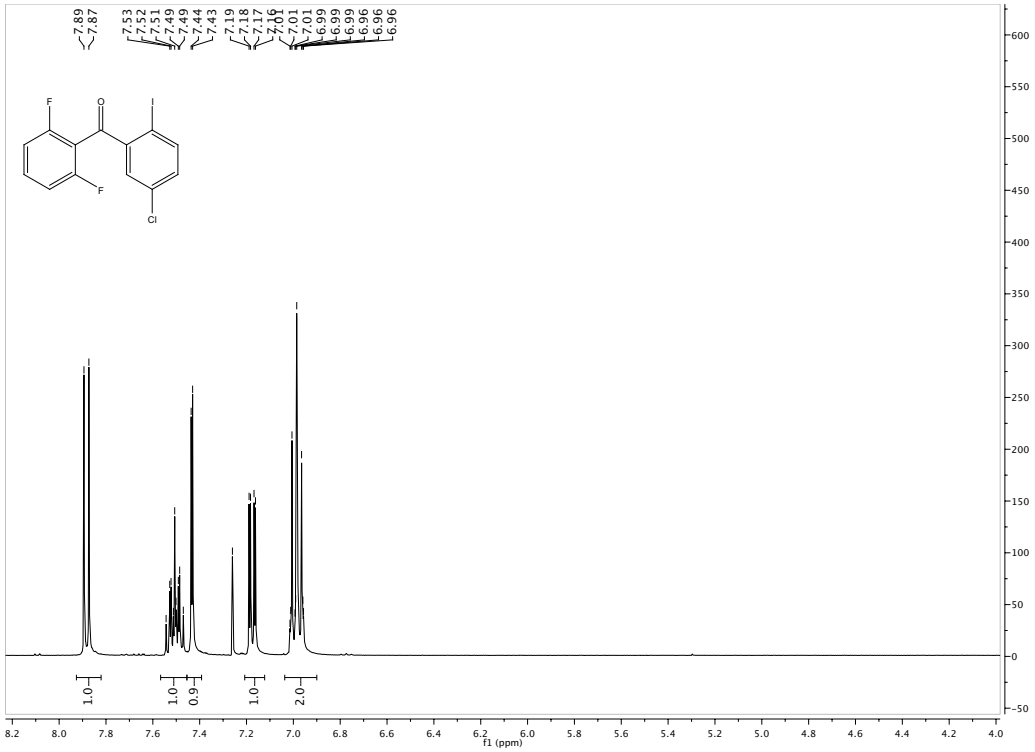


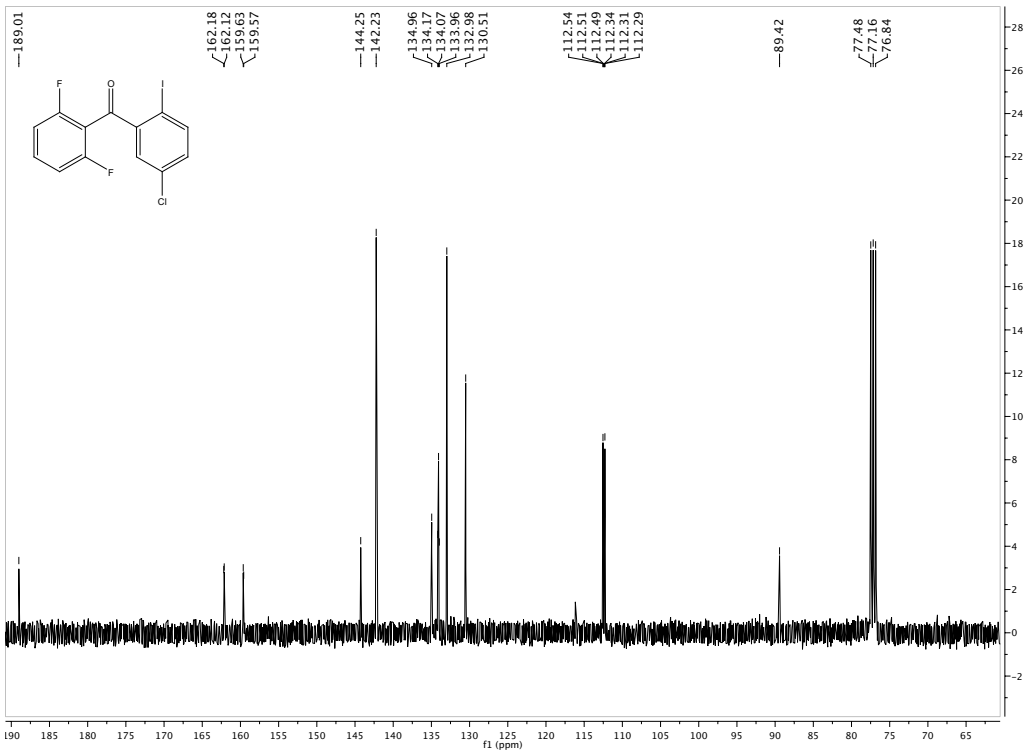
Spectra ^{1}H and ^{13}C of compound 2



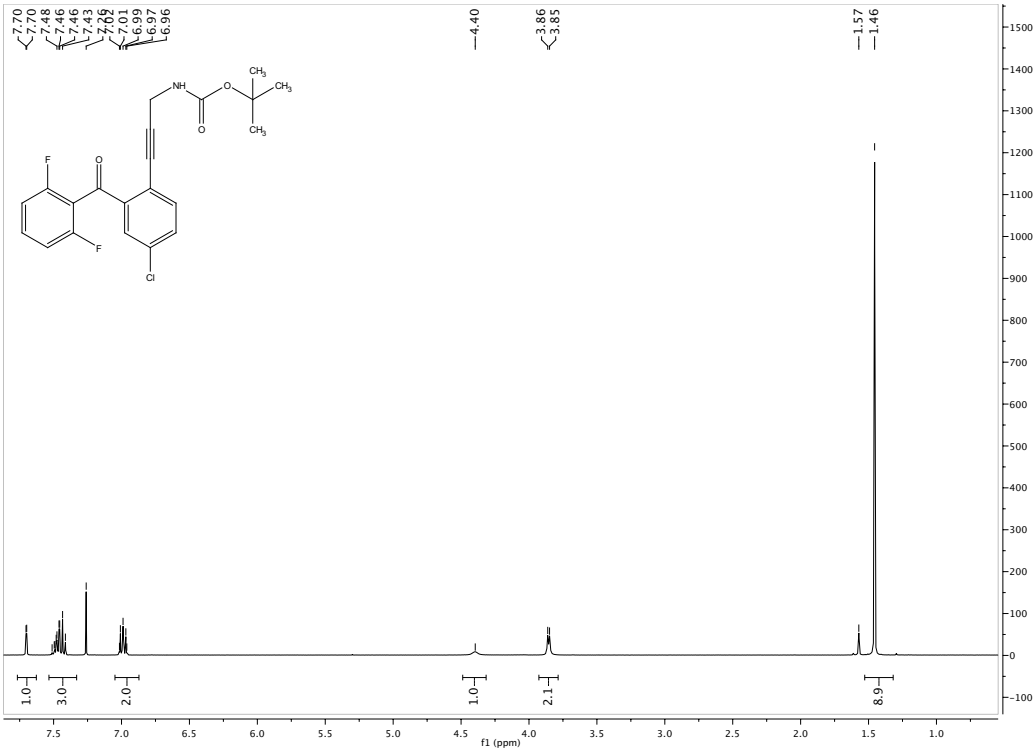


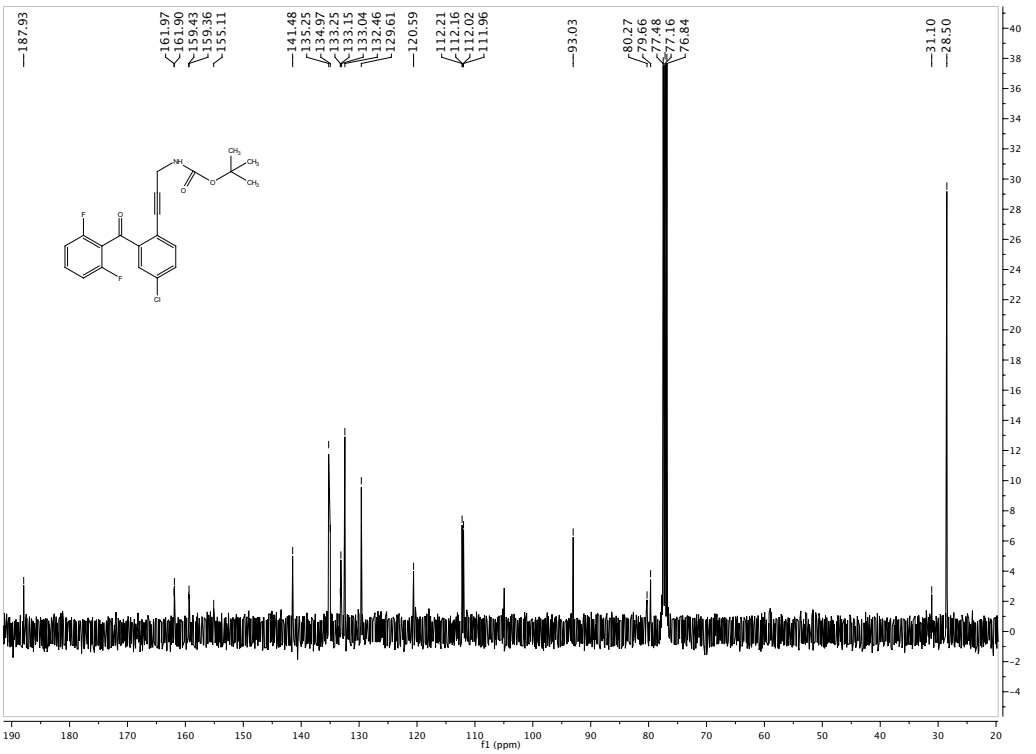
Spectra ^{1}H and ^{13}C of compound 3



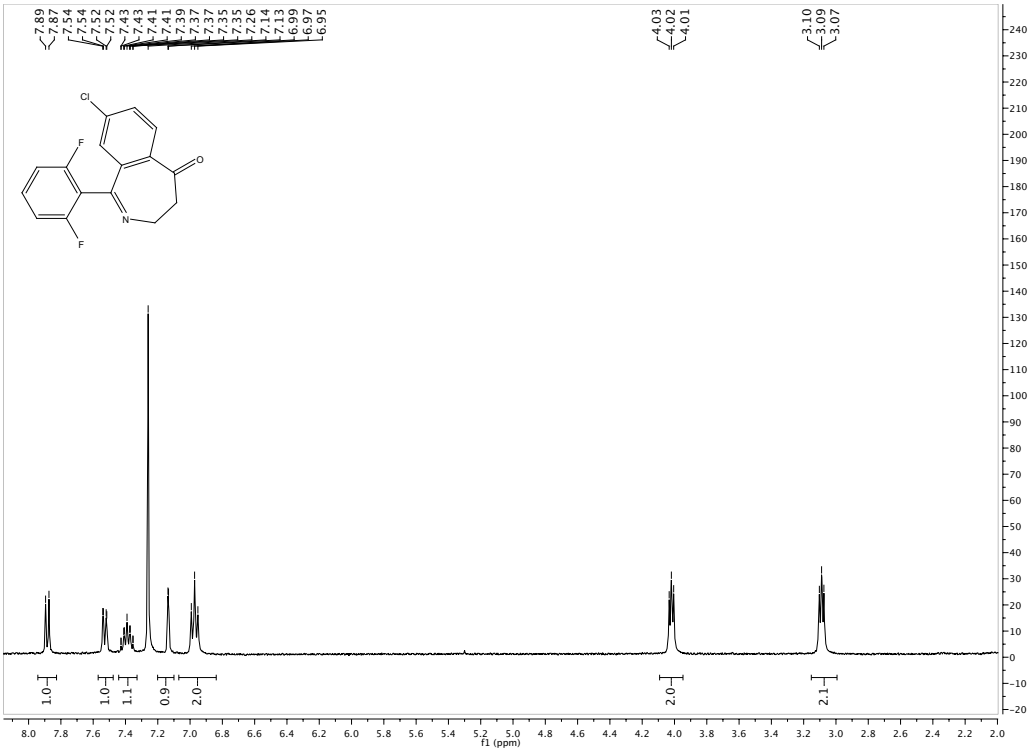


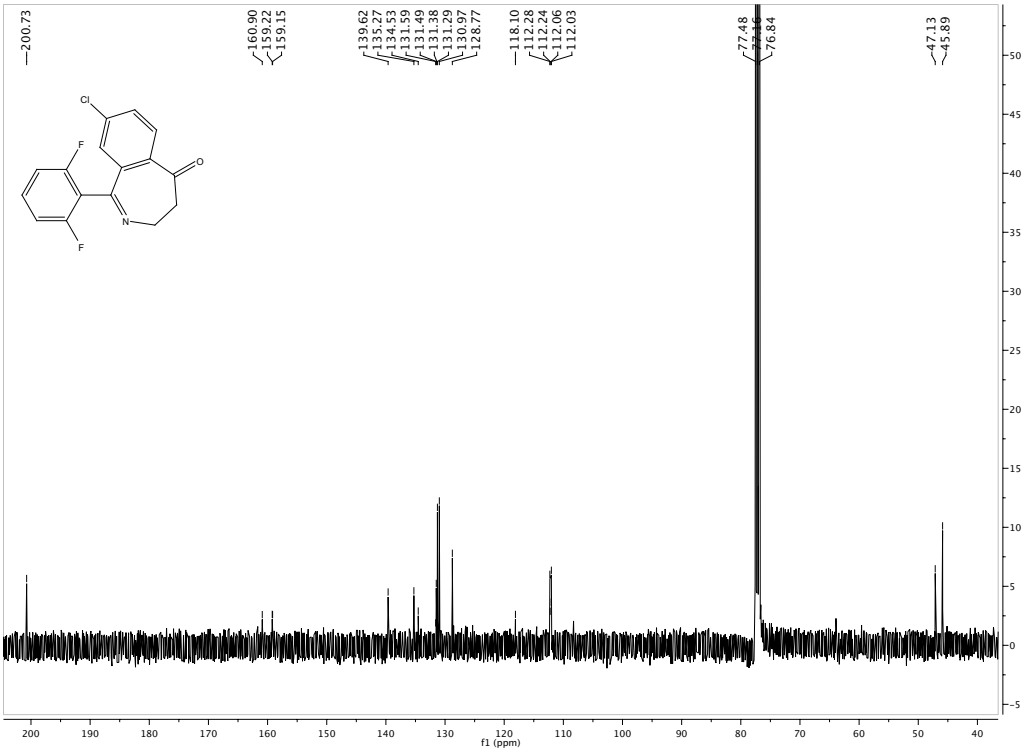
Spectra ^{1}H and ^{13}C of compound 4



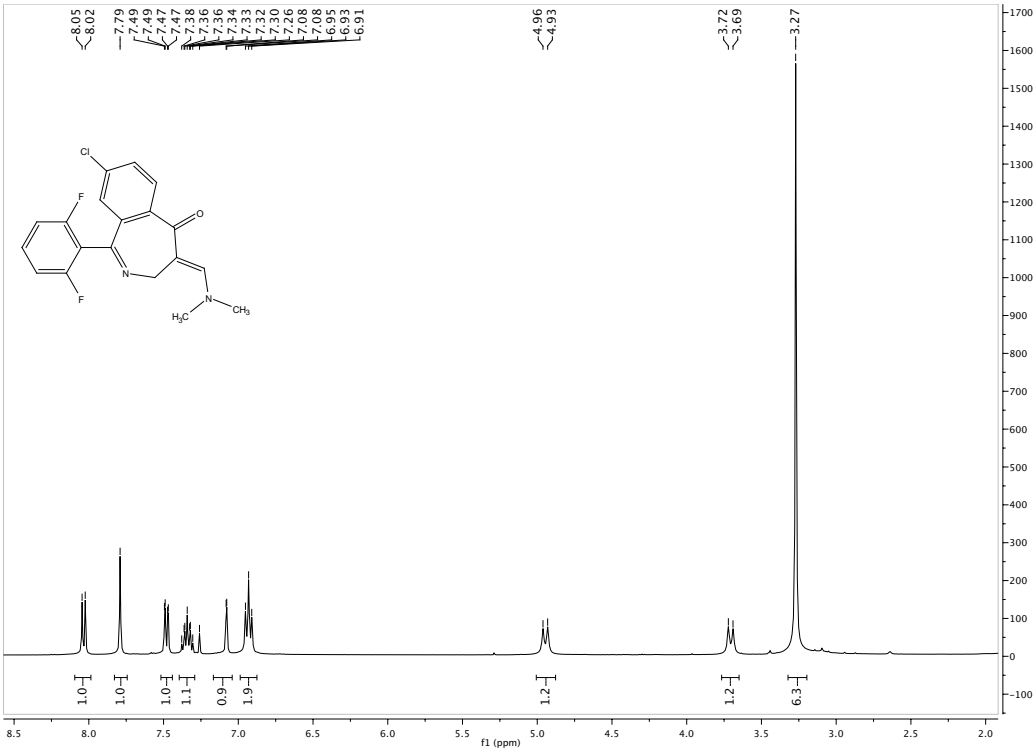


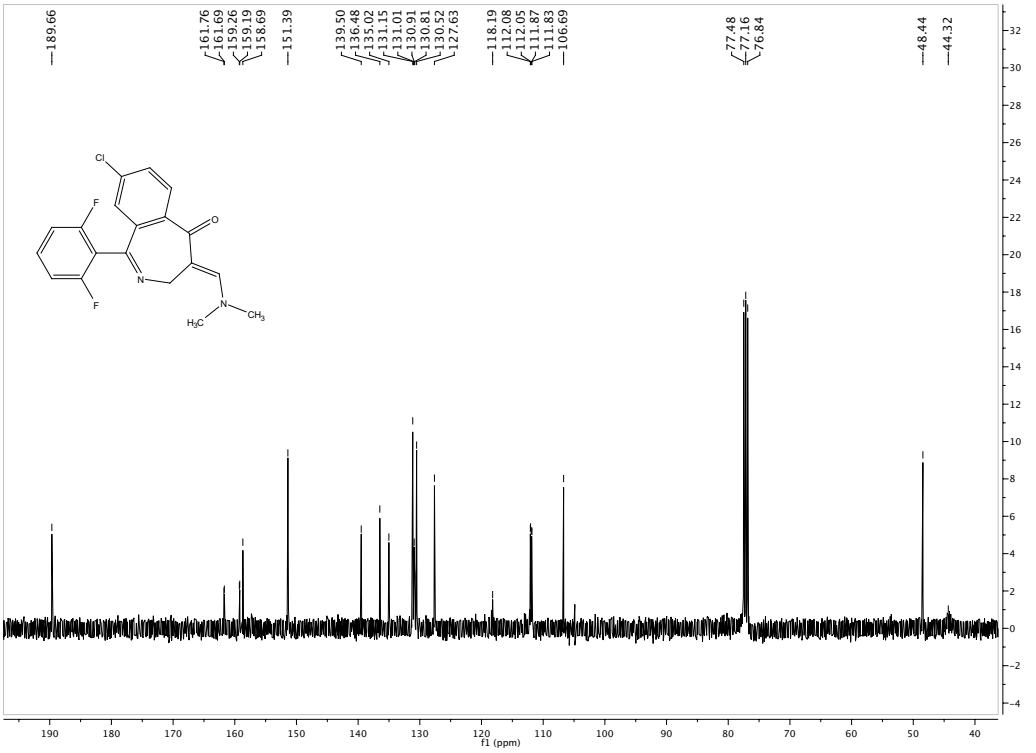
Spectra ^{1}H and ^{13}C of compound 5



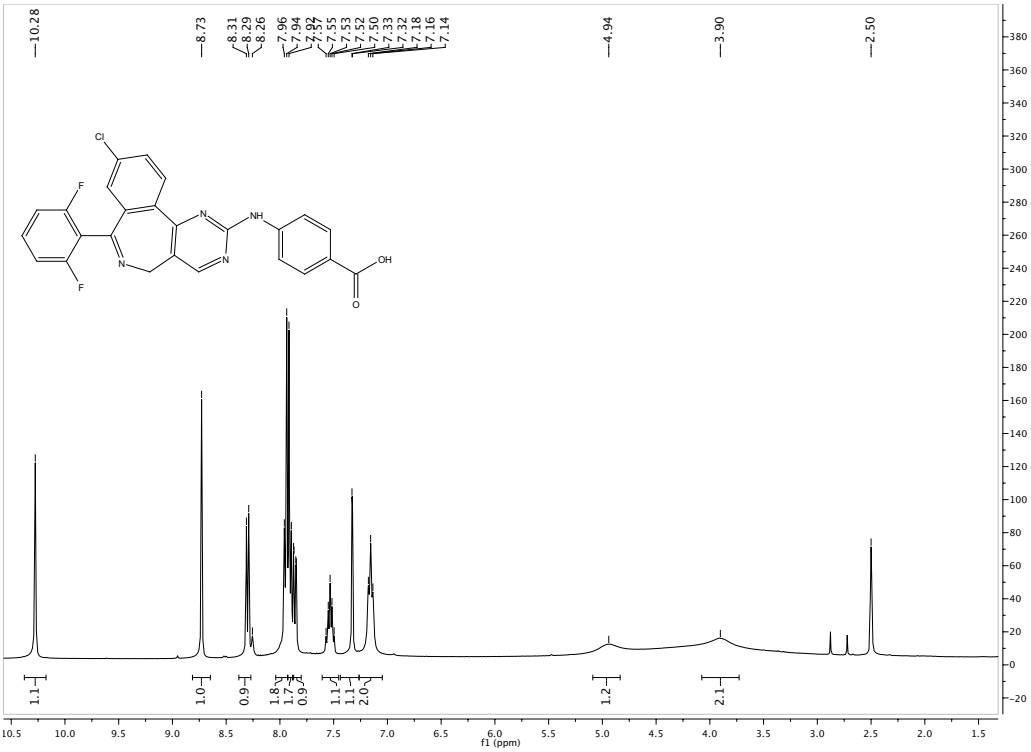


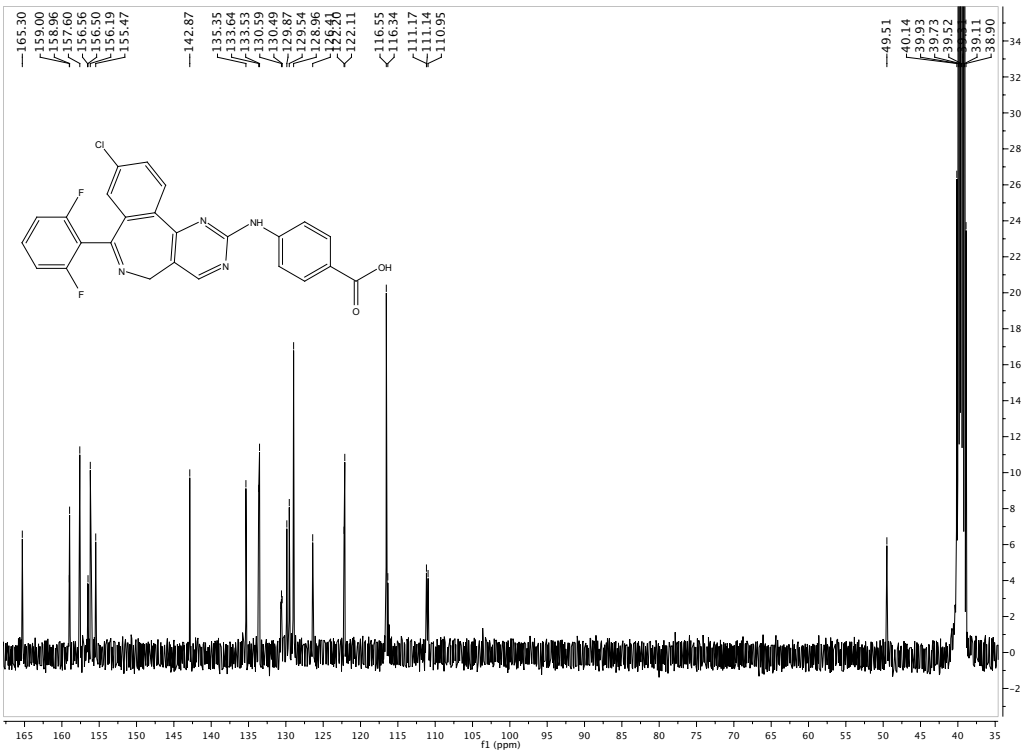
Spectra ^{1}H and ^{13}C of compound 6



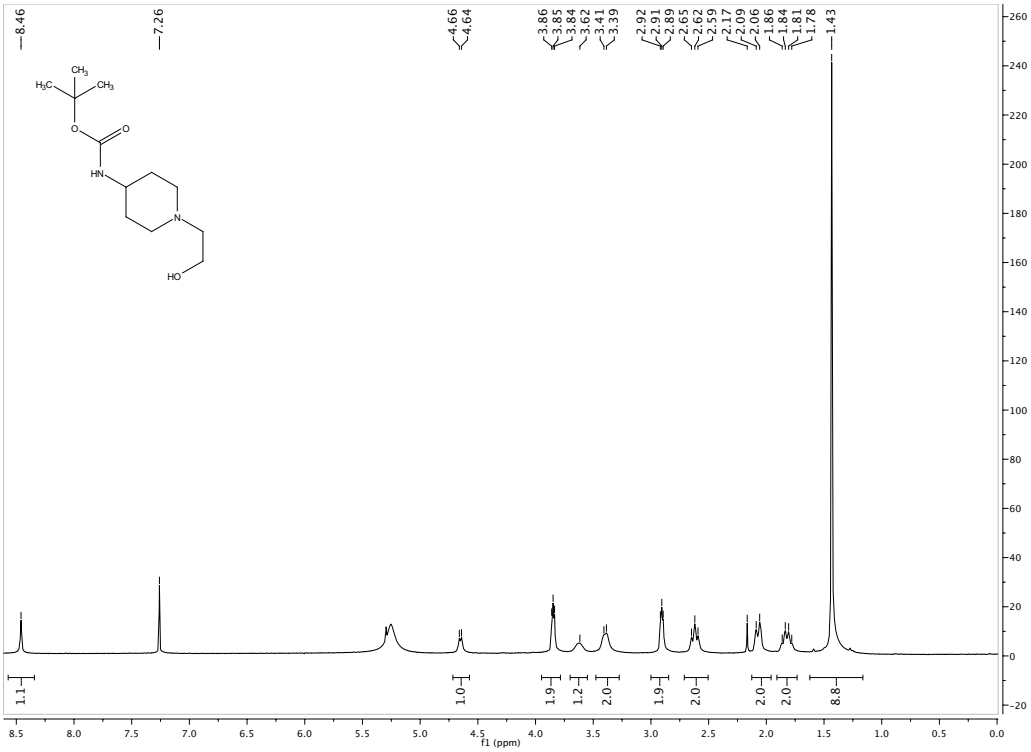


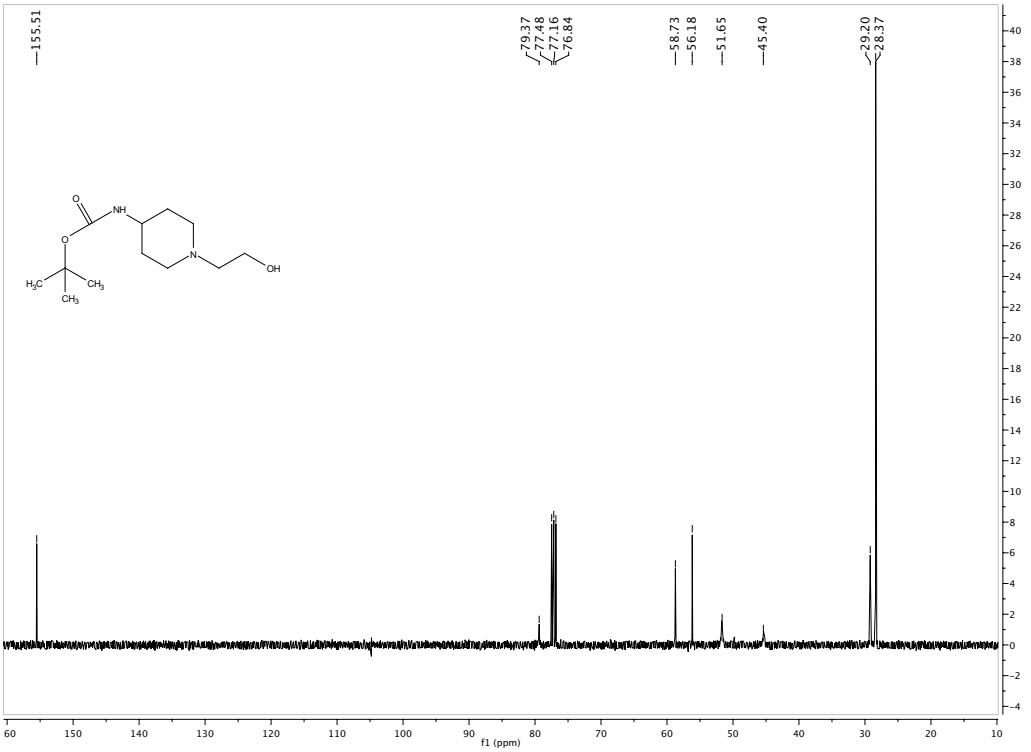
Spectra ^{1}H and ^{13}C of compound 7



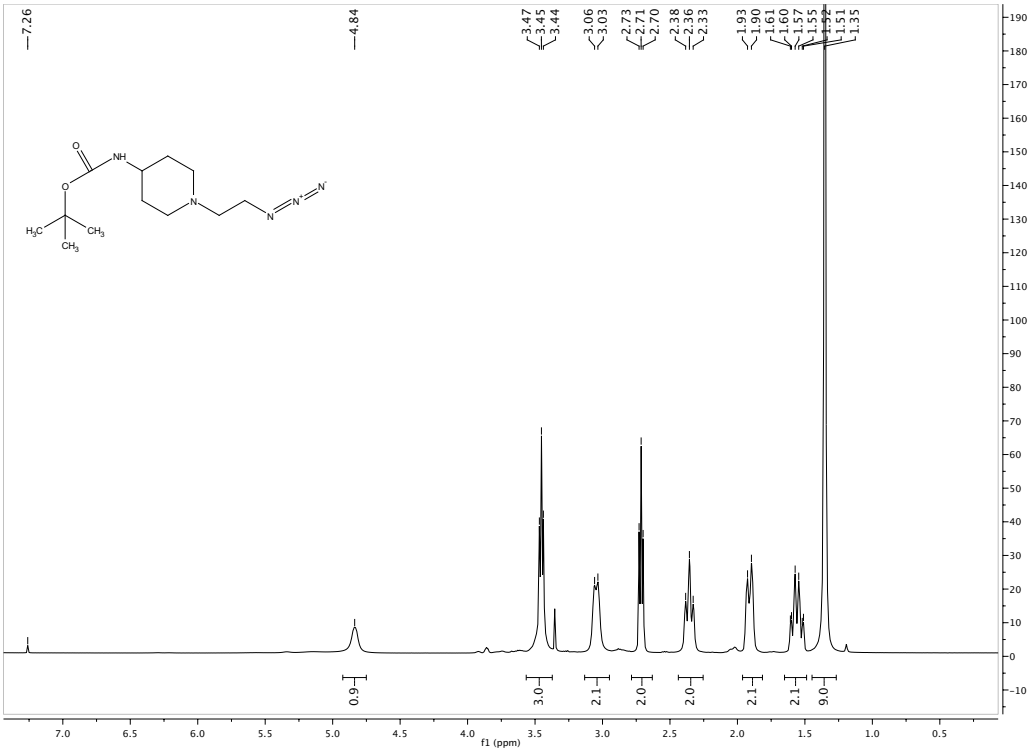


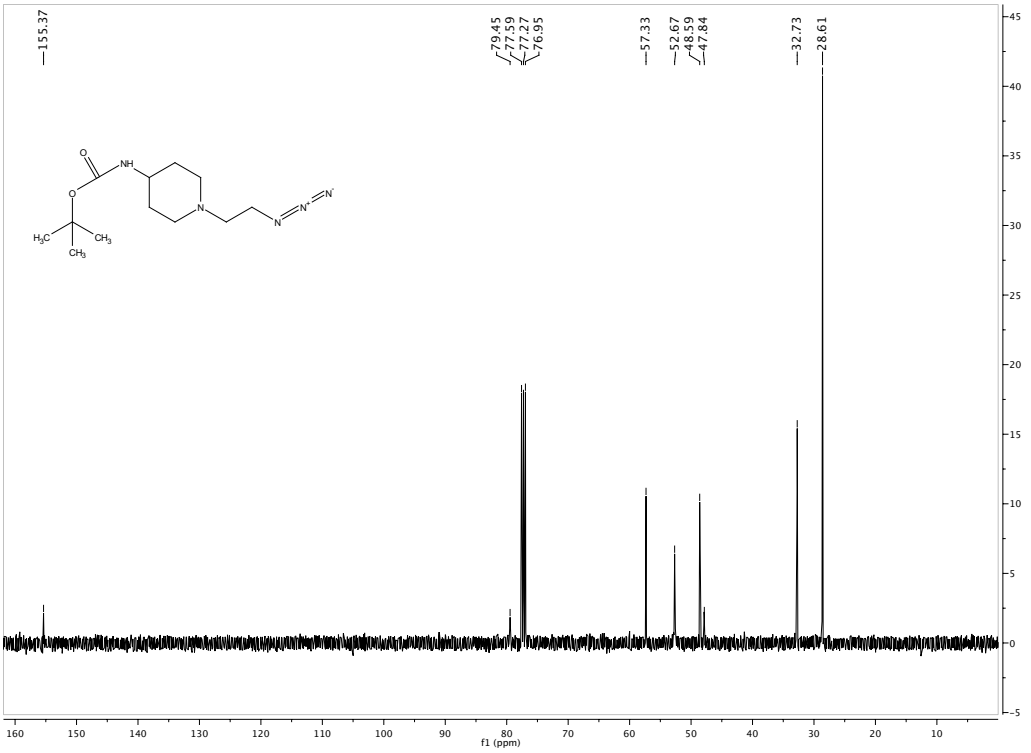
Spectra ^{1}H and ^{13}C of compound 8



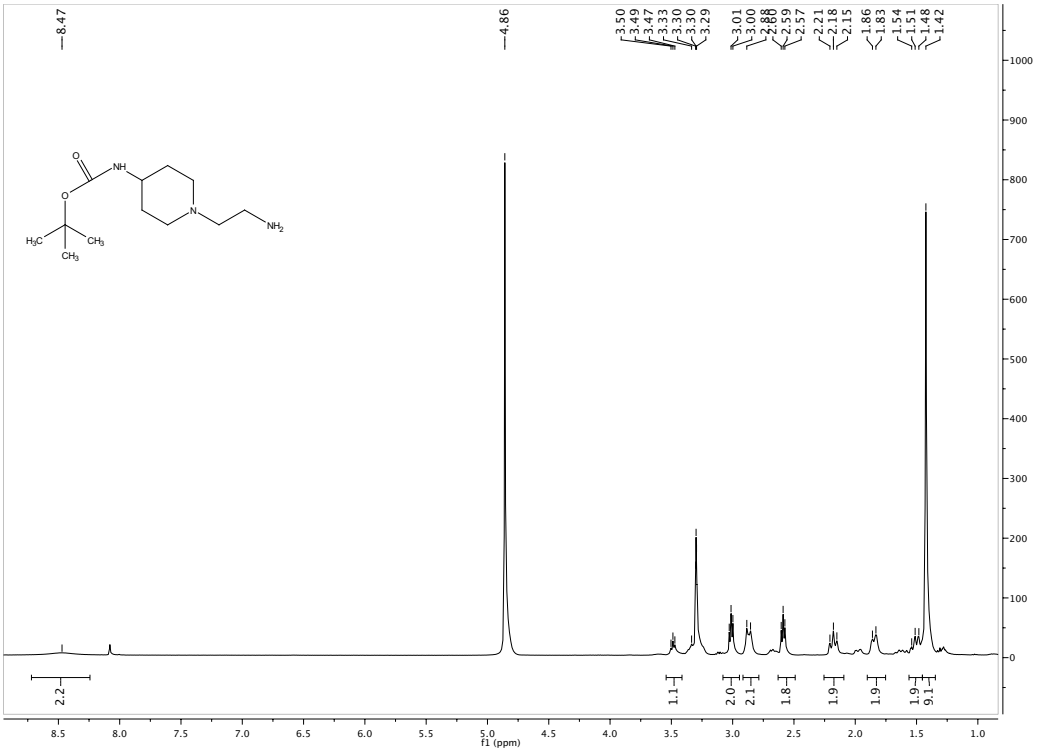


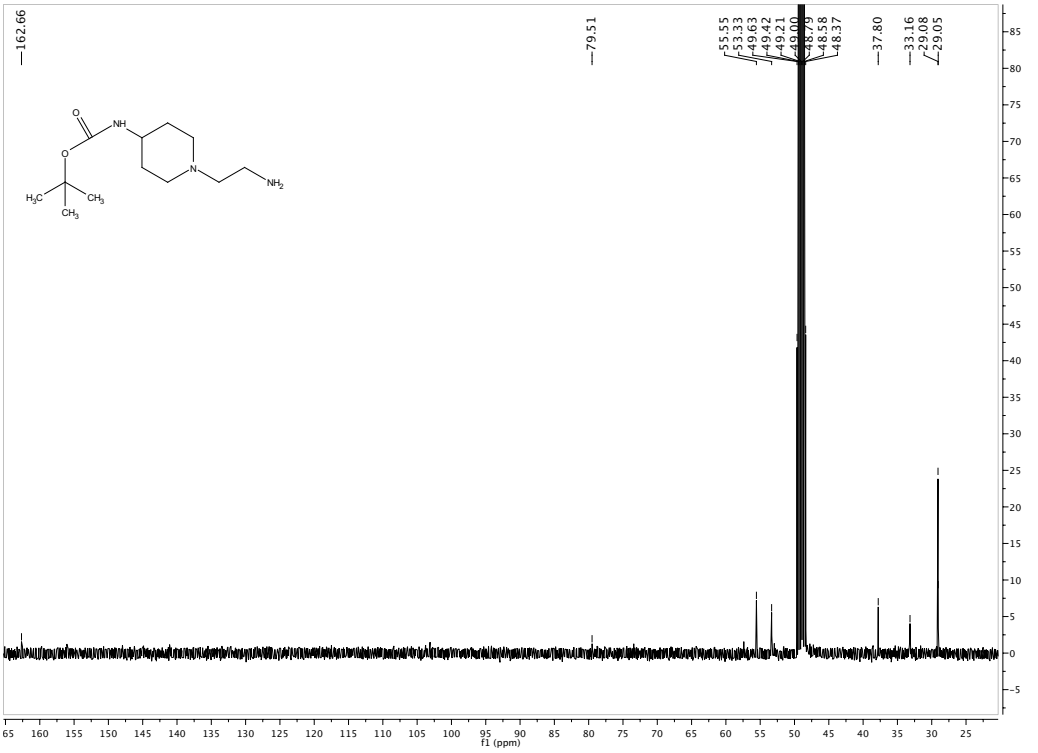
Spectra ^{1}H and ^{13}C of compound 9



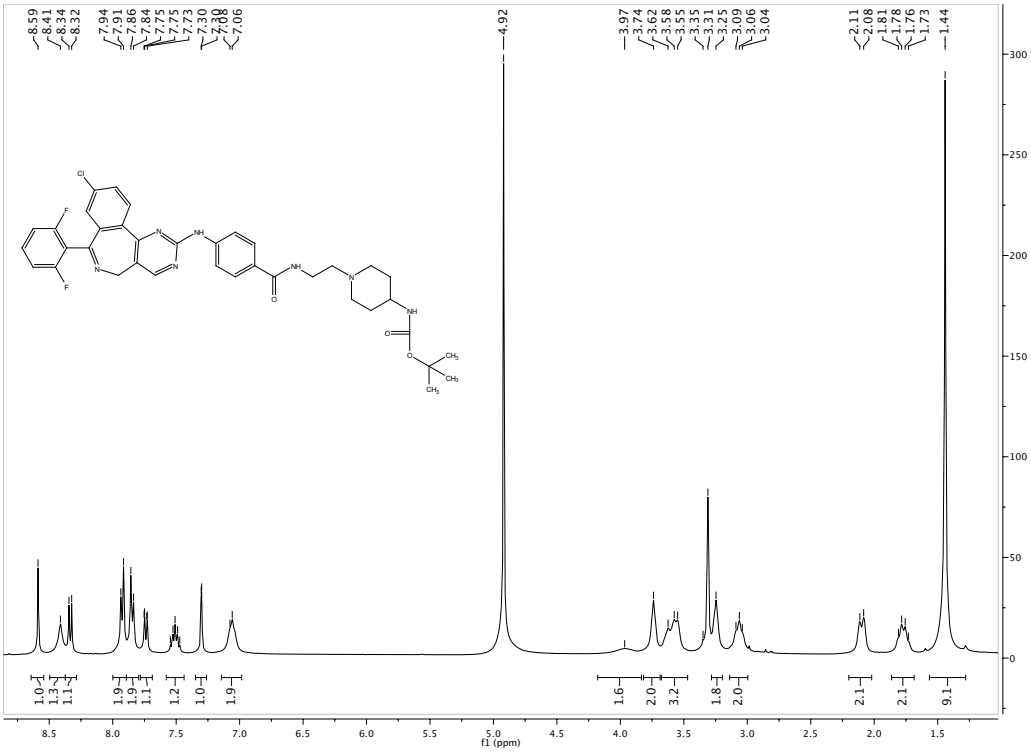


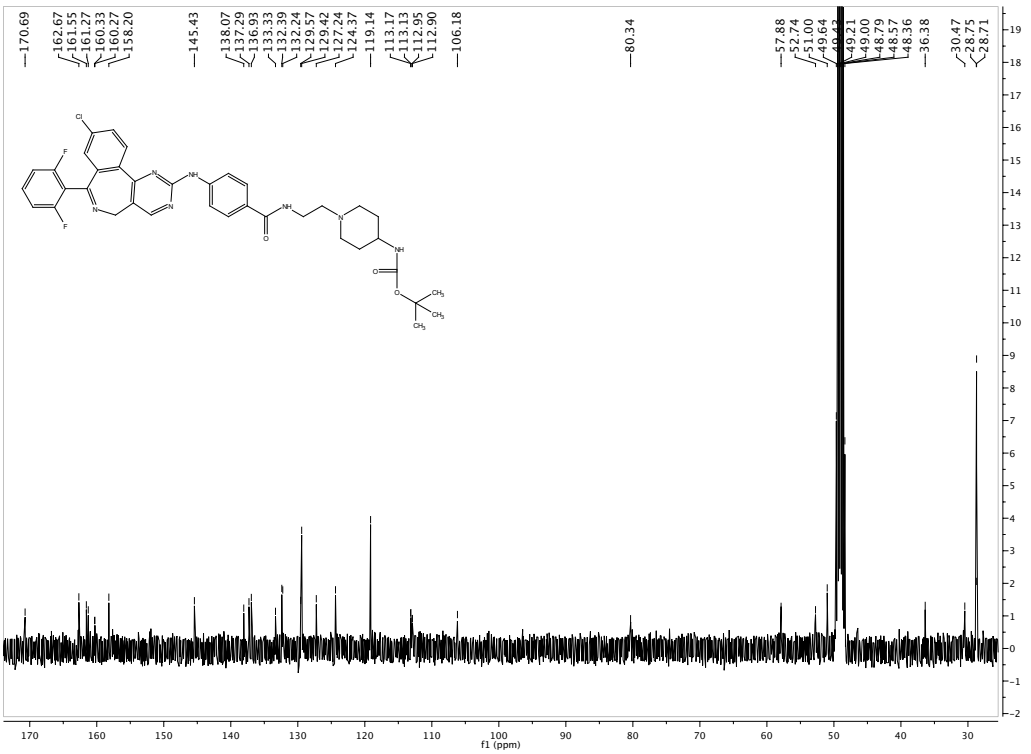
Spectra ^{1}H and ^{13}C of compound 10



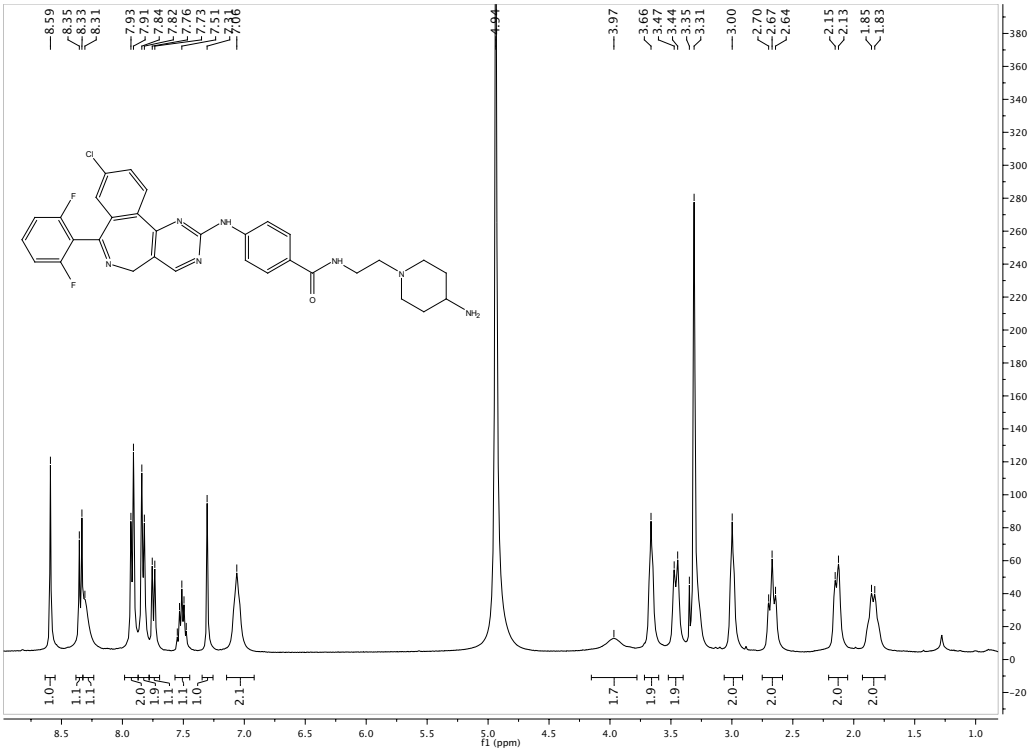


Spectra ^{1}H and ^{13}C of compound 11

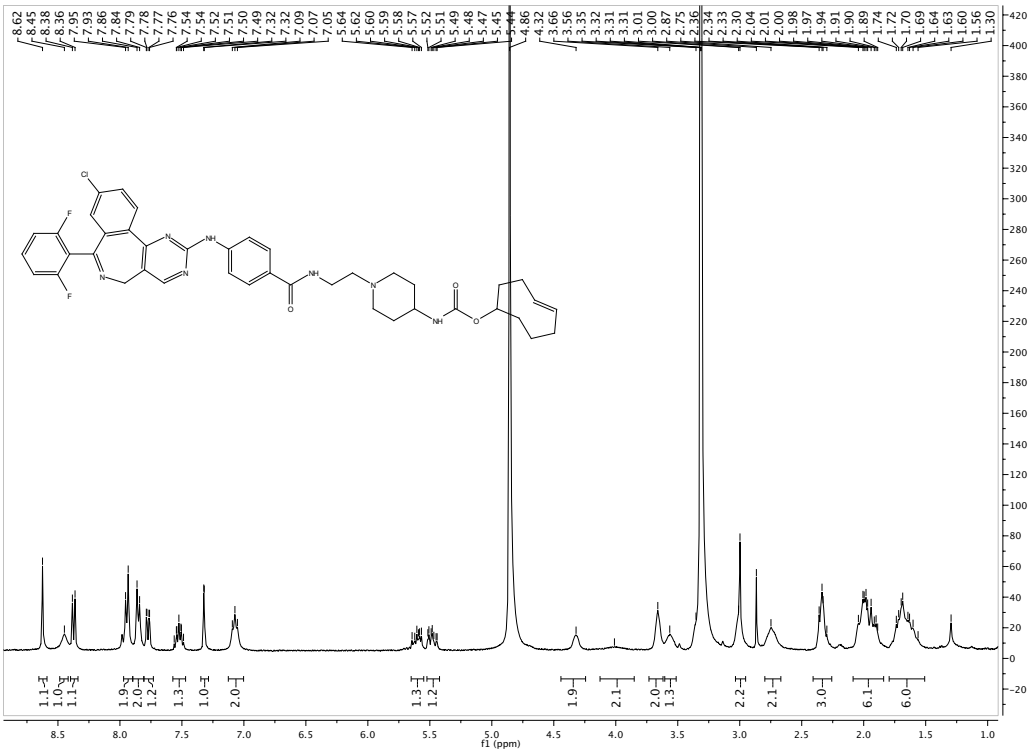


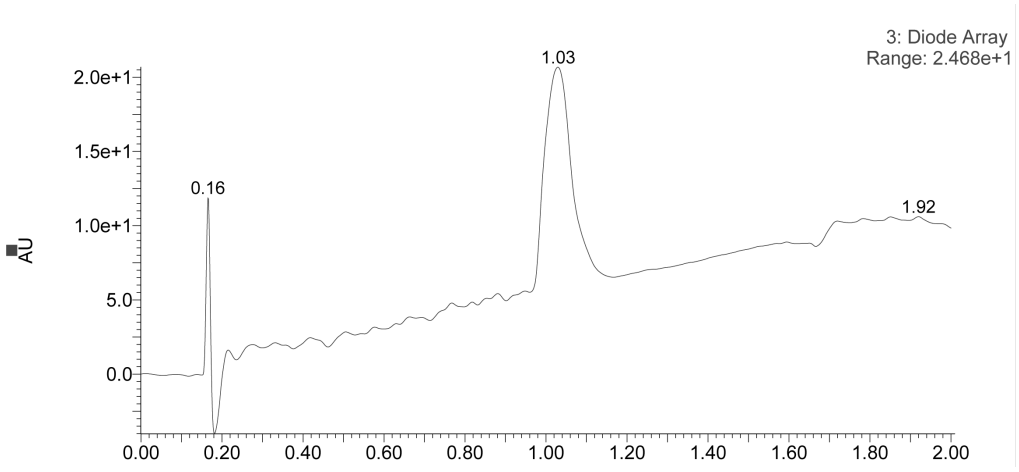


Spectra ^{1}H of compound 12



Spectra ^{1}H of compound 13





UV-HPLC trace of **13** (purity > 97%)

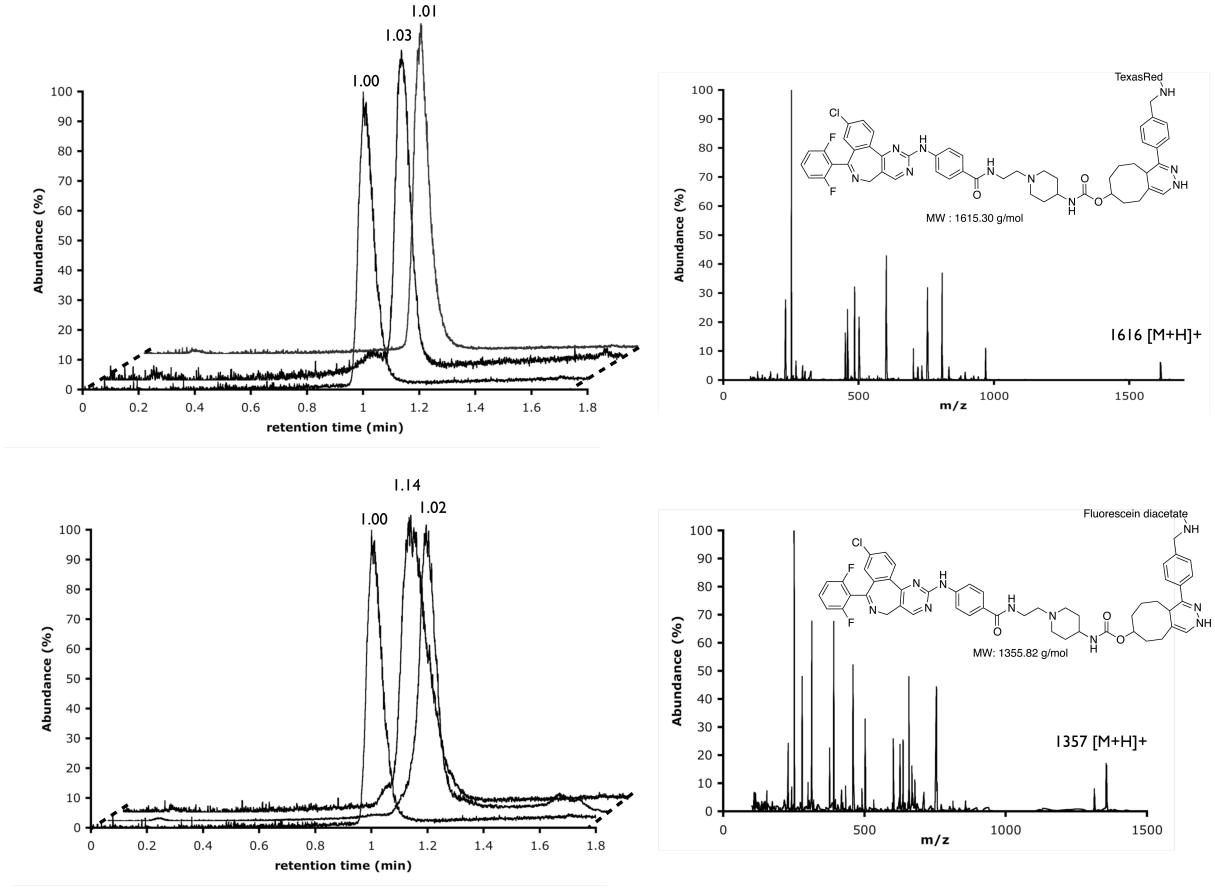


Figure S1. LESD HPLC and MS spectrum of bioorthogonal reaction between MLN8054-TCO **13** and Texas Red-Tz (TR-Tz) or FDA-Tz affording desired compound MLN8054-Fluorescent dye (crude reaction mixture in DMSO, not all isomers shown).

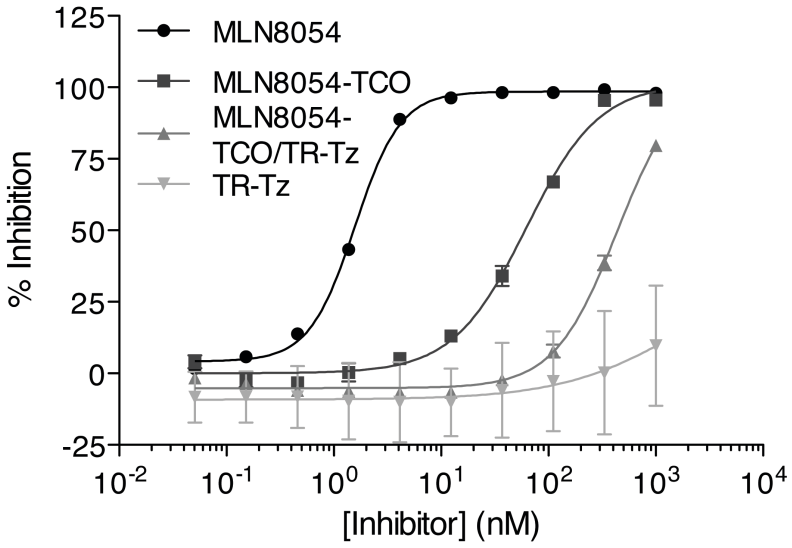


Figure S2. Inhibitory effect of MLN8054 **7**, MLN8054-TCO **13**, MLN8054-TCO **13** pre-reacted with Texas Red-Tz, and Texas Red-Tz on recombinant AKA. IC_{50} values were obtained using the z´-lyte serine/threonine kinase assay kit (Invitrogen). Data were fit to a sigmoidal dose-response curve using GraphPad (Prism).

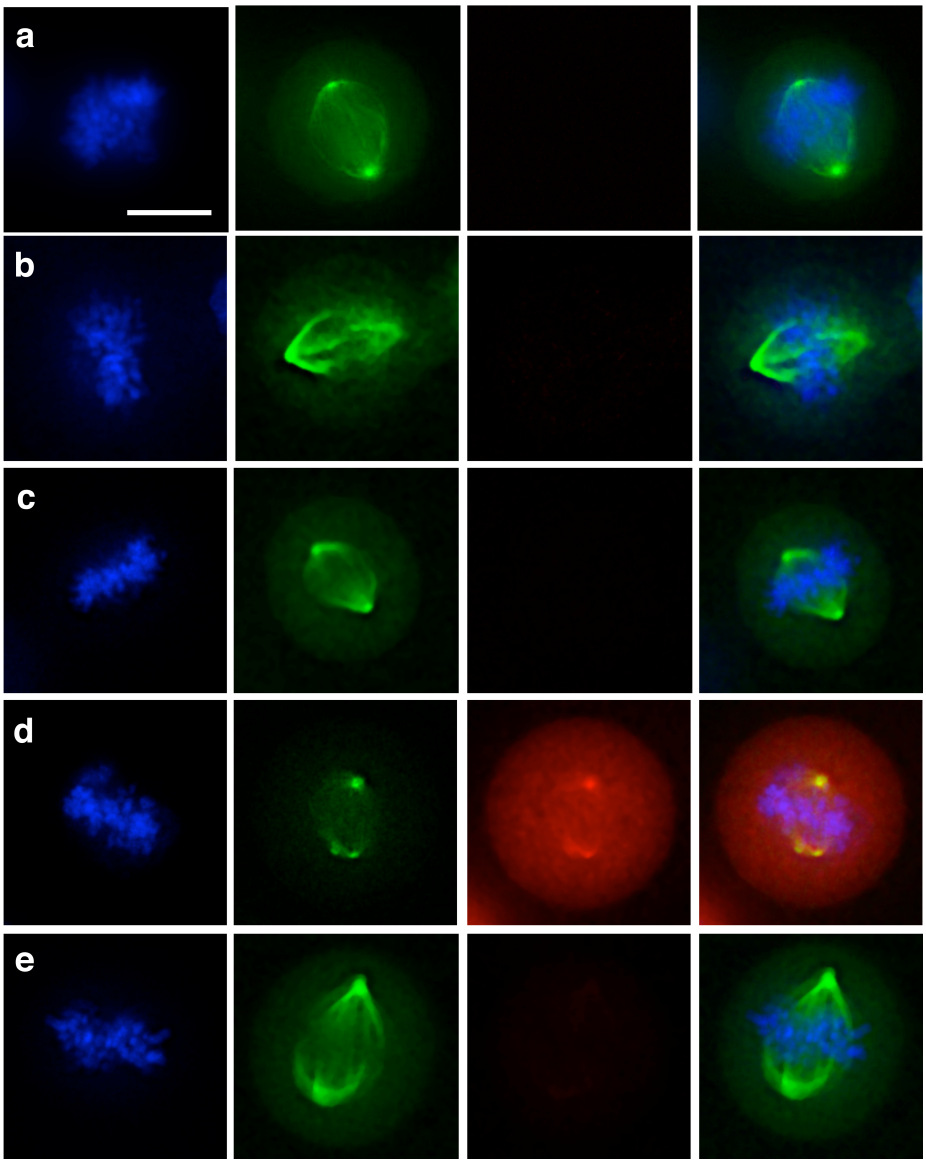


Figure S3. Colocalization between GFP-AKA and controls in PANC-1 Tet-On cells. Staining was done by first incubating cells with MLN8054-TCO **13** (1.5 μM, 0.1% DMSO) or the appropriate control at the same concentration for 20 minutes. Cells were then washed and stained with or without Texas Red-Tz (1 μM, 0.1% DMSO) for 20 minutes. Cells were treated with control (A), Texas Red-Tz alone (B), MLN8054 **7** without the TCO modification/Texas Red-Tz (C), MLN8054-TCO **13**/Texas Red-Tz (D), or MLN8054-TCO **13**/Texas Red-Tz pre-reacted outside of the cell (E). Blue: Hoechst, Green: GFP-AKA, Red: Texas Red-Tz. Scale bar: 10μm.

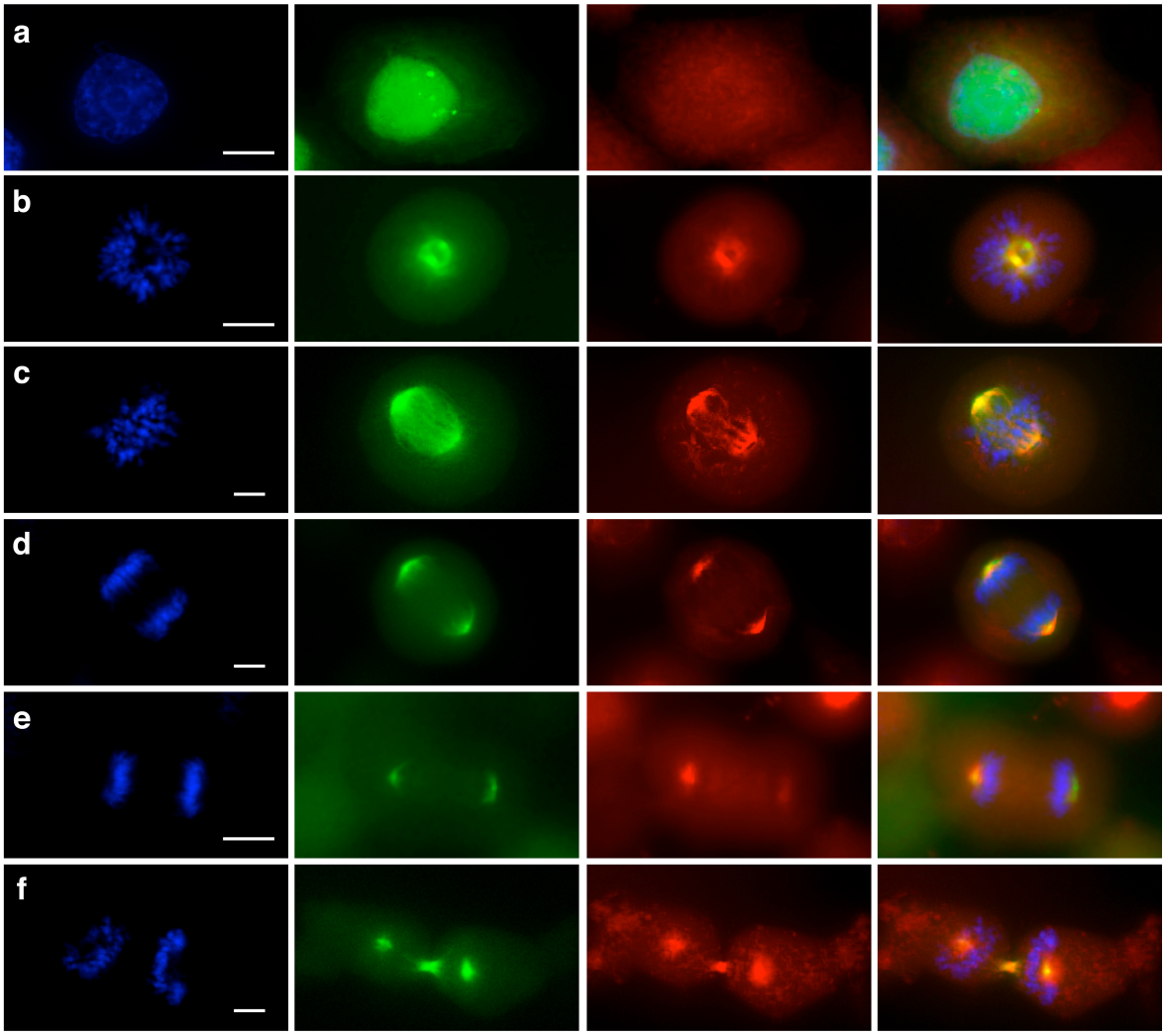


Figure S4. Colocalization between MLN8054-TCO **13** and GFP-AKA during mitosis in PANC-1 Tet-On cells. Cells were incubated for 20 minutes with 1.5 μM MLN8054-TCO **13** (0.1% DMSO), followed by a 20 minute incubation with 1 μM Texas Red-Tz (red, 0.1% DMSO). MLN8054-TCO **13**/Texas Red-Tz colocalized with GFP-AKA (green). Nuclei (blue) were stained using Hoechst and 40X images (1.6 zoom) were collected by DeltaVision deconvolution microscopy. PANC-1 cells are shown at each stage of progression through mitosis A) interphase/ G2, B) prophase, C) metaphase, D) anaphase, E) telophase, and F) cytokinesis. Scale bar: 10μm.

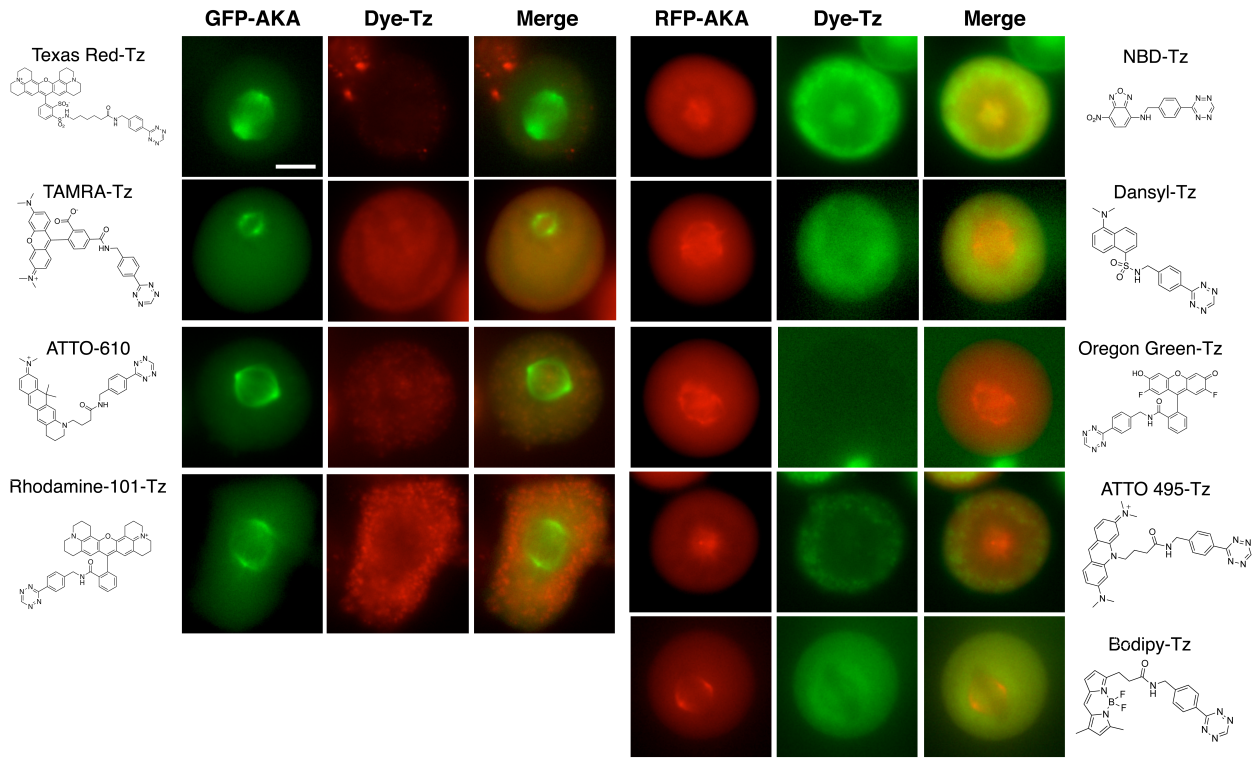


Figure S5. Library of fluorescently-tagged tetrazines for live cell imaging. Live cell colocalization of MLN8054-TCO **13**/Dye-Tz in PANC-1 Tet-On cells expressing GFP-AKA or RFP-AKA. Cells were incubated for 30 minutes with 1 μM MLN8054-TCO **13**. Cells were then washed and incubated for 30 minutes with 1.5 μM Dye-Tz for bioorthogonal reaction inside living cells. After washing, 40X images with 1.6 zoom were collected by DeltaVision deconvolution microscopy. Scale bar: 10 μm.

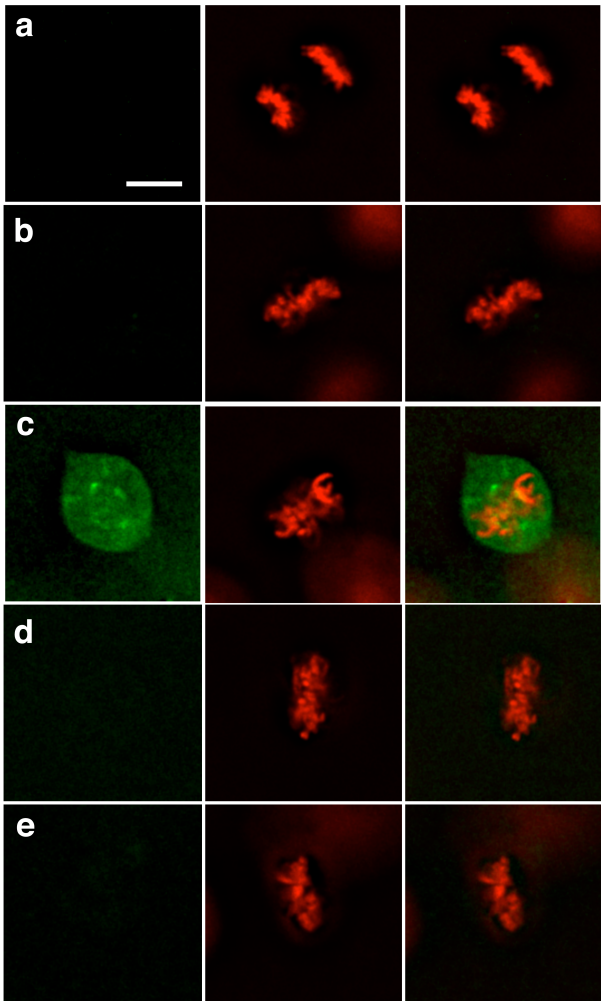


Figure S6. Localization of MLN8054-TCO **13** and controls in HT1080 H2B-Apple cells. Cells were treated for 30 min with 125 nM MLN8054-TCO **13** (0.1% DMSO) or the appropriate control at the same concentration. Following two wash steps, cells were incubated with control or FDA-Tz (1 μM, 0.1% DMSO). A) Control, B) FDA-Tz, C) MLN8054-TCO **13**/FDA-Tz, D) MLN8054-TCO **13** reacted outside the cell with FDA-Tz, and E) MLN8054 **7** (no TCO)/FDA-Tz. Green: MLN8054-TCO **13**, Red: H2B-mApple. Scale bar: 10μm.

Table S1. IC_{50} values of MLN8054 and its derivatives against recombinant AKA.

Compound	IC_{50}	+Error	-Error
MLN8054 **7**	1.6 nM	0.087	0.083
MLN8054[7]	4 nM	---	---
MLN8054-TCO **13**	61.9 nM	12.21	10.20
MLN8054-TCO **13**/Texas Red-Tz	437 nM	142.42	107.43
Texas Red-Tz	>>1 μM	---	---

References

[1] N. K. Devaraj, S. A. Hilderbrand, R. Upadhyay, R. Mazitschek, R. Weissleder, *Angew Chem Int Ed Engl* **2010**, 49, 2869.
[2] N. K. Devaraj, R. Upadhyay, J. B. Haun, S. A. Hilderbrand, R. Weissleder, *Angew Chem Int Ed Engl* **2009**, 48, 7013.
[3] N. K. Devaraj, R. Weissleder, S. A. Hilderbrand, *Bioconj Chem* **2008**, 19, 2297.
[4] S. Cheng, C. M. Tarby, D. D. Comer, J. P. Williams, L. H. Caporale, P. L. Myers, D. L. Boger, *Bioorg Med Chem* **1996**, 4, 727.
[5] M. J. Taylor, D. Perrais, C. J. Merrifield, *PLoS Biol.* **2011**, 9, e1000604.
[6] B. L. Millard, M. Niepel, M. P. Menden, J. L. Muhlich, P. K. Sorger, *Nat Methods* **2011**, 8, 487.
[7] M. G. Manfredi, J. A. Ecsedy, K. A. Meetze, S. K. Balani, O. Burenkova, W. Chen, K. M. Galvin, K. M. Hoar, J. J. Huck, P. J. LeRoy, E. T. Ray, T. B. Sells, B. Stringer, S. G. Stroud, T. J. Vos, G. S. Weatherhead, D. R. Wysong, M. Zhang, J. B. Bolen, C. F. Claiborne, *Proc Natl Acad Sci USA* **2007**, 104, 4106.