**Supporting Information**

# Exploiting Endogenous Enzymes for Cancer-Cell Selective Metabolic Labeling of RNA In Vivo

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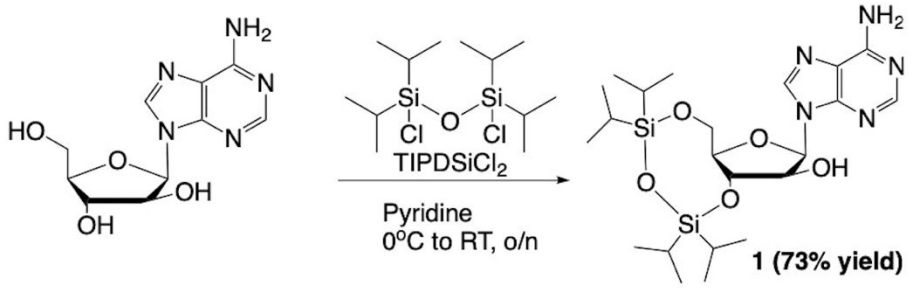
## 1. General

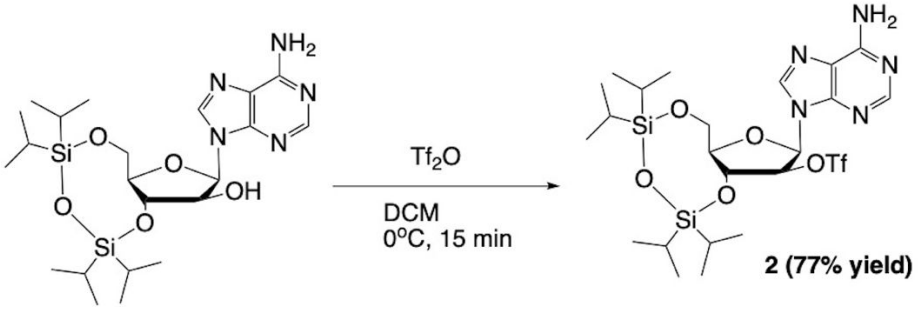
All reagents were purchased from commercial suppliers and were of analytical grade and used without further purification unless otherwise noted. Vidarabine was purchased from Cayman Chemicals (Cat#18149). Reaction progress was monitored by thin-layer chromatography on EMD 60 F254 plates, visualized with UV light. Compounds were purified via flash column chromatography using Sorbent Technologies 60 Å 230 x 400 mesh silica gel. Anhydrous solvents pyridine, dichloromethane (DCM), tetrahydrofuran (THF), dimethylformamide (DMF) were degassed and dried over molecular sieves. All reaction vessels were flame dried prior to use. NMR spectra were acquired with Bruker Advanced spectrometers. All spectra were acquired at 298 K. $^{1}$H-NMR spectra were acquired at 500 MHz. $^{13}$C-NMR spectra were acquired at 500 MHz. Chemical shifts are reported in ppm relative to residual non-deuterated NMR solvent, and coupling constants (J) are provided in Hz. All NMR spectra were analyzed using MestreNova software. Low and high-resolution electrospray ionization (ESI) mass spectra were collected at the University of California-Irvine Mass Spectrometry Facility.




**2. Synthesis**

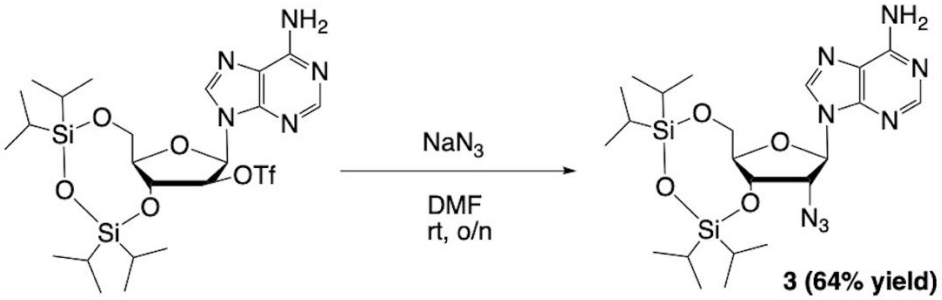
**Synthesis of (6a*R*,8*R*,9*S*,9a*S*)-8-(6-amino-9*H*-purin-9-yl)-2,2,4,4-tetraisopropyltetrahydro-6*H*-furo[3,2-*f*][1,3,5,2,4]trioxadisilocin-9-ol (1).**

Vidarabine (3.74mmol, 1eq) was added to a flame-dried flask and purged with nitrogen. The was flask was then placed in an ice bath and pyridine (40mL) was added. The reaction was spun for 5-10 minutes then TIPDSCl (11.23mmol, 3.59mL, 3eq) was added dropwise and the reaction was left to stir overnight. The pyridine was then coevaporated 3x with toluene, and the resulting residue was resuspended in DCM and washed 2x with water. The organic layer was dried over $MgSO_4$, filtered, concentrated, and purified by column (0 to 10% MeOH/DCM) to yield product in a 73% yield (1.3918g). $^1$H NMR (500MHz, $CDCl_3$) $\delta$ 8.12 (d, *J*=10.8Hz, 2H), 6.58 (s, 1H), 6.21 (d, *J*=5Hz, 1H), 4.64 (t, *J*=6.6Hz, 1H), 4.54 (t, *J*=7.7Hz, 1H), 4.05 (m, 2H), 3.84 (m, 1H), 1.10 (m, 28H). $^{13}$C NMR (500MHz, $CDCl_3$) $\delta$ 155.80, 152.58, 149.38, 140.01, 119.51, 84.06, 81.38, 76.46, 74.39, 61.51, 17.66, 17.56, 17.51, 17.22, 17.12, 17.07, 13.67, 13.21, 13.06, 12.55. HRMS: Theoretical 532.2388 [M+Na$^+$], Observed 532.2383 [M+Na$^+$].




**Synthesis of (6a*R*,8*R*,9*R*,9a*R*)-8-(6-amino-9*H*-purin-9-yl)-2,2,4,4-tetraisopropyltetrahydro-6*H*-furo[3,2-*f*][1,3,5,2,4]trioxadisilocin-9-yl trifluoromethanesulfonate (2).**

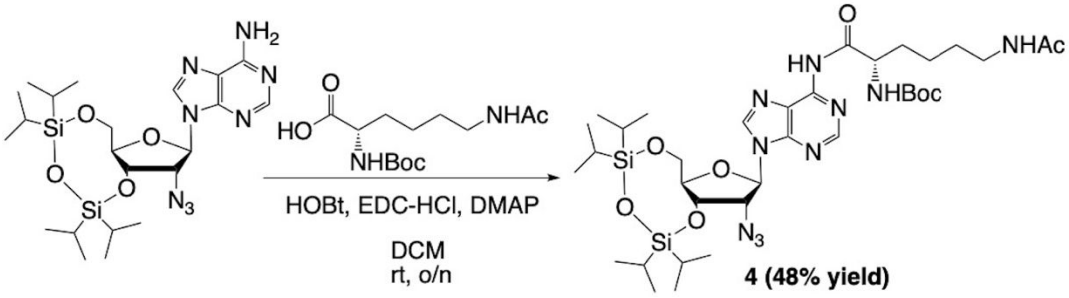
Triflic anhydride (3.01mmol, 0.505mL, 1.1eq) was added to a solution of compound **1** (2.73mmol, 1eq) and DMAP (19.11mmol, 7eq) in DCM (36mL) at 0°C under argon. The reaction was monitored by TLC until starting material was gone. Then the reaction was diluted with ice-cold 1% AcOH/DCM, and then washed with ice-cold sat. $NaHCO_3$, and washed with ice-cold brine. The organic layer was then dried over $MgSO_4$, filtered, concentrated, purified by column (0 to 10% MeOH/DCM) to give product in 77% yield (1.3476g). $^1$H NMR (500MHz, $CDCl_3$) $\delta$ 8.32 (s, 1H), 7.92 (s, 1H), 6.40 (d, *J*=7.6Hz, 1H), 5.48 (t, *J*=7.9Hz, 1H), 5.39 (t, *J*=8.6Hz, 1H), 4.22 (q, *J*=7.7Hz, 1H), 4.09 (dd, *J*=4.0Hz, 6.5Hz, 1H), 3.96 (m, 1H), 1.19 (m, 7H), 1.07 (m, 21H). $^{13}$C NMR (500MHz, $CDCl_3$) $\delta$ 155.67, 153.27, 149.65, 139.59, 119.86, 88.50, 81.23, 80.86, 74.39, 62.17, 17.47, 17.40, 17.36, 17.32, 16.89, 16.82, 16.79, 16.78, 13.27, 13.12, 13.03, 12.64.
HRMS: Theoretical 664.1880 [M+Na$^+$], Observed 664.1867 [M+Na$^+$].




**Synthesis of 9-((6a*R*,8*R*,9*R*,9a*S*)-9-azido-2,2,4,4-tetraisopropyltetrahydro-6*H*-furo[3,2-*f*][1,3,5,2,4]trioxadisilocin-8-yl)-9*H*-purin-6-amine (3).**

Compound **2** (0.791mmol, 1eq) was dissolved in dry DMF (20mL) followed by addition of $NaN_3$ (3.95mmol, 5eq). The reaction was left to stir overnight at room temperature. Then the reaction was diluted in EtOAc and washed 3x with water. Organic layer was then washed with brine and dried over $MgSO_4$, filtered, concentrated, and purified by column (isocratic 50/50 EtOAc/Hex) to give final product in a 64% yield (0.269g).

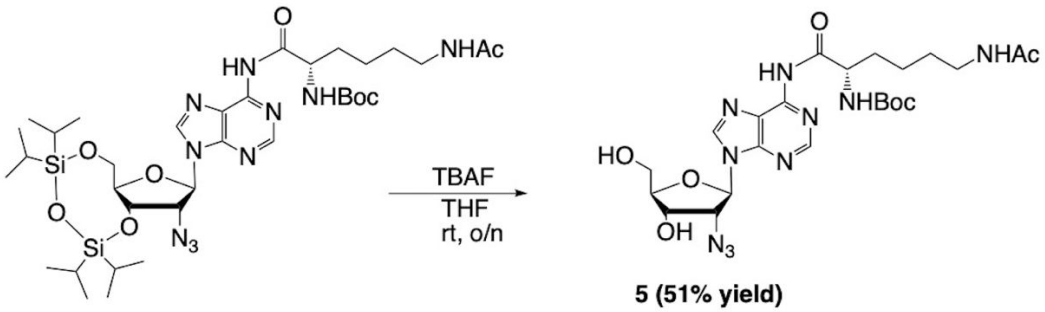
$^1$H NMR (500MHz, $CDCl_3$) $\delta$ 8.35 (s, 1H), 8.06 (s, 1H), 6.34 (s, 2H), 5.83 (s, 1H), 5.22 (q, *J*=4.8Hz, 1H), 4.67 (d, *J*=5.6Hz, 1H), 4.24 (dd, *J*=5.1, 2.3Hz, 1H), 4.17 (dt, *J*=5.3, 2.8Hz, 1H), 4.10 (dd, *J*=5.3, 2.8Hz, 1H), 1.13 (bs, 28H). $^{13}$C NMR (500MHz, $CDCl_3$) $\delta$ 155.80, 153.21, 149.01, 139.06, 120.34, 87.48, 81.77, 71.16, 65.42, 60.19, 17.45, 17.35, 17.31, 17.23, 17.07, 16.97, 16.89, 13.43, 12.99, 12.77, 12.74. HRMS: Theoretical 557.2452 [M+Na$^+$], Observed 557.2452 [M+Na$^+$].




**Synthesis of *tert*-butyl ((*S*)-6-acetamido-1-((9-((6a*R*,8*R*,9*R*,9a*S*)-9-azido-2,2,4,4-tetraisopropyltetrahydro-6*H*-furo[3,2-*f*][1,3,5,2,4]trioxadisilocin-8-yl)-9*H*-purin-6-yl)amino)-1-oxohexan-2-yl)carbamate (4).**

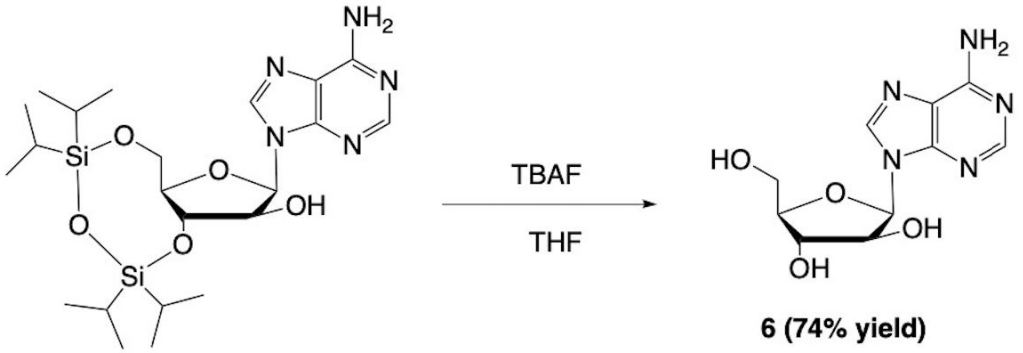
Compound 20 (0.879mmol, 1eq), N-Boc-N-Acyl-Lysine (1.54mmol, 1.75eq), EDC-HCl (1.76mmol, 2eq), HOBt (1.76mmol, 2eq), and DMAP (0.0879mmol, 0.1eq) were combined in a flask, purged with nitrogen, and then dissolved in dry DCM (20mL). The reaction was left to stir at room temperature overnight. Then the reaction was washed with 0.1N HCl (aq), water, and brine. The organic layer was dried over $MgSO_4$, filtered, concentrated, and purified by column (0 to 10% MeOH/EtOAc) to yield product as an off white solid (0.342g, 48% yield). $^1$H NMR (500MHz, $CDCl_3$) $\delta$ 10.08 (s, 1H), 8.68 (s, 1H), 8.30 (s, 1H), 6.50 (s, 1H), 5.81 (s, 2H), 5.21 (q, *J*=4.8Hz, 1H), 4.65 (d, *J*=5.5Hz, 1H), 4.15 (m, 3H), 3.22 (s, 2H), 1.95 (s, 3H), 1.74 (bs, 1H), 1.43 (m, 13H), 1.10 (m, 28H).

$^{13}$C NMR (500MHz, $CDCl_3$) $\delta$ 170.72, 152.46, 150.73, 149.11, 142.22, 87.62, 81.85, 71.18, 65.19, 60.10, 39.15, 28.34, 23.12, 22.79, 17.39, 17.28, 17.25, 17.18, 17.02, 16.91, 16.84, 13.36, 12.92, 12.69. HRMS: Theoretical 291.0593 [M+Na$^+$], Observed 291.0598 [M+Na$^+$].




**Synthesis of *tert*-butyl ((*S*)-6-acetamido-1-((9-((2*R*,3*R*,4*S*,5*R*)-3-azido-4-hydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-9*H*-purin-6-yl)amino)-1-oxohexan-2-yl)carbamate (12).**

Compound 21 (0.109mmol, 1eq) was dissolved in dry THF (5mL) followed by addition of TBAF (0.120mmol, 0.120mL, 1.1eq). The reaction was left to stir for 1h, then the solvent was evaporated and the residue was purified by column (0 to 10% MeOH/EtOAc) to yield final product as an off white compound (0.0314g, 51% yield). $^{1}$H NMR (500MHz, DMSO) $\delta$ 10.79 (s, 1H), 8.71 (s, 1H), 8.68 (s, 1H), 7.80 (t, *J*=5.5Hz, 1H), 7.12 (d, *J*=7.7Hz, 1H), 6.14 (d, *J*=5.6Hz, 1H), 6.08 (d, *J*=5.4Hz, 1H), 5.15 (t, *J*=5.9Hz, 1H), 4.70 (t, *J*=5.8Hz, 1H), 4.59 (q, *J*=5.0Hz, 1H), 4.38 (bs, 1H), 4.01 (q, *J*=4.0Hz, 1H), 3.72-3.68 (m, 1H), 3.61-3.56 (m, 1H), 3.00 (bs, 2H), 1.76 (s, 3H), 1.57 (bs, 2H), 1.38 (s, 13H). $^{13}$C NMR (500MHz, DMSO) $\delta$ 168.89, 155.64, 151.91, 151.50, 149.60, 142.55, 123.60, 85.92, 85.36, 78.12, 70.87, 64.53, 60.72, 57.50, 54.96, 31.07, 28.88, 28.23, 23.20, 23.08, 22.65, 19.23, 13.52. HRMS: Theoretical 585.2510 [M+Na$^{+}$], Observed 585.2504 [M+Na$^{+}$].




**Synthesis of (2*R*,3*S*,4*R*,5*R*)-5-(6-amino-9*H*-purin-9-yl)-4-azido-2-(hydroxymethyl)tetrahydrofuran-3-ol (6).**

Compound **3** (1.28mmol, 1eq) was dissolved in dry THF (10mL) followed by addition of 1M TBAF in THF (1.41mmol, 1.41mL, 1.1eq). The reaction was monitored by TLC and once starting material was gone the reaction was then dried down and purified by column (100% EtOAc to 10% MeOH/EtOAc) to yield product **6** as a white solid (0.267g, 74% yield). $^{1}$H NMR (500MHz, DMSO) $\delta$ 8.41 (s, 1H), 8.18 (s, 1H), 7.39 (s, 2H), 6.07 (m, 2H), 5.32 (t, *J*=5.7Hz, 1H), 4.67 (t, *J*=5.8Hz, 1H), 4.57 (q, *J*=4.7Hz, 1H), 4.03 (q, *J*=3.8Hz, 1H), 3.70 (m, 1H), 3.60 (m, 1H). $^{13}$C NMR (500MHz, DMSO) $\delta$ 156.65, 153. 18, 149.50, 139.92, 119.64, 86.60, 85.72, 71.62, 64.69, 61.56. HRMS: Theoretical 315.0930 [M+Na$^{+}$], Observed 315.0922 [M+Na$^{+}$].

## 3. Biochemical Methods

### Cell lines and culture conditions

DU145 cell lines, HeLa cell lines, and HEK cell lines were cultured in DMEM supplemented with 10% FBS, penicillin and streptomycin and grown at 37 °C, 5% $CO_2$. MCF7 cell lines were cultured in RPMI 1640 supplemented with 10% FBS, penicillin and streptomycin and grown at 37 °C, 5% $CO_2$. MCF10A cell lines were cultured in Mammary Epithelium Basal Medium supplemented with 5% Horse serum, 20 ng/mL EGF, 0.5 mg/mL Hydrocortisone, 100ng/mL Cholera toxin, 10μg/mL Insulin, peniciliin and streptomycin, and grown at 37 °C, 5% $CO_2$. MDAMB231 cell lines were cultured in DMEM with 110 mg/L Sodium pyruvate supplemented with 10% FBS, penicillin and streptomycin, and grown at 37 °C, 5% $CO_2$. K562 cell lines were cultured in RPMI 1640 supplemented with 2 mM L-Glutamine, 1 μM Imatinib, and 10% FBS, penicillin and streptomycin and grown at 37 °C, 5% $CO_2$. PC3 cell lines were cultured in F12k medium supplemented with 10% FBS, penicillin and streptomycin, and grown at 37 °C, 5% $CO_2$. LS174T cell lines were cultured in DMEM with 1 mM Sodium pyruvate and supplemented with 10% FBS, penicillin and streptomycin, and grown at 37 °C, 5% $CO_2$. HepG2 cell lines were cultured in DMEM with 1 g/L Glucose and 1mM Sodium pyruvate supplemented with 10% FBS, penicillin and streptomycin, and grown at 37 °C, 5% $CO_2$. CCD841CoN cell lines were cultured in DMEM with 1 mM Sodium pyruvate supplemented with 10% FBS, penicillin and streptomycin, and grown at 37 °C, 5% $CO_2$.

### Azido-nucleoside labeling of cellular RNA.

For the time course study, azido-nucleoside analogs were added to complete culture medium with a final concentration of 1 mM, at <1% DMSO, and was incubated at 0 h, 1 h, 5 h, 12 h, 16 h, and 2 4h. For titration of analog concentration, azido-nucleoside analogs were added to complete culture medium from 400 mM and 100mM with a final concentration of 1 mM, 500 μM, 250 μM, 100 μM, and 10 μM, at <1% DMSO, for an incubation of 5 h.

### Enzyme inhibitor treatment

<u>High concentration probe administration:</u> Trichostatin A and Z-FY-CHO (Fisher Scientific) were dissolved in nuclease-free DMSO to final concentration of 100 mM, respectively. TSA and Z-FY-CHO were added to complete culture medium to a final concentration of either 0.2 mM or 0.5 mM followed by addition of Lys2'$N_3$A or 2'$N_3$A to final concentration of 0.5 mM. The cells were incubated for 5 hours.

<u>Low concentration probe administration:</u> Trichostatin A and Z-FY-CHO (Fisher Scientific) were dissolved in nuclease-free DMSO to final concentration of 100 mM, respectively. TSA and Z-FY-CHO were added to complete culture medium to titrated final concentrations followed by addition of Lys2'$N_3$A to final concentration of 0.05 mM. The cells were incubated for 5 hours.

**RNA isolation and biotinylation via CuAAC.**
After labeling, total cellular RNA was harvested using Trizol Reagent (Invitrogen) following the manufacturer's instructions. Click reactions were prepared using 5 μg of total RNA, 1 mM biotin alkyne or biotin azide, and fresh 4.6 mM THPTA to a final concentration of 1 mM, fresh 10.6 mM Sodium ascorbate to a final concentration of 1.77 mM, and 12 mM $CuSO_4$ to a final concentration of 200 μM. The reactions were incubated at room temperature on shaker for 30 min. The reactions were purified using Qiagen RNAeasy Mini Purification kit according to the manufacturer instructions (Qiagen), and RNA was eluted in 12 μL of nuclease free water.

**HRP-streptavidin dot blot analysis.**
All gel reagents were from Bio-Rad. Equal amounts of column-purified RNA were applied onto Hybond-N+ membrane (GE Healthcare) as individual dots. RNA was transferred onto Hybond-N+ membrane using a standard vacuum blotter (Biometra, Analytic Jena Company), and UV-crosslinked to a membrane (Stratalinker UV crosslinker). Membranes were blocked followed by incubation with high sensitivity streptavidin-HRP (ThermoFisher Scientific). The membrane was washed twice in a 1:10 solution of blocking buffer and twice in Tris-saline buffer. It was then incubated in ECL Chemiluminescent Substrate (ThermoFisher Scientific) and imaged on a ChemiDoc MP imaging system (Bio-Rad).

**RNA fluorescence imaging via CuAAC.**
HEK cells were seeded at $2.5x10^5$ and grown on glass cover slips. Cells were treated with 1 mM Lys2'$N_3$A or 2'$N_3$A, and incubated for 5 hours. After labeling, cells were washed three times with DPBS, and fixed and permeabilized for 30 min at room temperature with 3.7% paraformaldehyde and 0.15% Triton-X100. Cells were then washed three times (7 min/each) on orbital shaker with DPBS, blocked with BSA (1 mg/ml in DPBS, 0.45% NaCl and 0.025% $NaN_3$) for 30 min at room temperature, washed twice with DPBS, and incubated with 500 μL of click solution (1 mM $CuSO_4$, 2 mM THPTA ligand, 10 mM Sodium ascorbate, and 15 μM Alkyne-Alexa 488) for 1 hour at 37 °C in the dark. Cells were washed three times for 5 min/each on an orbital shaker: twice with DPBS-0.1% Triton-X100 and once with DPBS. The DNA stain was prepared to a final concentration of 8.1 μM Hoechst 33342, trichloride, trihydrate (Invitrogen H3570) in DPBS and added to each section and left to incubate in the dark for 5 minutes. The sections were then washed 3x with DPBS for 5 minutes each. The slides were then mounted using Vectashield and slides were imaged via fluorescence confocal microscopy using a 40x oil immersion objective on a Leica 700 Carl Zeiss microscope.

**Co-culture RNA Fluorescence imaging via CuAAC**
MDAMB231 cells and HEK cells were seeded at $2.5x10^5$. HEK cells were transiently transfected with 5 μg mCherry on the following day using *jet*PRIME transfection reagent according to manufacturer's manual (Polyplus Transfection, France). MDAMB231 and HEkmCherry cells were mixed and grown on glass cover slips 24 hours post-transfection.

After 19 hours, cells were treated with 1 mM Lys2'$N_3$A or 2'$N_3$A, and incubated for 5 hours. After labeling, cells were washed three times with DPBS, and fixed and permeabilized for 30 min at room temperature with 3.7% paraformaldehyde and 0.15% Triton-X100. Cells were then washed three times (7 min/each) on orbital shaker with DPBS, blocked with BSA (1 mg/ml in DPBS, 0.45% NaCl and 0.025% $NaN_3$) for 30 min at room temperature, washed twice with DPBS, and incubated with 500 μL of click solution (1 mM $CuSO_4$, 2 mM THPTA ligand, 10 mM Sodium ascorbate, and 15 μM Alkyne-Alexa 488) for 1 hour at 37 °C in the dark. Cells were washed three times for 5 min/each on an orbital shaker: twice with DPBS-0.1% Triton-X100 and one with DPBS. The DNA stain was prepared to a final concentration of 8.1 μM Hoechst 33342, trichloride, trihydrate (Invitrogen H3570) in DPBS and added to each section and left to incubate in the dark for 5 minutes. The sections were then washed 3x with DPBS for 5 minutes each. The slides were then mounted using Vectashield and slides were imaged via fluorescence confocal microscopy using a 40x oil immersion objective on a Leica 700 Carl Zeiss microscope.

**IP Injection Experiment**

NOD/SCID mice were injected with MDAMB231 cells into mammary pads once on each side. Once tumors reached appreciable size, the mice received direct peritoneal cavity injections to the lower quadrant of the abdomen, lateral to the animal's midline. Injections of 100 μL of either DMSO, 2'N3A, or Lys2'N3A, at a concentration of 2 mg/mL once per 24 h over three days. After three days, the mice were sacrificed, and the tumors and organs were collected and placed in a solution of 4% paraformaldehyde. The tumors and organs were fixed for 1 h at 4 °C with gentle shaking. After 1 hour, the PFA was removed and the samples were washed in PBS buffer for 10 minutes at 4 °C with gentle shaking. The wash was repeated twice. After the third wash, the PBS was removed and the samples were place in a 30% sucrose solution and left at 4 °C with gentle shaking overnight. The samples were then removed from the sucrose solution and placed in Tissue Tek-OCT (VWR 25608-930) and frozen. The frozen blocks were sectioned on a ThermoScientific Cryostar NX50 Cryostat. Sections were cut at 20 μM thickness and placed onto VWR Superfrost Plus Micro slides (VWR 48311-703). Slides were placed at -80 °C until further needed.

**Direct Tumor Injection Experiment**

NOD/SCID mice were injected with MDAMB231 cells into mammary pads once on each side. Once tumors reached appreciable size, the mice received direct tumor injections of 100 μL of either DMSO, 2'N3A, or Lys2'N3A, at a concentration of 10 mg/mL once per 24 h over three days. After three days, the mice were sacrificed, and the tumors and organs were collected and placed in a solution of 4% paraformaldehyde. Slides and organs were prepared as described above (IP Injection Experiment).

**Tumor and Organ Slice Imaging**

Slides were removed from -80 °C and place in 60 °C incubator for 10 minutes and then washed 3x for 5 minutes each with DPBS to remove excess OCT. The slides were then dried and a hydrophobic barrier was drawn around each section with a PAP Pen. From this point on, all washes were done at room temperature with no shaking unless otherwise stated. The sections were then washed with 0.1% Triton in DPBS to permeabilize for 30 minutes. Then the sections were washed 2x with DPBS for 10 minutes each, followed by one wash of DPBS for 5 minutes. The sections were then washed with a 1 mg/mL solution

of BSA in DPBS for 35 minutes, followed by two washes of DPBS for 5 minutes each. The click chemistry cocktail was prepared to a final concentration of 1 mM $CuSO_4$, 2 mM THPTA, 10 mM Sodium ascorbate, and 15 μM Alex488-Alkyne and added to the sections, which were then left to incubate for 1 h at 37 °C in the dark. The click mix was then removed and the sections were washed 3x with 0.1% Triton DPBS for 5 minutes each followed by one wash with DPBS for 5 minutes each. The DNA stain was prepared to a final concentration of 8.1 μM Hoechst 33342, trichloride, trihydrate (Invitrogen H3570) in DPBS and added to each section and left to incubate in the dark for 5 minutes. The sections were then washed 3x with DPBS for 5 minutes each. The slides were then mounted using Vectashield and slides were imaged via fluorescence confocal microscopy using a 20x objective on a Leica 700 Carl Zeiss microscope.

**Tumor and Organ Slice Imaging with RNase A treatment**

Slides were removed from -80 °C and place in 60 °C incubator for 10 minutes and then washed 3x for 5 minutes each with DPBS to remove excess OCT. The slides were then dried and a hydrophobic barrier was drawn around each section with a PAP Pen. From this point on, all washes were done at room temperature with no shaking unless otherwise stated. The sections were incubated in 0.5% Triton-100 in 1x PBS pH 7.4 for 5 minutes and then washed 3x with DPBS for 5 minutes each. The sections were then incubated in a 0.2 mg/mL solution of RNase A in 0.5% Triton in 1x PBS pH 7.4 for 1h, followed by 3x washes with DPBS for 5 minutes each. The sections were then washed with 0.1% Triton in DPBS to permeabilize for 30 minutes. Then the sections were washed 2x with DPBS for 10 minutes each, followed by one wash of DPBS for 5 minutes. The sections were then washed with a 1 mg/mL solution of BSA in DPBS for 35 minutes, followed by two washes of DPBS for 5 minutes each. The click chemistry cocktail was prepared to a final concentration of 1 mM $CuSO_4$, 2 mM THPTA, 10 mM Sodium ascorbate, and 15 μM Alex488-Alkyne and added to the sections, which were then left to incubate for 1h at 37 °C in the dark. The click mix was then removed and the sections were washed 3x with 0.1% Triton DPBS for 5 minutes each followed by one wash with DPBS for 5 minutes each. The DNA stain was prepared to a final concentration of 8.1 μM Hoechst 33342, trichloride, trihydrate (Invitrogen H3570) in DPBS and added to each section and left to incubate in the dark for 5 minutes. The sections were then washed 3x with DPBS for 5 minutes each. The slides were then mounted using Vectashield and slides were imaged via fluorescence confocal microscopy using a 20x objective on a Leica 700 Carl Zeiss microscope.

**Isolation of RNA from organs and dot blot**

Mice were treated the same as the IP Injection Experiment above. Total RNA from organs was isolated using Trizol reagent (ThermoFisher). Extracted RNA was purified using RNeasy spin columns (Qiagen). Equal amounts of column-purified RNA were applied onto Hybond-N+ membrane (GE Healthcare) as individual dots. RNA was transferred onto Hybond-N+ membrane using a standard vacuum blotter (Biometra, Analytic Jena Company), and UV-crosslinked to a membrane (Stratalinker UV crosslinker). Membranes were blocked followed by incubation with high sensitivity streptavidin-HRP (ThermoFisher Scientific). The membrane was washed twice in a 1:10 solution of blocking buffer and twice in Tris-saline buffer. It was then incubated in ECL Chemiluminescent Substrate (ThermoFisher Scientific) and imaged on a ChemiDoc MP imaging system (Bio-Rad).

**MTT assay:**

MTT assay (Vybrant MTT Assay Kit, Thermofisher) was performed in 96-well plates with MDA-MB-231 cells seeded at densities of 10,000 cells per well. Cells were grown for 24 hours in phenol-red free media and treated with 1 mM of DMSO, 2'N3A, or Lys2'N3A, including untreated cells used for negative controls. Each treatment condition had a total of 6 wells per replicate. Following 24 hours of treatment, cell viability was measured by removing cell media and replacing it with 100 µl of phenol-red free media followed by addition of 10 µl of 12 mM MTT stock solution to each well. The cells were incubated in the MTT solution for 3 hours at 37 °C. Formazan salts were solubilized with 50 µl of DMSO per well and incubated for 30 min at 37 °C. The samples were homogenously mixed while avoiding formation of bubbles and the absorbances were measured at 540 nm using a Clariostar Plus microplate reader. Absorbance values from treated and untreated cells were used to calculate percentages of cell viability [100*(treated absorbance/untreated absorbance) = % cell viability]. For a total of 3 replicate MTT assays, the standard error of the mean (SEM) was calculated for measuring sample variability.

**Protein isolation and Immunoblot analysis**.

Cells were grown and isolated by pellet centrifugation. To each cell pellet, 200 µL RIPA lysis buffer, supplemented with 4 µL of proteinase inhibitor cocktail Set VII (EMB, Cat#: 539138-1ML), 2 µL Turbo DNase (Life Tech., Cat# AM2239), 2 µL RNase A (Thermo Fisher, Cat# FEREN0531) per milliliter of lysis buffer, was added. Cells were re-suspended and incubated on ice for 0.5 h then sonicated for 11 seconds (5 seconds ON, 1 second OFF) at 4 °C. WCE was cleared by centrifugation at 15 K rpm for 30min at 4 °C. The cleared lysate was subjected to BCA assay (BioSciences, Cat#786-570) to determine protein concentration according to Manufacturer instruction at 37 °C for 30 min and quantified using NanoDrop BCA program. SDS-PAGE analysis was performed: 5 µg of total protein was resolved in 4-20% gradient 10-well MiniProtean gel (Bio-Rad, Cat# 4561094).

Proteins were transferred to nitrocellulose membrane using Trans-Blot Turbo Transfer unit (Bio-Rad, Cat#1704150). Membranes were blocked with 1x PBST (1xPBS + 0.1%Tween-20) + 5% non-fat milk for 1h at room temperature and probed overnight with antibodies displayed below at 4 °C with fresh blocking buffer at 1 µg/mL. Blot was washed 3x10 min/each with 1x PBST at RT and incubated in 1x PBST+ 5% non-fat milk + secondary conjugated Horseradish peroxidase (1:20000 dilution) for 1 h at RT. Finally, blot was washed 3x10 min/each with 1x PBST at RT and imaged using ECL Chemiluminescent Substrate (Fisher Scientific, Cat# PI32106). Protein blots were subsequently stained in Pierce PageBlue Protein staining solution (Fisher Scientific, Cat#: PI24620) and imaged on a ChemiDoc MP imaging system (Bio-Rad).

**HPLC analysis of 2'N3A reaction with HDAC1 and Cathepsin L protease.** Human recombinant histone deacetylase 1 was used as a model HDAC enzyme (Active Motif; 31504). Human recombinant Cathepsin L protease was purchased from R&D systems

(952-CY-010). First, Lys-2'N3A (200 µg) and HDAC1 (0.1 µg) were incubated in buffer containing protein in reaction buffer including 25 mM Tris-HCl pH 8.0, 137 mM NaCl, 2.7 mM $MgCl_2$, 1 mM KCl and 0.1 mg/mL BSA for 30 min at 37 °C for three hours.

The solution was then filtered over a size-exclusion column and the supernatant lyophilized to dryness. To the dried pellet was added buffer solution for Cathepsin L protease (50 mM MES, 5 mM DTT, 1 mM EDTA, 0.005% (w/v) Brij-35, pH 6.0) and 0.001 µg of Cathepsin L protease was added and incubated at 37 $^{o}$C for 3 hours. The solution was spun down and decanted for HPLC loading.

Reactions were analyzed by analytical HPLC chromatography with the gradient from 0% to 50 % of acetonitrile in 0.1 M triethylammonium acetate buffer pH 7.0 over fifty two minutes. Product identities were confirmed by comparing retention times with controls.

**Extraction of cellular RNA and DNA for LC-MS/MS analysis of 2'N3A incorporation.** 2'N3A was added to complete culture medium with a final concentration of 1 mM and incubated for 16 hours. After labeling, total cellular RNA was harvested using Trizol Reagent (Invitrogen) following the manufacturer's instructions. Genomic DNA was extracted from cell pellets suspended in lysis buffer of 1M NaCl, 50mM Tris-HCl pH 8.0, 250 nM EDTA pH8.0, 10% SDS, 1mg/mL Proteinase K, and sterile water for 1 hour at 50°C. The DNA was extracted from the cell lysates via Phenol:Chloroform:Isoamyl Alcohol (25:24:1 v/v) (ThermoFisher, Cat# 15593031) following the manufacturers instructions. The aqueous phase extracted was then treated with RNase A to a final concentration of 20ug/mL at 37°C for 20 minutes. Finally, 0.5 volumes of 5M ammonium acetate and 2 volumes of ethanol were added to the DNA solution and precipitated overnight at -20°C. The precipitated DNA was pelleted via centrifugation for 10 minutes at 13K rpm before resuspension in nuclease free water for LC-MS/MS analysis.

**LC-MS/MS analysis of 2'N3A incorporation into cellular RNA and DNA.**
DNA or RNA extracts were digested to nucleosides by incubation with the Nucleoside Digestion Mix (New England Biolabs, Cat# M0649S) overnight at 37 °C. Digested DNA and RNA samples were directly analyzed by LC-MS/MS without purification LC-MS/MS analysis was performed in duplicate by injecting digested DNA or RNA on an Agilent 1290 UHPLC equipped with a G4212A diode array detector and a 6490A triple quadrupole mass detector operating in the positive electrospray ionization mode (+ESI). UHPLC was carried out on a Waters XSelect HSS T3 XP column (2.1 × 100 mm, 2.5 µm) with the gradient mobile phase consisting of methanol and 10 mM aqueous ammonium formate (pH 4.4). MS data acquisition was performed in the dynamic multiple reaction monitoring (DMRM) mode. Each nucleoside was identified in the extracted chromatogram associated with its specific MS/MS transition: dC $[M+H]^+$ at *m/z* 228→112, dG $[M+H]^+$ at *m/z* 268→152, dT $[M+Na]^+$ at *m/z* 265→149, dA $[M+H]^+$ at *m/z* 252→136, rC $[M+H]^+$ at *m/z* 244→112, rU $[M+Na]^+$ at *m/z* 267→135, rG $[M+H]^+$ at *m/z* 284→152, rA $[M+H]^+$ at *m/z* 268→136, and 2'N3A $[M+H]^+$ at *m/z* 293→136.

## 4. Supplementary Figures

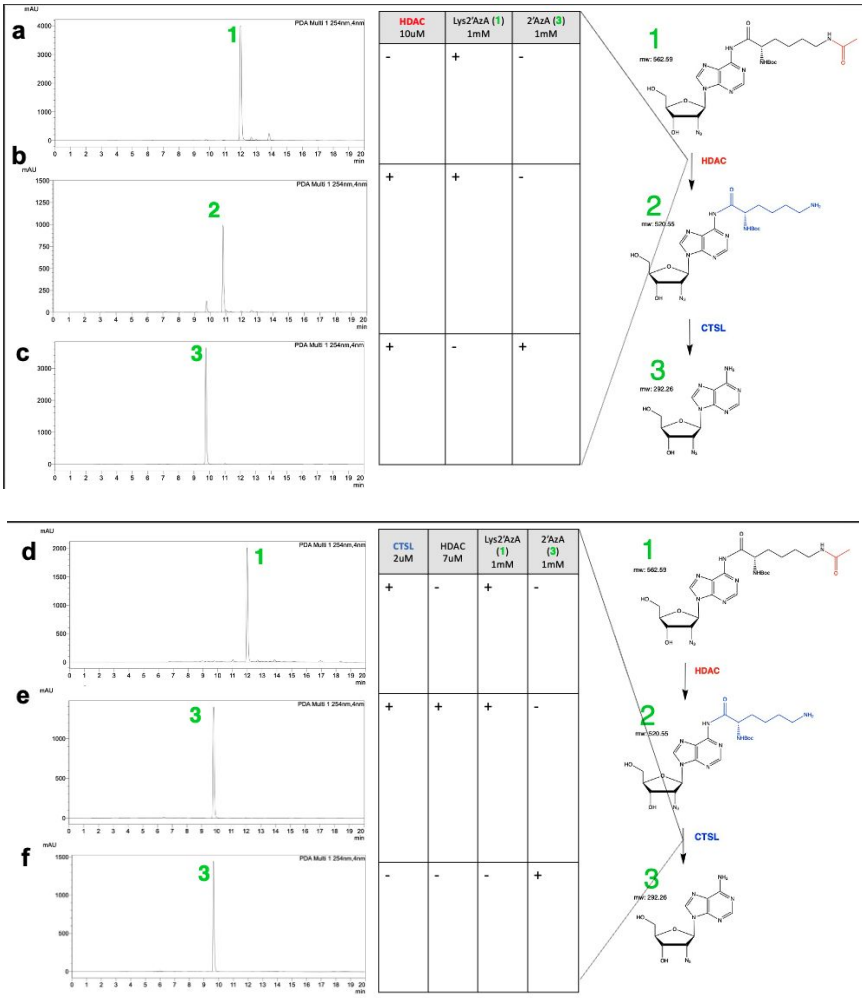



**Figure S1.** Reaction of 2'N3A with HDAC1 and Cathepsin L protease. **a.** HPLC trace of Lys-2'N3A control. **b.** Lys-2'N3A incubated with recombinant HDAC. **c.** HPLC trace of 2'N3A control. **d.** HPLC trace of reaction mixture of Lys-2'N3A after reaction with recombinant CTSL. **e.** HPLC trace of reaction mixture of Lys-2'N3A after reaction with recombinant HDAC and CTSL. **f.** HPLC trace of 2'N3A control.

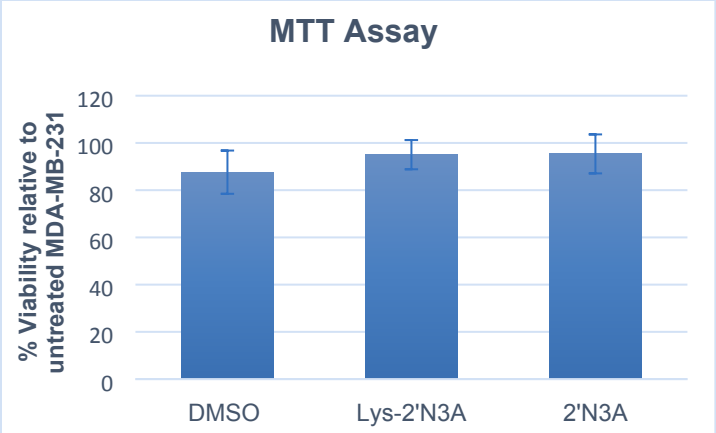



**Figure S2. MTT assay**. MTT assay was performed in 96-well plates with MDA-MB-231 cells seeded at densities of 10,000 cells per well. Cells were grown for 24 hours and then treated with 1 mM of DMSO, 2'N3A or Lys-2'N3A for 24 hours and then cell viability was measured by absorbance at 540 nm using a Clariostar Plus microplate reader. N = 3 replicate MTT assays.

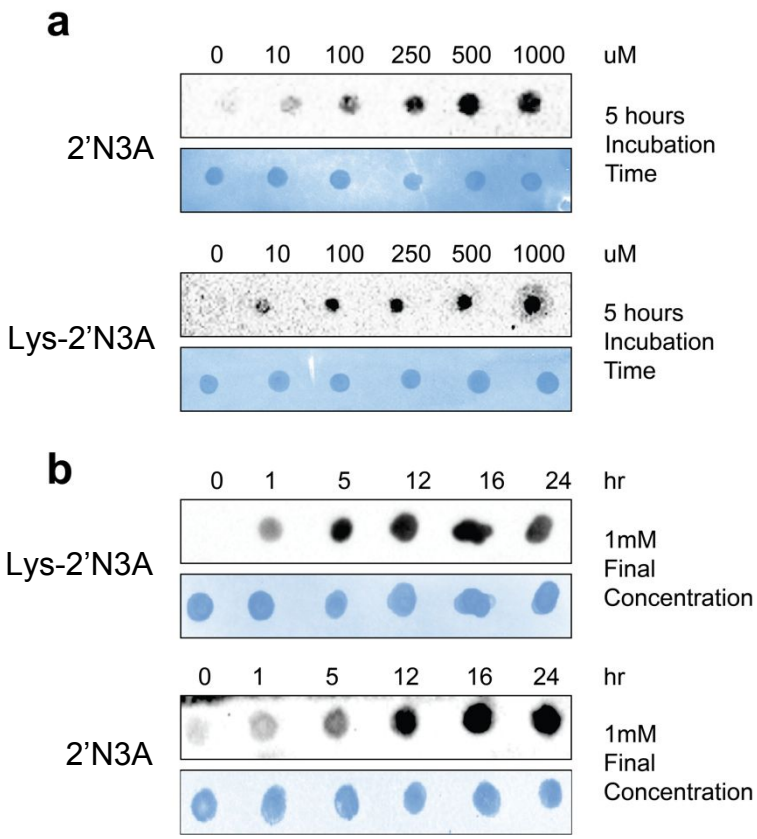



**Figure S3. Time and concentration courses comparing 2'N3A** and **Lys-2'N3A incorporation by dot-blot analysis in MDA-MB-231 cells**. **a.** Concentration titration of 2'N3A and Lys-2'N3A. MDA-MB-231 cells were incubated with increasing concentrations of each analog for 5 hours. RNA was isolated and biotinylated using CuAAC, followed by dot-blot analysis described in the methods. **b.** Time titration of 2'N3A and Lys-2'N3A. Cells were incubated at 1mM final concentration at increasing time intervals. RNA was isolated and biotinylated using CuAAC, followed by dot-blot analysis described in the methods.

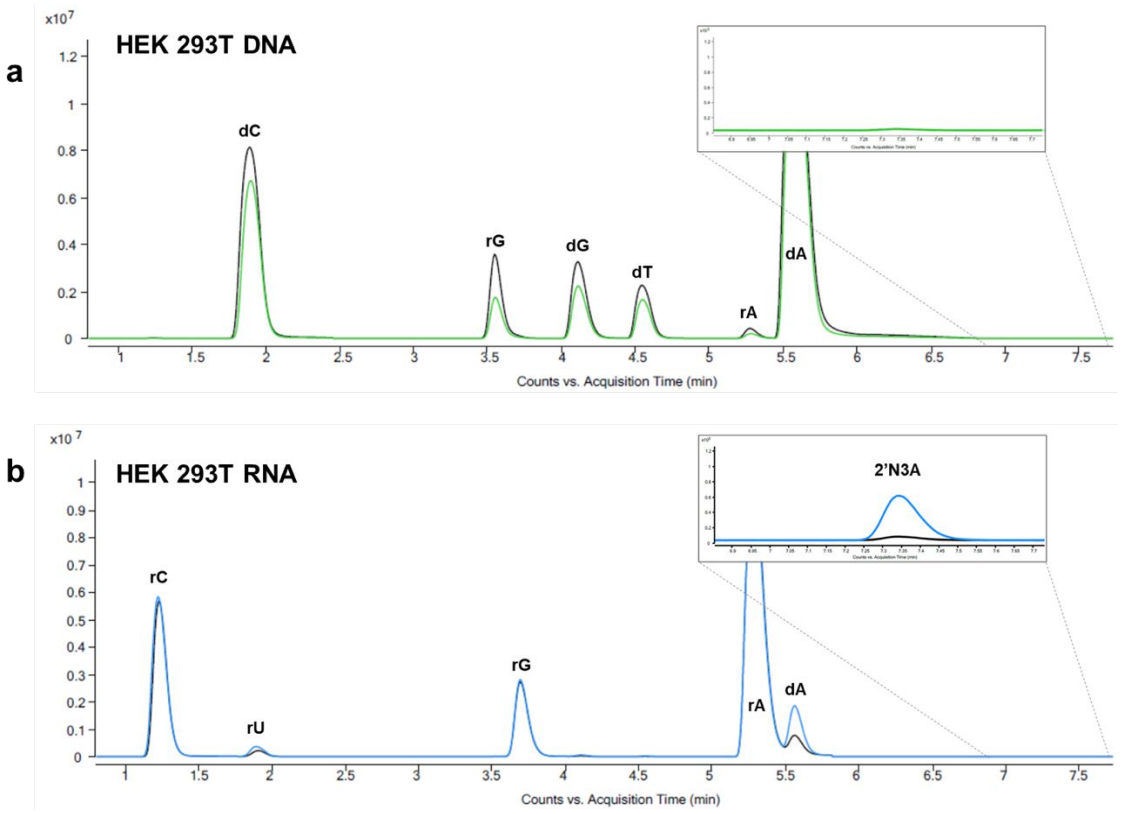
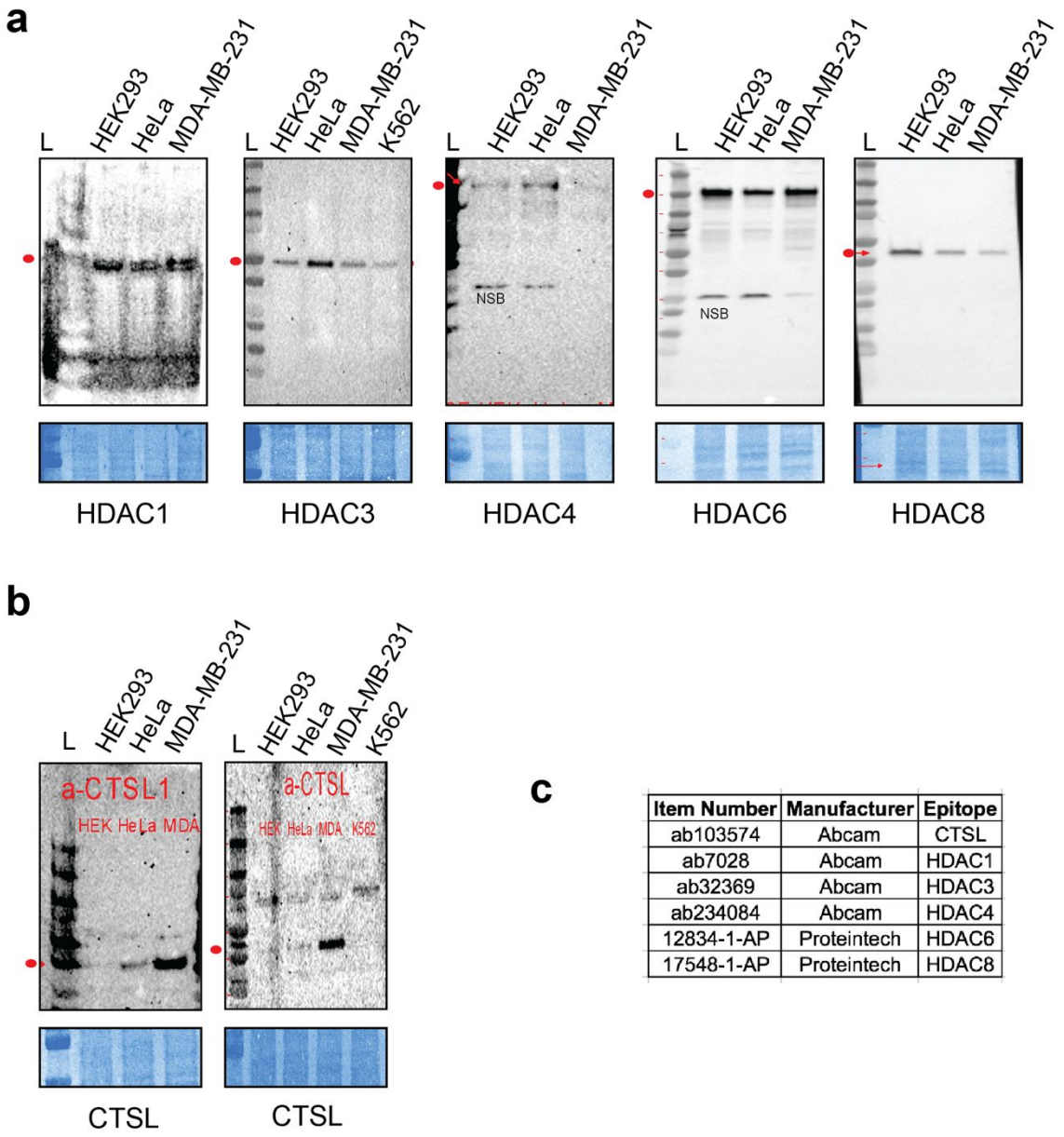



**Figure S4. LC-MS/MS analysis of 2'N3A incorporation into cellular RNA and DNA. a.** Digested DNA from HEK 293T cells that were incubated with 1 mM of 2'N3A (green) overlaid with digested DNA from cells treated with DMSO (black). **b.** Digested total RNA from HEK 293T cells that were incubated with 1 mM of 2'N3A (blue) overlaid with digested RNA from cells treated with DMSO (black). DNA and RNA nucleosides were resolved by reversed-phase HPLC and detected by dynamic multiple reaction monitoring (DMRM). Expansion of the overlaid total ion chromatograms (TIC) demonstrated the specific incorporation of 2'N3A on RNA only.




c

| Item Number | Manufacturer | Epitope |
|---|---|---|
| ab103574 | Abcam | CTSL |
| ab7028 | Abcam | HDAC1 |
| ab32369 | Abcam | HDAC3 |
| ab234084 | Abcam | HDAC4 |
| 12834-1-AP | Proteintech | HDAC6 |
| 17548-1-AP | Proteintech | HDAC8 |

**Figure S5. Western blot analysis**. **a.** Representative Western blots of the HDAC enzymes profiled herein. **b.** Representative Western blots for CTSL enzyme profiled herein. **c.** Antibodies used in this study for HDAC and CTSL. NSB is defined as Non-Specific Band.

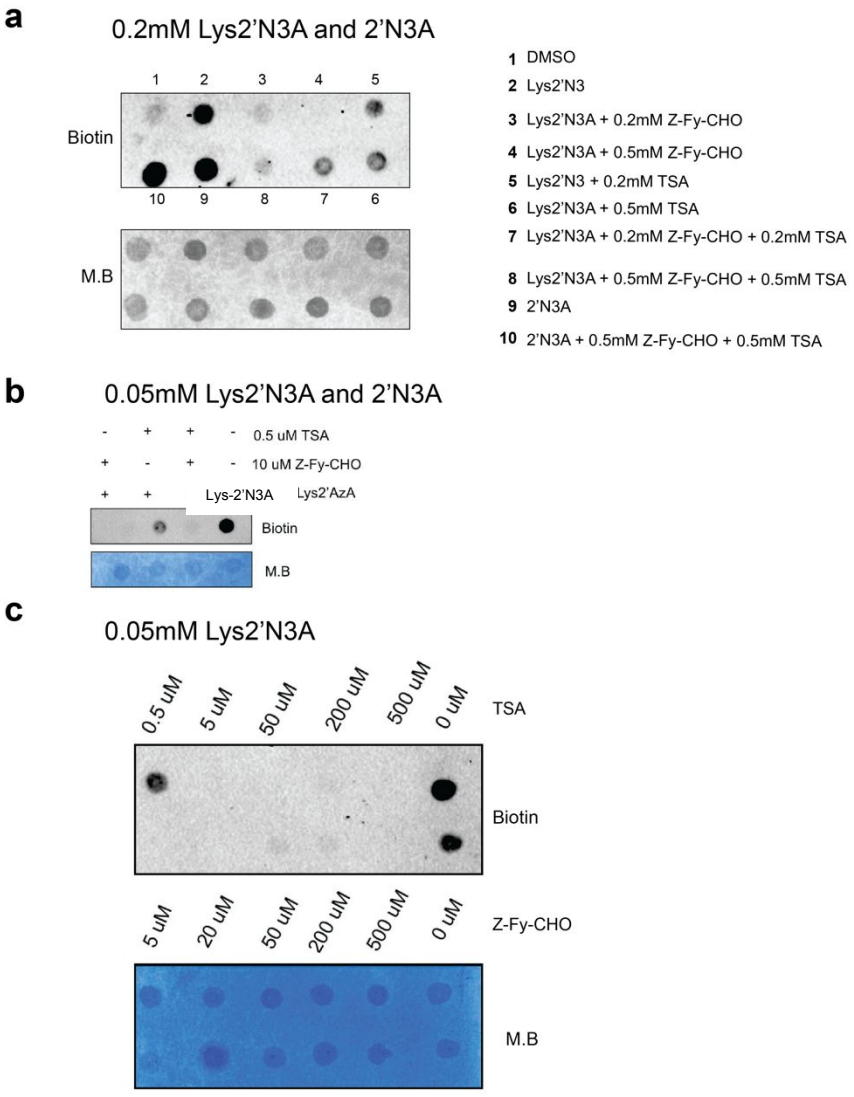



**Figure S6. Comparison of enzyme inhibition of RNA incorporation of liberated 2'N3A at different Lys2'N3A concentrations. a.** Dot-blot analysis demonstrating that HDAC and CTSL enzyme inhibitors suppress Lys-2'N3A uncaging in MDA-MB-231 cells. **b.** Incorporation of Lys-2'N3A at lower concentration is inhibited by HDAC and CTSL inhibitors. **c.** Inhibitor concentration titration against 0.05 mM of Lys-2'N3A.

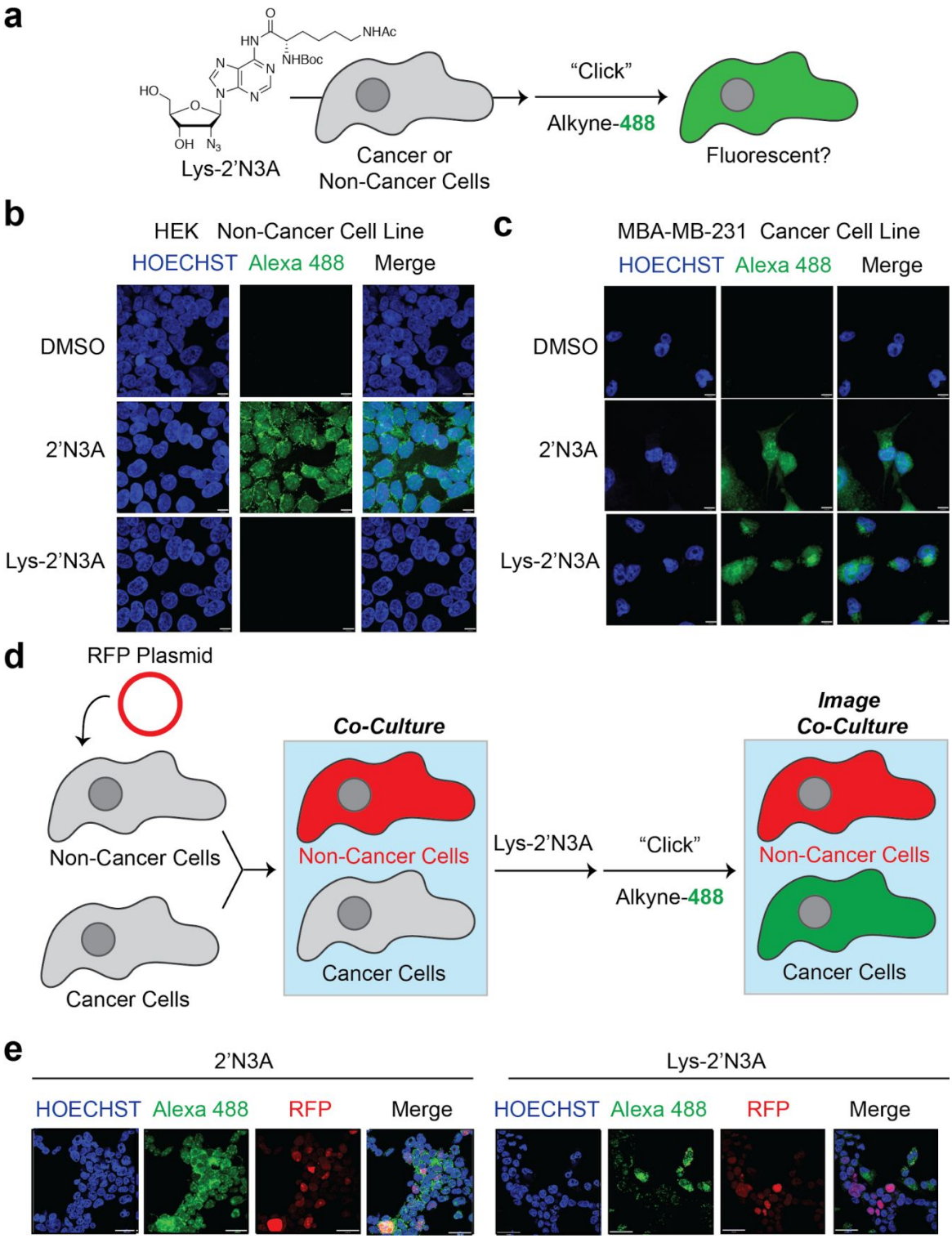



**Figure S6. Imaging cell-specific RNA labeling. a.** Outline of experiments used from monoculture to demonstrate RNA labeling and imaging. Cells were treated with 1 mM Lys2'N3A or 2'N3A for 5 hours. Cells were fixed with paraformaldehyde, lysed, and RNA was imaged using alkyne-Alexa Fluor 488. b. HEK293T cells. **c.** MDA-MB-231 cells. **d.** Schematic of co-culture imaging experiments. HEK293T cells were transfected with RFP

plasmid and co-cultured with non-transfected MDA-MB-231 cells for 24 hours. Cells were treated with 1 mM Lys2'N3A or 2'N3A for 5 hours. Cells were fixed with paraformaldehyde, lysed, and RNA was imaged using alkyne-Alexa Fluor 488. **e.** Imaging results of Lys2'N3A or 2'N3A with cell co-culture HEK and MDA-MB-231 cells. RFP denote HEK cells. Alexa 488 denote MDA-MB-231 cells.

**Figure S6 Note:** As demonstrated in **Fig. S6, b-c**, we observed a fluorescent signal for directly incorporated **2'N3A**, after labeling it with an alkyne-Alexa Fluor 488, in both cell types. However, we were only able to observe a fluorescent signal for uncaged labeled **Lys-2'N3A** in the cancer cell line. Having established the selective metabolic labeling in single cell type systems, we sought to test if **Lys-2'N3A** would incorporate into the RNA of cancer cells in complex mixtures. To test this, we established a co-culture system of cancerous MDA-MB-231 with non-cancerous HEK293T cells, in which the latter were transfected with red-fluorescent protein (RFP). **Lys-2'N3A** was added to the co-culture and cells were processed for imaging with alkyne-Alexa Fluor 488. As demonstrated in **Fig. S6, d & e**, we observed red and green signal (from RFP and Alexa 488, respectively) in mutually exclusive cells, suggesting that **Lys-2'N3A** is not incorporated into non-cancer cells even in co-culture situations. These results further demonstrate the cell-selectivity of cancer cell labeling for **Lys-2'N3A**.

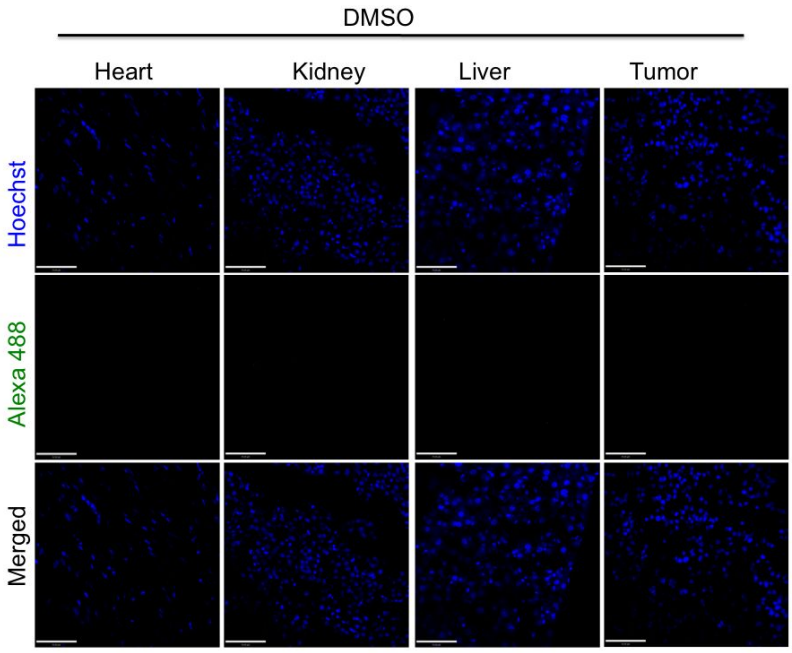


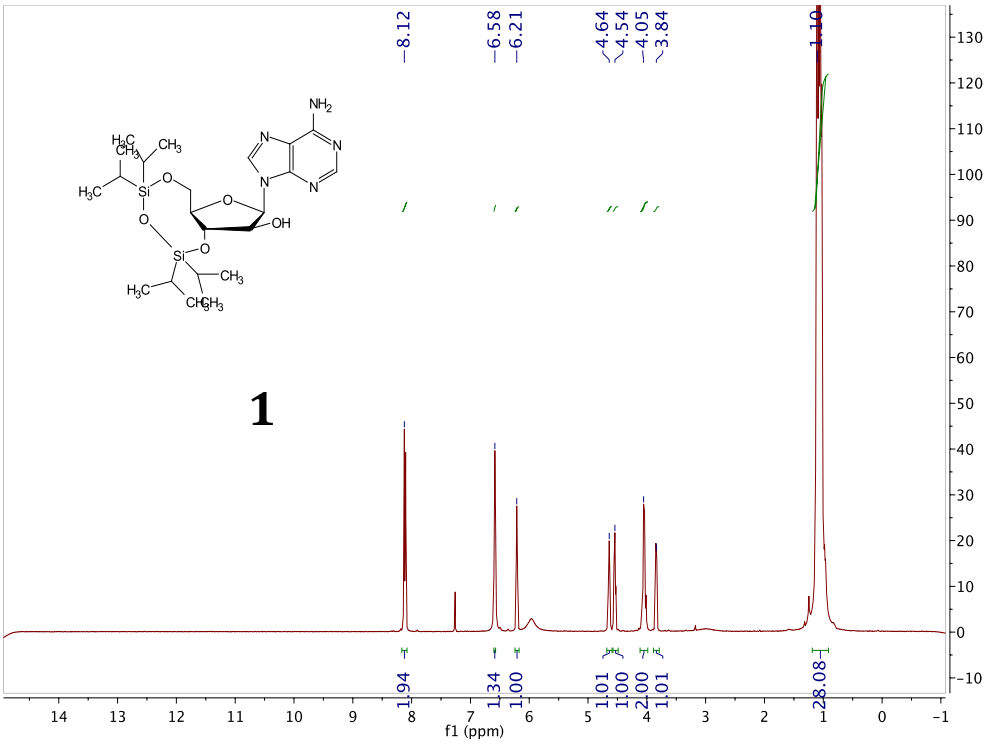

**Figure S7. Imaging cell-specific RNA labeling in vivo, DMSO negative control.** Imaging of isolated organs and tumor tissue from direct tumor injections of DMSO, Lys2'N3A and 2'N3A into mice.

| Cell type | Cancer Type | Cancerous/Non-Cancerous |
|---|---|---|
| HeLa | Cervical cancer | Cancer |
| HEK | Human Embryonic Kidney | Normal |
| MCF10A | human breast epithelial | Normal |
| CCD841 | Epithelial cells | Normal |
| PC3 | Prostate cancer | Cancer |
| DU145 | Prostate cancer | Cancer |
| MDA-MB-231 | Breast cancer | Cancer |
| MCF7 | Breast cancer | Cancer |
| LS174T | Colon adenocarcinoma | Cancer |
| K562 | Myelogenous leukemia | Cancer |
| HepG2 | Liver cancer | Cancer |

**Table S1. Cell types analyzed in this manuscript and their tissue.**

**5. NMR Spectra**

$^1$H NMR (500MHz, $CDCl_3$)



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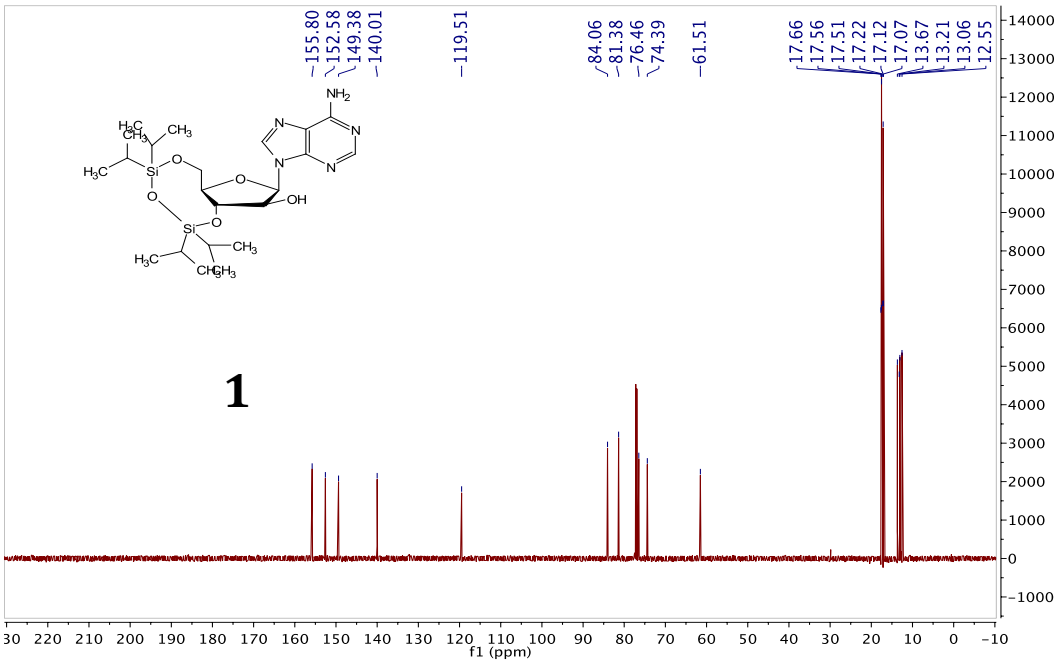

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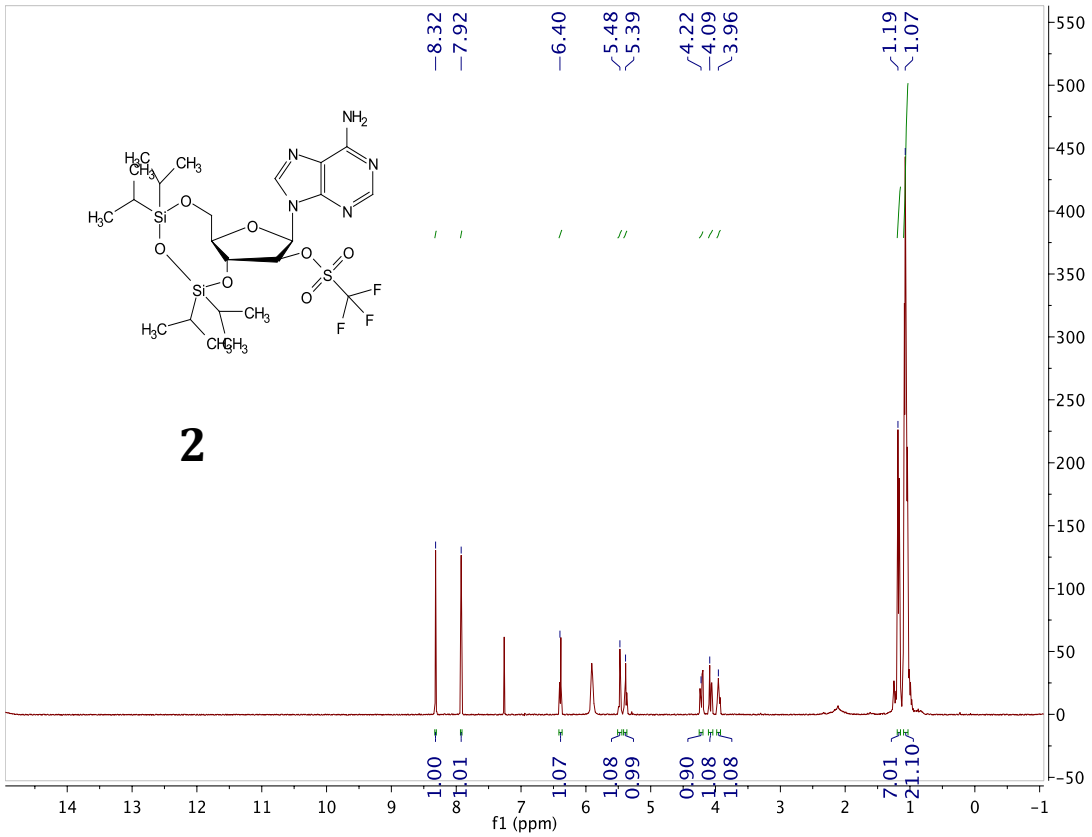

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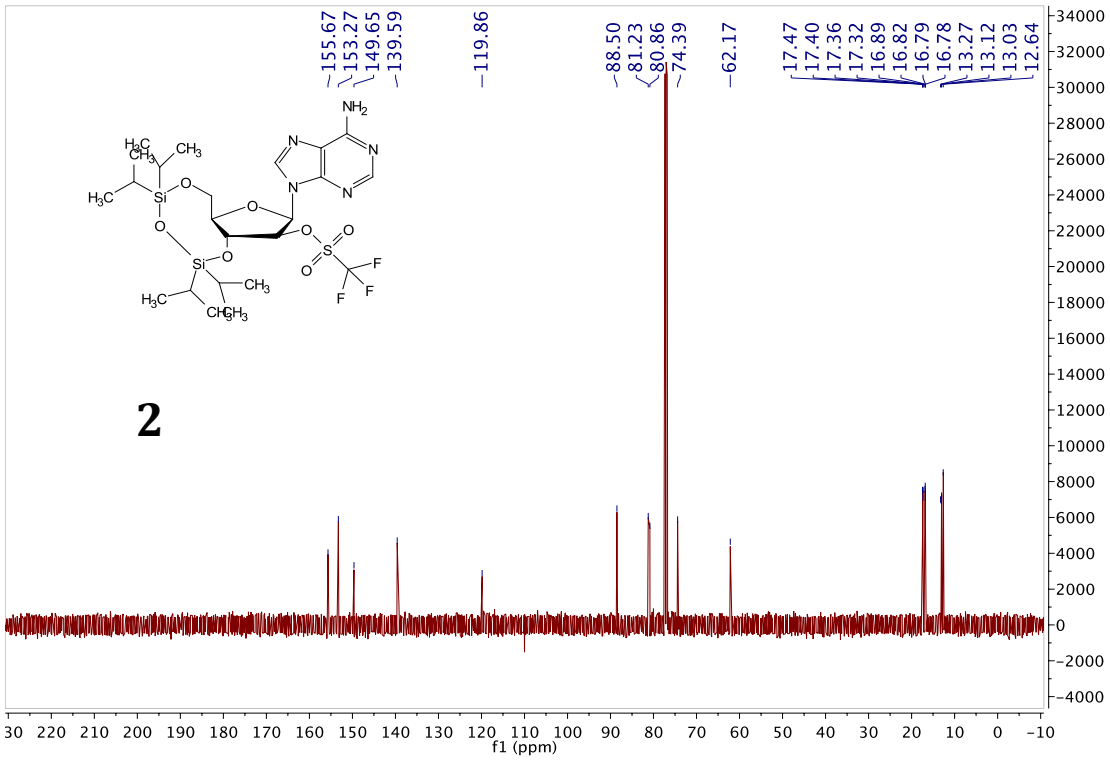

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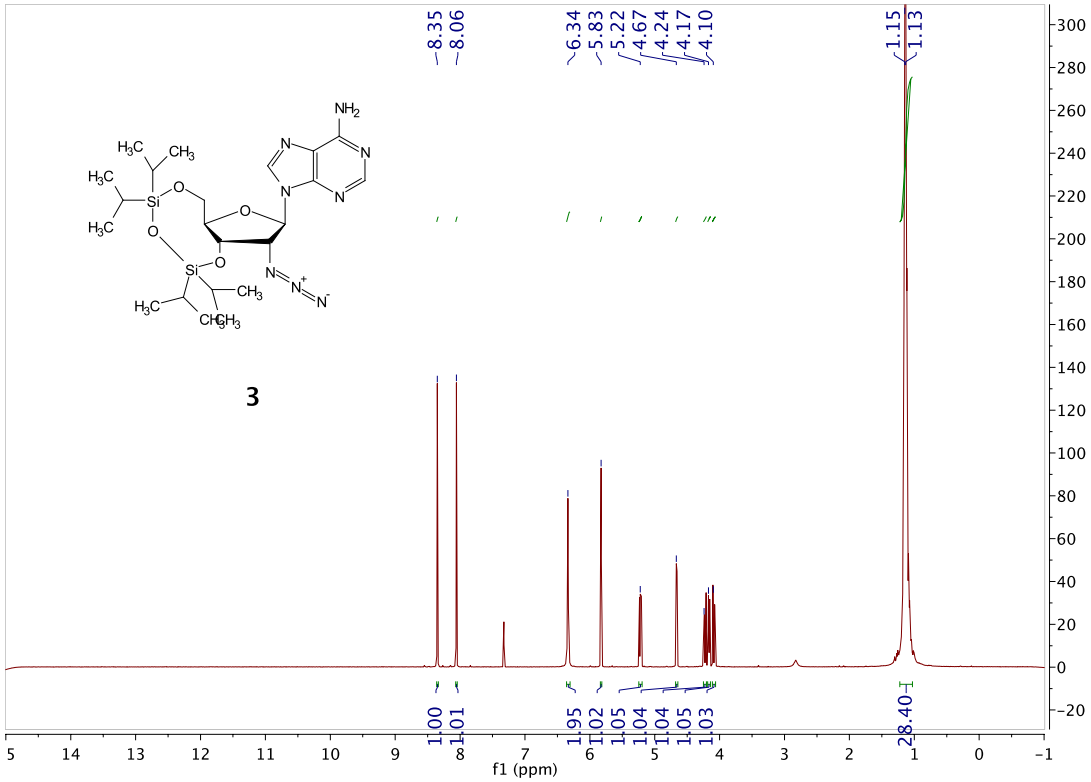
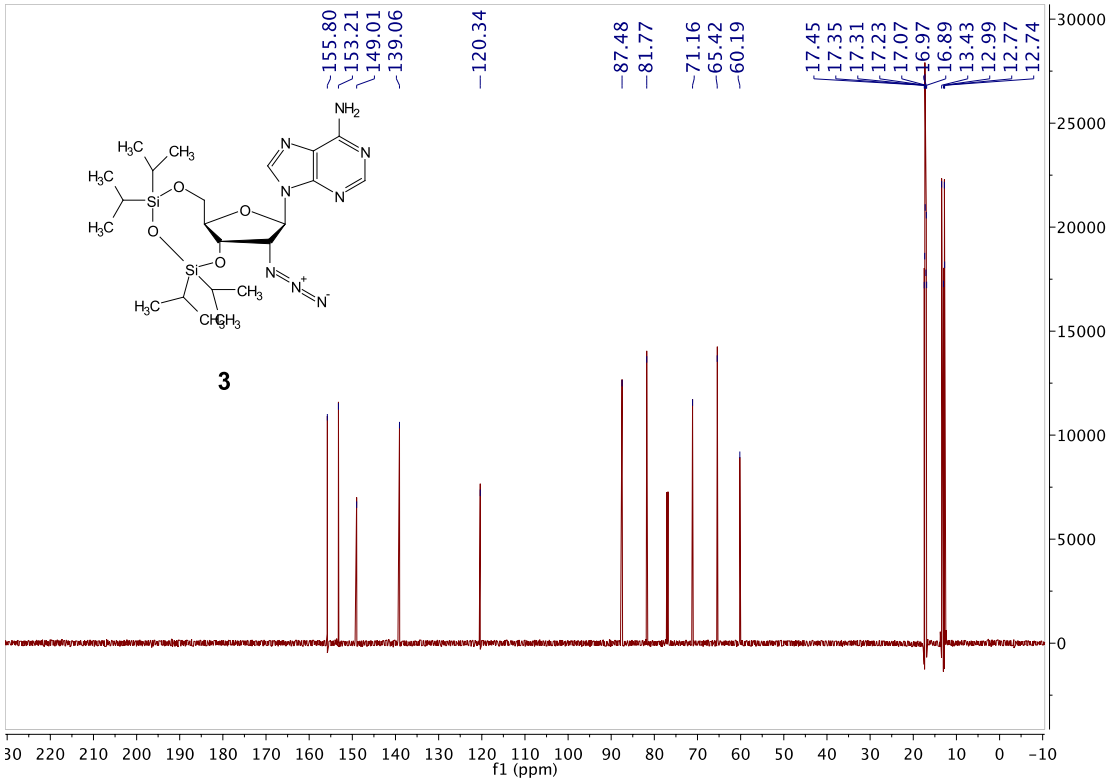



**3**

$^{13}C$ NMR (500MHz, $CDCl_3$)



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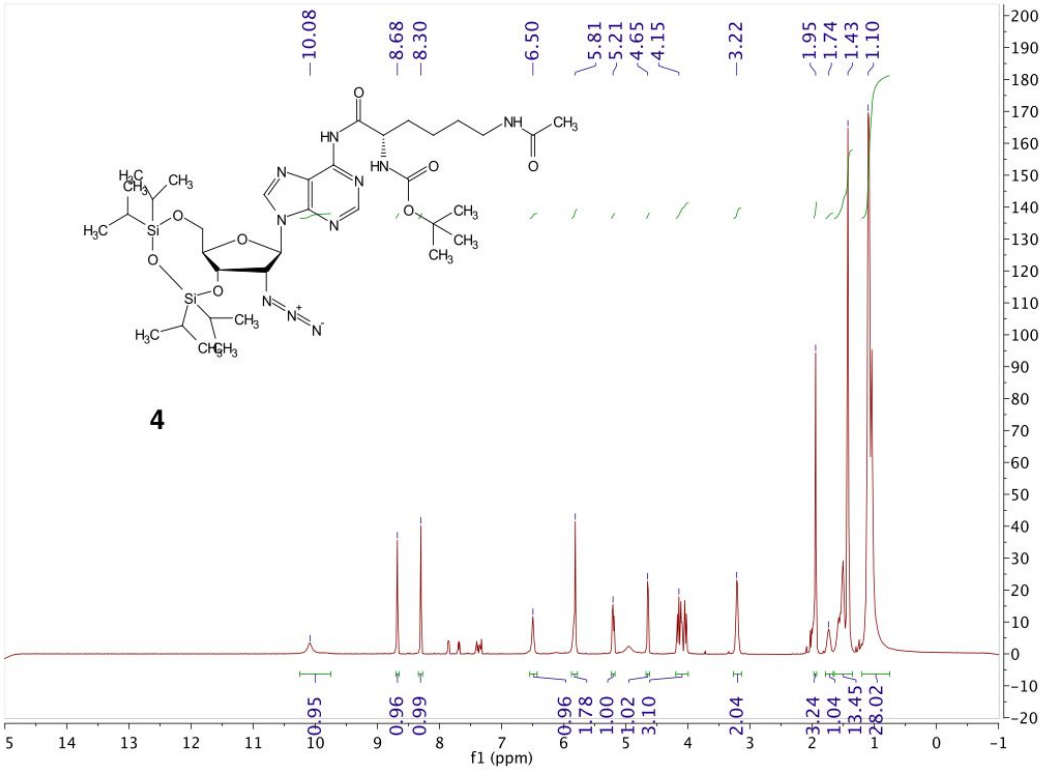

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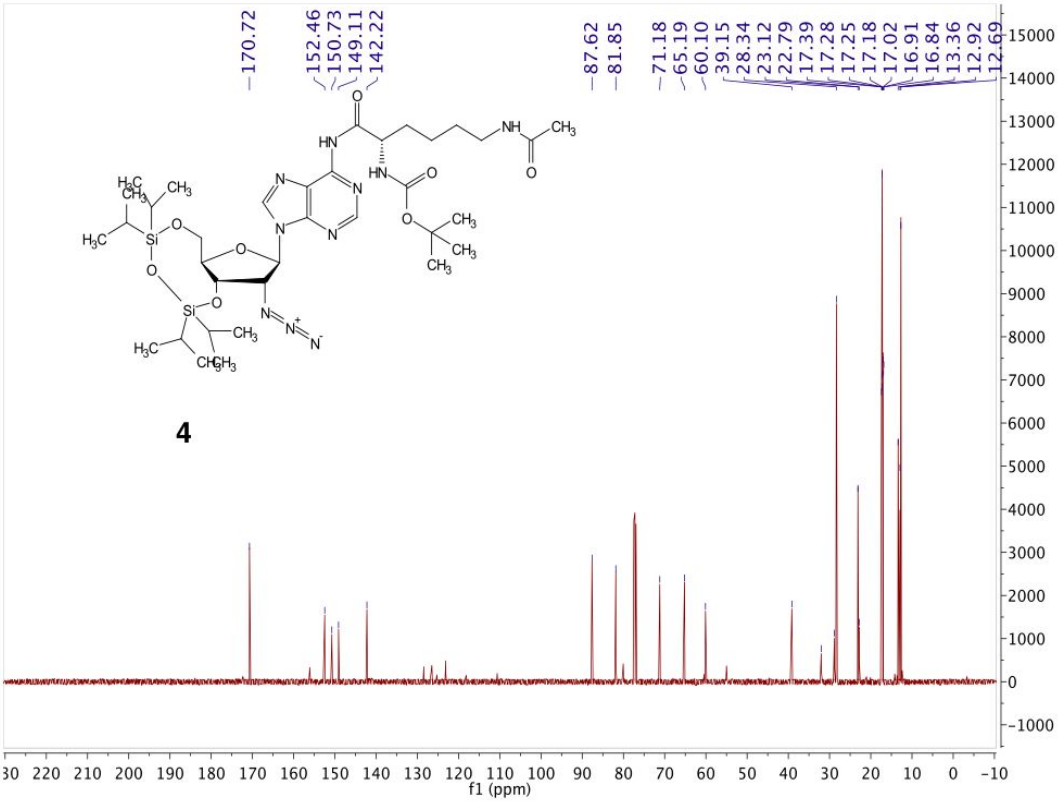
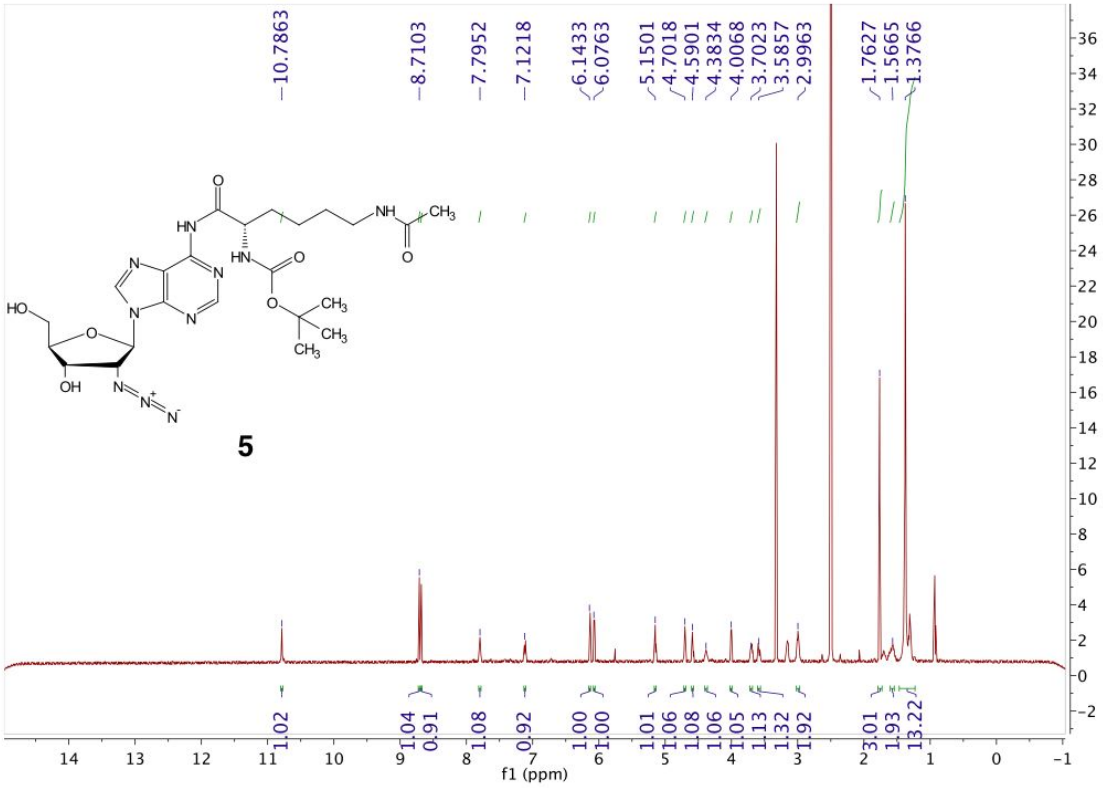


$^1$H NMR (500MHz, DMSO)




**5**

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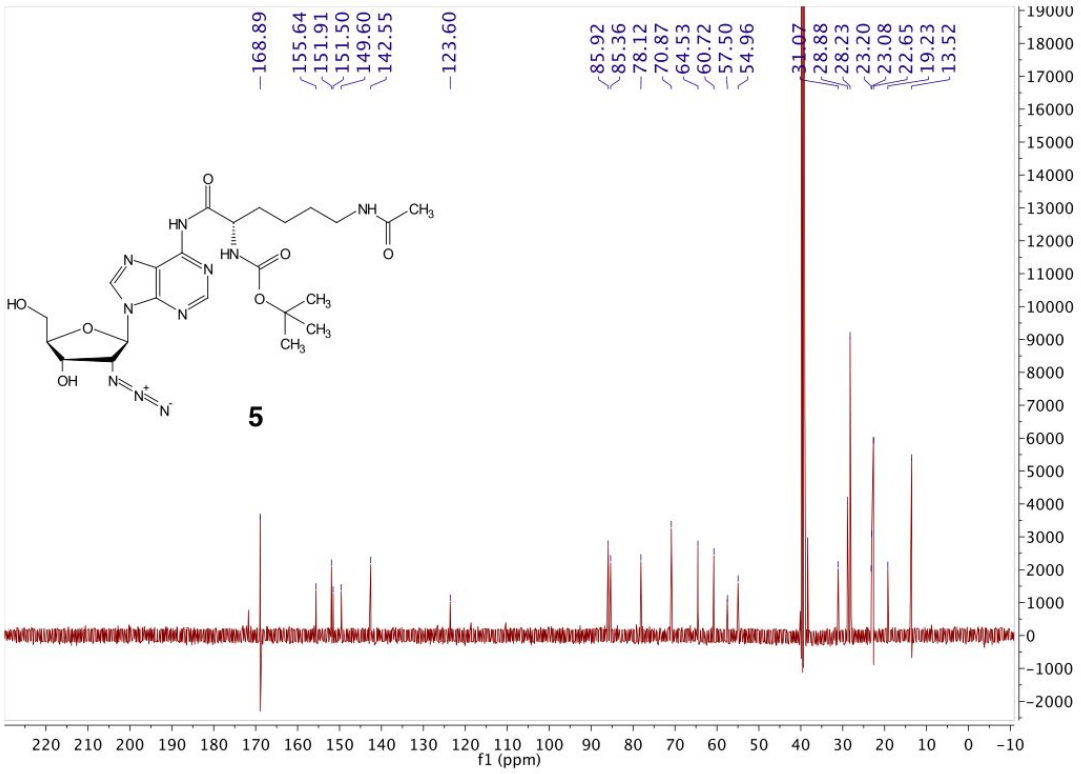

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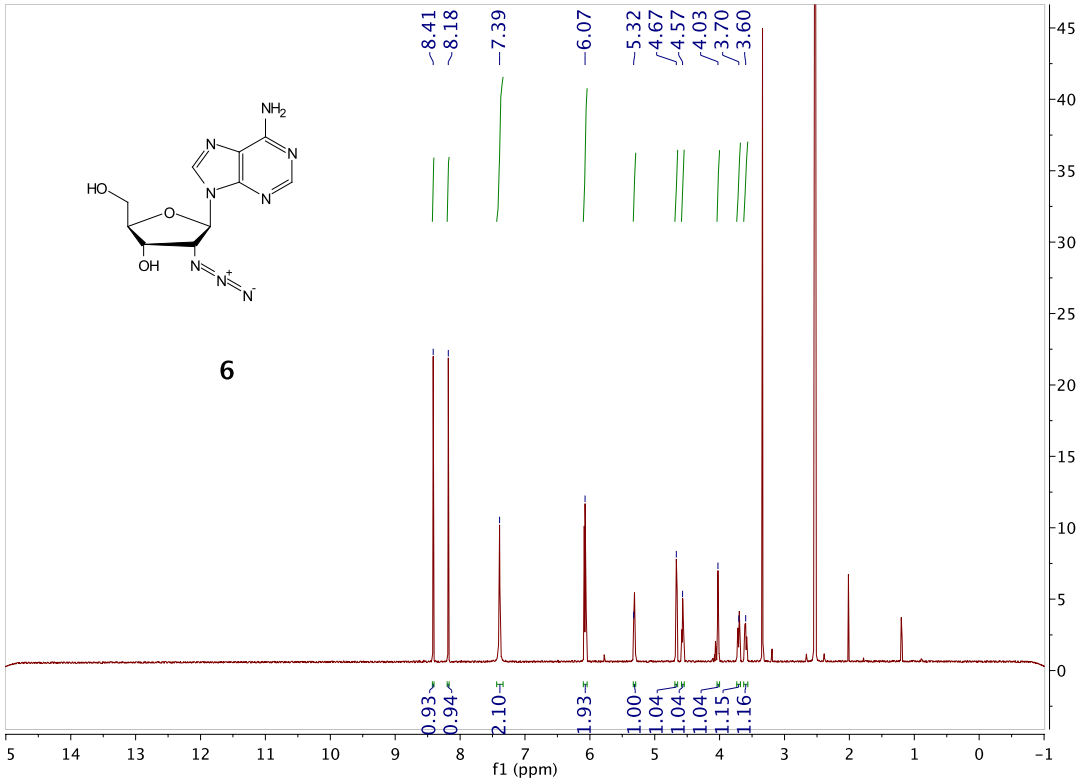

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