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SUPPORTING INFORMATION

Bio-orthogonal chemistry-based method for fluorescent labeling of ribosomal RNA in live mammalian cells.

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Materials and Methods

All chemicals were received from commercial sources and used without further purification. Recombinant ADK was purchased from R&D Systems. Chromatographic purifications of synthetic materials were conducted using SiliaSphere™ spherical silica gel with an average particle and pore size of 5 μm and 60 Å, respectively (Silicycle Inc, QC, Canada). Thin layer chromatography (TLC) was performed on SiliaPlate™ silica gel TLC plates with 250 μm thickness (Silicycle Inc, QC, Canada). Preparative TLC was performed using SiliaPlate™ silica gel TLC plates with 1000 μm thickness. HPLC purification was performed using Phenomenex Luna 5u C18 (2) semi-preparative column (250 x 10 mm). ^{1}H and ^{13}C NMR spectroscopy was performed on a Bruker NMR at 400 MHz (^{1}H) and 100 MHz (^{13}C). All ^{13}C NMR spectra were proton decoupled. Fluorescence microscopy experiments were carried out using Zeiss LSM 710 Pascal laser confocal microscope (Carl Zeiss Microscopy, Thornwood, NY, USA). Image acquisition and analyses were performed using Zeiss ZEN 2012 Confocal Microscopy Software (Release 2.02). Cell imaging experiments were done using commercially available fluorescent dyes: MitoTracker® Red CM-H2XRos, LysoTracker® Red DND-99 and Hoechst 33258 purchased from ThermoFisher Scientific. HeLa and HEK-239 cells were acquired from ATCC. Total cellular mRNA was isolated using magnetic mRNA isolation manufactured by New England BioLabs (cat.# S1550).

Fluorescent labeling of rRNA using IEDDA chemistry.

Either HeLa cells were grown to ~70% confluence in 35 mm MatTek glass bottom dishes in DMEM supplemented with 10% bovine serum albumin, penicillin and streptomycin. Compound **1** (100 μM) was added to the complete culture medium from a 100 mM stock in DMSO. After 24 h, the cells were washed with PBS and grown in fresh DMEM for 3 h. The cells were once again washed with PBS and incubated with DMEM containing 5 μM **OG-TZ** for 2 h. The cells were washed once with dye-free DMEM and incubated in dye-free DMEM suitable for fluorescence imaging.

Colocalization cell studies.

The colocolization studies were done using the following fluorescent dyes: MitoTracker® Red CM-H2XRos, LysoTracker® Red DND-99 and Hoechst 33258 based on the recommendations of the supplier's protocols. Stock solutions of each dye were prepared (MitoTracker – 200 μM in DMSO; LysoTracker 50 μM in DMSO; Hoechst 50 μM in DMSO). The protocol for fluorescent labeling of RNA with **1** and **OG-TZ**, described above, was followed. In addition to **OG-TZ**, 2

μL of either MitoTracker, LysoTracker or Hoechst was added to the 2 mL of DMEM. After 2 h incubation, the cells were washed with PBS and resuspend in 2 mL of dye-free DMEM.

Isolation of total cellular mRNA.

Total cellular mRNA was isolated using magnetic beads provided in a kit manufactured by New England BioLabs (cat.# S1550). The kit also included Lysis/Binding Buffer, Wash Buffer, Low Salt Buffer and Elution Buffer. The following procedure was followed:

1. Approximately $5x10^6$ cells were pelleted by centrifugation at 1,000 rpm for 5 minutes at 4°C. The cells were washed once with cold sterile 1X PBS (pH 7.4) and pelleted by centrifugation at 1,000 rpm for 5 minutes at 4°C. The cells were lysed upon addition of 1 mL of Lysis/Binding Buffer provided in the kit. Incubated at rt for 5 min with gentle agitation.
2. Magnetic beads containing poly(T) DNA strands were added to the cell lysate and agitated at rt for 10 min. The magnetic beads were pulled to the side with a strong magnet and the supernatant was removed. Added 1 mL of the Wash Buffer and mixed with agitation for 1 min. The magnetic beads were pulled to the side with a strong magnet and the supernatant was removed. Added 1 mL of the Low Salt Buffer to the beads and mixed with agitation for 1 min. The magnetic beads were pulled to the side with a strong magnet and the supernatant was removed. Added 1 mL of the Elution Buffer and vortexed gently to suspend beads. Incubated at 50 °C for 2 min with occasional agitation to elute poly(A)$^+$ RNA.

Ribosome Purification

Ribosomes were purified following a protocol by Fuchs et al. [*PNAS*, **2015**, *112*, 319-325] with the following modifications. HeLa cells at approximately 50% confluency were treated with 1 mM **1** in DMEM for 24 h. The cells were subsequently washed twice with PBS. Cells were scraped in PBS, and pelleted at 1000 rpm for 3 min. PBS was removed, and cells were lysed in lysis buffer containing 150 mM NaCl, 15 mM Tris•HCl (pH 7.5), 10 mM $MgCl_2$, 1% Triton X-100, 1 mg/mL heparin, 2 mM DTT. Cells were pelleted for 2h through a 30% sucrose cushion at 200,000g in a TLA120.2 rotor. The RNA from the ribosomal pellet was extracted using TRIzol following the manufacturer's protocol.

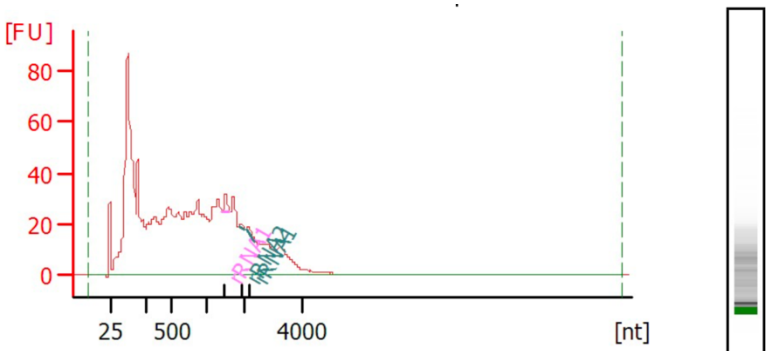
UHPLC-MS/MS analysis

HEK-293 cells (~$5x10^6$) were washed with PBS and total RNA was isolated using RNA Bee kit (AMS Biotechnology). The total RNA samples (100 ng each) were analyzed similarly to the previously described procedure (*Front. Genet.* **2018**, *9*, 32). Briefly, total cellular RNA was hydrolyzed to the composite mono-nucleosides via a two-step enzymatic hydrolysis. This

process involved the use of two enzymes: 18 h treatment with Nuclease p1 at 37 ºC, followed by a 2 h treatment with bacterial alkaline phosphatase at 37 ºC. The resulting nucleoside mixture was lyophilized and reconstituted in H_2O, containing 0.01% formic acid, to a final concentration of 1 ng/ul for subsequently UHPLC-MS/MS analysis. UHPLC-MS/MS analysis of RNA nucleosides was performed on a Waters XEVO TQ-S ™ (Waters, USA) triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source maintained at 500 °C and the capillary voltage was set at 1.5 kV with extraction cone of 14 V. Nitrogen flow was maintained at 1,000 L/h and desolvation temperature at 500 °C. The cone gas flow was set to 150 L/h and nebulizer pressure to 7 bars. Each individual nucleoside modification was characterized by single infusion in positive mode ionization over an *m/z* range of 100-500 amu. Further nucleoside characterization was produced by using Waters software part of Intellistart MS/MS method development where a ramp of collision and cone voltages is applied to find optimal collision energy parameters for all possible daughter ions. The incorporation ratio was calculated and normalized relative to canonical nucleosides. [^{13}C][^{15}N]-G (1 pg/μL) was used as an internal standard.

Cell viability assay

The colorimetric MTT assay, adopted from our previously published work, was used to evaluate the cytotoxicities of **1** and **EU** [*Chem. Commun.* **2016**, *52*, 6174]. Day one: using 96-well format, plated ~100 cells/well in 100 μL DMEM and incubated for 24 h. Day two: DMEM was removed and the cells were treated with variable concentrations of **1** and **EU** for 24 h. Day three: the medium was replaced with 100 μL of fresh DMEM and the cells were incubated for 72h. Day six: DMEM was removed and the cells were incubated with 100 μL of MTT solution (0.6 mg/mL in DMEM) per well for 4 h at 37 °C. The MTT solution was then replaced with 100 μL of DMSO containing 4% aqueous ammonia per well to dissolve the purple formazan crystals. After 30 min, the absorbance of each well at 550 nm was recorded using BioTek Synergy HT multi detection microplate reader. All MTT assays were done in sextuplicate. Data are expressed as means ± SEM, unless otherwise noted.



Overall Results for sample 1 : **Sample 2**

RNA Area:	1,841.3
RNA Concentration:	10,074 pg/µl
rRNA Contamination:	0.8 %

Figure S1. **Electropherogram images of mRNA-purified samples**. Electropherogram for total cellular mRNA purified using oligo-dT pull-down assay described above. The x-axis represents size distribution of each samples in nucleotides [nt] while the y-axis depicts signal intensity measured in fluorescence units [FU]. The rRNA contamination percentage was calculated using Bioanalyzer software.

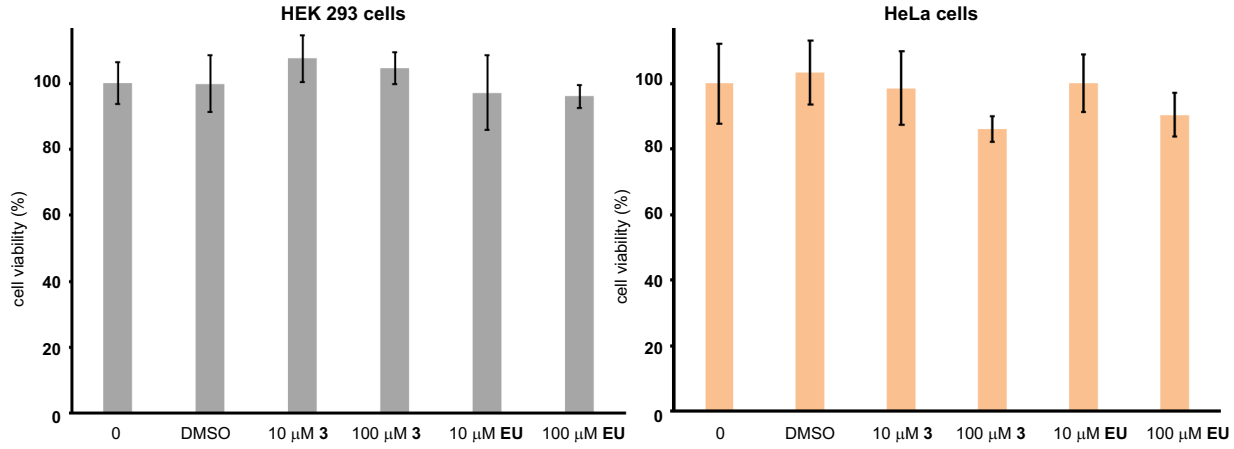


Figure S2. Viability of HEK 293 and HeLa cells treated with **1** or **EU**.

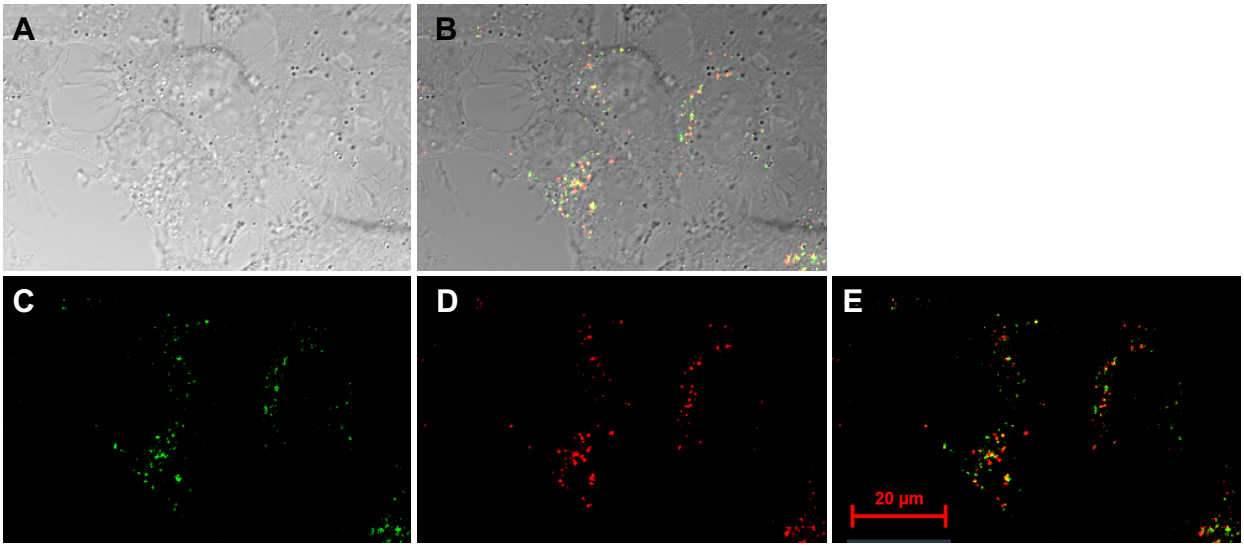
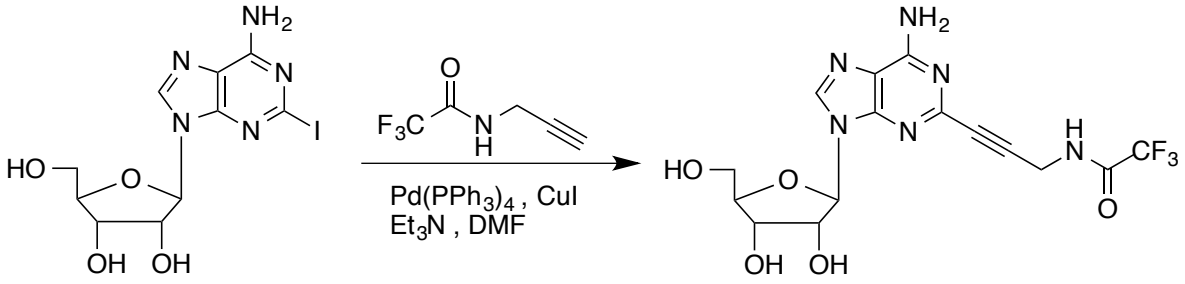


Figure S3. Zoom-in images of HeLa cells treated with **1** and subsequently **OG-Tz** and LysoTracker® Red DND-99. (**A**) Brightfield channel; (**B**) overlay of brightfield, red and green channels; (**C**) green chanel; (**D**) red chennal; (**E**) overlay of red and green channels.

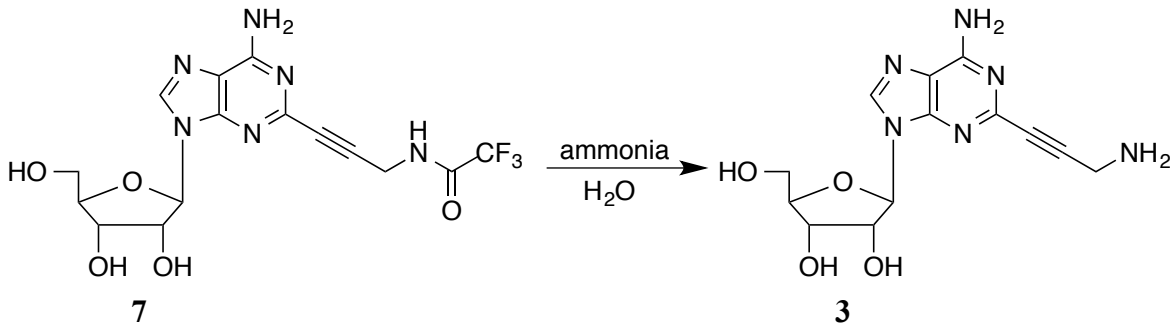


2 **7**

Scheme S1. Synthesis of compound **7**.

Combined 2-iodoadenosine (1.70 g, 4.32 mmol) and CuI (0.170 g, 0.892 mmol) in thoroughly degassed DFM (20.0 mL). Charged the *N*-propargyl trifluoroacetamide (3.66 g, 13.2 mmol), followed by $Pd(PPh_3)_4$ (0.508 g, 0.440 mmol) and N,N-diisopropylethylamine (DIPEA, 1.53 mL, 8.80 mmol). The reaction flask was evacuated and refilled with N_2 three times. Stirred at rt under N_2 atmosphere for 18 h. The reaction mixture was diluted with a 1:1 solution of MeOH:CH_2Cl_2 (18.0 mL) and AG-1-X8 resin (bicarbonate form, 3.00 g) was added. After stirring for 30 min, the crude product was filtered and the resin was once rinsed with a 1:1 solution of MeOH:CH_2Cl_2 (18.0 mL). The solvents were removed under reduced pressure, the crude product was re-dissolved in MeOH and deposited onto silica gel (15 g). Purified by flash chromatography using a gradient of MeOH in CH_2Cl_2 (5-10%). Yield = 1.49 g (82.8 %)

NMR: **^{1}H NMR** (*500 MHz, MeOD*) δ 8.37 (s, 1H), 5.95 (t, *J* = 13.2 Hz, 1H), 4.78 – 4.71 (m, 1H), 4.40 – 4.32 (m, 3H), 4.20 (dd, *J* = 5.0, 2.4 Hz, 1H), 4.00 – 3.73 (m, 2H);
^{13}C NMR (*125 MHz, MeOD*) δ 157.74, 157.44, 157.14, 156.85, 155.89, 148.79, 145.15, 141.61, 119.43, 117.15, 114.88, 112.59, 89.91, 86.69, 81.47, 80.12, 74.10, 71.12, 62.06, 28.92.
HRMS (*ESI*) *m/z*: calcd. for $C_{15}H_{15}F_3N_6NaO_5$ $[M+Na]^+$ 439.0954; found 439.0977

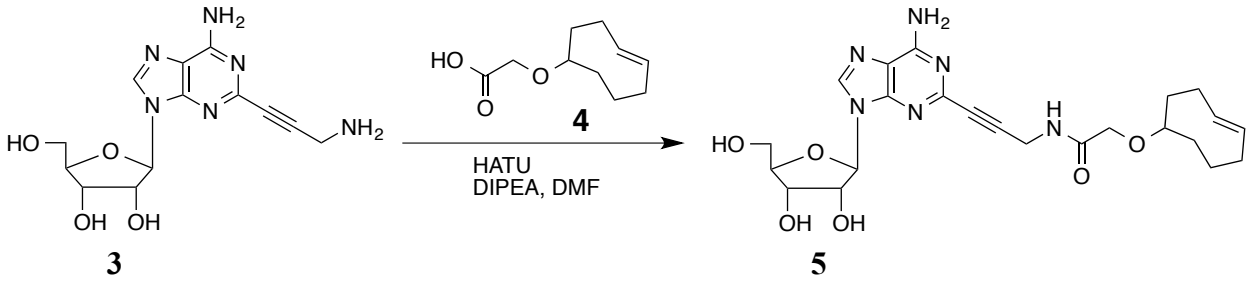


7 **3**

Scheme S2. Synthesis of compound **3**.

Compound **7** (0.508 g, 1.22 mmol) was dissolved in H_2O (8.5 mL). After complete dissolution, aqueous ammonium hydroxide (NH_4OH) (8.5 mL) was added to the reaction mixture. The reaction was stirred for 3 h at room temperature and then concentrated down to give the crude product as a yellow-orange residue. The crude product was redissolved in DI H_2O (10 mL) and AG50W-X8 resin (2.5 g) was added to the solution. The mixture was stirred for 15 min and filtered over a bed of AG50W-X8 resin (2.5 g). The resin was washed with DI H_2O and the product was then eluted off of the resin with DI H_2O/conc. NH_4OH, 4:1 (100 mL). The title product was obtained as a light tan solid after removal of the solvents under reduced pressure. Yield = 0.373 g (95.5 %).

NMR: **^{1}H NMR** (*400 MHz, D_2O*) δ 8.24 – 8.16 (m, 1H), 5.95 – 5.83 (m, 1H), 4.67 (s, 1H), 4.43 – 4.36 (m, 1H), 4.26 (d, *J* = 2.9 Hz, 1H), 3.96 – 3.77 (m, 2H), 3.70 (s, 2H) .
^{13}C NMR (*100 MHz, D_2O*) δ 154.88, 147.98, 144.90, 141.12, 118.46, 88.65, 85.75, 80.67, 73.83, 70.61, 61.56, 30.09.
HRMS (*ESI*) *m/z*: calcd. for $C_{13}H_{17}N_6O_4$ $[M+H]^+$ 321.1311; found 321.1371

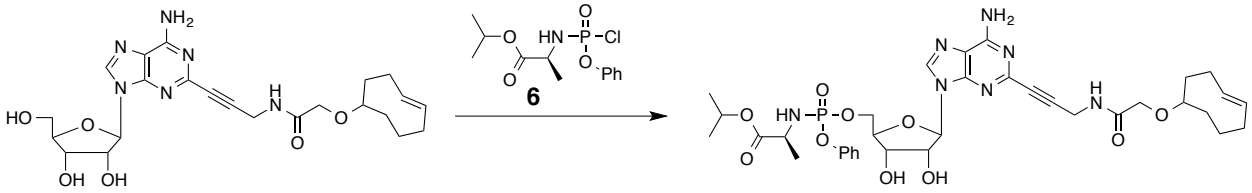


Scheme S3. Synthesis of compound **5**.

Compound **3** (0.200 g, 0.625 mmol) was dissolved in DMF (10.0 mL). Racemic axial isomer of TCO, **4**, (0.115 g, 0.625 mmol) and HATU (0.236 g, 0.625 mmol) were added and the resulting reaction mixture was stirred at rt under N_2 atmosphere for 18 h. The reaction mixture was concentrated under reduced pressure, re-dissolved in MeOH and deposited onto 5 g of SiO_2. Purified by preparative SiO_2 TLC using a 10% solution of MeOH in CH_2Cl_2 as a mobile phase. Yield = 0.123 g (40.5%)

Because of planar chirality of TCO, compound **5** was isolated as a mixture of diastereomers.

NMR: **^{1}H NMR** (*500 MHz, MeOD*) δ 8.35 (s, 1H), 5.96 (d, *J* = 6.2 Hz, 1H), 5.74 – 5.59 (m, 1H), 5.59 – 5.45 (m, 1H), 4.79 – 4.67 (m, 1H), 4.39 – 4.27 (m, 3H), 4.19 (q, *J* = 2.5 Hz, 1H), 4.03 – 3.97 (m, 2H), 3.96 – 3.75 (m, 2H), 3.70 (dd, *J* = 10.1, 4.6 Hz, 1H), 2.53 – 2.17 (m, 4H), 2.15 – 1.98 (m, 1H), 1.96 – 1.72 (m, 3H), 1.67 – 1.53 (m, 1H), 1.33 – 1.23 (m, 1H);
^{13}C NMR (*125 MHz, MeOD*) δ 171.34, 155.91, 148.66, 145.46, 141.34, 135.31, 131.06, 119.22, 89.88, 86.72, 81.88, 80.62, 76.32, 74.06, 71.10, 67.97, 62.08, 39.91, 33.97, 32.12, 29.28, 28.19, 27.55.
HRMS (*ESI*) *m/z*: calcd. for $C_{23}H_{31}N_6O_6$ $[M+H]^+$ 487.2305; found 487.2329



5 **1**

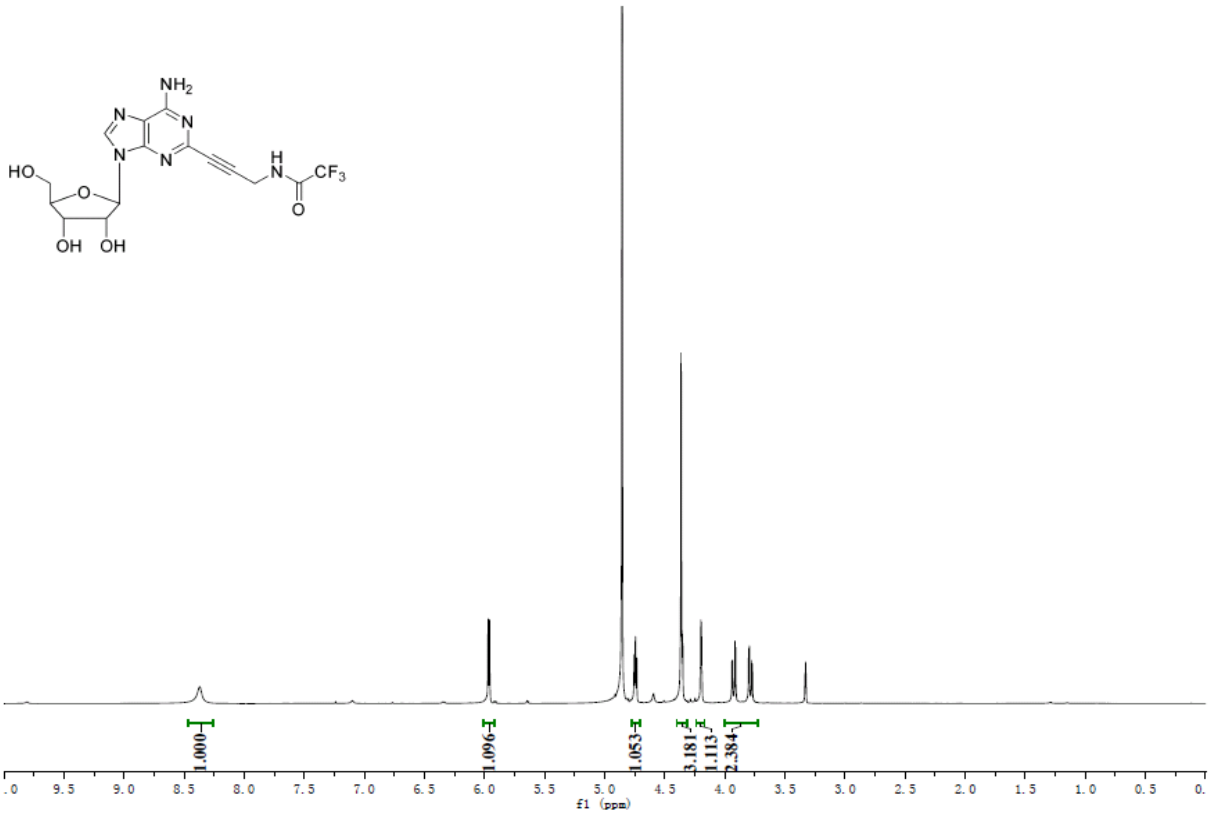
Scheme S5. Synthesis of compound **1**.

Compound **6** was synthesized as previously described (*J. Med. Chem.* **2014**, *57*, 1812-1825.)

Dissolved the compound **5** (0.123 g, 0.253 mmol) in a solution of trimethyl phosphate (1 mL) and THF (2 mL). Added *N*-methyl imidazole (0.104 g, 1.27 mmol) and cooled the resulting solution to 0 °C. A solution of compound **6** (0.155 g, 0.506 mmol) in THF (1 mL) was added dropwise. The resulting solution was slowly warmed up to rt and stirred for 1 h. The mixture was partitioned between ethyl acetate and water. The organic layer was concentrated and the title compound was purified by preparative SiO_2 TLC using a 10% solution of MeOH in EtOAc as a mobile phase. Yield = 0.0460 g (24.1%)

Because of planar chirality of TCO, compound **1** was isolated as a mixture of diastereomers.

NMR: **^{1}H NMR** (*400 MHz, CD_3OD*) δ 7.92 (s, 1H), 7.24 (s, 2H), 6.87 – 6.75 (m, 4H), 6.68 (s, 3H), 6.57 (s, 3H), 5.55 (d, *J* = 6.0 Hz, 1H), 5.29 – 5.09 (m, 2H), 4.31 (t, *J* = 5.5 Hz, 1H), 3.94 (s, 1H), 3.87 (s, 2H), 3.77 (s, 1H), 3.58 – 3.46 (m, 4H), 3.03 (s, 1H), 2.90 (s, 1H), 2.00 – 1.85 (m, 1H), 1.76 – 1.51 (m, 5H), 1.49 – 1.30 (m, 2H), 1.25 (d, *J* = 3.4 Hz, 1H), 1.06 (ddd, *J* = 32.8, 14.1, 8.2 Hz, 3H), 0.88 (t, *J* = 21.0 Hz, 5H), 0.76 (t, *J* = 6.4 Hz, 5H);
^{13}C NMR (*100 MHz, CD_3OD*) δ 172.78, 157.10, 149.85, 146.63, 142.56, 130.85, 130.40, 130.00, 123.73, 121.50, 121.45, 91.11, 87.96, 83.21, 83.12, 81.95, 75.42, 72.38, 69.64, 68.62, 63.34, 51.82, 34.83, 34.08, 33.78, 29.44, 26.54, 26.38, 23.35, 21.96, 21.88, 21.52, 21.47.
^{31}P NMR (*162 MHz, CD_3OD*) δ 0.02, -17.41;
HRMS (*ESI*) *m/z*: calcd. for $C_{35}H_{47}N_7O_{11}P$ $[M+OH]^-$ 772.3071; found 772.3075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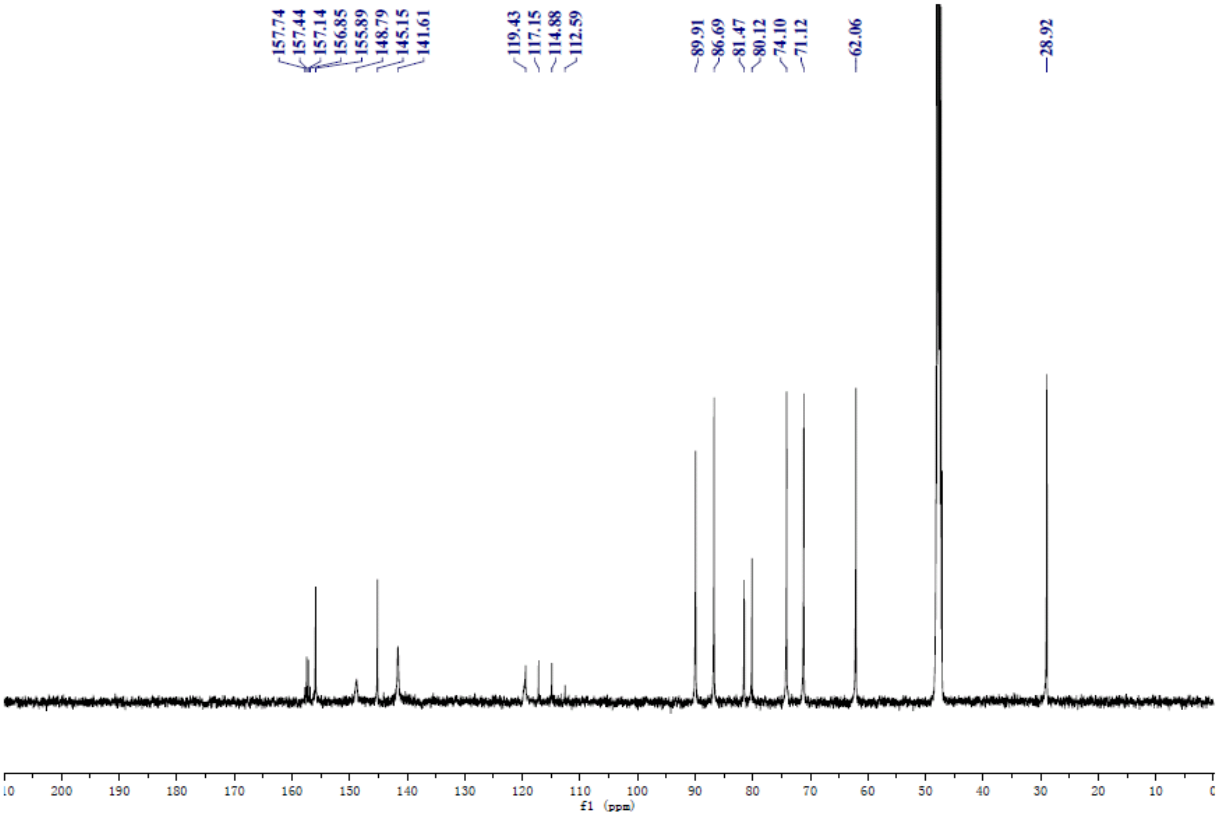
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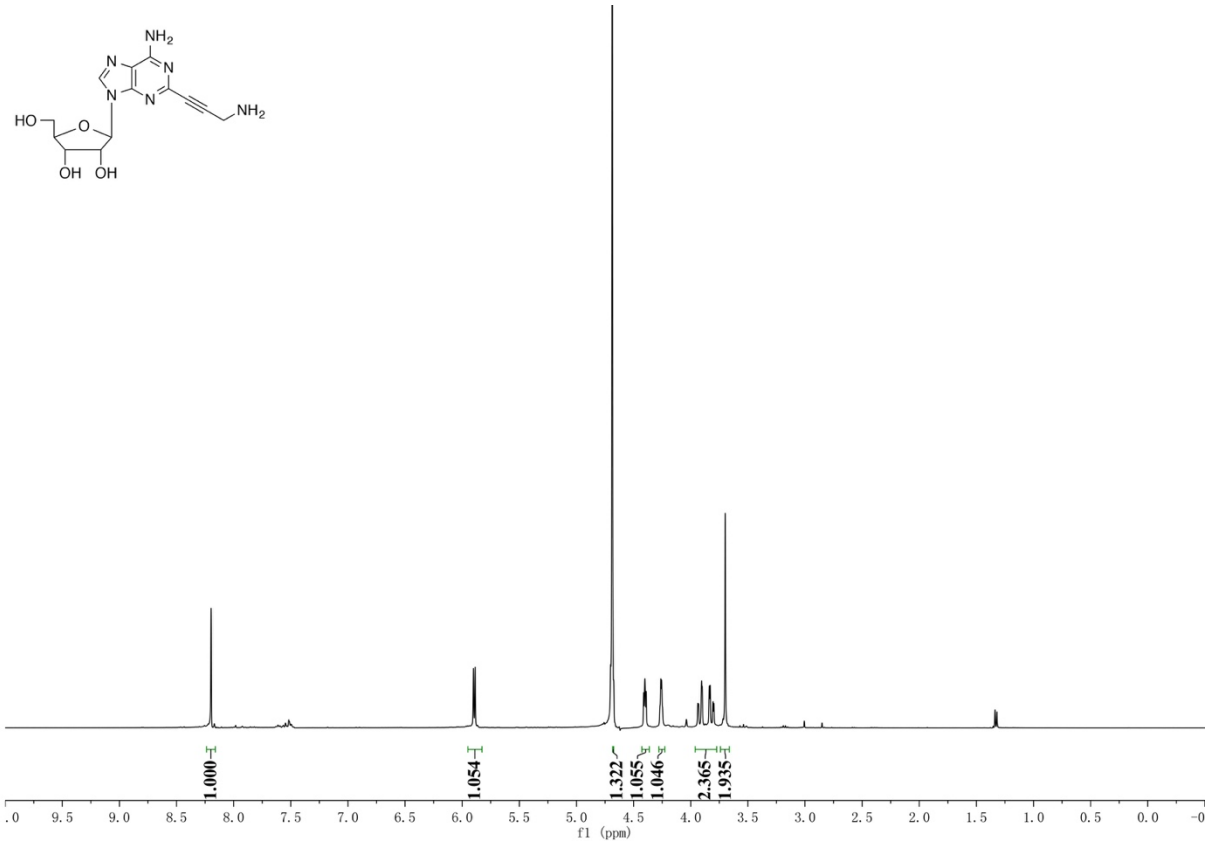
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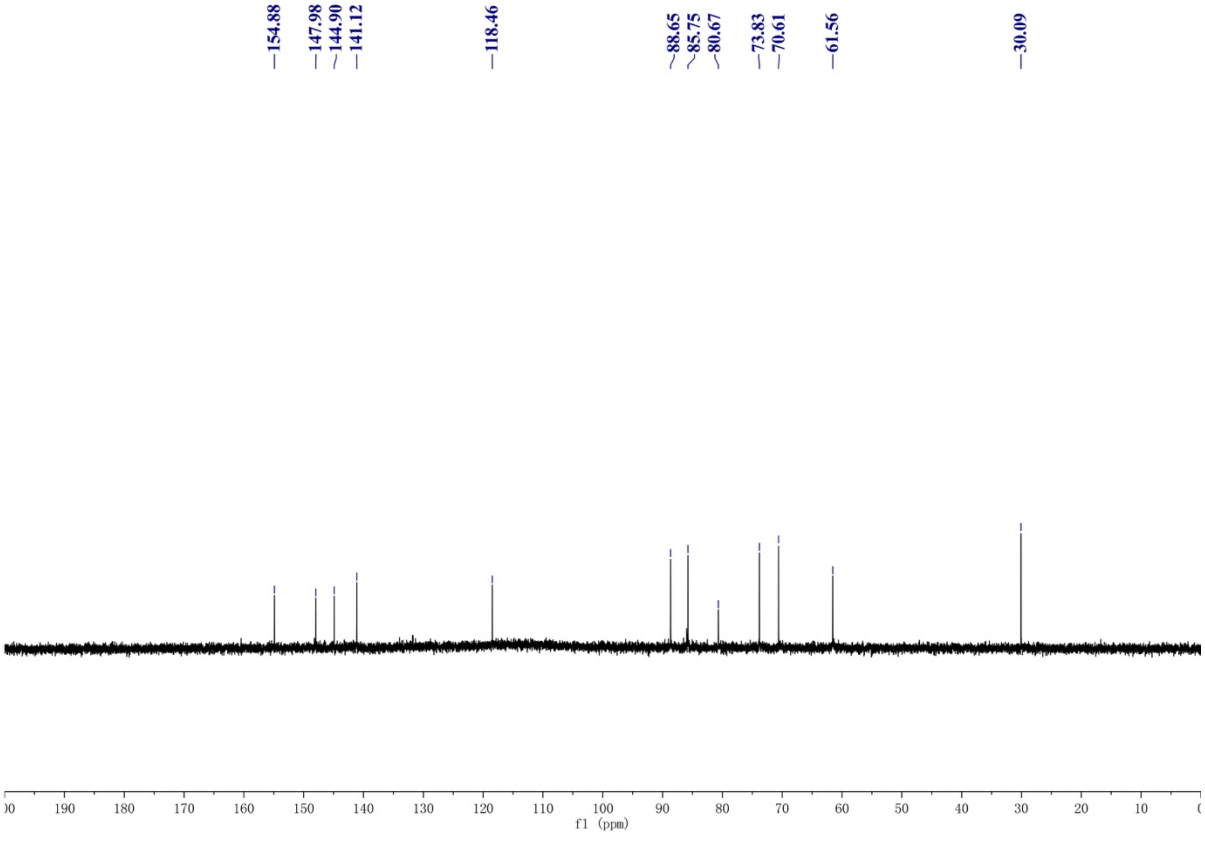
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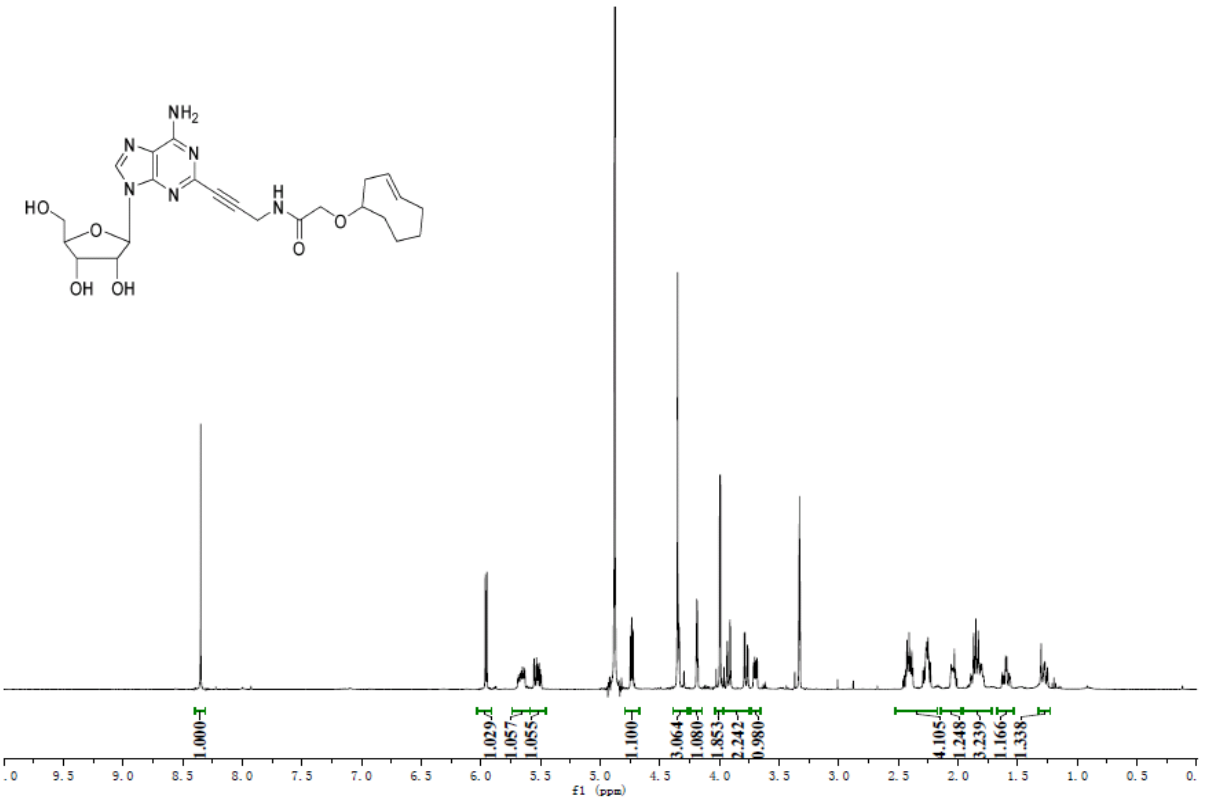
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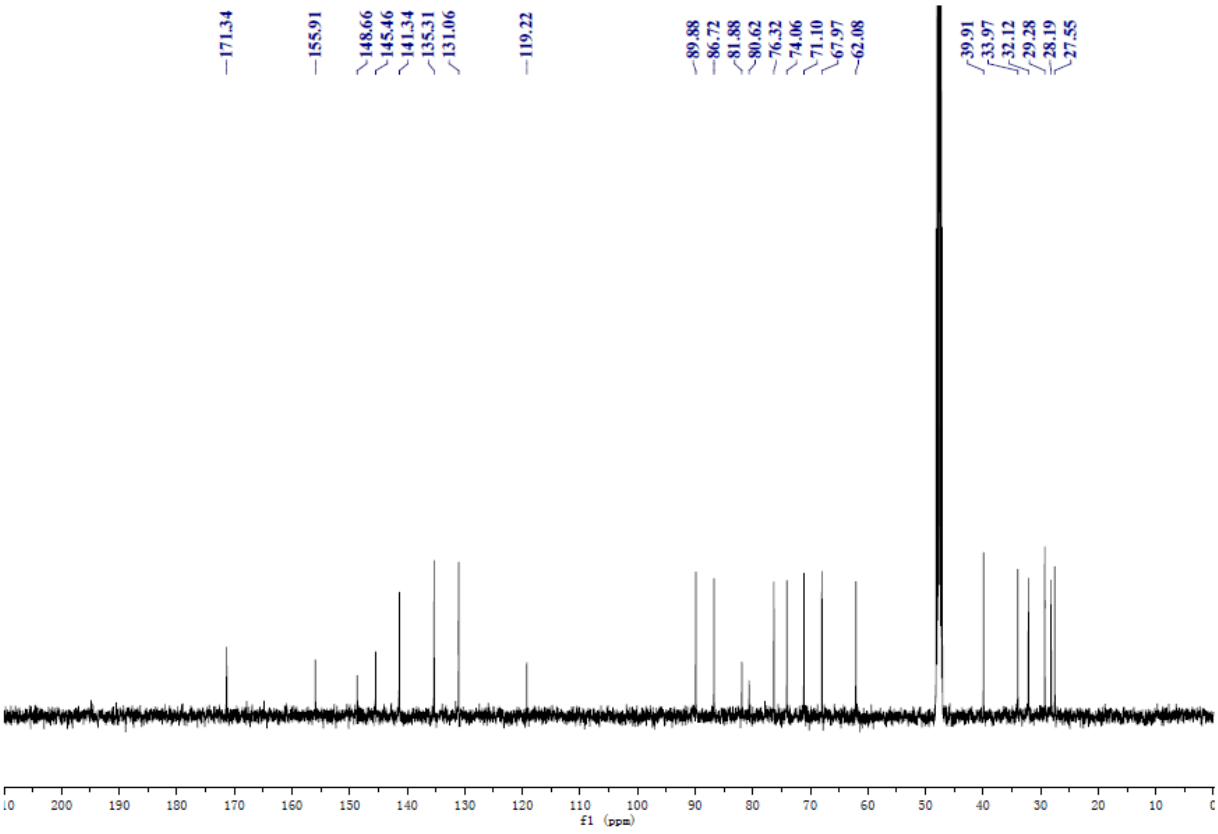
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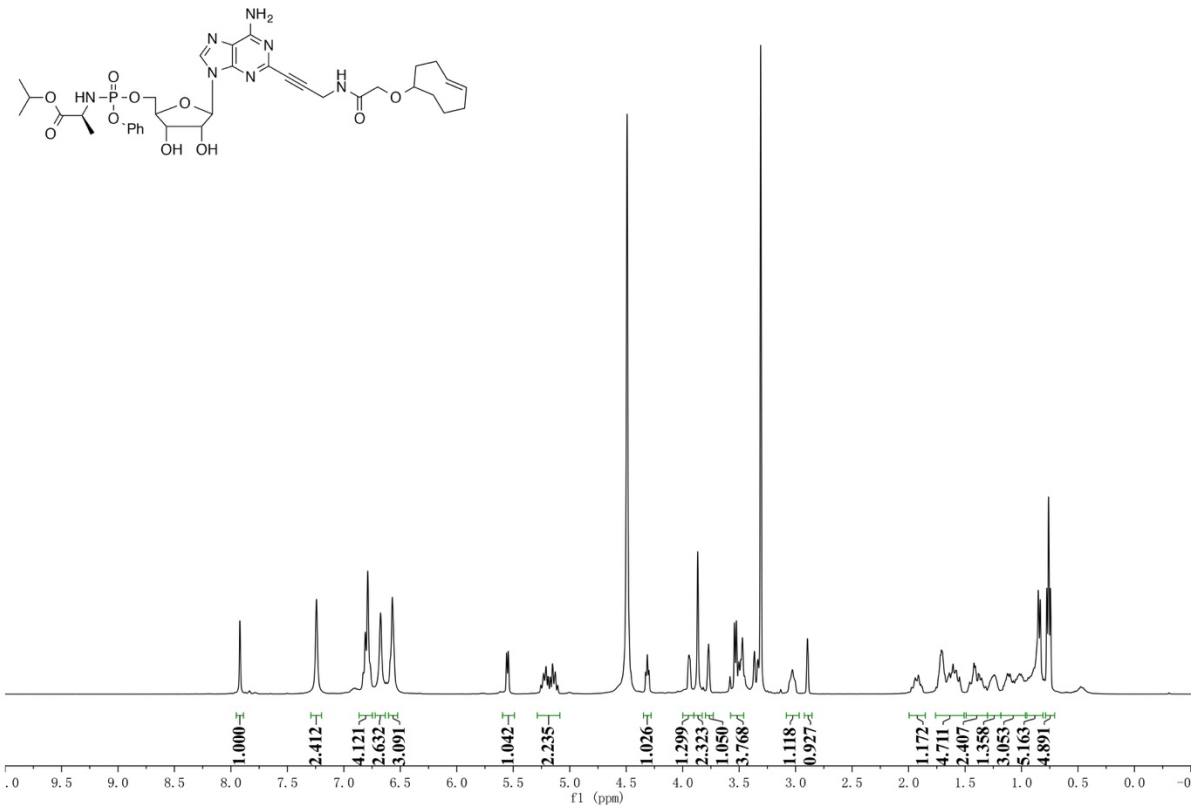
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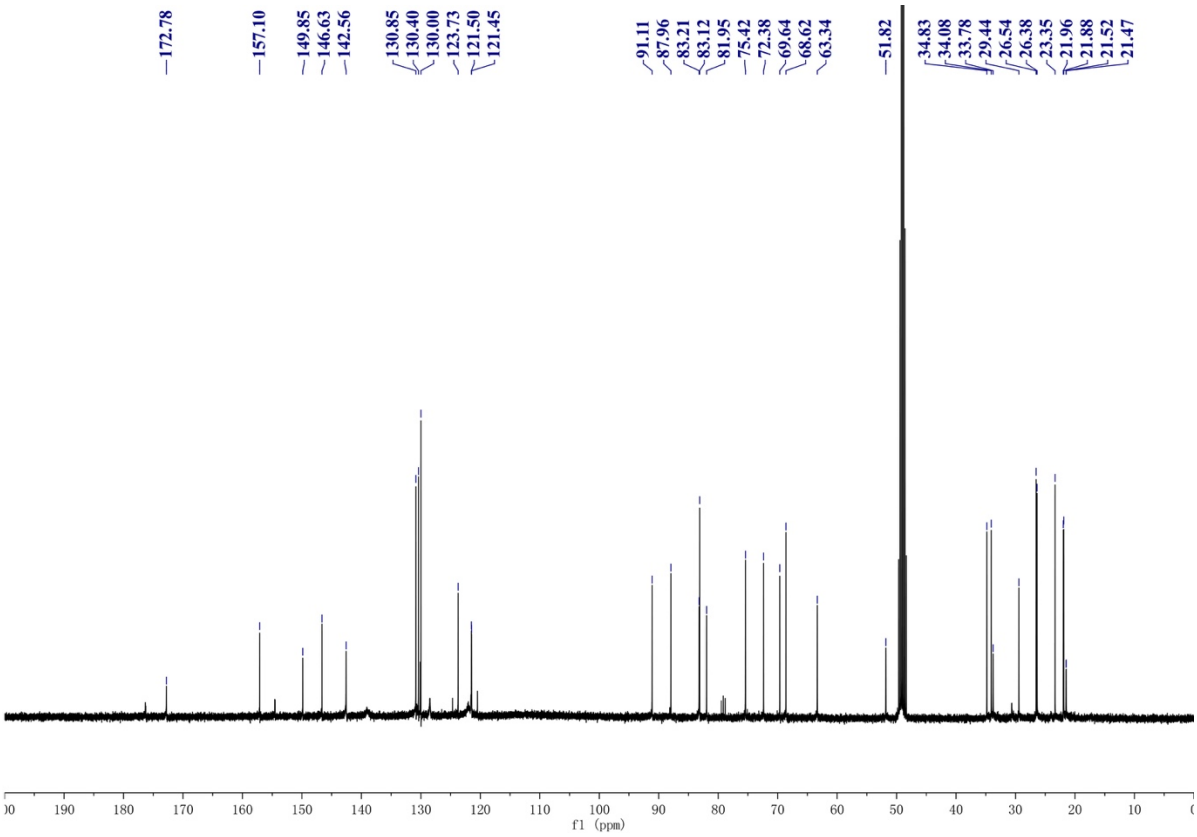
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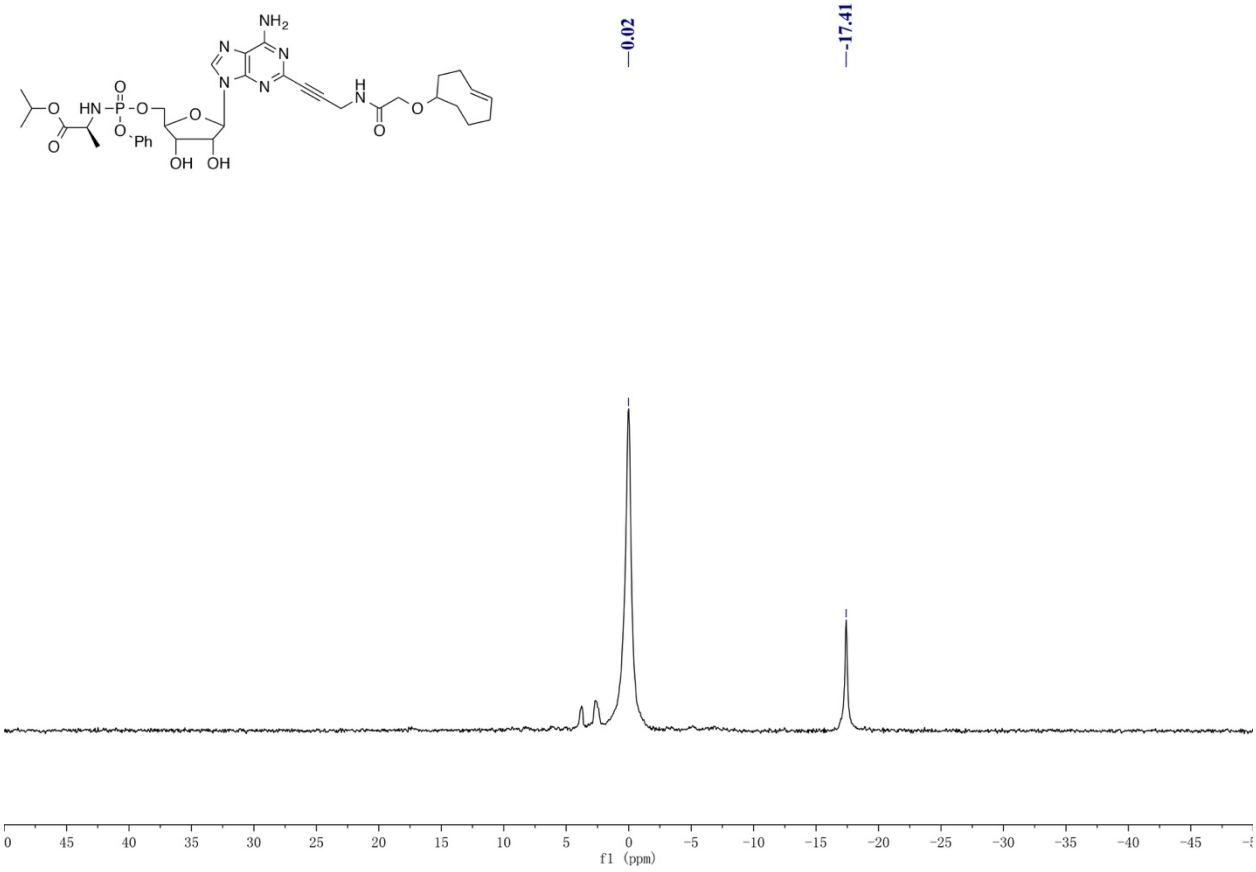
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