Inherently Emissive Puromycin Analogues For Live Cell Labelling

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1. Materials and Methods

Reagents were purchased from Sigma-Aldrich, TCI, Spectrum, Acros, Fisher Scientific, and VWR and used without further purification. Methyl 4-amino thiophene 3-carboxylate hydrochloride was purchased from Matrix Scientific. Azetidine hydrochloride and difluoroazetidine hydrochloride were purchased from Synthonix. Solvents were purchased from Sigma-Aldrich and Fisher Scientific and dried by standard techniques. NMR solvents were purchased from Cambridge Isotope Laboratories (Andover, MA). All reactions were monitored with analytical TLC (Merck Kieselgel 60 F254). Column Chromatography was carried out with a Teledyne ISCO Combiflash RF with silica gel particle size 40–63 µm. ^{1}H NMR and GDQCOSY spectra were obtained on either a Bruker Avance 300 MHz or Jeol ECA 500 MHz. ^{13}C NMR, GHSQC and GHMBC spectra and were taken on Varian VS 500 MHz. Mass spectra were obtained on an Agilent 6230 HR-ESI-TOF MS at the Molecular Mass Spectrometry Facility at the UCSD Chemistry and Biochemistry Department.

HPLC purification was carried out with an Agilent 1260 series system with a Sepax Bio-C18, 5µm, 300 Å, 10 × 250 mm column. 0.1% TFA stock solutions were prepared by dissolving 1 ml of TFA (Sigma-Aldrich, 99%) in 999 ml MilliQ water (or ACN) and filtered using Millipore type GNWP 0.2 µm filters before use. Each injection (100 µl) was subjected to a gradient (20 minutes, from 0.5 to 40% acetonitrile 0.1% TFA in water 0.1% TFA) followed by a flush (10 minutes). A flow rate of 3 mL / minute was used and the run was carried out at 25.00 ± 0.10 °C. Each run was monitored at 260, 280 and 380 nm with calibrated references at 600 nm and slit set at 1 nm.

Puromycin dihydrochloride was purchased from Sigma-Aldrich. DMEM, phenol-red free DMEM, and neurobasal medium (21103049) were purchased from Thermo-Fisher. B-27 plus neuronal culture system (A3653401) was purchased from Fisher. Fetal bovine serum (FB-01) was purchased from Omega Scientific. Penicillin:streptomycin (Pen-strep, 10,000 U/ml) was purchased from Gibco. CleanCap® EGFP mRNA (L-7601) was purchased from Trilink, and rabbit reticulocyte cell lysate (L4960) was purchased from Promega. 24-well plates (10062-896) were purchased from VWR and TC coverslips (NC9964441) were purchased from Fisher. Poly-D-Lysine was purchased from Neta Scientific. 1X Phosphate-buffered saline (PBS) with calcium and magnesium (21-030-CM) was purchased from Corning, and 4% Paraformaldehyde solution (PFA) in PBS (AAJ19943K2) was purchased from Fisher. Triton-X 100 (X100-5ML) was purchased from Sigma-Aldrich. Bovine-serum albumin was purchased from Sigma-Aldrich. Trysin-EDTA was purchased from Life Technologies. 10X RIPA buffer was purchased from Cell-Signaling Technologies and cOmplete Mini, EDTA-free proteinase inhibitor cocktail tablets were purchased from Sigma-Aldrich. Cycloheximide was purchased from AG scientific. Pierce BCA Protein Assay kits and NuPAGE LDS sample buffer were purchased from Fisher. Bolt 4 to 12%, Bis-Tris, 1.0 mm, Mini Protein Gels were purchased from Fisher. Celllight-lysosomes-GFP, BacMam 2.0 (C10596) and DRAQ5 5 mM solution was purchased from Thermo-Fisher. Anti-puromycin [3RH11] was purchased from Kerafast, and Human Antibody Against Ribosomal P Antigen (HPO-0100) was purchased from Immunovision. Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 was purchased from Fisher, and Donkey anti-human Cy3 was purchased from Jackson Immunoresearch. Seablock was purchased from Thermo-Fisher.

1.1. Abbreviations

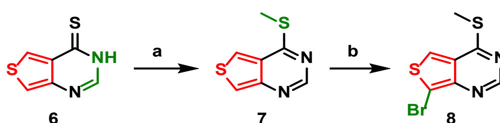
AcCl: acetyl chloride; ACN: acetonitrile; BCA: bicinchoninic acid; BCl_3: boron trichloride; $BF_3 \cdot OEt_2$: boron tifluoride diethyl etherate; BnBr: benzyl bromide; BSA: bovine serum albumin; CHX: cycloheximide; CO_2: carbon dioxide; DBDMH: 1,3-Dibromo-5,5-Dimethylhydantoin; DBU: 1,8-Diazabicyclo[5.4.0]undec-7-ene; DCM: dichloromethane; DIPEA: diisopropylethylamine; DMEM: Dulbecco's modified eagle medium; DMF: dimethyl formamide; DMSO: dimethyl sulfoxide; EDC: 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; EDTA: ethylenediaminetetraacetic acid; EtOAc: ethyl acetate; EtOH: ethanol; Et_3SiH: triethylsilane; GFP: green fluorescent protein; HCl: hydrochloric acid; HEK293T: human embryonic kidney 293T; $HNMe_2$: dimethylamine; HOBt: 1-hydroxybenzotriazole; H_2SO_4: sulfuric acid; K_2CO_3: potassium carbonate; LB: luria broth; LDS: lithium dodecyl sulfate; MeI: methyl iodide; MeOH: methanol; Me_3P: trimethylphosphine; $MgCl_2$: magnesium chloride; MIC: minimal inhibitory concentration; MFI: mean fluorescence intensity; mQ: Milliq water; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; *n*-BuLi: *n*-butyllithium; NaH: sodium hydride; NaN_3: sodium azide; NaOH: sodium hydroxide; PBS: phosphate-buffered saline; PCC: pyridinium chlorochromate; PDL: poly-D-lysine; PFA: paraformaldehyde; P_2O_5: phosphorous pentoxide; P_2S_5: phosphorous pentasulfide; rt: room temperature; PVDF: polyvinylidene fluoride; TBAI: tetrabutylammonium iodide; TBST: tris-buffered saline + tween 20; THF: tetrahydrofuran; TLC: thin layer chromatography; TFA: trifluoroacetic acid; Tf_2O: trifluoromethanesulfonic anhydride; UV: ultraviolet.

2. Synthetic procedures

Starting precursors **6** and **10** were synthesized based on previously published procedures.[1–3]

2.1. Synthesis of Nucleobase 8

Scheme S1. Synthesis of brominated thiomethyl purine analog **8**.



(a) MeI, K_2CO_3, DMF, RT, 1 hr, 78%. (b) DBDMH, DMF, 0 °C, 2.5 h, 45%.

4-(methylthio)thieno[3,4-*d*]pyrimidine (7)

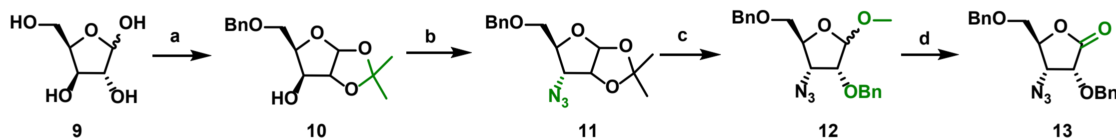
To a 2 L flask flame-dried and purged with argon was added thionated nucleobase **6** (14.5 g, 168 mmol, 1 eq), anhydrous DMF (375 ml) and K_2CO_3 (50 g, 362 mmol, 4.5 eq). MeI (34 g, 240 mmol, 15 ml, 3 eq) was added dropwise and the solution was allowed to stir for 1 hour at room temperature before adding another equivalent of MeI (11.3 g, 80 mmol, 5 ml, 1 eq) dropwise. The reaction was stirred for another 30 minutes at room temperature before deemed complete by TLC. The solution was evaporated to dryness and the crude was partitioned between DCM and water. The organic phase was washed 3 times with water, dried with sodium sulfate, filtered, and evaporated to dryness. The remaining crude was subjected to column chromatography with a gradient of 0–3% MeOH in DCM to yield a light-brownish solid (12 g, 78%). ^{1}H NMR (500 MHz, chloroform-*d*) δ 8.64 (s, 1H), 8.01 (d, *J* = 3.3 Hz, 1H), 7.83 (d, *J* = 3.3 Hz, 1H), 2.70 (s, 3H). ^{13}C NMR (126 MHz, Chloroform-*d*) δ 168.23, 151.87, 148.20, 127.48, 120.15, 117.74, 12.08. ESI-HRMS calculated for $C_7H_7N_2S_2$ $[M+H]^+$ 183.0045, found 183.0047.

7-bromo-4-(methylthio)thieno[3,4-*d*]pyrimidine (8)

To a 1 L flame-dried round-bottom flask purged with argon was added nucleobase **13** (12 g, 66 mmol) and anhydrous DMF (92 ml). The solution was cooled to 0 °C in an ice bath and 1,3-Dibromo-5,5-Dimethylhydantoin (2.35 g, 8.2 mmol, 0.12 eq) was added slowly. The solution was stirred at 0 °C for one hour before adding another eighth of an equivalent of 1,3-Dibromo-5,5-Dimethylhydantoin (2.35 g, 8.2 mmol, 0.12 eq). After stirring for 15 minutes at 0 °C more 1,3-Dibromo-5,5-Dimethylhydantoin (2.35 g, 8.2 mmol, 0.12 eq) was added, then following another 15 minutes at 0 °C a final eighth of an equivalent (total 0.5 eq) was added. The reaction was stirred on ice for another hour before deemed complete by TLC. The reaction was quenched with saturated sodium thiosulfate and partitioned between EtOAc and water. The aqueous phase was washed 4 times with EtOAc and the organic phases were combined, dried with sodium sulfate, filtered, and evaporated to dryness. The crude halogenated nucleobase was subjected to column chromatography using a gradient of 0–20% EtOAc in hexanes to yield a yellowish solid (7.8 g, 45%). ^{1}H NMR (500 MHz, chloroform-*d*) δ 8.72 (s, 1H), 8.06 (d, *J* = 3.7 Hz, 1H), 2.72 (s, 3H). ^{13}C NMR (126 MHz, chloroform-*d*) δ 169.21 (s), 152.50 (s), 145.38 (s), 127.40 (s), 120.41 (s), 106.56 (s), 12.22 (d, *J* = 3.3 Hz). ESI-HRMS calculated for $C_7H_6N_2BrS_2$ $[M+H]^+$ 260.9150, found 260.9149.

2.2. Synthesis of 3'-azido-3'deoxy lactone 13

Scheme S2. Synthesis of azidolactone **13**



(a) i. H_2SO_4, Acetone, RT, 2.5 hr; ii. TBAI, DIPEA, BnBr, 90 °C, 22h, 65%. (b) i. Tf_2O, Pyridine, DCM, 0 °C, 2h; ii. NaN_3, DMF, RT, 48 hr, 53%. (c) i. AcCl, MeOH, RT, ON; ii. NaH, BnBr, TBAI, DMF, 0 °C to RT, ON, 83%. (d) i. HCl, H_2O, Dioxane, 60 °C, ON; ii. PCC, DCM, RT, ON, 78%.DMF, RT, 1 hr, 78%.

(5*R*,6*S*)-5-((benzyloxy)methyl)-2,2-dimethyltetrahydrofuro[2,3-*d*][1,3]dioxol-6-ol (10)

Compound **10** was synthesized according to a previously published procedure.[3] Briefly, D-xylose (50 g, 333 mmol) was dissolved in a solution of sulfuric acid (50 ml) in acetone (1.3 L). The solution was allowed to stir for 30 minutes at room temperature using a water bath to keep the temperature constant. A solution of sodium carbonate (65 g, 613 mmol, 1.8 eq) in water (560 ml) was added slowly at room temperature and allowed to stir for 2.5 hours. Solid sodium carbonate was then added (35 g, 330 mmol, 1 eq) and the solution was stirred for 20 minutes. The resulting solid was filtered, and the filtrate was evaporated to dryness leaving a crude oil. To the crude was added DIPEA (85.23 g, 660 mmol, 115 ml, 2 eq), benzyl bromide (90 g, 526 mmol, 62.5 ml, 1.6 eq) and tetrabutylammonium iodide (36.75 g, 100 mmol, 0.3 eq). The reaction mixture was stirred at 90 °C for 5 hours. Both DIPEA (85.23 g, 660 mmol, 115 ml, 2 eq) and benzyl bromide (90 g, 526 mmol, 625 ml, 1.6 eq) were added again, and the reaction was stirred at 90 °C for 17 hours. The reaction mixture was then cooled to room temperature and partitioned between DCM and water. The aqueous phase was washed with DCM 6 times and the organic phases were combined, dried over sodium sulfate, filtered, and evaporated to dryness. The resulting residue was subjected to column chromatography with a gradient of 0 to 45% EtOAc in hexanes to yield a clear oil (60.7 g, 65% over 2 steps) that was recrystallized from toluene/pentane. ^1H NMR (500 MHz, chloroform-*d*) δ 7.36 – 7.28 (m, 5H), 5.95 (d, J = 3.7 Hz, 1H), 4.58 (ABq, J = 11.9 Hz, 2H), 4.48 (d, J = 3.7 Hz, 1H), 4.26 – 4.20 (m, 2H), 3.90 (dd, J = 10.9, 4.1 Hz, 1H), 3.86 (dd, J = 10.9, 3.6 Hz, 1H), 3.68 (d, J = 3.0 Hz, 1H), 1.47 (s, 3H), 1.29 (s, 3H).^{13}C NMR (126 MHz, Chloroform-*d*) δ 137.15, 128.60, 128.09, 127.93, 111.59, 104.84, 85.30, 78.18, 76.29, 74.05, 68.16, 26.77, 26.19. ESI-HRMS calculated for $C_{15}H_{20}O_5Na$ $[M+Na]^+$ 303.1203, found 303.1202.

(5*S*,6*R*)-6-azido-5-((benzyloxy)methyl)-2,2-dimethyltetrahydrofuro[2,3-*d*][1,3]dioxole (11)

Compound **10** (60.7 g, 217 mmol) was azeotroped with toluene 3 times, then dissolved in anhydrous DCM. Anhydrous pyridine (43 ml) was added, and the solution was cooled to 0 °C with an ice bath and trifluoromethanesulfonic anhydride (91.9 g, 326 mmol, 60.9 ml, 1.5 eq) was added dropwise via a flame-dried addition funnel. Following addition, the reaction was allowed to stir at 0 °C for 2 hours. The reaction was then partitioned between DCM and water. The organic phase was dried over sodium sulfate, filtered and evaporated to dryness yielding an oil that was immediately reacted further. The crude triflate was azeotroped with toluene 3 times and purged with argon, then dissolved in anhydrous DMF (400 ml). Sodium azide (60.6 g, 933 mmol, 4.3 eq) was added and the reaction was stirred at room temperature for 2 days. The reaction was concentrated and partitioned between EtOAc and water. The organic phase was washed 5 times with water, dried over sodium sulfate, filtered, and evaporated to dryness. The crude oil was subjected to column chromatography with a gradient of 0–30% EtOAc in hexanes to yield a yellowish oil (35.5g, 53% over 2 steps). ^{1}H NMR (500 MHz, chloroform-*d*) δ 7.38 – 7.27 (m, 5H), 5.82 (d, *J* = 3.6 Hz, 1H), 4.71 (dd, *J* = 4.2, 3.6 Hz, 1H), 4.60 (ABq, *J* = 12.1 Hz, 2H), 4.21 – 4.17 (m, 1H), 3.80 (dd, *J* = 11.3, 2.5 Hz, 1H), 3.64 (dd, *J* = 11.4, 3.6 Hz, 1H). 3.62 (dd, *J* = 9.6, 4.7 Hz, 1H). 1.57 (s, 3H), 1.36 (s, 3H). ^{13}C NMR (126 MHz, chloroform-*d*) δ 137.59 (s), 128.48 (s), 127.86 (s), 127.79 (s), 113.06 (s), 104.13 (s), 79.77 (d, *J* = 20.4 Hz), 77.26 (s), 73.70 (s), 67.53 (s), 60.43 (s), 26.41 (s), 26.40 (s). ESI-HRMS calculated for $C_{15}H_{19}N_{3}O_{4}Na$ $[M+Na]^{+}$ 328.1268, found 328.1271.

(2*S*,3*R*,4*R*)-3-azido-4-(benzyloxy)-2-((benzyloxy)methyl)-5-methoxytetrahydrofuran (12)

Compound **11** (35.5 g, 232 mmol) was dissolved in MeOH (305 ml) and acetyl chloride (4.51 g, 57.5 mmol, 4.1 ml) was added dropwise. The solution was allowed to stir at room temperature overnight, then evaporated to dryness and partitioned between DCM and saturated $NaHCO_3$. The aqueous phase was extracted with EtOAc twice and the organic phases were combined, dried over sodium sulfate, and evaporated to dryness leaving an oily crude that was dried on high vacuum overnight. After purging with argon, the riboside was dissolved in anhydrous DMF (594 ml) and tetrabutylammonium iodide (429 mg, 1.16 mmol, 0.01 eq). The solution was brought to 0 °C with an ice bath and sodium hydride (5.58 g, 232 mmol, 2 eq) was added slowly under an argon stream. The solution was allowed to stir for 15 minutes at 0 °C. Benzyl bromide (39.8 g, 232 mmol, 2 eq) was added dropwise via an addition funnel. The reaction was left to warm to room temperature overnight. The reaction was quenched with 45 ml saturated NH_4Cl solution and concentrated. The remaining crude was partitioned between EtOAc and water. The aqueous phase was extracted with EtOAc 3 times, and the organic phases were combined, dried with sodium sulfate, and concentrated. Column chromatography of the resulting residue with a gradient of 0 to 30% EtOAc in hexanes yielded an oil with two separable isomers that were combined for the next reaction (35.5 g, 83% over 2 steps). ^{1}H NMR (500 MHz, chloroform-*d*) δ 7.46 – 7.30 (m, 10H), 4.92 (s, 1H), 4.73 (s, 2H), 4.63 (d, *J* = 1.4 Hz, 2H), 4.35 (dt, *J* = 7.5, 4.9 Hz, 1H), 4.00 (d, *J* = 4.8 Hz, 1H), 3.84 (dd, *J* = 7.5, 4.9 Hz, 1H), 3.68 – 3.58 (m, 2H). ^{13}C NMR (126 MHz, Chloroform-*d*) δ 137.91, 137.25, 128.52, 128.41, 127.98, 127.71, 127.63, 106.09, 82.50, 79.74, 77.49, 73.41, 72.79, 70.95, 61.45, 55.14. ESI-HRMS calculated for

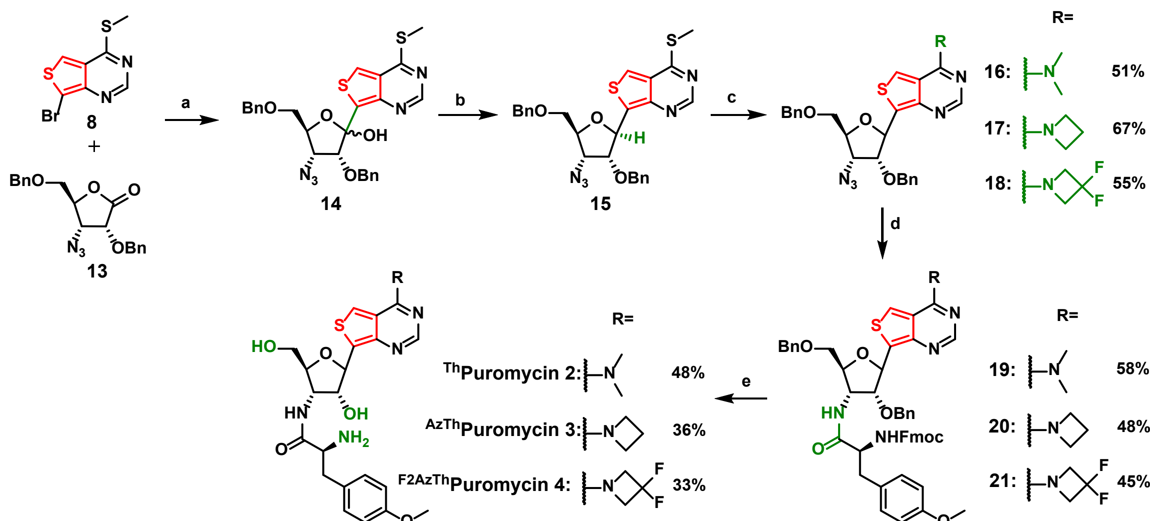
$C_{20}H_{23}N_3O_4Na$ [M+Na]$^+$ 392.1581, found 328.1578. 1H NMR (300 MHz, CDCl3) δ 7.45 – 7.29 (m, 10H), 4.88 (d, *J* = 4.2 Hz, 1H), 4.71 (ABq, *J* = 12.2 Hz, 2H), 4.57 (ABq, *J* = 12.1 Hz, 2H), 4.14 (dd, *J* = 7.3 Hz, 3.5 Hz, 1Hz), 3.94 (ABq, *J* = 7.5, 3.9 Hz, 2H), 3.60 (d, *J* = 3.6 Hz, 2H), 3.46 (s, 3H). ^{13}C NMR (126 MHz, chloroform-*d*) δ 137.64, 137.17, 128.52, 128.48, 128.12, 128.09, 127.85, 127.65, 101.81, 80.73, 78.33, 77.49, 73.61, 72.94, 69.83, 59.62, 55.31. ESI-HRMS calculated for $C_{20}H_{23}N_3O_4Na$ [M+Na]$^+$ 392.1581, found 392.1581.

(3*R*,4*R*,5*S*)-4-azido-3-(benzyloxy)-5-((benzyloxy)methyl)dihydrofuran-2(3H)-one (13)

Compound **12** (13.6 g, 37 mmol) was dissolved in a mixture of dioxane (170 ml) and water (108 ml). 37% HCl (54 ml) was added to the solution slowly over 20 minutes and the solution was stirred at 60 °C overnight. The reaction was then cooled in an ice bath and quenched with saturated $NaHCO_3$. The mixture was poured into a separatory funnel and extracted 3 times with EtOAc. The organic phases were combined, dried with sodium sulfate, filtered, and concentrated to yield an oil. The crude hemi-acetal was then coevaporated once with DCM and 3 times with toluene, then purged with argon and dissolved in anhydrous DCM (297 ml). Pyridinium chlorochromate (12 g, 55.5 mmol, 1.5 eq) was added and the reaction was stirred at room temperature overnight. Upon reaction completion the solution was filtered over a celite cake and evaporated to dryness. The crude residue was subjected to column chromatography with a gradient of 0-40% EtOAc in hexanes to yield the lactone as an off-yellow oil (9.7 g, 74% over 2 steps). ^{1}H NMR (500 MHz, chloroform-*d*) δ 7.40 – 7.13 (m, 10H), 5.02 (d, *J* = 12.0 Hz, 1H), 4.78 (d, *J* = 12.0 Hz, 1H), 4.62 (d, *J* = 6.2 Hz, 1H), 4.47 (dd, *J* = 50.1, 11.8 Hz, 2H), 4.35 (dd, *J* = 3.9, 2.2 Hz, 1H), 4.14 (dd, *J* = 6.2, 1.5 Hz, 1H), 3.68 (dd, *J* = 11.1, 2.6 Hz, 1H), 3.61 (dd, *J* = 11.1, 2.3 Hz, 1H). ^{13}C NMR (126 MHz, chloroform-*d*) δ 172.95 (s), 136.77 (s), 136.43 (s), 128.65 (s), 128.61 (s), 128.34 (s), 128.19 (s), 128.18 (s), 127.70 (s), 80.65 (d, *J* = 33.8 Hz), 74.16 (s), 73.78 (s), 73.29 (s), 68.90 (s), 60.21 (s). ESI-HRMS calculated for $C_{19}H_{19}N_3O_4Na$ [M+Na]$^+$ 376.1268, found 376.1265.

2.3. Synthesis of Puromycin Analogues 2, 3, and 4

Scheme S3. Synthesis of Puromycin Derivatives **2**, **3**, and **4** from nucleobase **8** and azidolactone **13**



(a) n-BuLi, THF, –78°C, 27%. (b) $BF_3{\cdot}OEt_2$, Et_3SiH, DCM, -78°C to RT, 55%. (c) RH_2Cl, DBU, DMSO, RT. (d) Fmoc-O-methyl-L-tyrosine, EDC, HOBt, $(Me)_3P$, THF. (e) i. Piperidine, DMF, RT; ii. BCl_3, DCM, HPLC.

(3*R*,4*R*,5S)-4-azido-3-(benzyloxy)-5-((benzyloxy)methyl)-2-(4-(methylthio)thieno[3,4-*d*]pyrimidin-7-yl)tetrahydrofuran-2-ol (14)

To a 100 ml flame-dried round bottom flask purged with argon was added nucleobase **8** (1 g, 3.8 mmol), anhydrous THF (10 ml), azidolactone **13** (1.6 g, 4.6 mmol, 1.2 eq), and activated 3 Å molecular sieves. The solution was allowed to dry overnight and was then brought to –78° C in a dry-ice acetone bath. *n*-BuLi (2.4 M in hexanes, 1.9 ml, 4.6 mmol, 1.2 eq) was added slowly dropwise. The reaction was allowed to stir for 2 hours at –78° C, then quenched with 1 ml saturated ammonium chloride. The solution was partitioned between EtOAc and water. The organic phase was dried with sodium sulfate, filtered, and evaporated to dryness. The crude hemi-ketal was subjected to column chromatography using a gradient of 0–30% EtOAc in hexanes to yield a yellowish solid (550 mg, 27%). The product was crudely purified and used immediately in the next reaction.

7-((2*R*,3*R*,4*R*,5*S*)-4-azido-3-(benzyloxy)-5-((benzyloxy)methyl)tetrahydrofuran-2-yl)-4-(methylthio)thieno[3,4-*d*]pyrimidine (15)

To a 25 ml round bottom flask that was flame-dried and purged with argon was added hemi-ketal **14** (530 mg, 1.0 mmol) and anhydrous DCM (12 ml). Triethylsilane (690 mg, 5.9 mmol, 950 µl, 6 eq) was added and the solution was allowed to cool to -78 °C in a dry ice acetone bath. $BF_3 \cdot Et_2O$ (420 mg, 3.0 mmol, 370 µl, 3 eq) was added dropwise. The reaction was initially allowed to stir at -78 °C for 1 hour before stirring at room temperature for 2 hours, then quenched with triethylamine (506 mg, 5 mmol, 700 µl, 5 eq). The solution was partitioned between EtOAc and water, then extracted. The aqueous phase was washed with EtOAc and the organic phases were combined, dried with sodium sulfate, filtered, and evaporated to dryness. The crude glycoside was subjected to column chromatography using a gradient of 0–15% EtOAc in hexanes to yield the desired product as a yellowish solid (284 mg, 55%). ^{1}H NMR (500 MHz, chloroform-*d*) δ 8.62 (s, 1H), 7.87 (s, 1H), 7.38 – 7.20 (m, 10H), 5.91 (d, *J* = 4.3 Hz, 1H), 4.76 (ABq, *J*= 12.2 Hz, 2H), 4.64 (ABq, *J* = 12.0 Hz, 2H), 4.44 (t, *J* = 4.9 Hz, 1H), 4.32 (dt, *J* = 6.9, 3.5 Hz, 1H), 3.97 (dd, *J* = 6.8, 5.3 Hz, 1H), 3.81 (dd, *J* = 10.9, 3.1 Hz, 1H), δ 2.70 (s, 1H). ^{13}C NMR (126 MHz, Chloroform-d) δ 168.14, 151.15, 144.35, 137.94, 137.32, 134.76, 128.43, 128.27, 128.21, 128.01, 127.82, 127.73, 127.65, 118.58, 84.15, 80.46, 78.20, 73.59, 72.40, 69.64, 61.01, 11.97. ESI-HRMS calculated for $C_{26}H_{26}N_5O_3S_2$ $[M+H]^+$ 520.1472, found 520.1474.

7-((3*R*,4*R*,5*S*)-4-azido-3-(benzyloxy)-5-((benzyloxy)methyl)tetrahydrofuran-2-yl)-*N*,*N*-dimethylthieno [3,4-*d*]pyrimidin-4-amine (16)

To a 10 ml round-bottom flask that was flame-dried and purged with argon was added protected nucleoside **15** (45 mg, 0.1 mmol) and anhydrous DMSO (1 ml). Dimethylammonium hydrochloride (184 mg, 2.3 mmol, 26 eq) was added followed by DBU (490 mg, 3.6 mmol, 450 µl, 37 eq). The reaction was allowed to stir at room temperature overnight. Upon proof of completion the solution was partitioned between EtOAc and water and extracted. The organic phase was washed with water 6 times, dried with sodium sulfate, filtered, and evaporated to dryness. The crude was subjected to column chromatography using a gradient of 0–2% MeOH in DCM to yield the desired product as a yellowish film (23 mg, 51%). ^{1}H NMR (500 MHz, chloroform-*d*) δ 8.29 (s, 1H), 7.89 (s, 1H), 7.38 – 7.26 (m, 10H), 5.93 (d, *J* = 3.8 Hz, 1H), 4.82 (Abq, *J* = 12.2 Hz, 2H), 4.65 (ABq, *J* = 12.2 Hz, 2H), 4.39 (dd, *J* = 5.4, 3.9 Hz, 1H), 4.34 – 4.30 (m, *J* = 7.3, 4.0, 3.2 Hz, 1H), 3.92 (dd, *J* = 7.4, 5.4 Hz, 1H), 3.82 (dd, *J* = 10.9, 3.1 Hz, 1H), 3.73 (dd, *J* = 10.9, 4.2 Hz, 1H), 3.48 (s, 6H).^{13}C NMR (126 MHz, chloroform-*d*) δ 157.94 (s), 153.20 (s), 148.42 (s), 138.08 (s), 137.60 (s), 130.46 (s), 128.40 (s), 128.26 (s), 128.06 (s), 127.78 – 127.54 (m), 121.38 (s), 120.76 (s), 83.87 (s), 80.04 (s), 78.74 (s), 73.55 (s), 72.11 (s), 69.67 (s), 60.88 (s), 40.48 (s). ESI-HRMS calculated for $C_{27}H_{29}N_6O_3S$ $[M+H]^+$ 517.2016, found 517.2012.

4-(azetidin-1-yl)-7-((3*R*,4*R*,5*S*)-4-azido-3-(benzyloxy)-5-((benzyloxy)methyl)tetrahydrofuran-2-yl)thieno[3,4-*d*]pyrimidine (17)

To a flame-dried 10 ml round bottom flask under argon was added protected nucleoside **15** (89 mg, 0.17 mmol) and anhydrous DMSO (3.4 ml). Azetidine hydrochloride (458 mmol, 5 mmol, 29 eq) was added along with DBU (1.08 g, 7.13 mmol, 1 ml, 42 eq) and the reaction was allowed to stir overnight. The reaction was partitioned between EtOAc and water and extracted. The organic phase was washed with water 6 times, dried over sodium sulfate, filtered, and evaporated to dryness. Column chromatography of the crude nucleoside using a gradient of 0-2% MeOH in DCM yielded the desired product as a brownish solid (63 mg, 70%). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.26 (s, 1H), 7.66 (s, 1H), 5.87 (d, *J* = 4.1 Hz, 1H), 4.85 – 4.74 (m, 2H), 4.69 – 4.58 (m, 2H), 4.40 (dd, *J* = 5.4, 4.1 Hz, 1H), 4.30 (ddd, *J* = 7.2, 4.1, 3.1 Hz, 1H), 3.94 (dd, *J* = 7.2, 5.4 Hz, 1H), 3.85 – 3.68 (m, 2H), 2.56 (tt, *J* = 8.4, 6.9 Hz, 2H). ^{13}C NMR (126 MHz, Chloroform-*d*) δ 156.20, 153.21, 146.48, 138.05, 137.52, 131.16, 128.44, 128.30, 128.08, 127.75, 127.69, 127.65, 119.68, 83.89, 80.23, 78.47, 73.57, 73.56, 72.25, 72.23, 69.67, 60.93, 53.09, 50.97, 29.75, 17.16. ESI-HRMS calculated for $C_{28}H_{29}N_6O_3S$ $[M+H]^+$ 529.2016, found 529.2016.

7-((3*R*,4*R*,5*S*)-4-azido-3-(benzyloxy)-5-((benzyloxy)methyl)tetrahydrofuran-2-yl)-4-(3,3-difluoroazetin-1-yl)thieno[3,4-*d*]pyrimidine (18)

To a flame-dried 25 ml round bottom flask under argon was added protected nucleoside **15** (92 mg, 0.18 mmol) and anhydrous DMSO (1.7 ml). Difluoroazetidine hydorchloride (600 mg, 4.6 mmol, 26 eq) and DBU (1 g, 6.6 mmol, 0.9 ml, 37 eq) were added sequentially. The reaction was allowed to stir overnight at room temperature. The reaction solution was then partitioned between EtOAc and water. The organic phase was washed with water 6 times, dried over sodium sulfate, filtered, and evaporated to dryness. The crude nucleoside was subjected to column chromatography using a gradient of 0–40% EtOAc in hexanes to yield the desired product as a yellowish solid (57 mg, 57%). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.34 (s, 1H), 7.59 (s, 1H), 7.36 – 7.22 (m, 13H), 5.90 (d, *J* = 3.8 Hz, 1H), 4.80 (dd, *J* = 13.5, 8.4 Hz, 7H), 4.70 – 4.60 (m, 2H), 4.42 (t, *J* = 4.7 Hz, 1H), 4.32 (dt, *J* = 7.2, 3.4 Hz, 1H), 3.94 (t, *J* = 6.4 Hz, 1H), 3.77 (ddd, *J* = 52.9, 10.9, 3.4 Hz, 2H). ^{13}C NMR (126 MHz, Chloroform-*d*) δ 156.64 (d, *J* = 4.1 Hz), 153.07, 146.95, 138.00, 137.45, 133.06, 128.42, 128.29, 128.05, 127.76, 127.69, 127.62, 120.24, 118.39, 117.98, 115.80, 113.62, 83.98, 80.24, 78.52, 73.56, 72.27, 69.56, 63.70 – 62.61 (m), 60.84. ESI-HRMS calculated for $C_{28}H_{27}F_2O_3N_6S$ $[M+H]^+$ 565.1828, found 565.1822.

(9*H*-fluoren-9-yl)methyl ((2*S*)-1-(((2*S*,3*R*,4*R*)-4-(benzyloxy)-2-((benzyloxy)methyl)-5-(4-dimethylamino) thieno[3,4-*d*]pyrimidin-7-yl)tetrahydrofuran-3-yl)amino)-3-(4-methoxyphenyl)-1-oxopropan-2-yl)carbamate (19)

To a 10 ml round bottom flask that was flame-dried and purged with argon was added protected nucleoside **16** (54 mg, 0.10 mmol) and THF (1.1 ml) and water (100 µl). A solution of trimethylphosphine in THF (1 M, 312 µl, 3 eq) was added dropwise and the solution was stirred at room temperature until disappearance of starting material was observed 30 minutes later. The solution was then evaporated to dryness and the crude aminonucleoside was coevaporated 3 times with acetonitrile and left to dry on high vacuum for 3 hours. Subsequently, in a separate flame-dried and argon-purged 10 ml round bottom flask was added Fmoc-O-methyl-L-tyrosine (57 mg, 0.14 mmol, 1.3 eq) and anhydrous THF (4 ml). The solution was brought to 0 °C in an ice bath and HOBt (26 mg, 0.19 mmol, 1.7 eq) was added. Following 15 minutes of stirring on ice, EDC·HCl (37 mg, 0.19 mmol, 1.7 eq) was added. The newly formed suspension was stirred at 0 °C for 30 minutes before adding a cooled solution of the crude aminonucleoside in THF (1 m) dropwise. The reaction was allowed to warm to room temperature overnight. The solution was then rotorvapped to dryness, then dissolved in DCM and extracted with water. The aqueous phase was washed with DCM 3 times and the organic phases were combined, dried with sodium sulfate, filtered, and evaporated to dryness. The protected antibiotic was crudely purified using column chromatography with a gradient of 0–5% MeOH in DCM to yield the desired product as a slightly impure cream solid (54 mg, 58%) that was immediately subjected to deprotection.

(9*H*-fluoren-9-yl)methyl ((2*S*)-1-(((2*S*,3*R*,4*R*)-5-(4-(azetidin-1-yl)thieno[3,4-*d*]pyrimidin-7-yl)-4-(benzyloxy)-2-((benzyloxy)methyl)tetrahydrofuran-3-yl)amino)-3-(4-methoxyphenyl)-1-oxopropan-2-yl)carbamate (20)

To a flame-dried 10 ml round-bottom flask was added azetidine-modified nucleoside **17** (55 mg, 0.10 mmol) and THF (1.1 ml). Trimethylphosphine (1 M in THF, 0.34 ml, 3.3 eq) was added along with water (0.1 ml). The reaction was stirred for 30 minutes at room temperature until all starting material dissappeared by TLC. The solution was then evaporated to dryness, coevaporated 3 times with acetonitrile, and reconstituted in anhydrous THF (1 ml). Simultaneously, in a separate flame-dried round bottom flask under argon was added Fmoc-*O*-methyl-L-tyrosine (62 mg, 0.15 mmol, 1.4 eq) and anhydrous THF (4 ml), The solution was cooled to in an ice bath to 0 °C and HOBt (26 mg, 0.19 mmol, 1.9 eq) was added. After stirring under ice for 15 minutes, EDC·HCl (37 mg, 0.19 mmol, 1.9 eq) was added. The reaction was allowed to stir at 0 °C for 30 minutes. A chilled solution of the 3'-amino-3'-deoxy nucleoside in THF prepared previously was added dropwise to the newly formed activated ester. The reaction was allowed to warm to room temperature overnight. Upon proof of completion the reaction was evaporated to dryness and the crude was partitioned between DCM and water. The aqueous phase was washed 3 times with DCM and the organic phases were combined, dried over sodium sulfate, filtered, and evaporated to dryness. The protected antibiotic was crudely purified using column chromatography with a gradient of 0–5% MeOH in DCM to yield the desired product as cream solid (45 mg, 48%) and immediately subjected to deprotection.

(9*H*-fluoren-9-yl)methyl ((2*S*)-1-(((2*S*,3*R*,4*R*)-4-(benzyloxy)-2-((benzyloxy)methyl)-5-(4-(3,3-difluoroazetidin-1-yl)thieno[3,4-*d*]pyrimidin-7-yl)tetrahydrofuran-3-yl)amino)-3-(4-methoxyphenyl)-1-oxopropan-2-yl)carbamate (21)

To a flame-dried 10 ml round-bottom flask was added difluoroazetidine-modified nucleoside **18** (57 mg, 0.10 mmol) and THF (1.1 ml). Trimethylphosphine (1 M in THF, 0.34 ml, 3.3 eq) was added along with water (0.1 ml). The reaction was stirred for 30 minutes at room temperature until all starting material dissappeared by TLC. The solution was then evaporated to dryness slowly while keeping the solution cool, coevaporated 3 times with cold acetonitrile, and reconstituted in chilled anhydrous THF (1 ml). Simultaneously, in a separate flame-dried round bottom flask under argon was added Fmoc-*O*-methyl-L-tyrosine (62 mg, 0.15 mmol, 1.4 eq) and anhydrous THF (4 ml), The solution was cooled to in an ice bath to 0 °C and HOBt (26 mg, 0.19 mmol, 1.9 eq) was added. After stirring under ice for 15 minutes, EDC·HCl (37 mg, 0.19 mmol, 1.9 eq) was added. The reaction was allowed to stir at 0 °C for 30 minutes. A chilled solution of the 3'-amino-3'-deoxy nucleoside in THF prepared previously was added dropwise to the newly formed activated ester. The reaction was allowed to warm to room temperature overnight. Upon proof of completion the reaction was evaporated to dryness and the crude was partitioned between DCM and water. The aqueous phase was washed 3 times with DCM and the organic phases were combined, dried over sodium sulfate, filtered, and evaporated to dryness. The antibiotic was crudely purified using column chromatography with a gradient of 0–5% MeOH in DCM to yield the desired product as cream solid (43 mg, 45%) that was immediately subjected to deprotection.

(2*S*)-2-amino-N-((2*S*,3*S*,4*R*)-5-(4-(dimethylamino)thieno[3,4-*d*]pyrimidin-7-yl)-4-hydroxy-2-(hydroxy methyl)tetrahydrofuran-3-yl)-3-(4-methoxyphenyl)propenamide (2)

To a flame-dried 25 ml round-bottom flask under argon was added protected Thpuromycin **19** (38 mg, 0.04 mmol) and a solution of 20% piperidine in DMF (1 ml). The reaction was allowed to stir at room temperature for 15 minutes, then evaporated to dryness. The crude was coevaporated 3 times with heptane and left to dry on high vaccuum overnight, then subsequently flushed with argon and dissolved in anhydrous DCM (2.5 ml) The solution was brought to –78 °C with a dry-ice acetone bath and BCl_3 (1M in DCM, 0.4 ml, 0.4 mmol, 10 eq) was added dropwise. The solution was allowed to stir at –78 °C for 1 hour, then at room temperature for another hour. Upon proof of reaction completion with ESI-MS, the reaction was quenched with methanol and evaporated to dryness. The crude antibiotic was purified using reverse-phase HPLC (0-40% ACN + 0.1% TFA in water + 0.1% TFA over 22 minutes) to yield the desired product as a white solid (10 mg, 48% over 2 steps). ^{1}H NMR (500 MHz,) δ 8.61 (s, 1H), 8.08 (s, 1H), 7.13 – 7.07 (m, 2H), 6.92 – 6.83 (m, 2H), 5.03 (d, *J* = 5.5 Hz, 1H), 4.26 (dd, *J* = 6.5, 5.5 Hz, 1H), 4.13 – 4.09 (m, 2H), 3.67 (d, *J* = 1.4 Hz, 6H), 3.63 (ddd, *J* = 6.8, 4.1, 2.6 Hz, 1H), 3.53 (dd, *J* = 12.4, 2.6 Hz, 1H), 3.42 (s, 3H), 3.37 (dd, *J* = 12.5, 4.2 Hz, 1H), 3.17 – 2.87 (m, 2H). $_{13}$C NMR (126 MHz, D_2O) δ 169.13 (s), 163.03 (s), 162.75 (s), 158.29 (s), 151.09 (s), 147.25 (s), 130.63 (s), 130.47 (s), 126.27 (s), 118.28 (s), 117.29 (s), 114.97 (s), 114.28 (s), 82.14 (s), 79.11 (s), 74.96 (s), 60.76 (s), 55.13 (s), 54.36 (s), 51.98 (s), 42.46 (s), 41.44 (s), 35.98 (s). ESI-HRMS calculated for $C_{23}H_{30}N_5O_5S$ $[M+H]^+$ 488.1962, found 488.1955.

(2*S*)-2-amino-N-((2*S*,3*S*,4*R*)-5-(4-(azetidin-1-yl)thieno[3,4-*d*]pyrimidin-7-yl)-4-hydroxy-2-(hydroxymethyl)tetrahydrofuran-3-yl)-3-(4-methoxyphenyl)propenamide (3)

To a flame-dried 25 ml round-bottom flask under argon was added protected antibiotic **20** (35 mg, 0.04 mmol) and a solution of 20% piperidine in DMF (1 ml). The reaction was allowed to stir at room temperature for 15 minutes, then evaporated to dryness. The crude was coevaporated 3 times with heptane and left to dry on high vaccuum overnight, then subsequently flushed with argon and dissolved in anhydrous DCM (2.5 ml) The solution was brought to –78 °C with a dry-ice acetone bath and BCl_3 (1M in DCM, 0.4 ml, 0.4 mmol, 10 eq) was added dropwise. The solution was allowed to stir at –78 °C for 1 hour, then at room temperature for another hour. Upon proof of reaction completion with ESI-MS, the reaction was quenched with methanol and evaporated to dryness. The crude antibiotic was purified using reverse-phase HPLC (0-40% ACN + 0.1% TFA in water + 0.1% TFA over 22 minutes) to yield the desired product as a white solid (7 mg, 36% over 2 steps). ^{1}H NMR (500 MHz, D_2O) δ 8.28 (s, 1H), 8.01 (s, 1H), 7.12 – 7.05 (m, 2H), 6.89 – 6.80 (m, 2H), 5.03 (d, *J* = 5.4 Hz, 1H), 4.82 – 4.74 (m, 2H), 4.46 – 4.37 (m, 2H), 4.24 (dd, *J* = 6.4, 5.4 Hz, 1H), 4.15 – 4.04 (m, 2H), 3.65 (s, 3H), 3.62 (ddd, *J* = 6.8, 4.2, 2.5 Hz, 1H), 3.54 – 3.31 (m, 2H), 3.18 – 2.84 (m, 2H), 2.52 (p, *J* = 7.7 Hz, 2H). ^{13}C NMR (126 MHz, Deuterium Oxide) δ 169.11, 162.84 (q, *J* = 35.6 Hz), 158.28, 153.44, 147.47, 133.42, 130.45, 128.50, 126.25, 125.97, 119.61, 117.31, 114.98, 114.25, 112.65, 82.08, 79.18 (d, *J* = 2.5 Hz), 75.14, 60.75, 55.28 – 55.07 (m), 54.84, 54.36, 52.99, 51.94, 35.97, 16.14. ESI-HRMS calculated for $C_{24}H_{30}N_5O_5S$ $[M+H]^+$ 500.1962, found 500.1960.

(2*S*)-2-amino-N-((2*S*,3*S*,4*R*)-5-(4-(3,3-difluoroazetidin-1-yl)thieno[3,4-*d*]pyrimidin-7-yl)-4-hydroxy-2-(hydroxymethyl)tetrahydrofuran-3-yl)-3-(4-methoxyphenyl)propenamide (4)

To a flame-dried 25 ml round-bottom flask under argon was added protected antibiotic **21** (43 mg, 0.05 mmol) and a solution of 20% piperidine in DMF (1 ml). The reaction was allowed to stir at room temperature for 15 minutes, then evaporated to dryness. The crude was coevaporated 3 times with heptane and left to dry on high vaccuum overnight, then subsequently flushed with argon and dissolved in anhydrous DCM (2.5 ml) The solution was brought to –78 °C with a dry-ice acetone bath and BCl_3 (1M in DCM, 0.5 ml, 0.5 mmol, 10 eq) was added dropwise. The solution was allowed to stir at –78 °C for 1 hour, then at room temperature for another hour. Upon proof of reaction completion with ESI-MS, the reaction was quenched with methanol and evaporated to dryness. The crude antibiotic was purified using reverse-phase HPLC (0-40% ACN + 0.1% TFA in water + 0.1% TFA over 22 minutes) to yield the desired product as a white solid (8 mg, 33% over 2 steps). ^{1}H NMR (500 MHz, D_2O) δ 8.36 (s, 1H), 8.20 (s, 1H), 7.15 – 7.08 (m, 2H), 6.91 – 6.85 (m, 2H), 5.26 (t, *J* = 11.1 Hz, 2H), 5.07 (d, *J* = 5.4 Hz, 1H), 4.88 (t, *J* = 11.4 Hz, 2H), 4.31 – 4.22 (m, 1H), 4.11 (ddd, *J* = 8.2, 6.2, 1.4 Hz, 2H), 3.67 (s, 3H), 3.64 (ddd, *J* = 6.6, 4.1, 2.4 Hz, 1H), 3.54 (dd, *J* = 12.5, 2.6 Hz, 1H), 3.36 (dd, *J* = 12.5, 4.1 Hz, 1H), 3.19 – 2.85 (m, 2H). ^{13}C NMR (126 MHz, Deuterium Oxide) δ 176.12, 169.14, 162.91 (q, *J* = 35.6 Hz), 158.30, 155.83, 149.29, 132.88, 130.47, 128.27, 126.27, 125.86, 119.62, 117.65, 117.30, 116.78, 114.98, 114.62, 114.28, 112.66, 112.46, 104.99, 82.14, 79.23 (d, *J* = 2.2 Hz), 75.14, 64.49 (dt, *J* = 97.5, 30.0 Hz), 60.67, 55.69 – 55.07 (m), 54.36, 51.92, 38.83, 35.98, 35.38. ESI-HRMS calculated for $C_{24}H_{28}F_2N_2O_5S$ $[M+H]^+$ 536.1774, found 536.1774.

3. Structural analysis

3.1. Experimental summary

Single crystal X-ray diffraction studies were carried out on a Bruker Pt 135 CCD diffractometer equipped with Cu K_{α} radiation (λ = 1.54178).

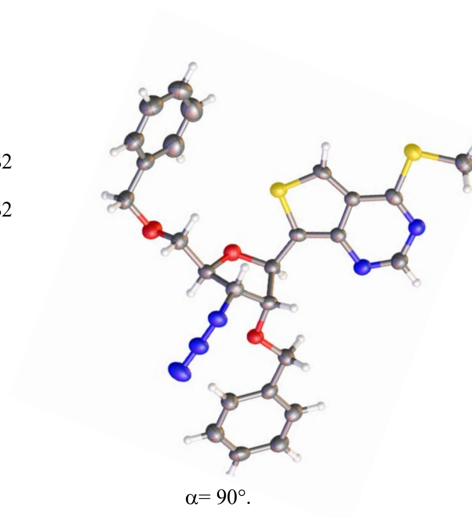
A 0.135 × 0.045 × 0.040 mm light yellow colorless crystal was mounted on a Cryoloop with Paratone oil. Data were collected in a nitrogen gas stream at 100(2) K using ϕ and ϖ scans. Crystal-to-detector distance was 45 mm using exposure time 10, 15 and 60s s with a scan width of 1.25°. Data collection was 99.4% complete to 63.776° in θ.

A total of 14046 reflections were collected covering the indices, –5<=h<=5, –14<=k<13, –46<=l<=45. 4065 reflections were found to be symmetry independent, with a R_{int} of 0.0739. Indexing and unit cell refinement indicated a Primitive, Orthorhombic lattice. The space group was found to be $P2_12_12_1$. The data were integrated using the Bruker SAINT Software program and scaled using the SADABS software program. Solution by direct methods (SHELXT) produced a complete phasing model consistent with the proposed structure.

All nonhydrogen atoms were refined anisotropically by full-matrix least-squares (SHELXL-2014). All carbon bonded hydrogen atoms were placed using a riding model. Their positions were constrained relative to their parent atom using the appropriate HFIX command in SHELXL-2014. All other hydrogen atoms (H-bonding) were located in the difference map (N-H, O-H). There positions were refined using “riding” model. Crystallographic data are summarized in Table S1.

3.2. X-ray crystal structures

Table S1. Crystal data and structure refinement for compound **15**



Identification code	tor153	
Empirical formula	C26 H25 N5 O3 S2	
Molecular formula	C26 H25 N5 O3 S2	
Formula weight	519.63	
Temperature	100.0 K	
Wavelength	1.54178 Å	
Crystal system	Orthorhombic	
Space group	$P2_12_12_1$	
Unit cell dimensions	a = 4.7605(4) Å	α= 90°.
	b = 12.8199(9) Å	β= 90°.
	c = 40.759(3) Å	γ = 90°.
Volume	2487.5(3) $Å^3$	
Z	4	
Density (calculated)	1.388 Mg/m^3	
Absorption coefficient	2.263 mm^{-1}	
F(000)	1088	
Crystal size	0.135 x 0.045 x 0.04 mm^3	
Crystal color, habit	light yellow plank	
Theta range for data collection	2.168 to 63.776°.	
Index ranges	-5<=h<=5, -14<=k<=13, -46<=l<=45	
Reflections collected	14046	
Independent reflections	4065 [R(int) = 0.0739]	
Completeness to theta = 63.776°	99.4 %	
Absorption correction	Semi-empirical from equivalents	
Max. and min. transmission	0.5829 and 0.4428	
Refinement method	Full-matrix least-squares on F^2	
Data / restraints / parameters	4065 / 0 / 327	
Goodness-of-fit on F^2	1.056	
Final R indices [I>2sigma(I)]	R1 = 0.0590, wR2 = 0.1439	
R indices (all data)	R1 = 0.0878, wR2 = 0.1597	
Absolute structure parameter	0.04(2)	
Extinction coefficient	0.0038(6)	
Largest diff. peak and hole	0.318 and -0.368 e.$Å^{-3}$	

4. Absorption and emission spectroscopy assays and data

Table S2. Photophysical and chemical properties of puromycin (**1**) and analogues **2**–**4**.

Table S2. Photophysical Properties of Antibiotics **1**–**4**

Antibiotic	Solvent	λ_{abs} [a]	ε [a]	λ_{em} (Φ)[a]	Stokes Shift[a]
1	PBS	274	1.18[b]	n/a	n/a
2	PBS	354	1.03[c]	424 (0.004)	4.67±0.16
3	PBS	354	1.11[c]	417 (0.21)	4.21±0.07
4	PBS	353	0.90[c]	419 (0.31)	4.44±0.07

[a] λ_{abs}, ε, λ_{em} and Stokes shift are reported in nm, 10^4 M^{-1} cm^{-1}, nm and 10^3 cm^{-1} respectively. All the photophysical values reflect the average over three independent measurements. No detectable emission was observed for puromycin under these conditions. [b] Previously reported.[4] Aqueous extinction coefficients of the corresponding nucleobases reported previously.[1]

4.1. General

Absorption spectra were measured on a Shimadzu UV-2450 spectrophotometer setting the slit at 1 nm and using a resolution of 0.5 nm. All measurements were carried out in a 1 cm four-sided quartz cuvette from Helma, and the spectra were corrected for the blank. Emission and excitation spectra were measured on a Horiba Fluoromax-4 equipped with a cuvette holder with a stirring system setting the excitation slit at 1 nm and the emission slit at 3 nm, the resolution at 1 nm and the integration time 0.1 s. Emission spectra were measured exciting at 350 nm. Both instruments were equipped with a thermostat-controlled ethylene glycol-water bath fitted to specially designed cuvette holder and the temperature was kept at 25.00 ± 0.10 °C.

4.2. Fluorescence quantum yield

The samples' concentration were adjusted to have an optical density lower than 0.1 at the excitation wavelength (λ_{ex}). The fluorescence quantum yield (Φ) were evaluated based on an external standard, quinine sulfate in 0.5 M H_2SO_4 (0.546, λ_{ex} 350 nm) by using the following equation.

$$\Phi = \Phi_{STD} \frac{I}{I_{STD}} \frac{OD_{STD}}{OD} \frac{n^2}{n_{STD}^2}$$

Where Φ_{STD} is the fluorescence quantum yield of the standard, I and I_{STD} are the integrated area of the emission band of the sample and the standard respectively, OD and OD_{STD} are the optical density at the excitation wavelength for the sample and the standard respectively and n and n_{STD} are the solvent refractive index of the sample and the standard solutions respectively.

5. Dose-Dependent Inhibition of GFP Translation *In Vitro*

The dose-dependent inhibition of GFP was assessed using a rabbit reticulocyte cell lysate system, RNAsin ribonuclease inhibitor (40 u/ml) and CleanCap eGFP mRNA (trilink). Reactions were performed in Corning Costar black 96-well plates, non-sterile, flat-bottom with a total volume of 25 µl, in the presence of 0, 0.1, 0.5, 1, 2, 4, or 10 µM of puromycin **1** or antibiotics **2**, **3**, or **4**. Reactions were initiated through the addition of 1 µg of mRNA to each reaction solution, and quickly transferred to a pre-warmed TECAN spark plate reader at 30 °C. Fluorescence at 410 nm (**2**, **3**, **4**) and at 535 nm (GFP) were measured by exciting samples at 350 nm or 488 nm, respectively. Measurements were taken every 3 minutes for 200 minutes.

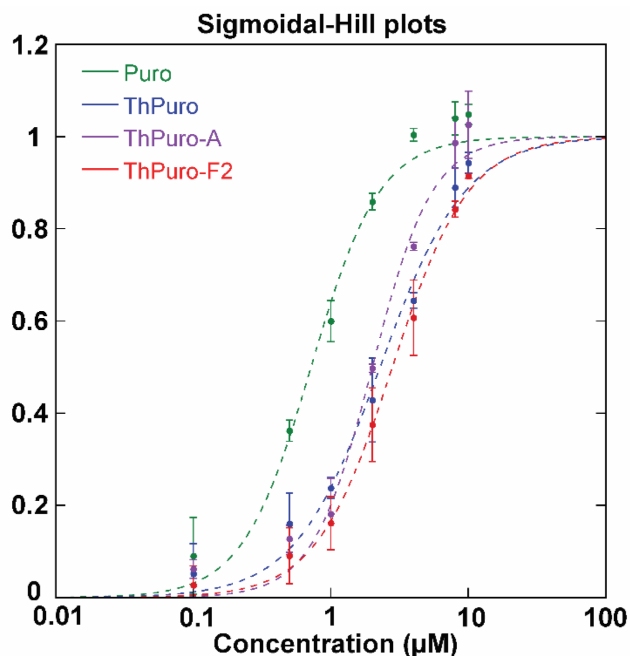


Figure S1. Sigmoidal-Hill plots of GFP fluorescence (8000 s from Figure 3) vs antibiotic dose for **1**–**4**.

6. Cell Culture

6.1. Bacterial Cell Culture and MIC determination

Compounds were tested using the broth double-dilution method in 96-well plates (Corning).[5] For inoculum preparation, overnight starter cultures incubated at 37 °C were diluted into fresh Luria Broth (LB) and allowed to grow to the exponential phase (OD600 = 0.5–0.7). Cultures were then diluted again in LB to obtain an optical density of 0.008 (OD600). Tested compounds were added to LB to form mother liquors (128 µL of stock solution in 1,122 µL of LB) at a concentration of 256 µg/mL. Next, 100 µL serial double dilutions of the tested compounds (256, 128, 64, 32, 16, 8, and 4 µg/mL) were prepared in LB in flat-bottomed 96-well microplates. An equal volume (100 µL) of bacterial suspension in LB was added to each well for a final volume of 200 µL and a final OD600 of 0.0004. Control wells containing only LB (blanks) or only bacteria in LB were also prepared. After 18 h, MTT (50 µL of a 1 mg/mL solution in H2O) was added to each well followed by additional incubation at 37 °C for 2 h. The MIC value was defined as the lowest concentration of compound in which no bacterial growth was observed. Each drug concentration was tested in triplicate and results were reproduced in two independent experiments.

6.2. HEK293T Cell Culture

HEK293T cells were a generous gift from the lab of Dr. Jeffrey D. Esko (UCSD, SoM). Cells were grown under a 5% CO_2 atmosphere in air with 100% relative humidity at 37 °C, using DMEM with 10% v/v fetal bovine serum and 1% Pen-strep as a growth medium. All experiments use PBS with calcium and magnesium unless otherwise stated.

6.3. Primary Rat Hippocampal Neuron Cultures

Rat dissociated hippocampal cultures prepared from postnatal day 1 pups were plated onto poly-D-lysine coated glass coverslips at a density of 45,000 cells/cm^2. Cultures were maintained in B27 supplemented Neurobasal media (Invitrogen) until 14 days *in vitro* as previously described.[6–8]

7. Western Blot Immunostaining

HEK293T cells were seeded into 24-well tissue culture plates at a density of 200,000 cells/well and incubated at 37 °C with a 5% CO_2 atmosphere overnight. The growth medium was aspirated, and the cells were washed with PBS. 200 µl of cycloheximide solution (0, 5, or 50 µg/ml) diluted in growth medium was added to the cells. The cells were incubated with the translation inhibitor for 1 hour at 37 °C with a 5% CO_2 atmosphere, then aspirated and washed with PBS. 200 µl of a 10 µM solution of the corresponding blank (water) or antibiotic (puromycin, **2**, **3**, or **4**) in growth medium was added. The cells were incubated with the antibiotic solutions for 30 minutes, 2 h or 4 h at 37 °C with a 5% CO_2 atmosphere, after which the cells were washed with PBS. 100 µl of trypsin-EDTA was added and the cells were trypsinized for 5 minutes at 37 °C with a 5% CO2 atmosphere. The lifted cells were diluted with 600 µl of growth medium and transferred to microcentrifuge tubes and centrifuged for 1 minute at 14,000 rpm. The supernatant was discarded, and the cells were washed with PBS and pelleted again using centrifugation. The PBS was removed and the cell pellets were suspended in 100 µl 1× RIPA buffer to lyse the cells. 100 µl 1× RIPA buffer with an EDTA-free proteinase inhibitor cocktail tablet was added and the solutions were centrifuged to remove insoluble cellular debris. The concentration of proteins within the cell lysates was assayed using the BCA assay, and appropriate aliquots of the cell lysates were concentrated via speedvac to obtain 15 µg of total protein. The pellet was then reconstituted in 8 µl of water, and 4 µl of 3:1 NuPAGE LDS sample buffer: β-mercaptoethanol was added to resolubilize everything remaining. The solutions were boiled in a heat block set to 115 °C for 15 minutes. After the samples had cooled, they were loaded onto 4-12% Bis-Tris gels and ran at 100 V for 2 hours. The gels were removed and washed with water for 5 minutes, then blotted onto PVDF membranes using a power blotter (Thermo). The membrane was washed with water for 5 minutes, then blocked with seablock fish serum for 1 hour. The membrane was then incubated with anti-puromycin [3RH11] (1:1000 in 3% BSA in TBST) overnight at 4 °C. The membrane was washed with TBST buffer for 5 minutes at room temperature, then incubated with alexafluor 488-tagged goat anti-mouse secondary antibody (1:15,000 in 3% BSA in TBST) for 1 hour at room temperature. Lastly, the membrane was washed with TBST for 5 minutes and imaged.

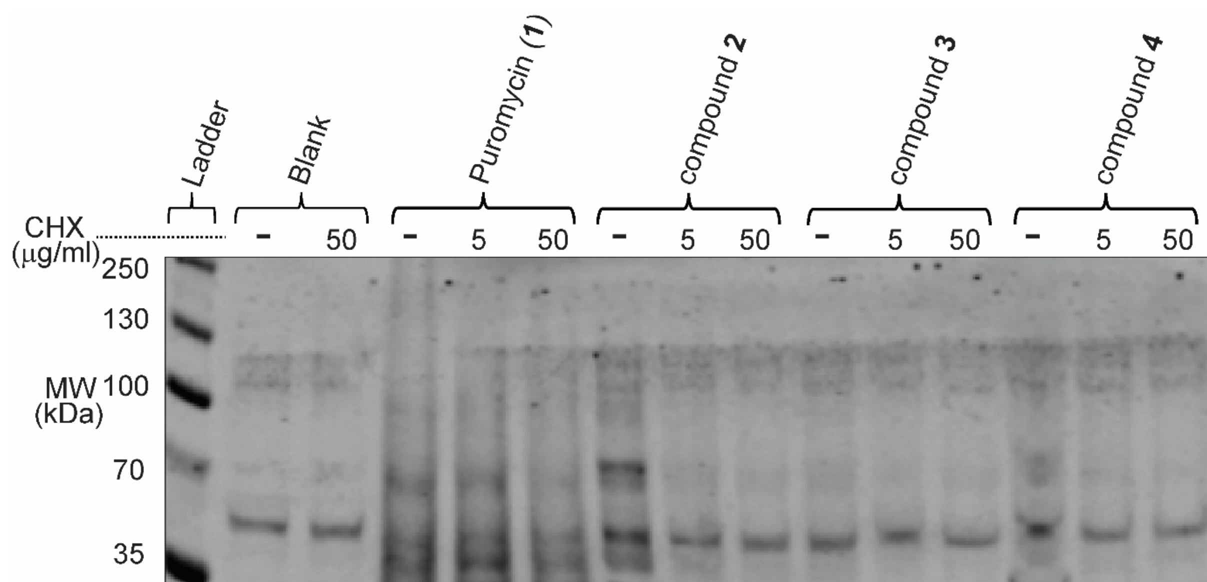


Figure S2. Western blot analysis of total protein isolated from HEK293T cells following treatment with antibiotics **1**–**4**, indicating the presence of truncated peptides terminated with **1**–**4**. Reduction of truncated peptides is observed upon co-treatment of antibiotics **2**–**4** with increased concentrations of translocation inhibitor cycloheximide, as observed with native puromycin (**1**).[9] Additionally, band intensities vary depending on the identity of the antibiotic truncating the peptides (lanes 4, 7, 10, 13).

8. Live-Cell Imaging of HEK293T Cells

HEK293T cells were seeded into sterile 24-well culture plates at a density of 200,000 cells per well and allowed to grow to confluence overnight. The growth medium was aspirated and the cells were then washed with PBS. 200 μl of a 10 μM solution of the corresponding antibiotic **1**–**4** in growth medium was added to the wells. Cells were incubated with the solutions for either 30 minutes, 2 h, or 4 h, at which point the solutions were aspirated off, and the cells were washed with PBS (with calcium and magnesium). The cells were suspended in phenol-red free DMEM and imaged using an EVOS cell imaging system under 40X magnification. The images were then converted to 8-bit in ImageJ with the brightness adjusted to the following values: blue channel: min 21, max 58; brightfield: min 54, max 118.

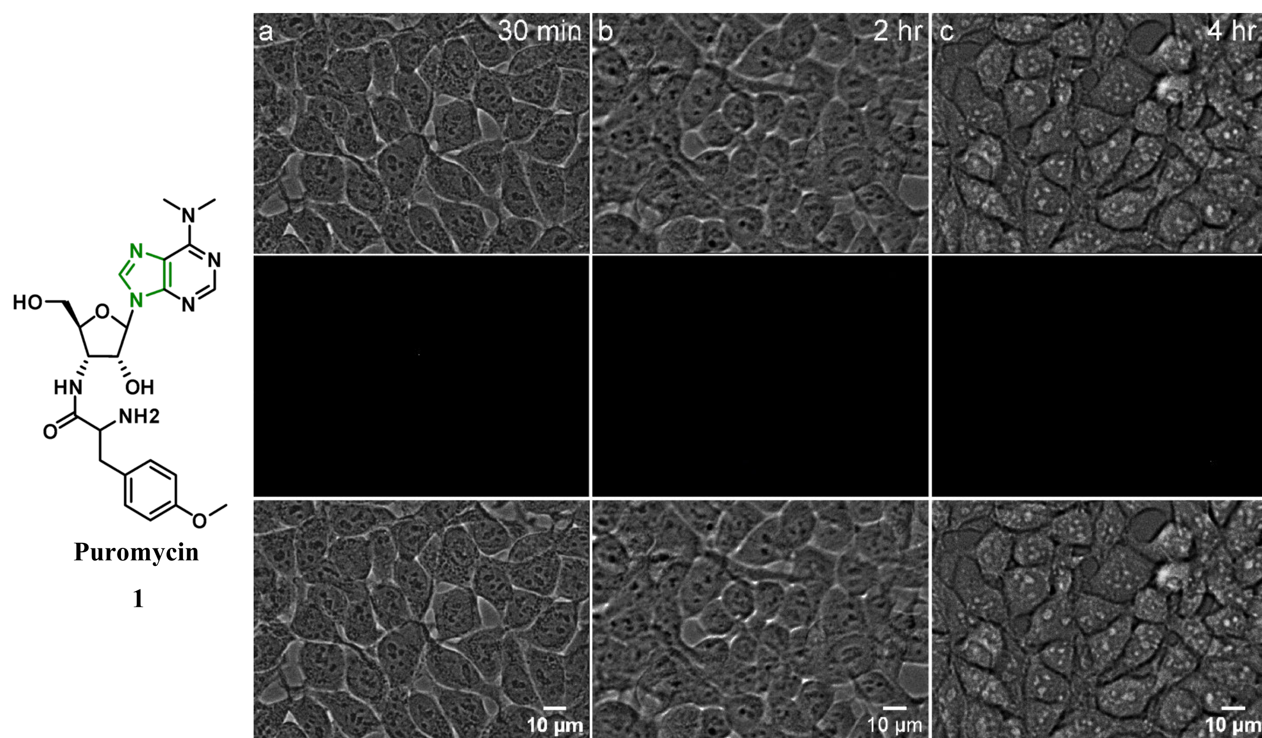


Figure S3. Brightfield (top row), DAPI channel (360 nm, middle row) and overlay (bottom row) images taken of HEK293T cells incubated with puromycin (compound **1**) at 30 min (left column), 2 hours (middle column) and 4 hours (right column). No cellular autofluorescence was observed under these conditions.

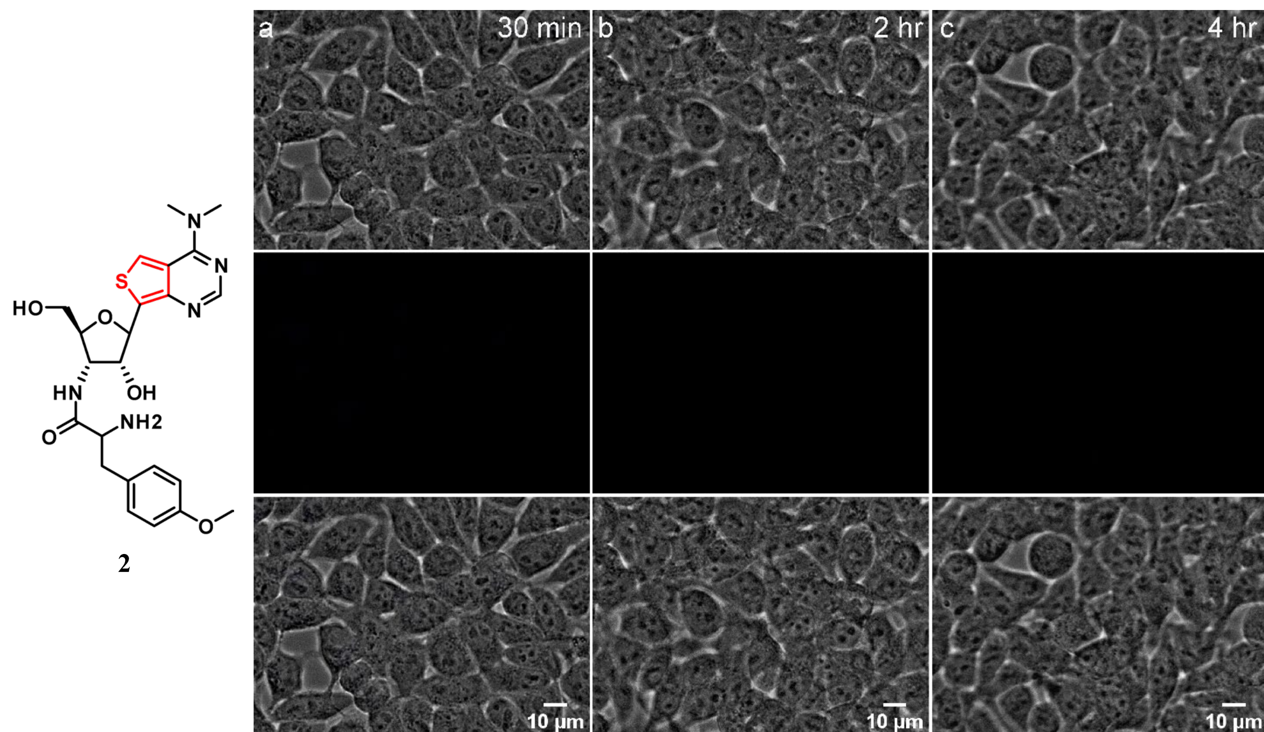


Figure S4. Brightfield (top row), DAPI channel (middle row) and overlay (bottom row) images taken of HEK293T cells incubated with Thpuromycin (compound **2**) at 30 min (left column), 2 hours (middle column) and 4 hours (right column).

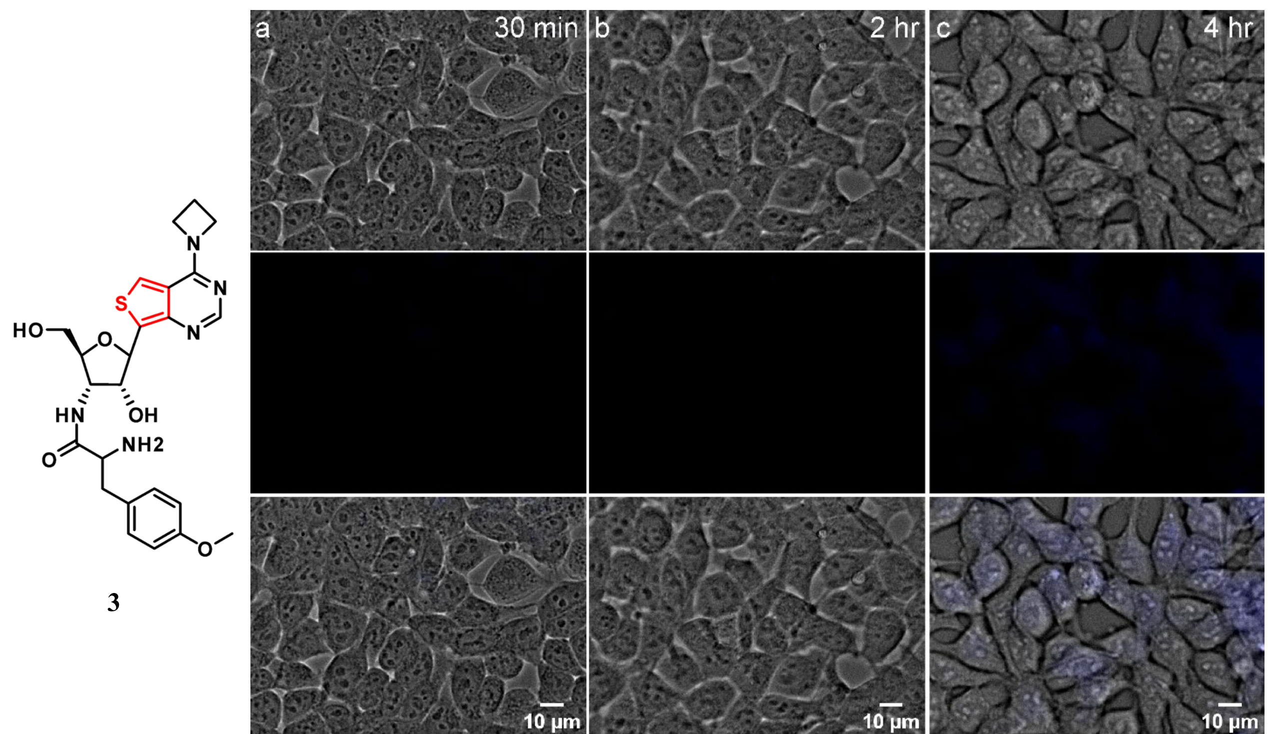


Figure S5. Brightfield (top row), DAPI channel (middle row) and overlay (bottom row) images taken of HEK293T cells incubated with compound **3** at 30 min (left column), 2 hours (middle column) and 4 hours (right column).

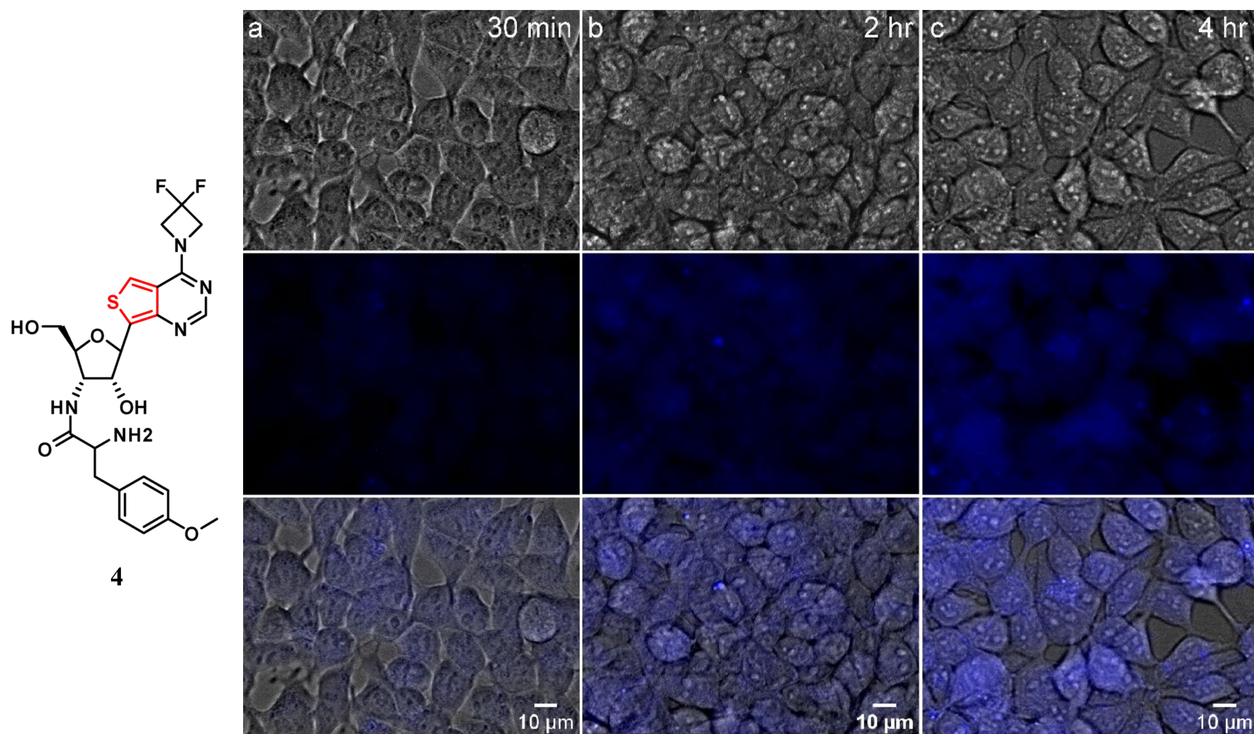


Figure S6. Brightfield (top row), DAPI channel (middle row) and overlay (bottom row) images taken of HEK293T cells incubated with compound **4** at 30 min (left column), 2 hours (middle column) and 4 hours (right column). Compound **4** is the brightest analogue as reported in table S2.

9. Live-Cell Imaging of LAMP1-GFP expressing HEK293T cells treated with compound 4

9.1. General

HEK293T cells were seeded into sterile 24-well culture plates at a density of 250,000 cells per well incubated for 3 hours under a 5% CO_2 atmosphere with 100% relative humidity at 37 °C. Once the cells had visibly adhered to the wells, 75 µl of Celllight-lysosomes-GFP, BacMam 2.0 reagent (Thermo) was added to the cells and gently mixed with pipetting. The cells were then incubated overnight under a 5% CO_2 atmosphere with 100% relative humidity at 37 °C. After checking the cells under an EVOS microscope (GFP channel) to confirm proper LAMP1-GFP expression, The cells were aspirated and washed with phenol-red free DMEM and PBS. A solution of either water in growth medium (prepared by 4:5 dilution of growth medium in water) or compound **4** in growth medium (prepared by 1:5 dilution of 50 µM compound **4** in growth medium) was added to the cells. The cells were then incubated again for 4 hours under a 5% CO_2 atmosphere with 100% relative humidity at 37 °C, then washed with phenol-red free DMEM and suspended in phenol-red free DMEM for imaging using an EVOS cell imaging system under 20X magnification. The images were then converted to 8-bit in ImageJ with the brightness adjusted to the following values: blue channel: min 21, max 58; brightfield: min 54, max 118; green channel: min 5, max 85.

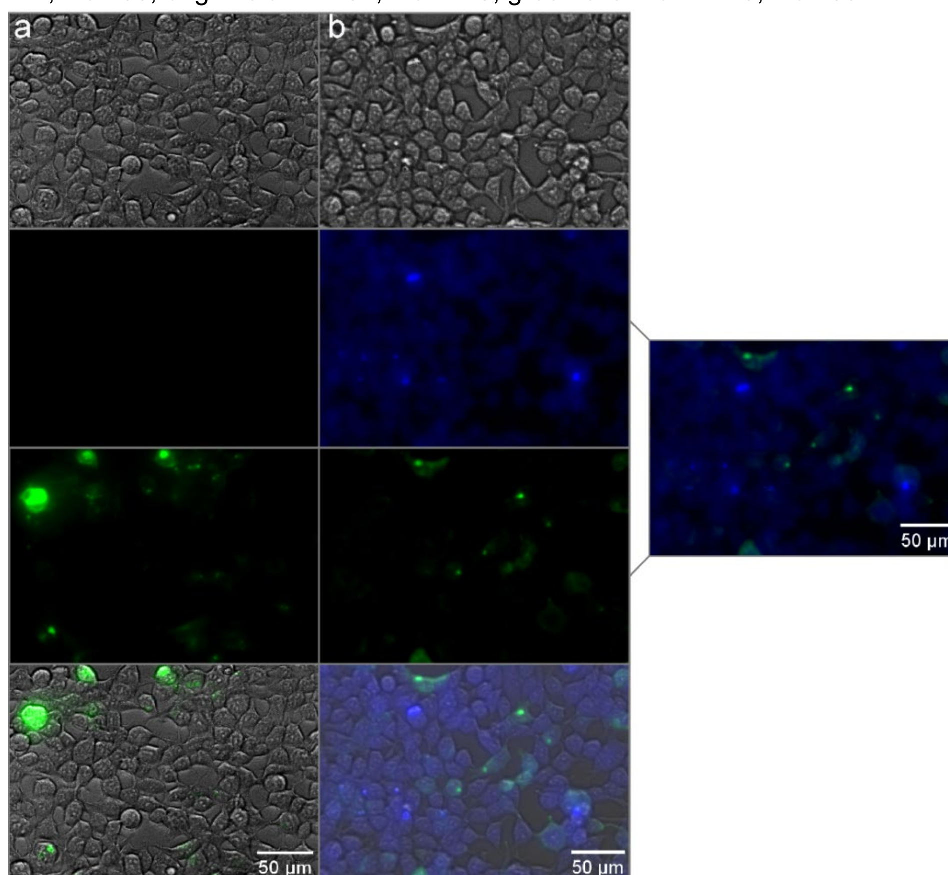


Figure S7. Live-cell images of HEK293T expressing LAMP1-GFP that were treated with (a) water or (b) compound **4** for 4 hours. Brightfield (top), DAPI channel (second row, for imaging compound **4**), GFP channel (third row, for imaging LAMP1-GFP) and overlay images of Brightfield, DAPI and GFP channels (bottom). To the right: overlay image compound **4** fluorescence and LAMP1-GFP.

9.2. Colocalization Analysis

Immunofluorescence images were analyzed using the JaCOP and colocalization finder plugins in ImageJ. Images from the DAPI and GFP channels (for analyzing fluorescence from compound **4** and LAMP1-GFP, respectively) were loaded into ImageJ and converted into 8-bit for analysis. ImageJ is publicly available through Fiji.

Table S3. Colocalization analysis of figure S7

M1	M2	Mander's coefficient (M1; M2)	Pearson's coefficient (r)
Compound **4**	LAMP1-GFP	0.346; 0.994	0.137

10. Live-Cell Imaging of Primary Rat Hippocampal Neurons

Half of the growth media of neuronal cell cultures was removed and replaced with an equivalent volume of 60 μM solution of difluoroazetidine-modified antiobiotic **4** diluted in growth medium (for an active concentration of 30 μM). The neurons were incubated with compound **4** for 4 hours at 37 °C with a 5% CO_2 atmosphere, then washed with growth medium and PBS (quickly). The cells were then quickly suspended in phenol-red free DMEM and imaged using an EVOS cell imaging system under 20X magnification.

11. HEK293T Immunofluorescence Experiments

11.1. General

TC coverslips were added to the wells of a 24-well tissue culture plate, and 500 µl of PDL solution (50 mg/ml) was added. Following incubation for 45 minutes, the PDL solution was removed, and the plate was left to sterilize under UV light for 20 minutes. The wells were then washed with PBS and HEK293T cells were seeded at a density of 200,000 cells/well. Cells were allowed to grow to confluence overnight. The growth medium was aspirated, and the cells were washed with PBS. 200 µl of cycloheximide solution (0, 5, or 50 µg/ml) diluted in growth medium was added to the cells. The cells were incubated with the translation inhibitor for 1 hour at 37 °C with a 5% CO_2 atmosphere, then aspirated and washed with PBS. 200 µl of a 10 µM solution of the corresponding blank (water) or antibiotic (puromycin, **2**, **3**, or **4**) in growth medium was added. The cells were incubated with the antibiotic solutions for 30 minutes, 2 h or 4 h at 37 °C with a 5% CO_2 atmosphere, after which the cells were washed with PBS and treated with 500 µl fixation solution (4% PFA, 4% sucrose, and 5 mM $MgCl_2$ in PBS) for 15 minutes. The cells were washed with PBS and permeabilized with 500 µl 0.1% Triton X-100 in PBS for 1 minute, then blocked with 500 µl 1% BSA in PBS for 1 hr at room temperature with shaking. The solution was aspirated and a 500 µl solution of anti-puromycin [3RH11] and HPO-0100 (1:1000 and 1:3000 in 0.1% BSA in PBS) was added to the cells and incubated overnight at 4 °C with shaking. The cells were washed with PBS and a 500 µl solution of Goat anti-mouse AlexaFluor 488 and Donkey anti-human Cy3 (1:1000 and 1:500 in 0.1% BSA in PBS) was added. Following incubation for 2 hours at room temperature with shaking, the cells were washed with PBS and treated with a 500 µl solution of 5 µM DRAQ5. The cells were shaken with the solution for 30 minutes at room temperature protected from light. The DRAQ5 solution was removed and the cells were washed with PBS (note: staining intensity may be affected by subsequent washes). The coverslips were then extracted and mounted onto microscope slides using prolong gold antifade mountant. Once dried, the slides were imaged on a Keyence BZ-X700 microscope at 20X magnification. The images were then converted to 8-bit in ImageJ with the brightness adjusted to the following values: blue channel: min 5.1354, max 32.8073; green channel: min 1.8458, max 38.1771;red channel: min 0.3320, max 84.6680; purple channel: min 1.992, max 169.341. Blue channel is not shown in this experiment as no signal was observed. Note: Binding affinity between anti-puromycin antibody and the different compounds may differ.

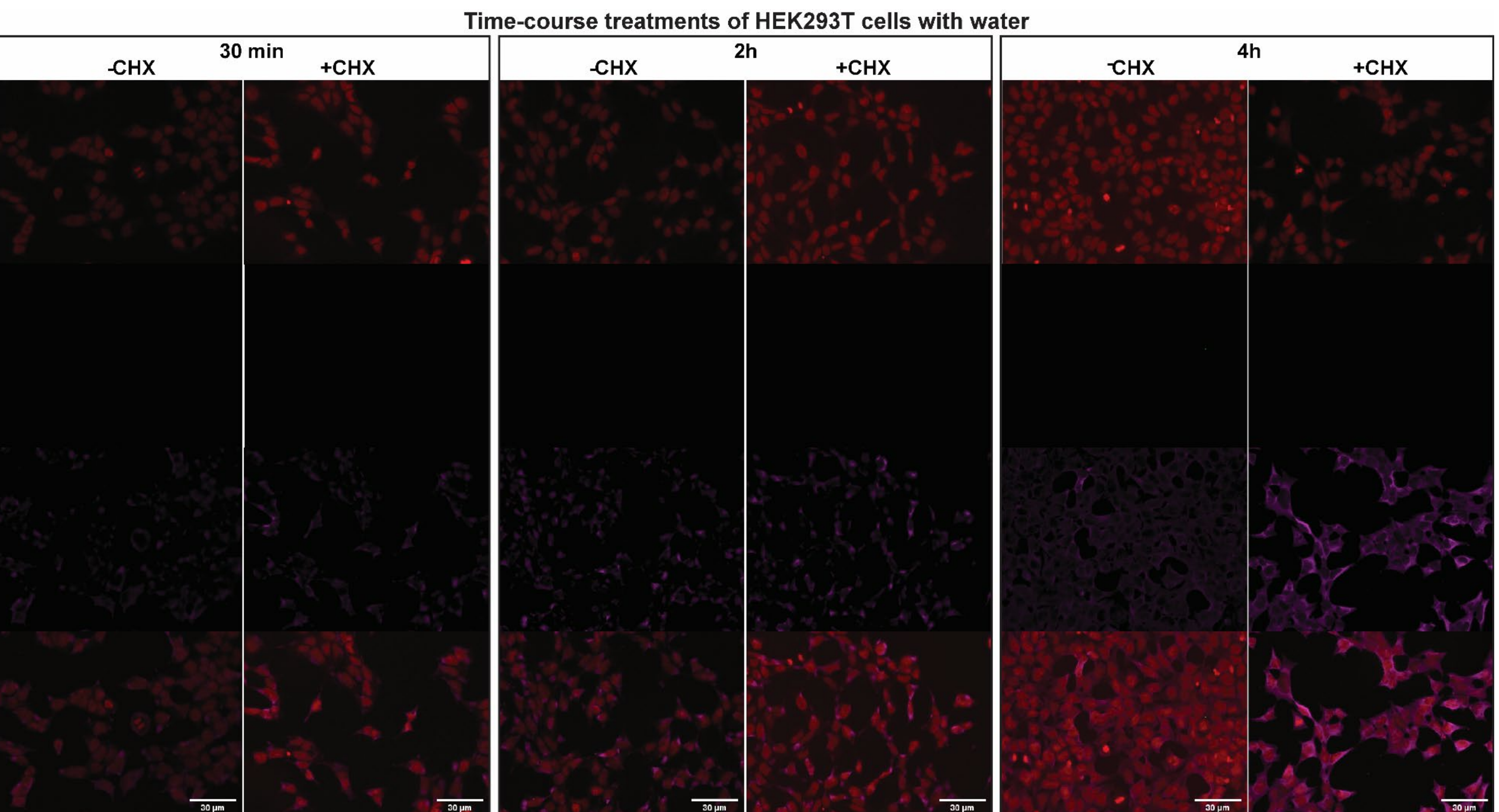


Figure S8. DRAQ5 (top row), anti-puromycin antibody/GAM-AF488 (second row), HPO-0100/DAH-Cy3 (third row) and overlay images (bottom row) of fixed HEK293T cells treated with water (negative control) at 30 minutes without cycloheximide (first column) and with cycloheximide (second column), 2 hours without cycloheximide (third column) and with cycloheximide (fourth column) and 4 hours without cycloheximide (fifth column) and with cycloheximide (sixth column).

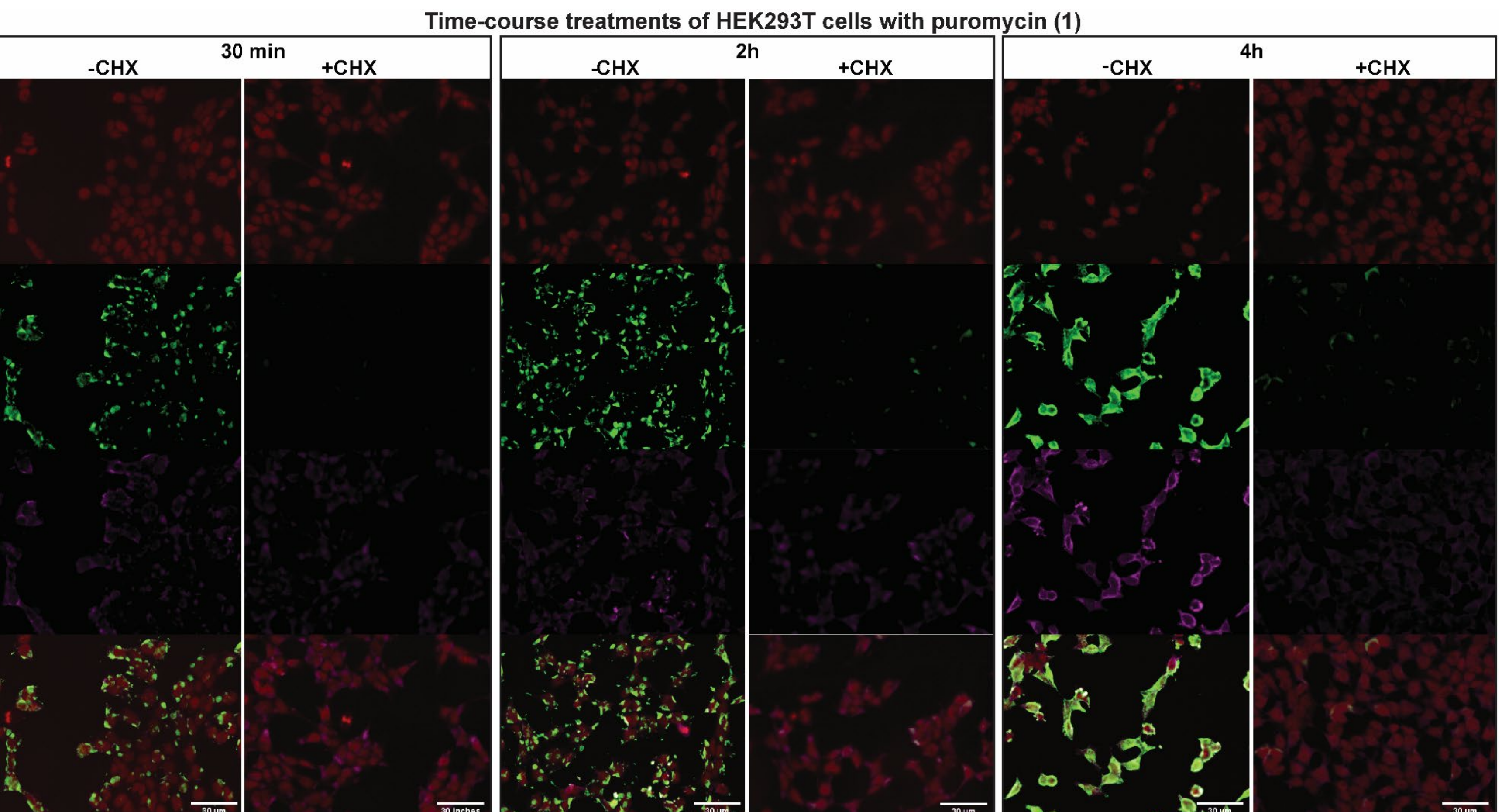


Figure S9. DRAQ5 (top row), anti-puromycin antibody/GAM-AF488 (second row), HPO-0100/DAH-Cy3 (third row) and overlay images (bottom row) of fixed HEK293T cells treated with puromycin (antibiotic **1**) at 30 minutes without cycloheximide (first column) and with cycloheximide (second column), 2 hours without cycloheximide (third column) and with cycloheximide (fourth column) and 4 hours without cycloheximide (fifth column) and with cycloheximide (sixth column).

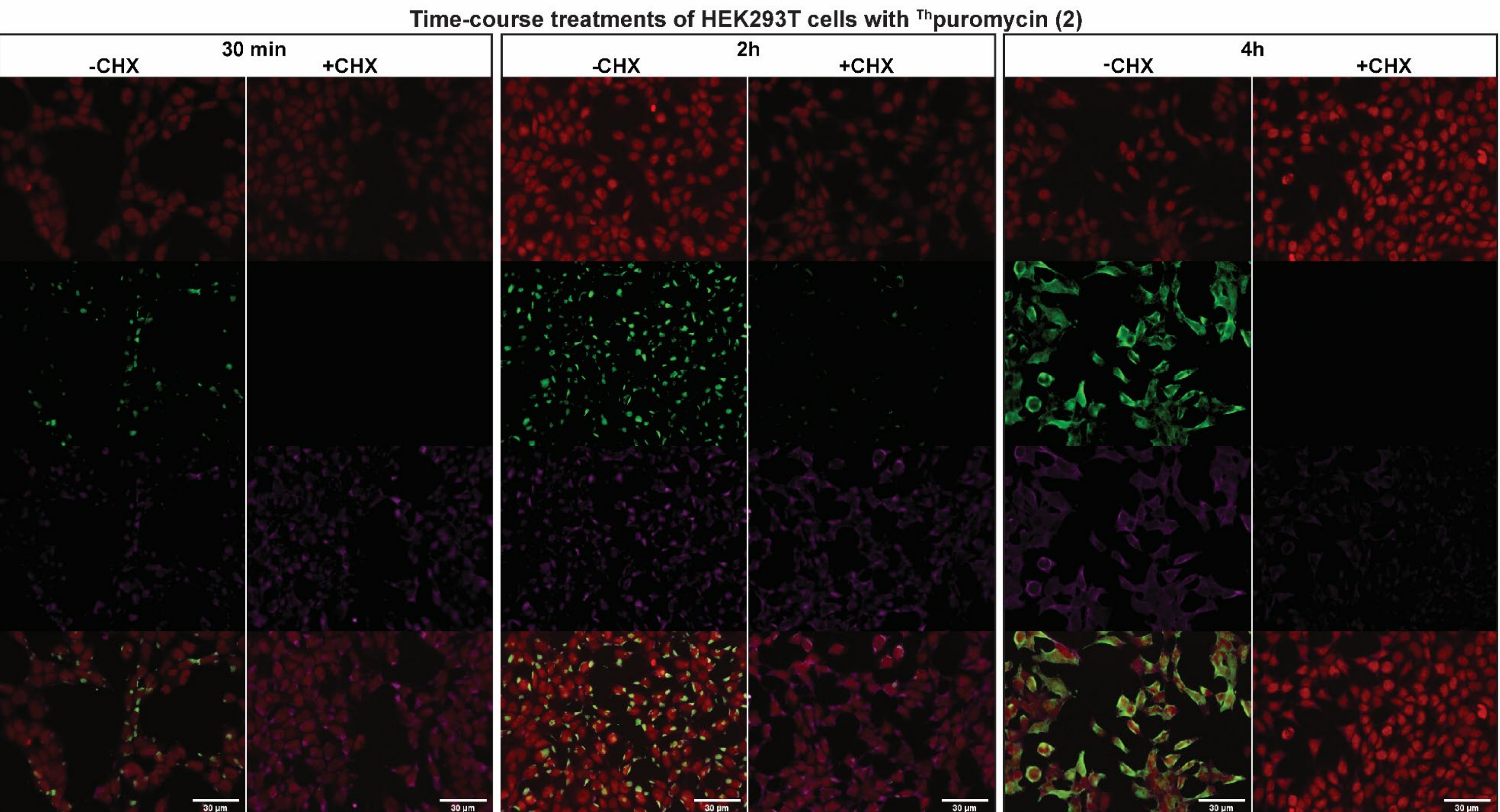


Figure S10. DRAQ5 (top row), anti-puromycin antibody/GAM-AF488 (second row), HPO-0100/DAH-Cy3 (third row) and overlay images (bottom row) of fixed HEK293T cells treated with antibiotic **2** at 30 minutes without cycloheximide (first column) and with cycloheximide (second column), 2 hours without cycloheximide (third column) and with cycloheximide (fourth column) and 4 hours without cycloheximide (fifth column) and with cycloheximide (sixth column).

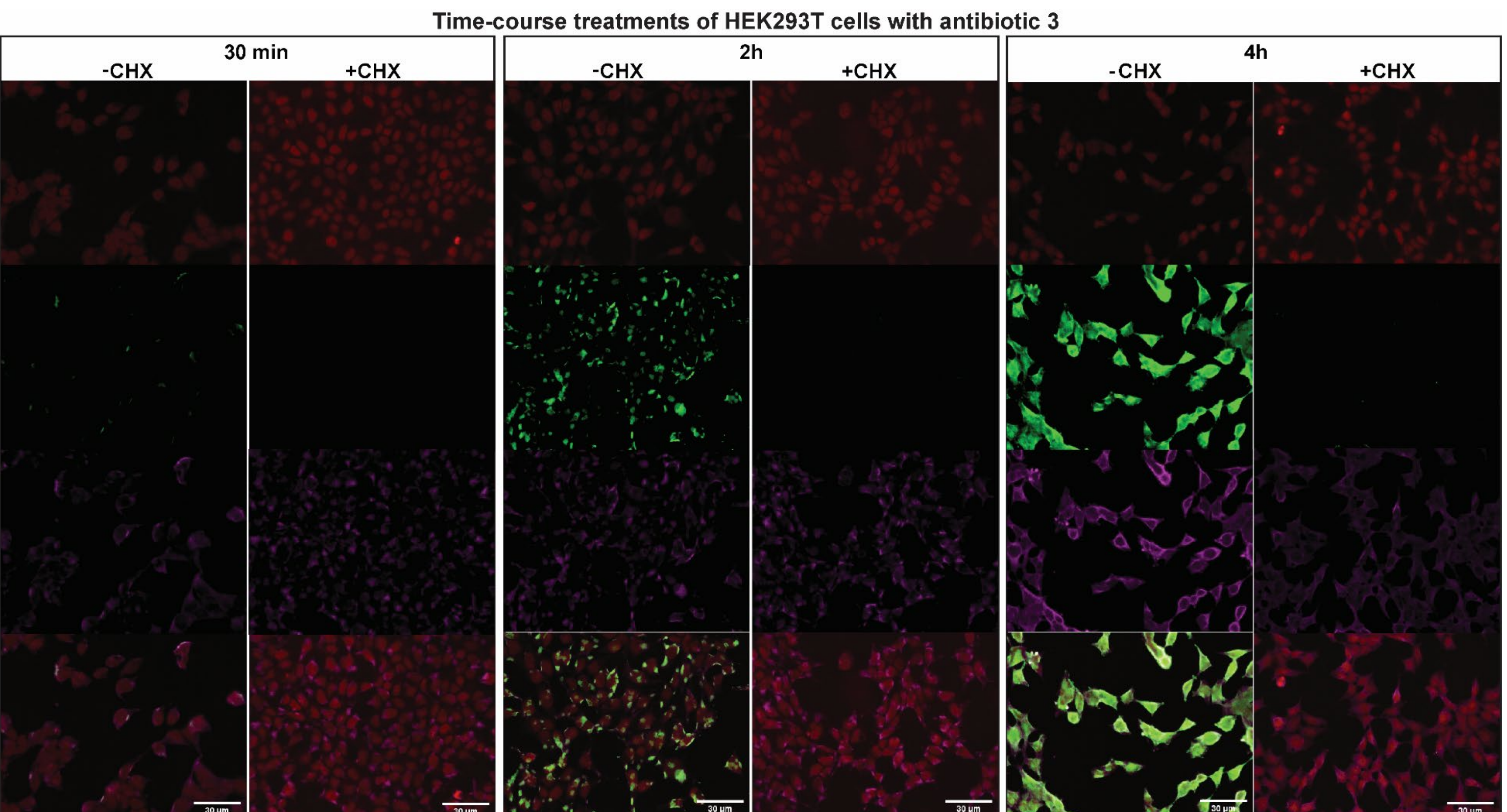


Figure S11. DRAQ5 (top row), anti-puromycin antibody/GAM-AF488 (second row), HPO-0100/DAH-Cy3 (third row) and overlay images (bottom row) of fixed HEK293T cells treated with azetidine-modified antibiotic **3** at 30 minutes without cycloheximide (first column) and with cycloheximide (second column), 2 hours without cycloheximide (third column) and with cycloheximide (fourth column) and 4 hours without cycloheximide (fifth column) and with cycloheximide (sixth column).

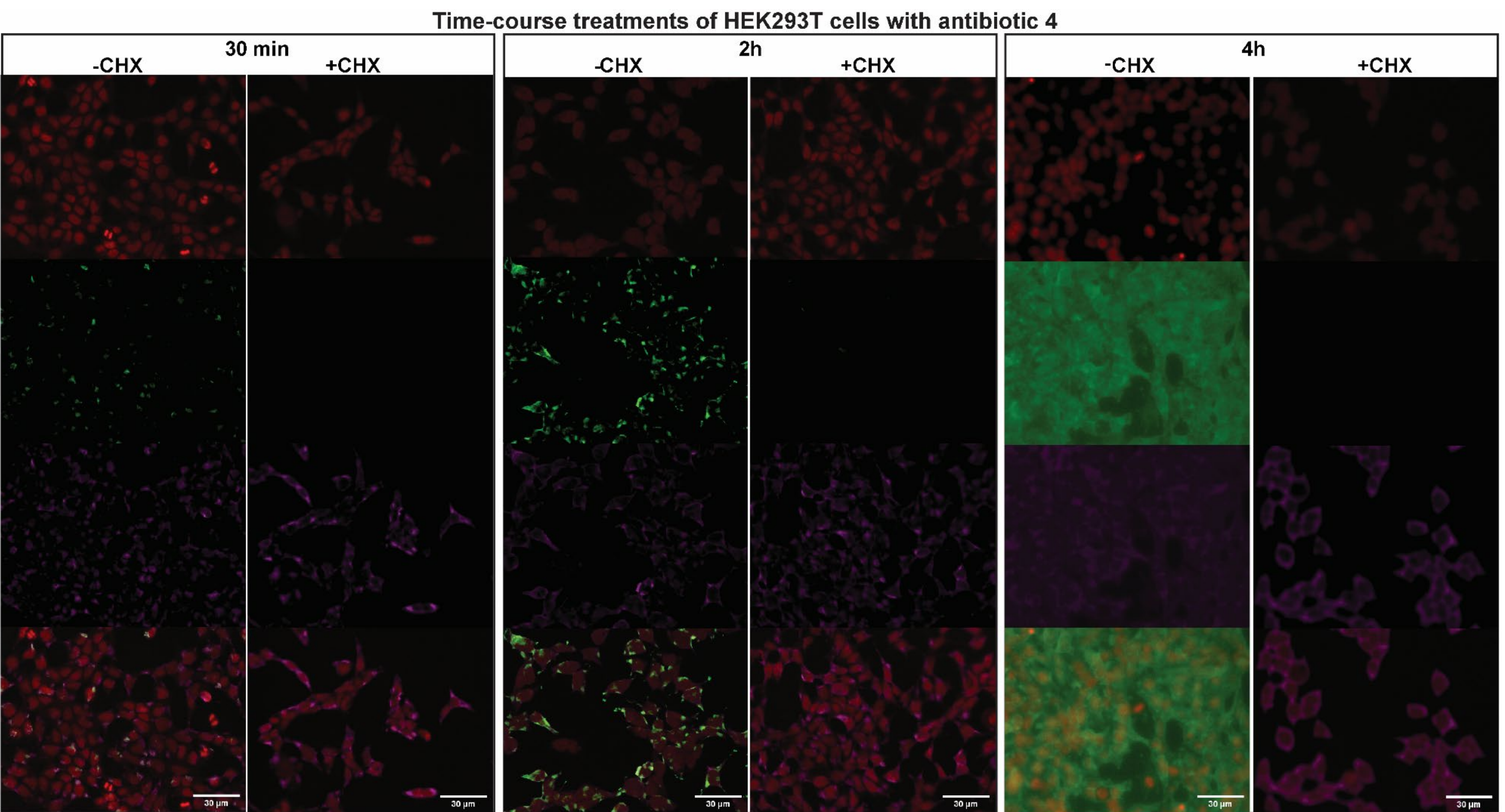


Figure S12. DRAQ5 (top row), anti-puromycin antibody/GAM-AF488 (second row), HPO-0100/DAH-Cy3 (third row) and overlay images (bottom row) of fixed HEK293T cells treated with difluoroazetidine-modified antibiotic **4** at 30 minutes without cycloheximide (first column) and with cycloheximide (second column), 2 hours without cycloheximide (third column) and with cycloheximide (fourth column) and 4 hours without cycloheximide (fifth column) and with cycloheximide (sixth column).

11.2. Colocalization Analysis

Immunofluorescence images were analyzed using the JaCOP and colocalization finder plugins in ImageJ. Images from the GFP and TexasRed channels (for analyzing fluorescence from goat anti-mouse alexafluor 488 and donkey anti-human Cy3, respectively) were loaded into ImageJ and converted into 8-bit for analysis. ImageJ is publicly available through Fiji.

Table S4. Colocalization analysis of figures S8–S12

Figure	Time	±CHX	Mander's coefficient (M1; M2)	Pearson's coefficient (r)
S8	**30 m**	+CHX	0; 0	0.0
		–CHX	0; 0	0.0
	2 h	+CHX	0; 0	0.0
		–CHX	0; 0	0.0
	4 h	+CHX	0; 0	0.0
		–CHX	1.0; 0	0.002
S9	**30 m**	+CHX	1.0; 0.098	0.4
		–CHX	0.998; 0.749	0.864
	2 h	+CHX	1.0; 0.161	0.504
		–CHX	0.998; 0.683	0.798
	4 h	+CHX	1.0; 0.137	0.436
		–CHX	0.999; 0.934	0.897
S10	**30 m**	+CHX	1.0; 0.001	0.098
		–CHX	1.0; 0.511	0.768
	2 h	+CHX	1.0; 0.113	0.434
		–CHX	0.999; 0.667	0.853
	4 h	+CHX	1.0; 0.001	0.058
		–CHX	0.999; 0.873	0.854
S11	**30 m**	+CHX	0; 0	0.0
		–CHX	1.0; 0.148	0.507
	2 h	+CHX	1.0; 0.004	0.099
		–CHX	0.999; 0.681	0.85
	4 h	+CHX	1.0; 0.008	0.103
		–CHX	0.999; 0.95	0.904
S12	**30 m**	+CHX	0; 0	0.0
		–CHX	1.0; 0.308	0.582
	2 h	+CHX	1.0; 0.007	0.142
		–CHX	0.995; 0.607	0.804
	4 h	+CHX	0; 0	0.0
		–CHX	1.0; 1.0	0.874

12. Primary Rat Hippocampal Neuron Immunofluorescence Experiments

12.1. General

Half of the growth media of neuronal cell cultures was removed and replaced with an equivalent volume of 20 μM solution of difluoroazetidine-modified antibiotic **4** diluted in growth medium (for an active concentration of 10 μM. The neurons were then incubated with the antibiotic for 4 hours at 37 °C with a 5% CO2 atmosphere, then washed with growth medium and PBS (quickly) and fixed with fixation solution (4% PFA, 4% sucrose, and 5 mM $MgCl_2$ in PBS) for 15 minutes. The cells were washed with PBS and permeabilized with 500 µl 0.1% Triton X-100 in PBS for 1 minute, then blocked with 500 µl 1% BSA in PBS for 1 hr at room temperature with shaking. The solution was aspirated and a 500 µl solution of anti-puromycin [3RH11] and HPO0100 (1:1000 and 1:3000 in 0.1% BSA in PBS) was added to the cells and incubated overnight at 4 °C with shaking. The cells were washed with PBS and a 500 µl solution of Goat anti-mouse AlexaFluor 488 and Donkey anti-human Cy3 (1:1000 and 1:500 in 0.1% BSA in PBS) was added. Following incubation for 2 hours at room temperature with shaking, the cells were washed with PBS and treated with a 500 µl solution of 5 µM DRAQ5. The cells were shaken with the solution for 30 minutes at room temperature protected from light. The DRAQ5 solution was removed and the cells were washed with PBS (note: staining intensity may be affected by subsequent washes). The coverslips were then extracted and mounted onto microscope slides using prolong gold antifade mountant. Once dried, the slides were imaged on a Keyence BZ-X700 microscope under 20X magnification. Image workup was performed according to section 10.1.

12.2. Colocalization Analysis

Immunofluorescence images were analyzed using the JaCOP and colocalization finder plugins in ImageJ. Images from the DAPI, GFP and TexasRed channels (for analyzing fluorescence from compound **4**, goat anti-mouse alexafluor 488, and donkey anti-human Cy3, respectively) were loaded into ImageJ and converted into 8-bit for analysis. ImageJ is publicly available through Fiji.

Table S5. Colocalization analysis of Figure 5e

M1	M2	Mander's coefficient (M1; M2)	Pearson's coefficient (r)
Anti-Puromycin	HPO-0100	0.723; 0.999	0.329
Compound **4**	Anti-Puromycin	0.985; 0.997	0.388
Compound **4**	HPO-0100	0.829; 0.999	0.488

13. Supplementary figures

13.1 NMR spectroscopy

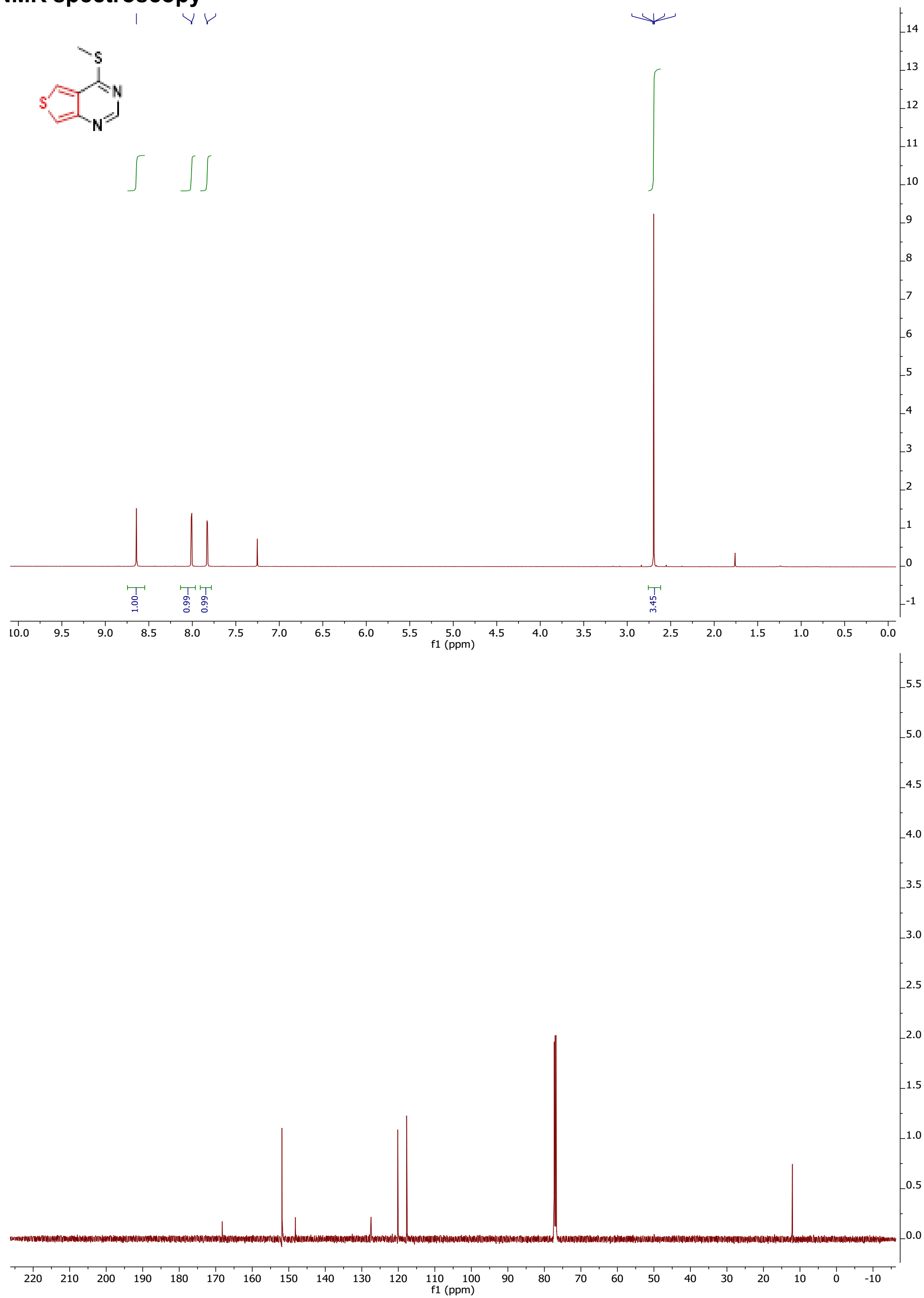


Figure S13. ^1H and ^{13}C NMR spectra of compound **7**.

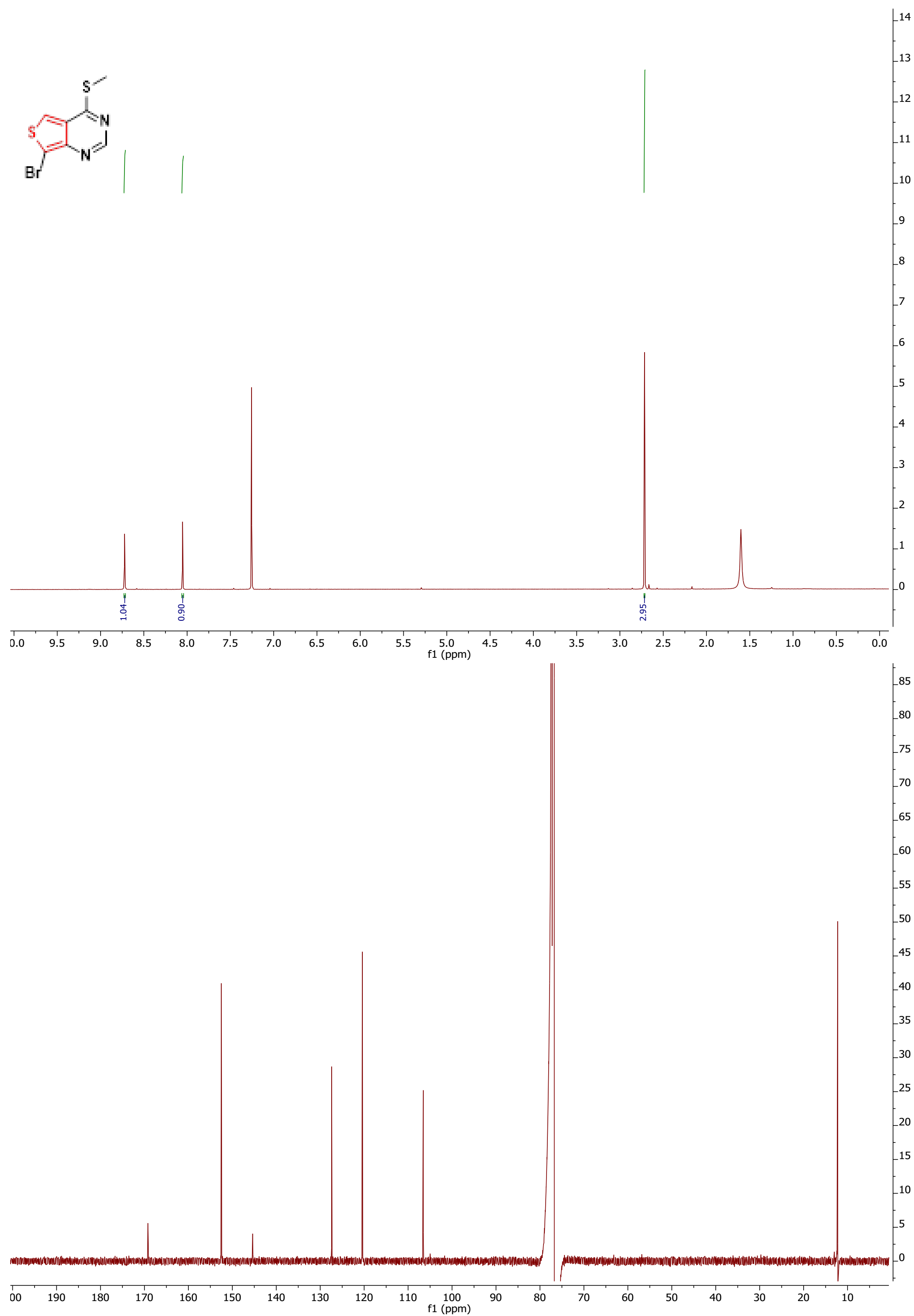


Figure S14. ^{1}H and ^{13}C NMR spectra of compound **8**.

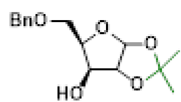


Figure S15. ^{1}H and ^{13}C NMR spectra of compound **10**.

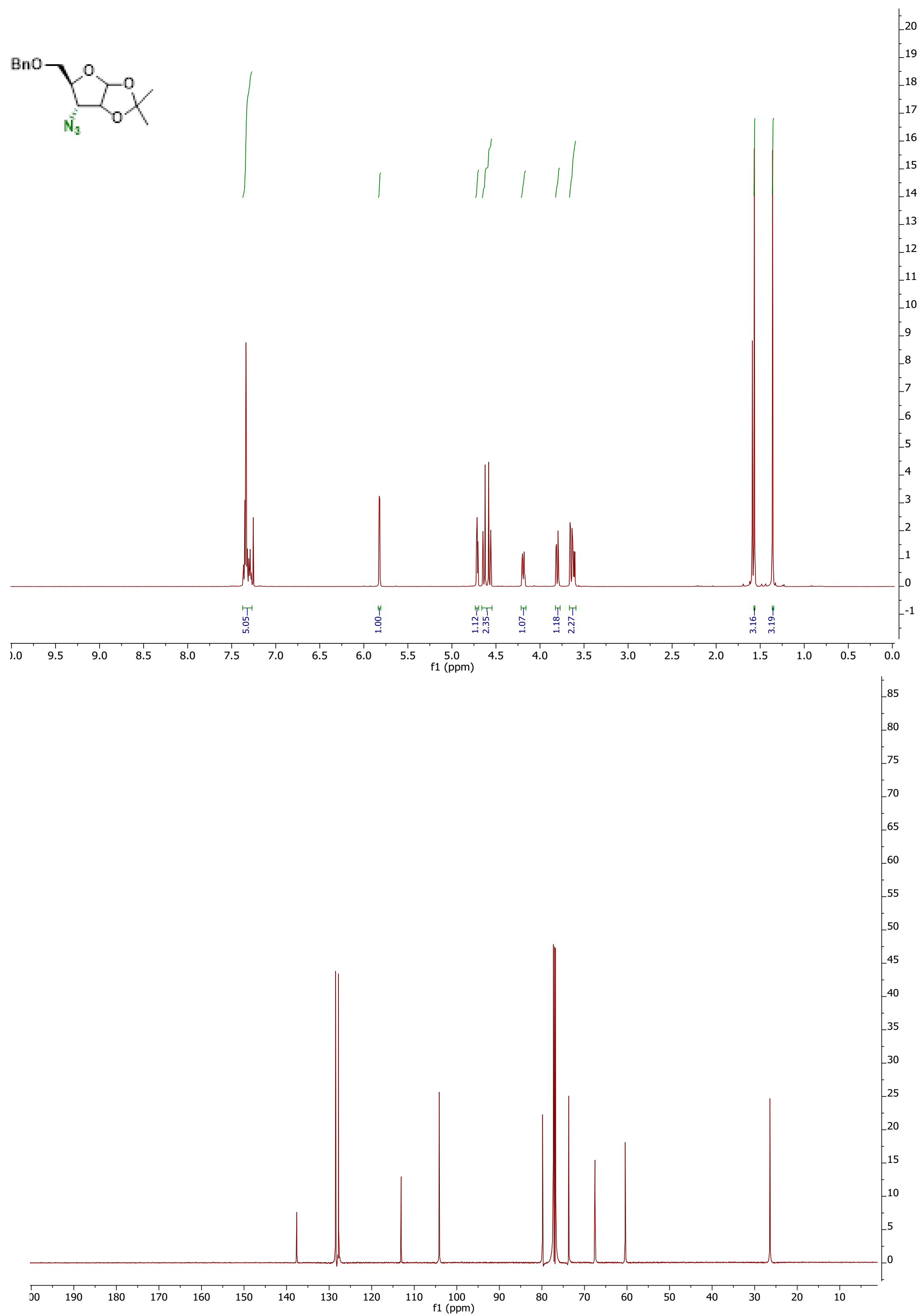


Figure S16. ^{1}H and ^{13}C NMR spectra of compound **11**.

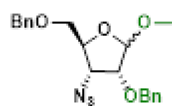


Figure S17. ^{1}H and ^{13}C NMR spectra of compound **12**.

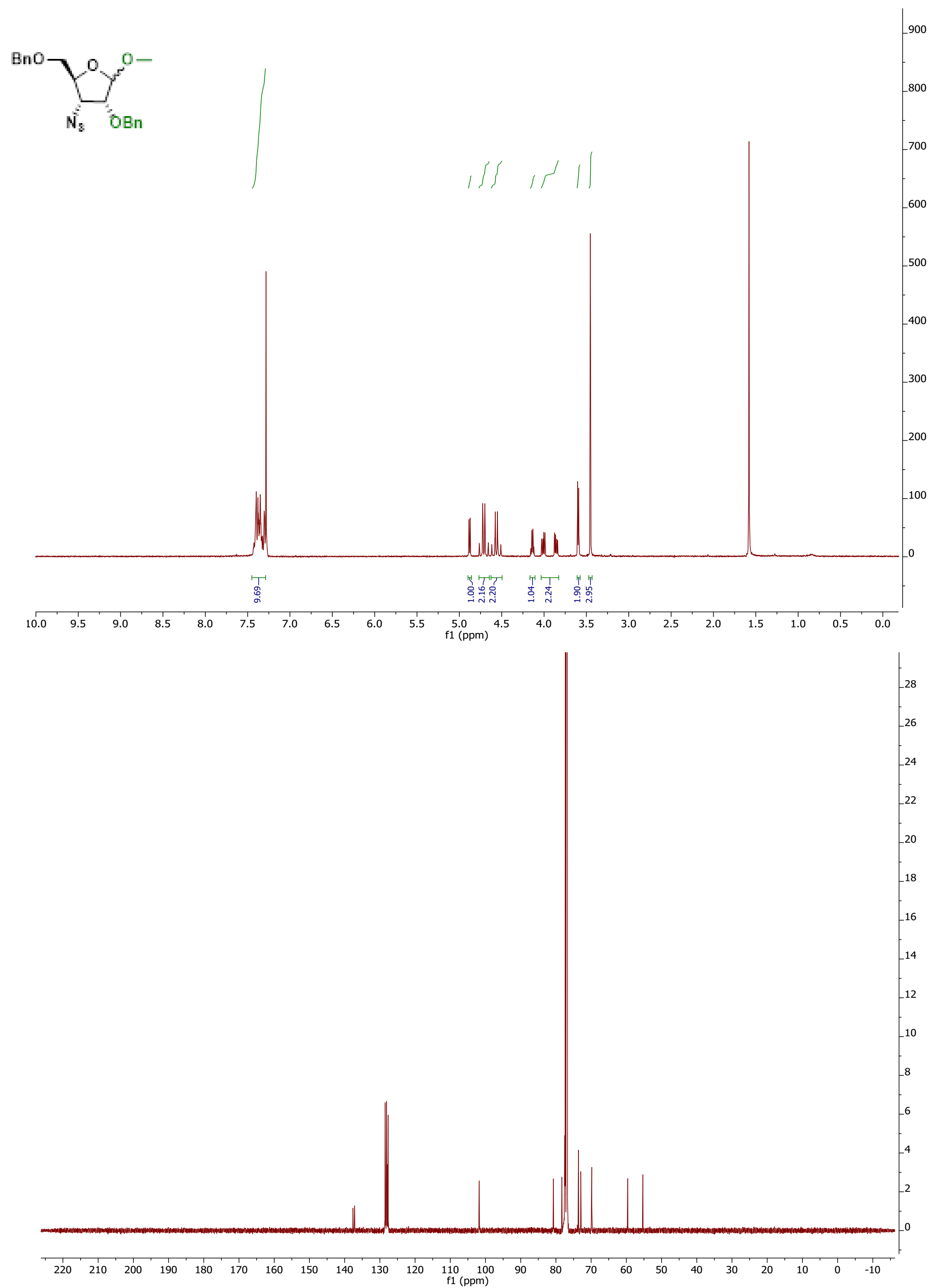


Figure S18. ^{1}H and ^{13}C NMR spectra of compound **12**.

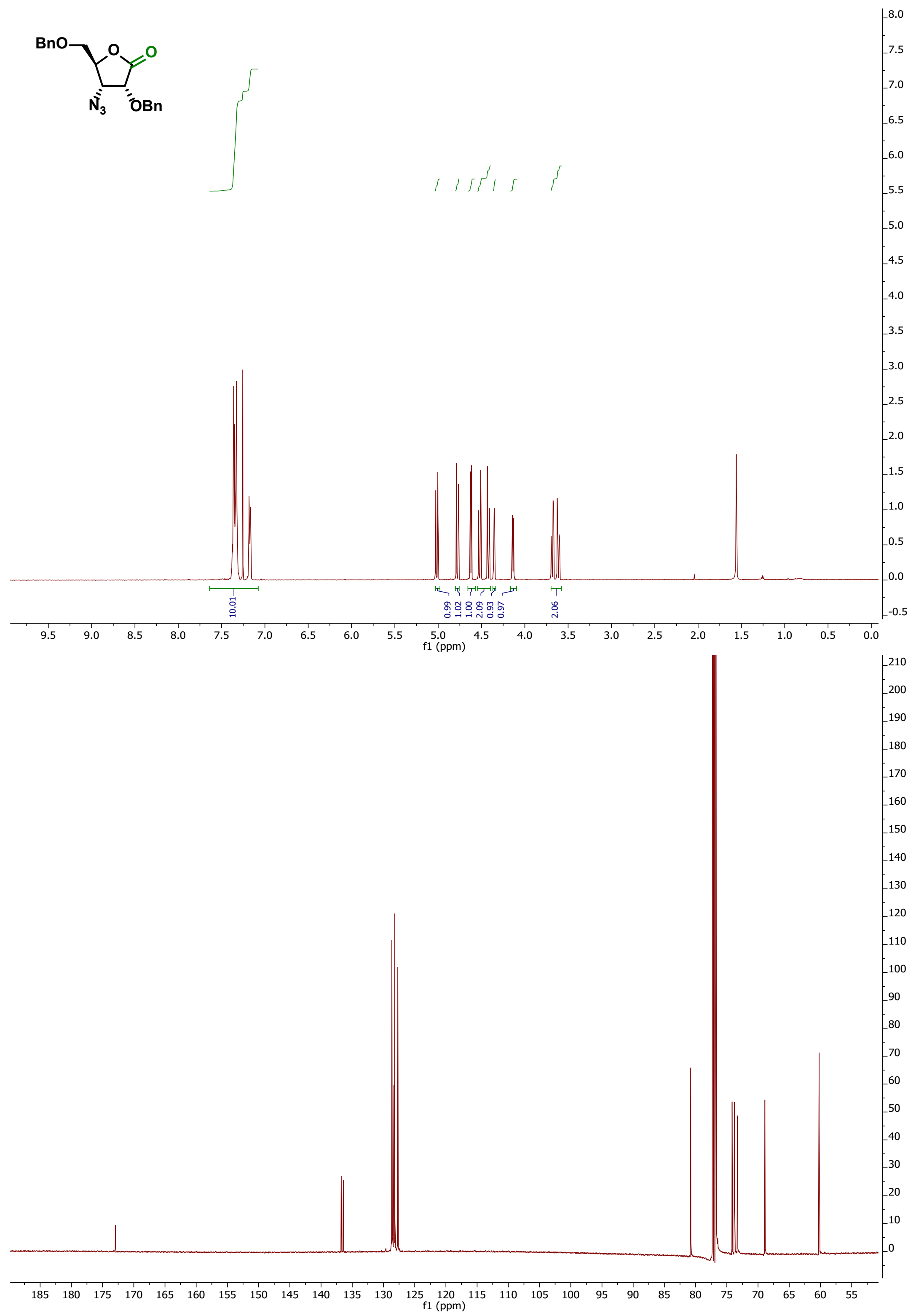


Figure S19. ^{1}H and ^{13}C NMR spectra of compound **13**.

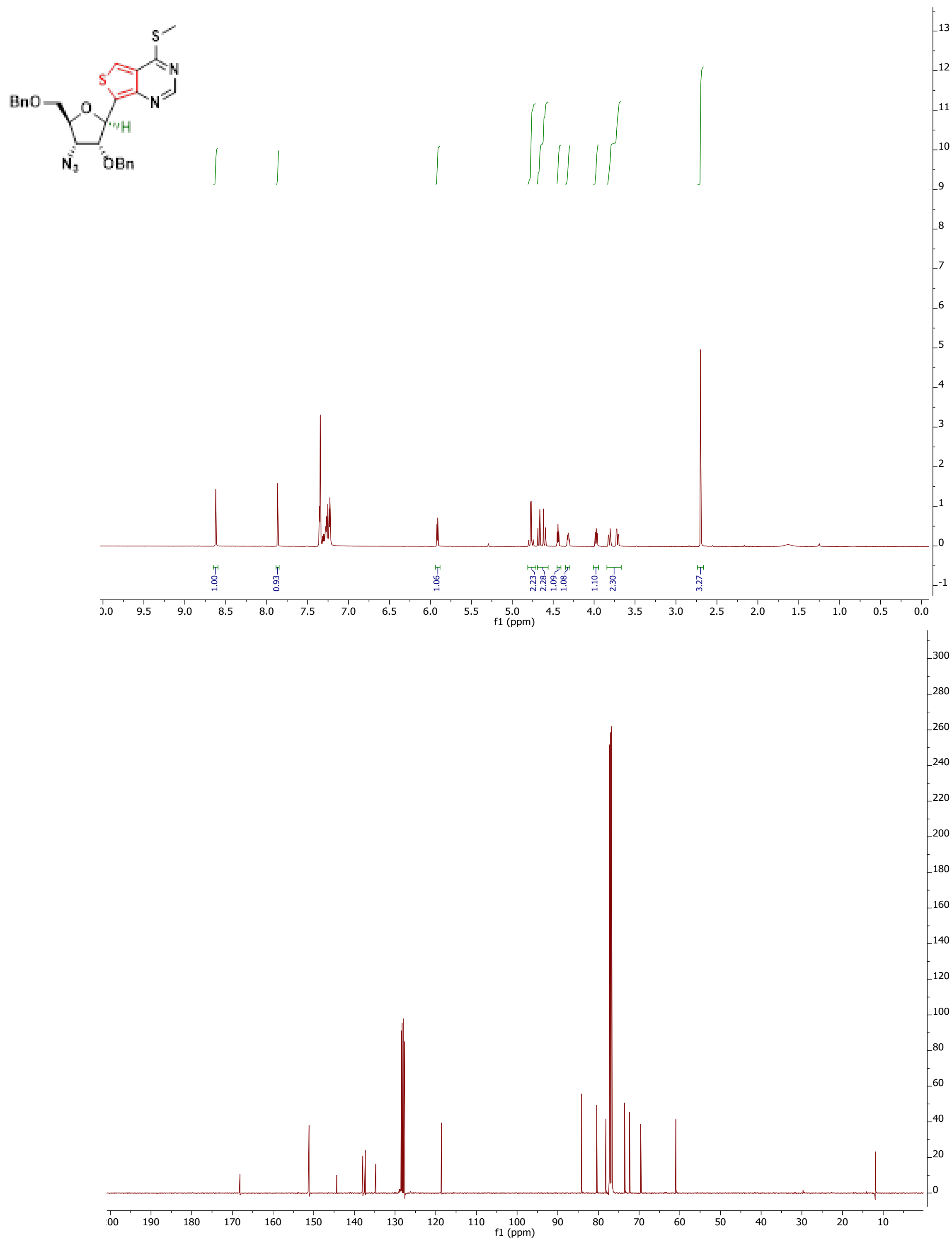


Figure S20. ^{1}H and ^{13}C NMR spectra of compound **15**.

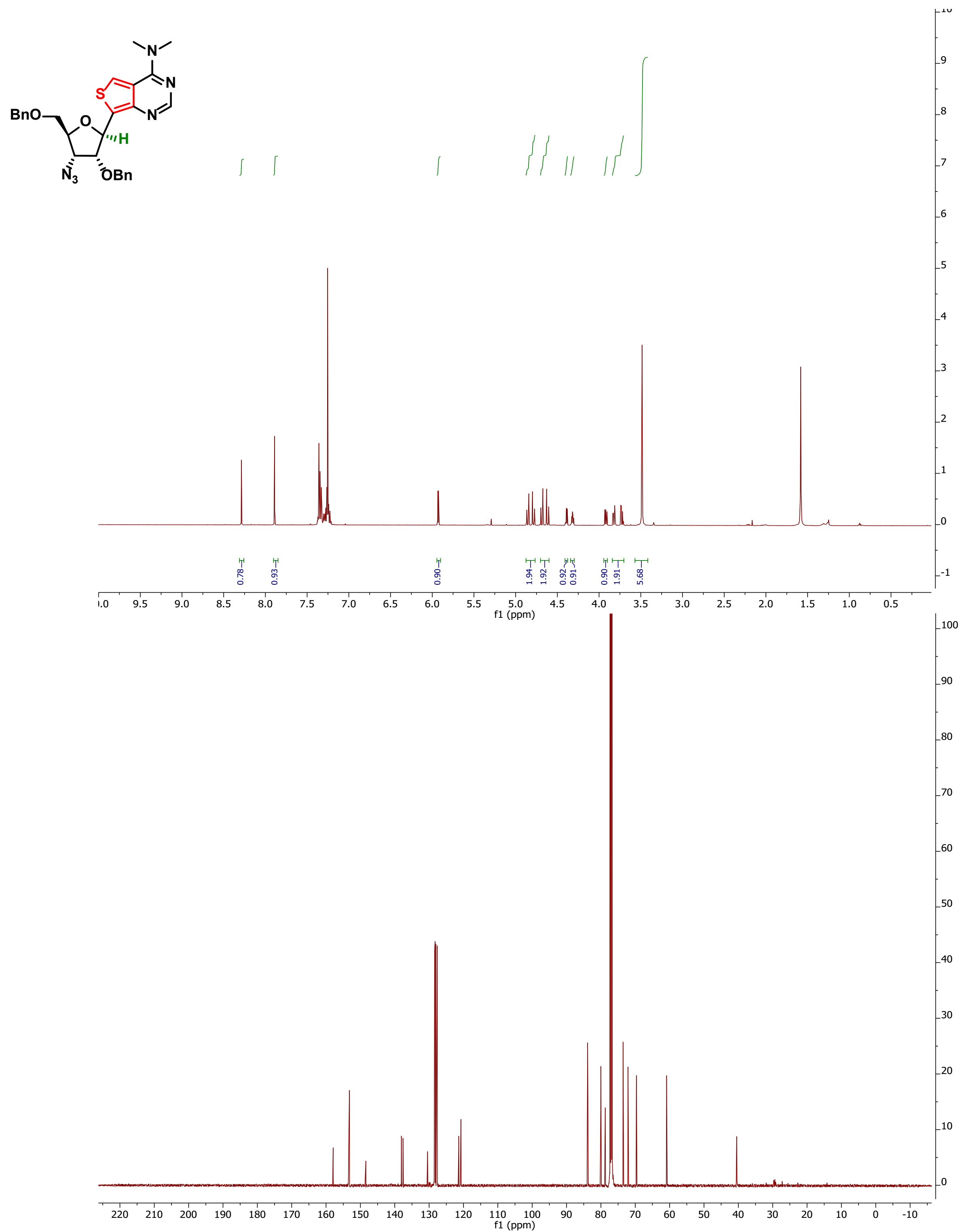


Figure S21. ^{1}H and ^{13}C NMR spectra of compound **16**.

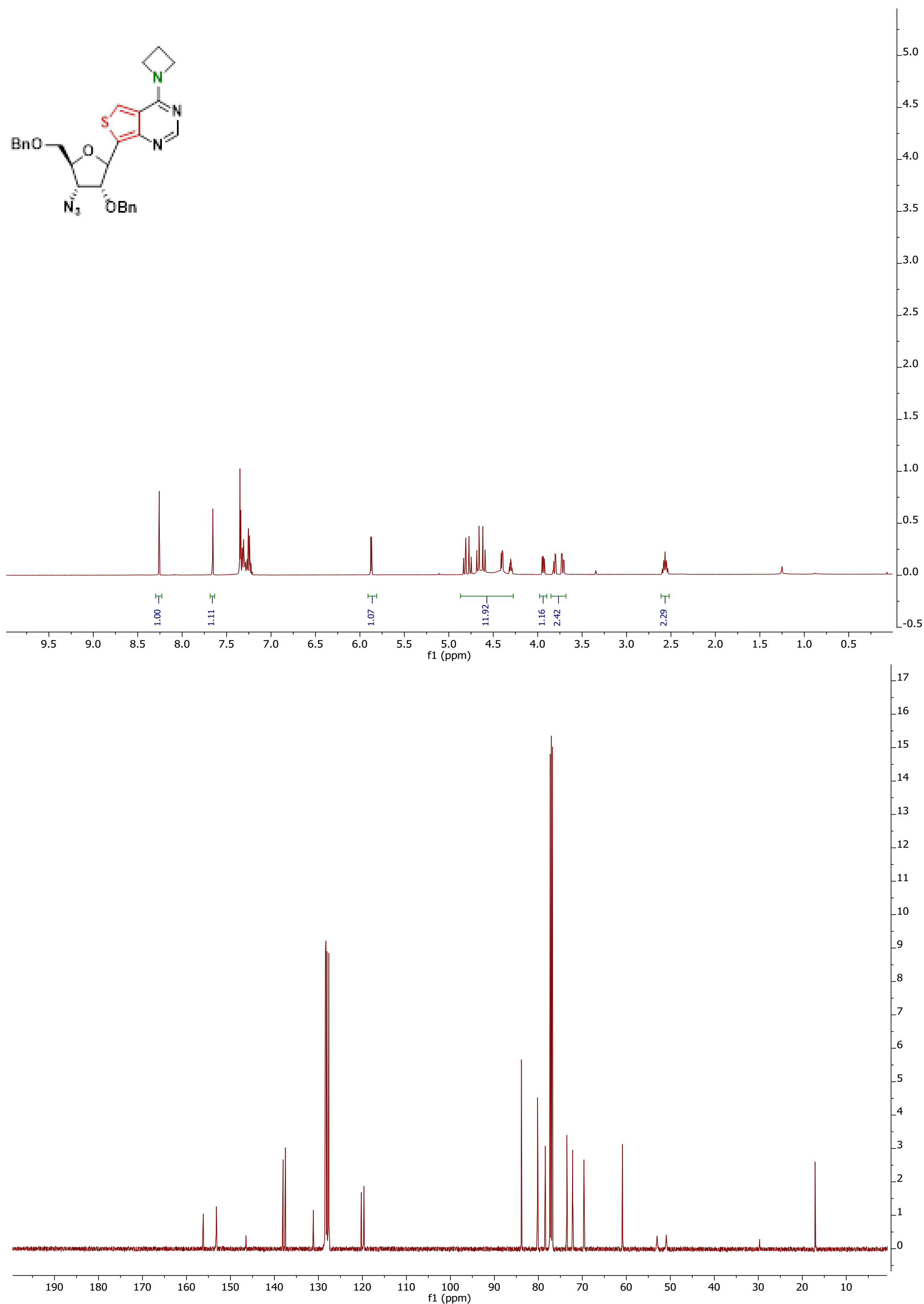


Figure S22. ^{1}H and ^{13}C NMR spectra of compound **17**.

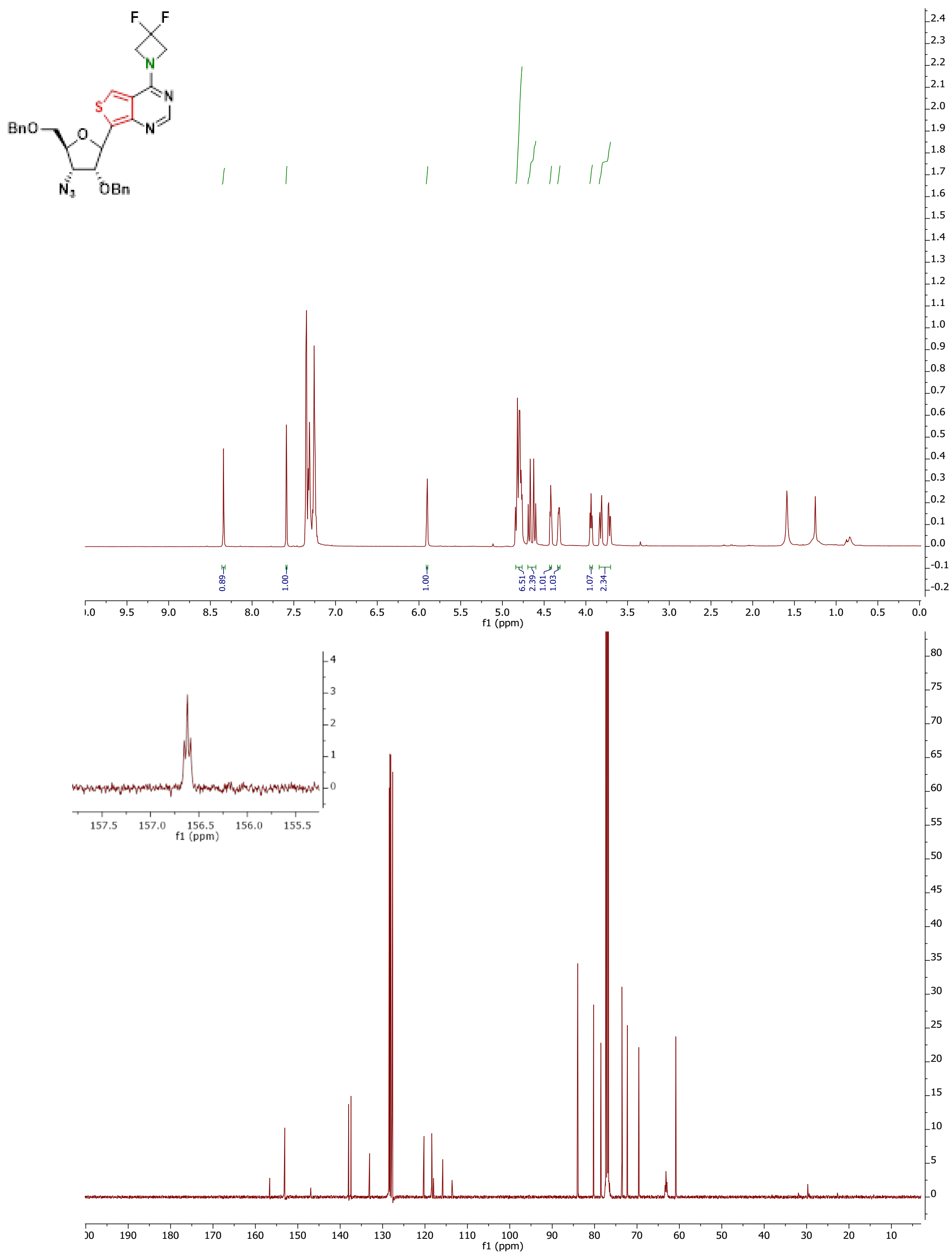


Figure S23. ^{1}H and ^{13}C NMR spectra of compound **18**.

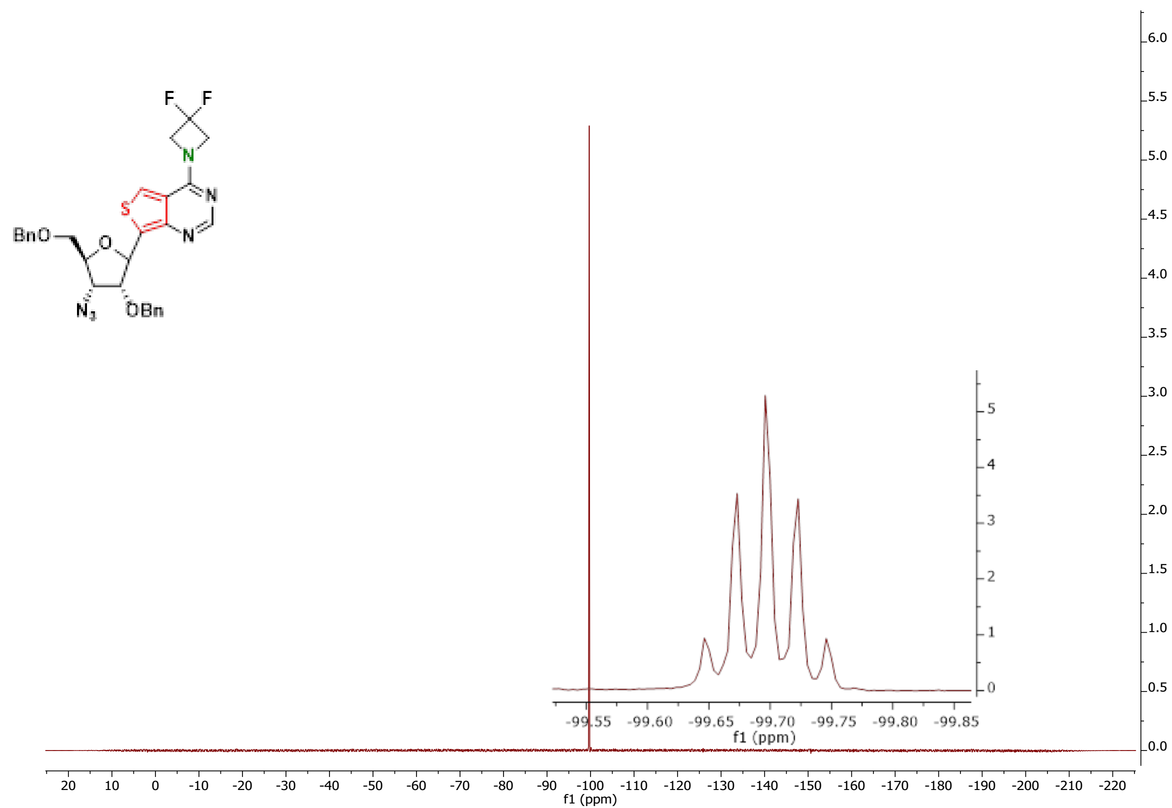


Figure S24. ^{19}F NMR spectra of compound **18**.

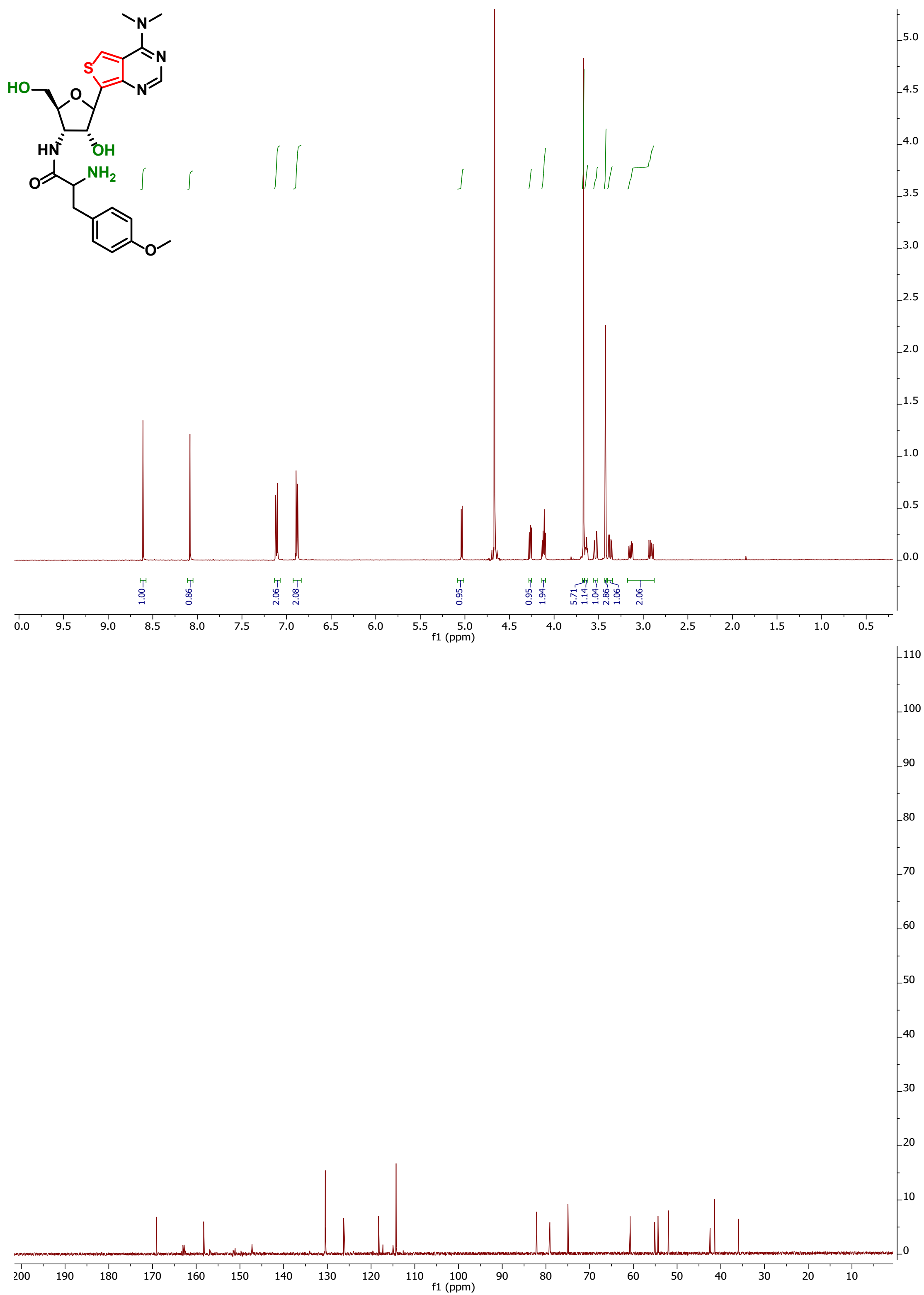


Figure S25. ^{1}H and ^{13}C NMR spectra of compound **2**.

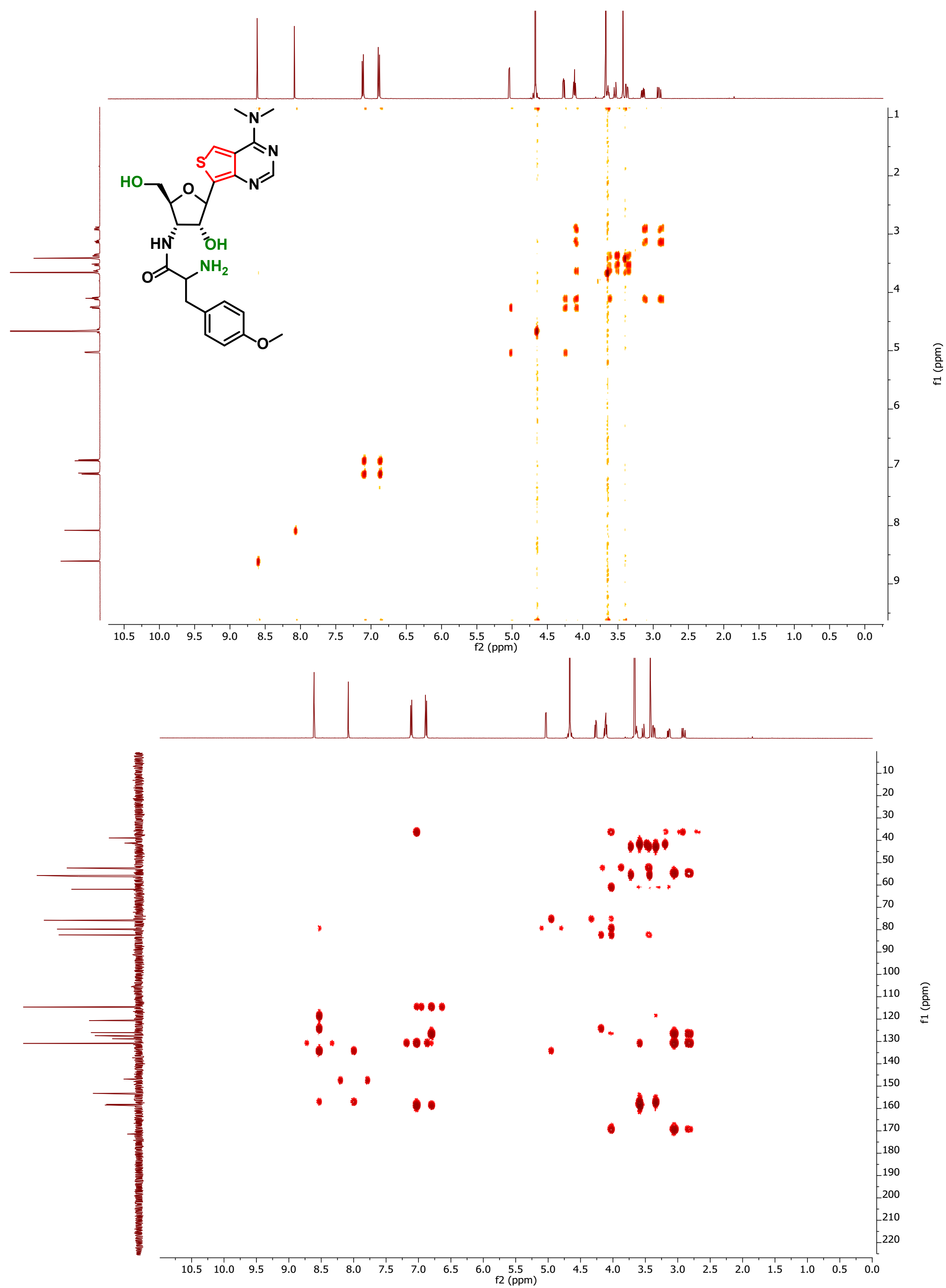


Figure S26. COSY and HMBC spectra of compound **2**.

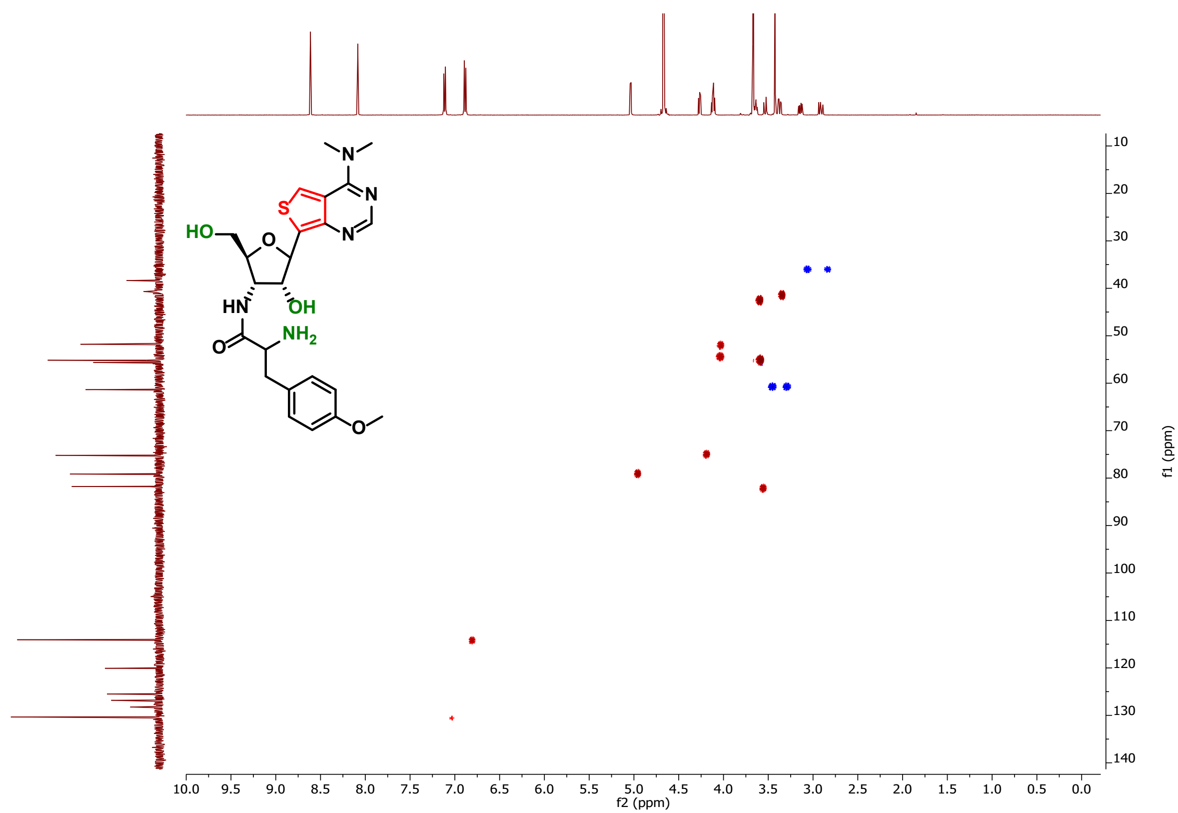


Figure S27. HSQC spectra of compound **2**.

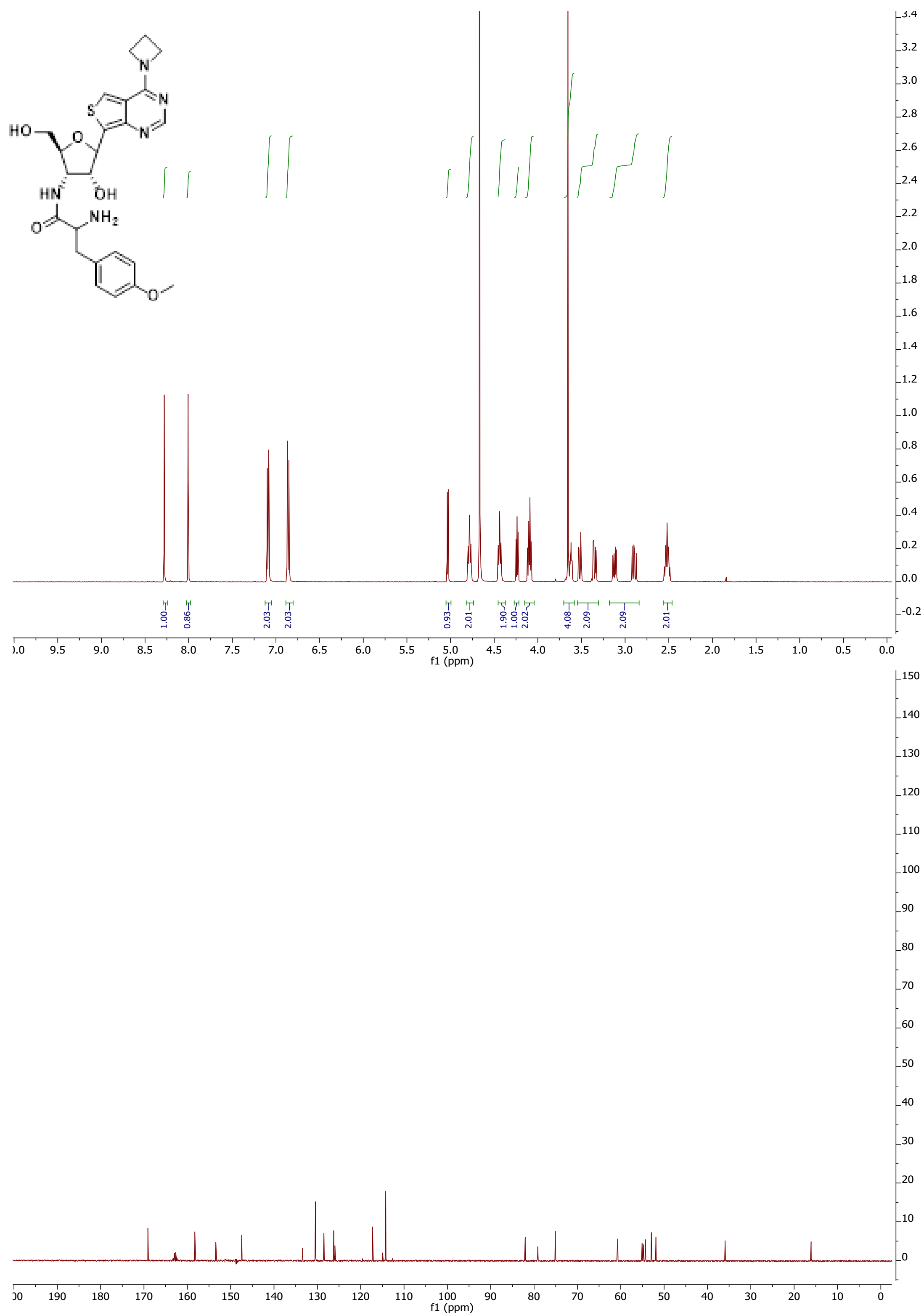


Figure S28. ^{1}H and ^{13}C NMR spectra of compound **3**.

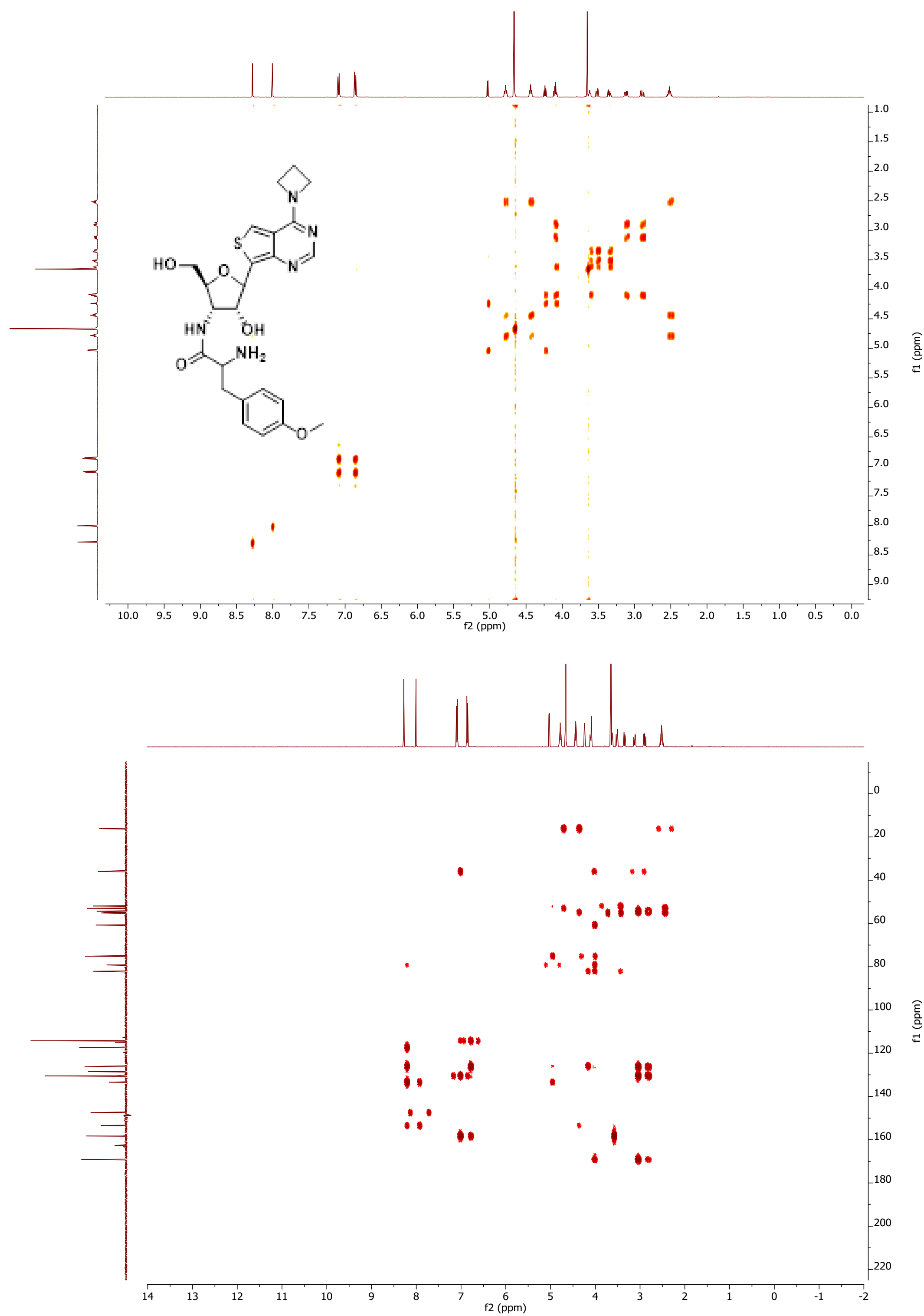


Figure S29. COSY and HMBC spectra of compound **3**.

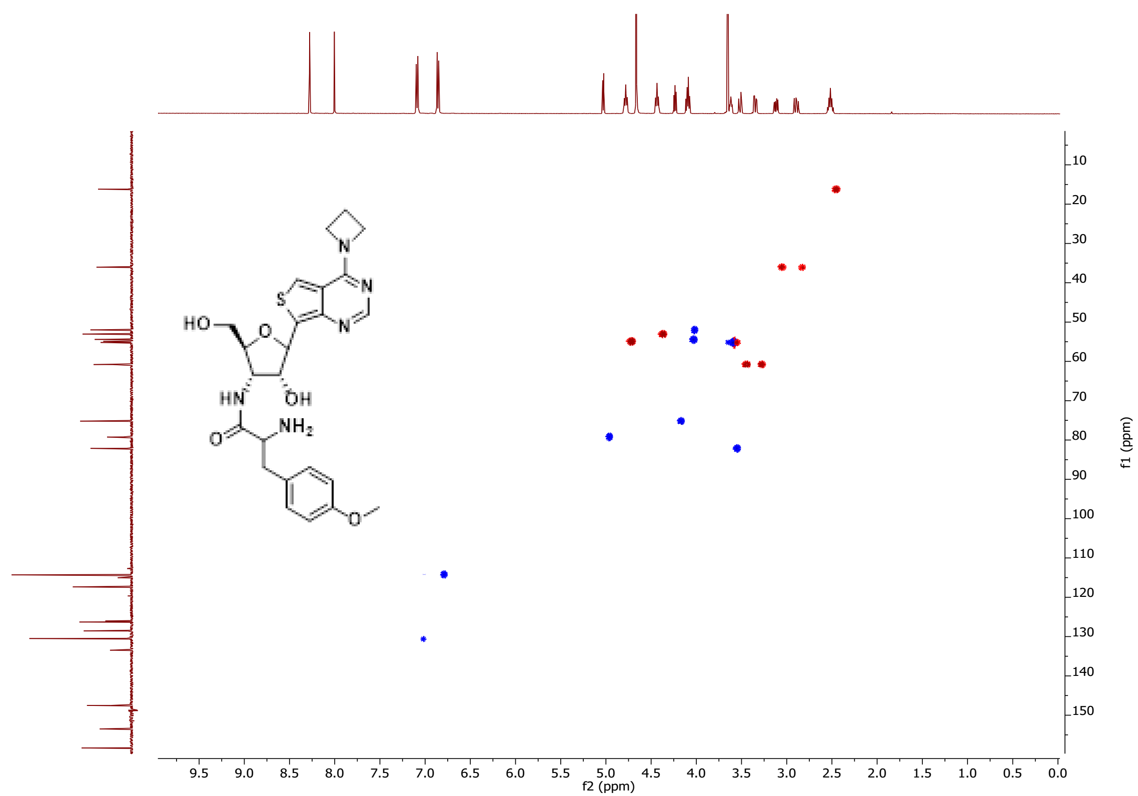


Figure S30. HSQC spectra of compound **3**.

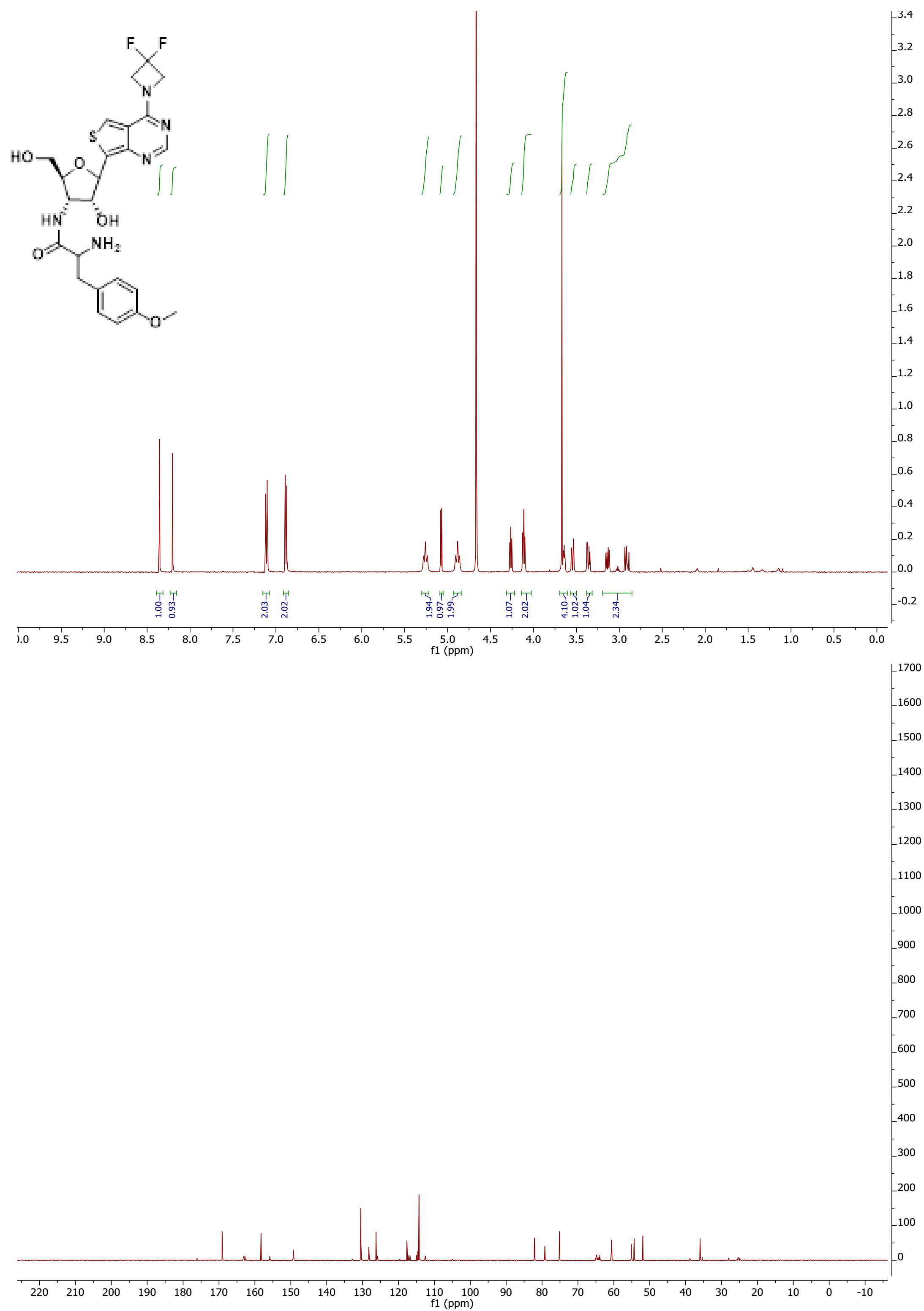


Figure S31. ^{1}H and ^{13}C NMR spectra of compound **4**.

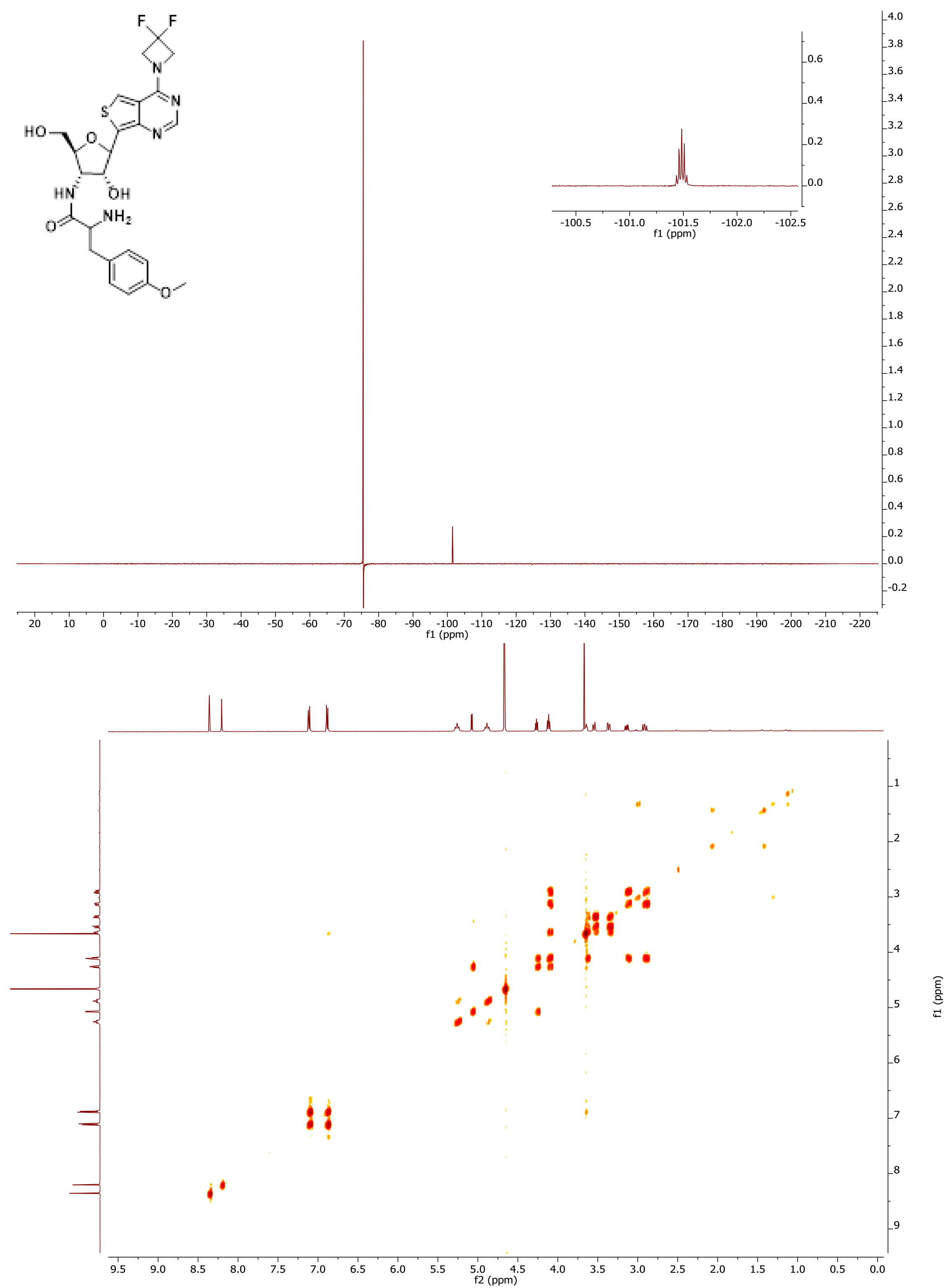


Figure S32. ^{19}F NMR and COSY spectra of compound **4**.

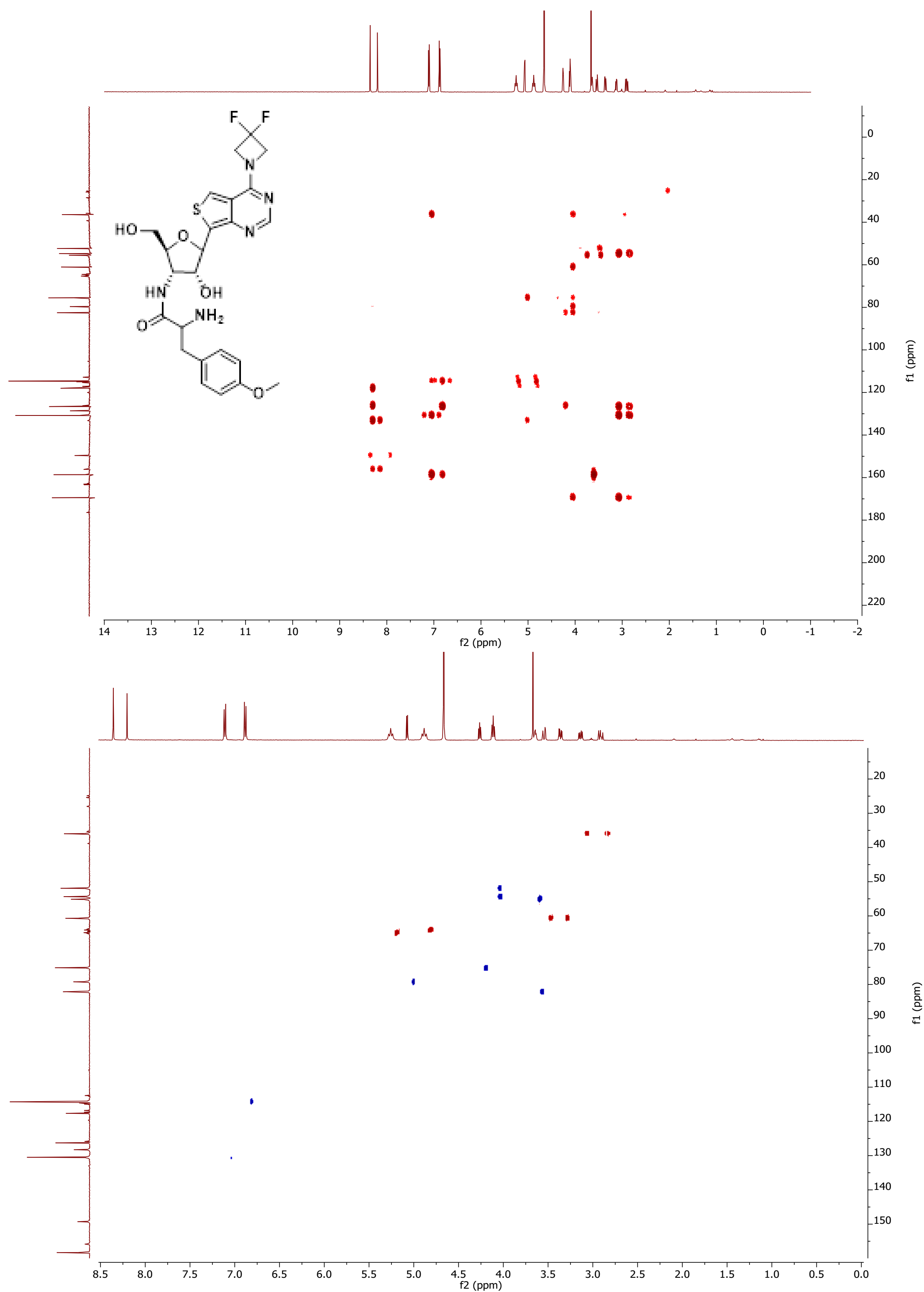


Figure S33. HMBC and HSQC spectra of compound **4**.

14. Supplementary references

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