Supporting Information: Selective Inhibition of DNA Polymerase β by a Covalent Inhibitor

Shelby C. Yuhas[a], Daniel J. Laverty[a], Huijin Lee[a], Ananya Majumdar[b] and Marc M. Greenberg*[a]

[a]Johns Hopkins University, Department of Chemistry, 3400 N. Charles St., Baltimore, MD 21218, United States

[b]Johns Hopkins University, Biomolecular NMR Center, 3400 N. Charles St., Baltimore, MD 21218, United States

mgreenberg@jhu.edu

Contents:

General Methods

Modified oligonucleotides were synthesized on an Applied Biosystems Incorporated 394 oligonucleotide synthesizer. Oligonucleotide synthesis reagents including 5'-phosphorylation reagent (Solid CPR II), SIMA HEX (dichloro diphenyl fluorescein) phosphoramidite, THF abasic site analogue (dSpacer), TAMRA phosphoramidite, and BHQ phosphoramidite were purchased from Glen Research (Sterling, VA). Oligonucleotides containing only native nucleotides were purchased from Integrated DNA Technologies (Coralville, IA) and were purified by 20% denaturing polyacrylamide gel electrophoresis (PAGE) (See below). Oligonucleotides were characterized using a Bruker AutoFlex III Maldi-TOF/TOF system.

All chemicals were purchased from Sigma Aldrich, Fisher, or Alfa and were used without further purification. Small quantities of all library compounds (Chart S1) were purchased from Sigma Aldrich but were from a variety of vendors. Pol η was purchased from EnzyMax. Sybr Gold was purchased from ThermoFisher. Trypsin, dNTPs, terminal deoxynucleotide transferase, Klenow exo$^-$, and T4 polynucleotide kinase were obtained from New England Biolabs. Radionuclides were from Perkin Elmer. Poly-Prep columns were from BioRad. C18-Sep-Pak cartridges were obtained from Waters. Zip-Tips were purchased from Millipore. Quantification of radiolabeled oligonucleotides was carried out using a Molecular Dynamics Phosphorimager 840 equipped with ImageQuant.

UPLC-MS/MS analyses were carried out on a Waters Acquity/Xevo-G2 UPLC-MS system equipped with an ACQUITY UPLC HSS T3 Column (100 Å, 1.8 μm, 2.1 mm x 100 mm). Masses were obtained via deconvolution using MassLynx 4.2 software or BioPharmaLynx 1.3.2 software.

Well plates used for organic solvents and photolyses were obtained from VWR. Well plates used for fluorescence assays were obtained from Corning (CLS3825, 384 well plates, for homogenous luminescent and HTRF assays). Fluorescence data were collected on a Varian Cary Eclipse fluorescence spectrophotometer equipped with a well plate attachment. Fluorescence anisotropy measurements were conducted using an AVIV Biomedical Model ATF 107 spectrofluorometer at the Center for Molecular Biophysics at Johns Hopkins University.

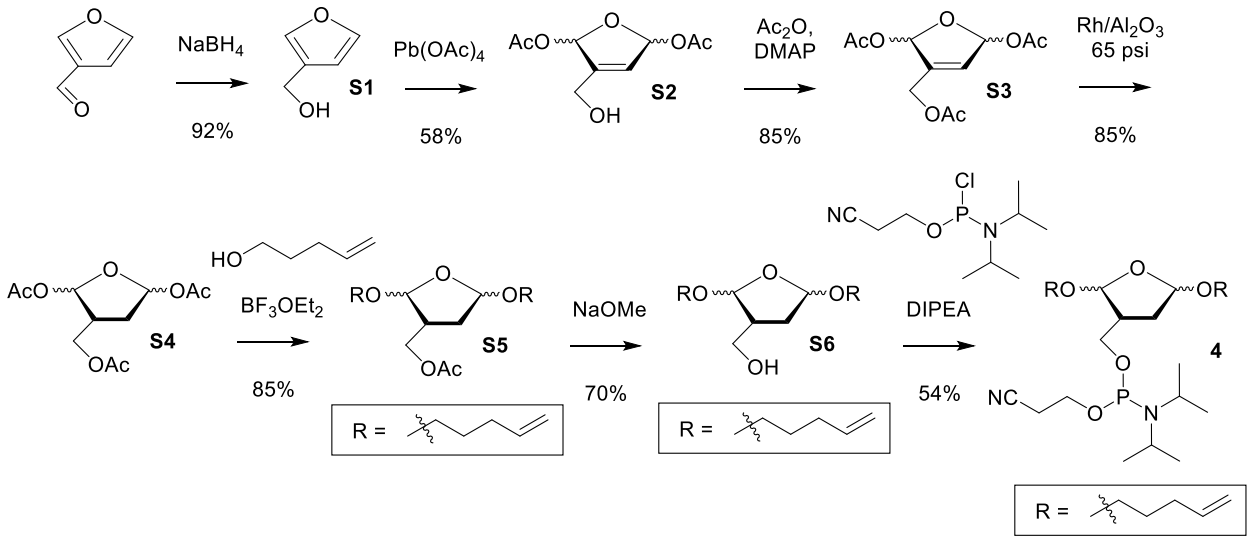
The following items were generous gifts from colleagues. Plasmids for the 8 kDa and 31 kDa domains were from Dr. Sam Wilson, NIH. The Pol β domains were prepared using previously reported conditions.[1,2] Pol λ plasmid was from Professor Zucai Suo, Florida State University. Pol λ was expressed and purified using previously reported conditions.[3,4]

Dulbecco's Modified Eagle Medium (DMEM) with high glucose was obtained from ThermoFisher. Antibiotic antimycotic solution (penicillin, streptomycin, and amphotericin B), and fetal bovine serum (FBS) were obtained from MilliporeSigma. PBS buffer was obtained from Quality Biological. Cells were counted using a BioRad TC20 cell counter.

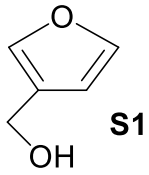
All small molecules synthesized were characterized using a Bruker Avance 400 MHz Spectrometer or an Avance Neo 800 MHz spectrometer at Johns Hopkins University.

All R_f values provided in TLC information correspond to the product unless otherwise explicitly stated.

Scheme S1. Synthesis of bis-pentene DOB phosphoramidite (4).



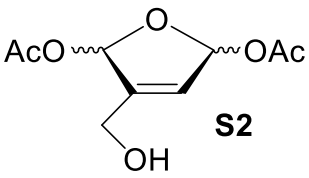
Preparation of S1[5,6]



S1

Commercially available 3-furaldehyde (4.38 mL, 5 g, 52.4 mmol, 1 eq) was added to a mixture of 10:1 Et_2O/H_2O (59 mL). The mixture was cooled to 0 °C and stirred. Small aliquots of NaBH4 (3.27 g, 86.4 mmol, 6.65 eq) were added to the mixture carefully over a period of 15 min. The reaction was stirred in air for 30 min. After 30 min, TLC (4:1 Hex/EtOAc, $R_f = 0.3$) showed the complete conversion of the starting material ($R_f = 0.5$, UV active, did not stain with PAA) to a slightly more polar spot that was not UV active and stained dark purple with PAA. The reaction was quenched with water until there was no more bubbling. The ether layer was washed with water (2 x 50 mL) and the combined aqueous layers were extracted with ether (6 x 40 mL). The final organic layer was washed with brine (1 x 50 mL), dried with MgSO4, and concentrated under vacuum at 0 °C to yield 4.714 g (91.7%) of a yellow liquid. The product was volatile, so the rotary evaporatory water bath was cooled to 0 °C during concentration. NMR data showed the crude product was pure. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.36 (s, 2H), 6.38 (s, 1H), 4.46 (d, J = 4.9 Hz, 2H), 2.92 (d, J = 4.9 Hz, 1H).

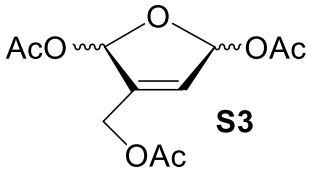
Preparation of S2[5,6]



S2

Without purification, **S1** (474.6 mg, 4.84 mmol, 1 eq) was combined with $Pb(OAc)_4$ (3.22 g, 7.22 mmol, 1.5 eq). Glacial acetic acid (12.5 mL) was added to the flask, which was then flushed with Argon. The reaction was stirred at 25 °C for 21 h. When the reaction was complete by TLC (3:7 EtOAc/DCM, R_f = 0.4, stained with PAA), AcOH was removed via vacuum. Ether (50 mL) was added to the resulting residue and the precipitate was triturated with ether and removed. The filtrate was concentrated under vacuum and purified by column chromatography (7:3 Hexanes/EtOAc) to give 518 mg (58%) of a 2:1 mixture of diastereomers (**S2**). ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.90 (dd, *J* = 1.0, 4.7 Hz, 1H), 6.69 (dd, *J* = 1.0, 4.7 Hz, 1H), 6.15 – 6.05 (m, 1H), 4.30 (q, *J* = 1.0 Hz, 2H), 2.19 – 2.07 (m, 6H).

Preparation of S3



S3

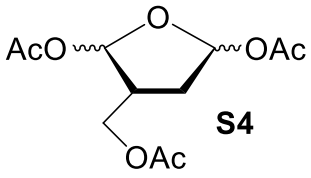
Compound **S2** (150 mg, 0.685 mmol, 1 eq, 500 mM) and DMAP (8.6 mg, 0.07 mmol, 0.1 eq) were added to a flask. The contents were flushed with Argon and dissolved in DCM (1.25 mL). The mixture was cooled to 0 °C. Acetic anhydride (0.33 mL, 3.5 mmol, 5 eq) was added slowly to the flask via syringe. Pyridine (0.68 mL, 8.56 mmol, 12.5 eq) was added dropwise to the solution via syringe. After 2 h, the reaction was confirmed complete by TLC (6:4 EtOAc/DCM, R_f = 0.6, stained with PAA) and was quenched with sat. $NaHCO_3$ until the pH was neutral. The mixture was diluted with EtOAc (5 mL) and washed with water (2 x 5 mL). The aqueous layer was extracted with EtOAc (2 x 10 mL), the combined organic layers were washed with brine (1 x 30 mL), dried over Na_2SO_4, and concentrated under vacuum. The crude residue was purified by column chromatography (5:1 DCM/EtOAc) to yield 167 mg (85%) of **S3**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.83 (s, 1H), 6.70 –

6.56 (m, 1H), 6.22 – 5.89 (m, 1H), 4.83 – 4.57 (m, 2H), 2.11 – 1.96 (m, 9H).

^{13}C NMR ($CDCl_3$) 166.4, 152.3, 138.1, 111.6, 85.9, 85.8, 62.3, 38.1, 12.4, 9.2.

ESI-TOF m/z calculated for $C_{11}H_{14}O_7$ (M + H) – 258.0740, 258.0703 observed.

Preparation of S4



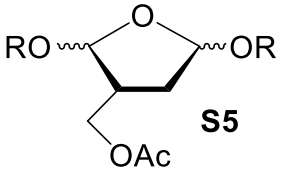
S4

Compound **S3** (150 mg, 0.69 mmol, 1 eq) was dissolved in EtOAc (17 mL). Rhodium on alumina catalyst (75.5 mg) was added to the pressure bottle equipped with a regulator. The vial was pressurized with H_2 to 70 psi, purged three times, and stirred at 25 ^{o}C for 2-4 h. After venting the pressure bottle, TLC (1:1 Hex/EtOAc, R_f = 0.4, stained with PAA) showed the starting material was no longer present. When complete, the reaction mixture was passed through celite to remove the Rh catalyst. The filtrate was concentrated under vacuum to give 127.5 mg (85%) of pale, yellow compound **S4**. No purification was needed. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.25 – 6.15 (m, 2H), 4.06 (m, 1H), 4.03 – 3.91 (m, 1H), 2.71 – 2.56 (m, 1H), 2.38 (m, 1H), 2.04 – 1.88 (m, 9H), 1.83 – 1.67 (m, 1H).

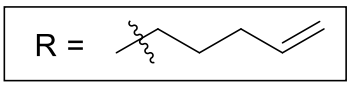
^{13}C NMR ($CDCl_3$) 170.7, 169.9, 169.5, 98.5, 96.4, 62.1, 41.6, 32.7, 21.5, 20.9, 20.7.

ESI-TOF m/z calculated for $C_{11}H_{16}O_7$ (M + H) – 260.0896, 260.0900 observed.

Preparation of S5



S5

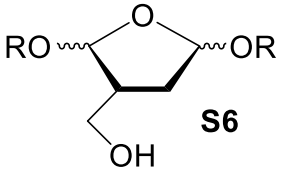


BF_3•etherate was distilled from CaH_2 under vacuum and kept under Argon. Compound **S4** (750 mg, 2.88 mmol, 1 eq, 160 mM) was dissolved in DCM (18 mL) and cooled to 0 ^{o}C. 4-Pentenol (1.86 mL, 1.49 g, 17.3 mmol, 6 eq) was added to the reaction. BF_3•etherate (8.14 mL, 1.67 M) was slowly added to the solution over a period of 15-20 min (until diluted to 28 mL, 600 mM). After 30 min, the reaction was incomplete when analyzed by TLC (7:3 Hex/Et_2O, stained with PAA) but additional products began to appear, so the reaction was

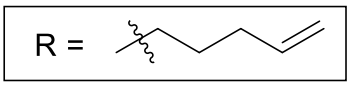
quenched with sat. $NaHCO_3$ (5 mL), diluted with DCM (20 mL), and washed with sat. $NaHCO_3$ (1 x 15 mL). The aqueous layer was extracted with DCM (4 x 20 mL). The organic layer was washed with water (1 x 30 mL), brine (1 x 30 mL), and dried over Na_2SO_4. The residue was concentrated under vacuum and purified by column chromatography (8:2 hex/EtOAc) to give 228.6 mg (25.4%) of **S5a** and 519 mg (57.7%) of **S5b** (totaling 83%), which were each a mixture of diastereomers. **S5a** ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.82 – 5.65 (m, 2H), 5.21 – 4.84 (m, 6H), 4.24 – 3.99 (m, 2H), 3.75 – 3.57 (m, 2H), 3.45 – 3.22 (m, 2H), 2.61 (m, 1H), 2.42 – 2.17 (m, 1H), 2.08 – 2.01 (m, 4H), 1.99 (d, *J* = 9.5 Hz, 3H), 1.93 – 1.74 (m, 1H), 1.67 – 1.50 (m, 4H). **S5b** ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.91 – 5.68 (m, 2H), 5.10 – 4.83 (m, 6H), 4.15 – 3.88 (m, 2H), 3.73 (m, 2H), 3.55 – 3.32 (m, 2H), 2.65 (ddd, *J* = 5.5, 7.4, 13.5 Hz, 1H), 2.39 (s, 1H), 2.32 – 2.18 (m, 1H), 2.16 – 2.08 (m, 4H), 2.05 (s, 3H), 1.81 (ddd, *J* = 5.5, 7.4, 13.5 Hz, 1H), 1.73 – 1.59 (m, 4H). **S5a** and **S5b** ^{13}C NMR ($CDCl_3$) 170.9, 170.8, 138.20, 138.18, 138.15, 114.8, 106.9, 106.2, 104.7, 104.2, 103.5, 103.2, 67.6, 67.5, 67.4, 67.3, 67.2, 67.0, 64.8, 64.5, 63.3, 43.8, 43.2, 40.7, 34.7, 34.2, 33.4, 30.4, 30.3, 29.9, 28.9, 28.8, 20.9, 20.8.

ESI-TOF m/z calculated for $C_{17}H_{28}O_5$ (M + H) – 312.1937, 312.1942 observed.

Preparation of S6[6]



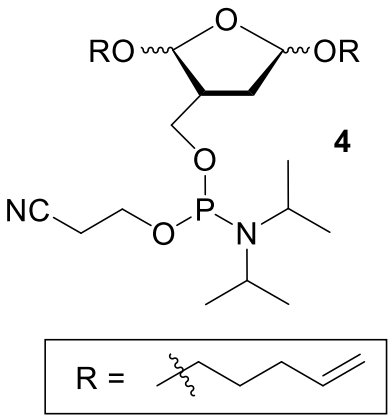
S6



The diastereomerically enriched mixture (**S5a** or **S5b**) (82 mg, 0.26 mmol, 1 eq, 150 mM) was dissolved in MeOH (1.7 mL). Sodium methoxide stock solution (700 mM) was prepared by dissolving Na metal (122 mg, 5.3 mmol) in MeOH (7.5 mL). An aliquot of NaOMe (700 mM, 0.3 mL) was added to the reaction slowly (effectively diluting NaOMe to 100 mM). After 2 h, TLC (6:4 Hex/EtOAc, R_f = 0.2, stained with PAA) confirmed the reaction was complete by the disappearance of **S5**. The reaction was quenched with a few drops of AcOH until neutral

pH. The reaction was diluted with DCM (20 mL) and washed with H_2O (2 x 15 mL). The aqueous layer was extracted with DCM (4 x 20 mL) and the combined organic layers were washed with brine (1 x 30 mL) and dried over Na_2SO_4. The reaction was concentrated under vacuum to give 49.3 mg (70%) of **S6**. No purification was needed. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.91 – 5.65 (m, 2H), 5.23 – 4.88 (m, 6H), 3.93 – 3.71 (m, 2H), 3.72 – 3.61 (m, 2H), 3.49 – 3.31 (m, 2H), 2.63 – 2.46 (m, 1H), 2.38-2.23 (ddd, *J* = 5.0, 9.5, 13.3 Hz, 1H), 2.26 – 2.13 (m, 1H), 2.13 – 2.03 (m, 4H), 1.82 (ddd, *J* = 1.5, 7.8, 13.3 Hz, 1H), 1.65 (dddt, *J* = 1.5, 5.0, 7.8, 9.5 Hz, 4H).

Preparation of 4



Compound **S6** (58.2 mg, 0.22 mmol) was azeotropically dried with pyridine (2 x 0.5 mL). The reagent was cooled to 0 °C. DIPEA (0.18 mL, 133 mg, 0.88 mmol, 4 eq) was added to the cold starting material and the reactants were dissolved in DCM (2.1 mL, 100 mM). 2-Cyanoethyl-N, N-diisopropylchlorophosphoramidite (0.06 mL, 60.4 mg, 0.26 mmol. 1.2 eq) was added and the cold mixture stirred with periodic monitoring by TLC (7:1 Hex/EtOAc, R_f = 0.3, stained with PAA). After 2 h, TLC showed complete conversion to the phosphoramidite. The reaction was diluted with freshly distilled EtOAc (10 mL). The organic layer was washed with saturated bicarbonate solution (2 x15 mL) and the aqueous layers were extracted with distilled EtOAc (2 x 20 mL). The combined organic layers were washed with brine (1 x 25 mL) and dried over Na_2SO_4. The organic layer was concentrated under vacuum and purified by column chromatography (7:1 distilled hexanes/distilled EtOAc) yielding 55.9 mg (54%) of **4**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.80 (dddt, *J* = 4.1, 8.2, 13.5, 16.0 Hz, 2H), 5.18 – 4.93 (m, 6H), 3.86 – 3.72 (m, 2H), 3.64 (dtd, *J* = 2.1, 6.0, 8.2 Hz, 2H), 3.59 – 3.51 (m, 2H), 3.34 (dddd, *J* = 2.1, 4.1,

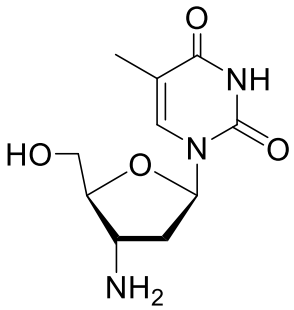
9.4, 16.0 Hz, 2H), 2.71 – 2.52 (m, 2H), 2.30 (ddd, *J* = 6.0, 9.4, 13.5 Hz, 2H), 2.11 – 2.02 (m, 4H), 1.93 – 1.76 (m, 1H), 1.65 – 1.55 (m, 6H), 1.17 (m, 12H).

^{13}C NMR (400 MHz, $CDCl_3$): δ 170.92, 170.91, 138.28, 138.25, 138.23, 114.75, 114.73, 114.69, 114.68, 114.64, 106.9, 105.7, 105.2, 104.5, 103.8, 102.6, 67.6, 67.3, 66.6, 64.5, 63.4, 44.8, 43.8, 43.2, 42.8, 34.7, 30.0, 30.3, 28.8, 20.9, 20.8.

^{31}P NMR (400 MHz, $CDCl_3$) δ 147.87.

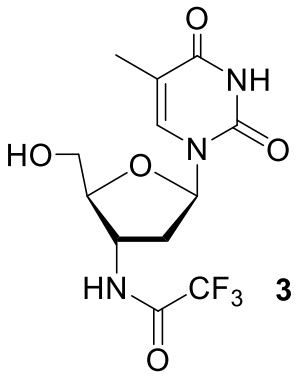
ESI-TOF m/z calculated for $C_{24}H_{43}N_2O_5P$ (M + H) – 471.2910, 471.2887 observed.

Preparation of 3[7,8]



precursor to **3**

AZT (2.19, 8.2 mmol) was dissolved in 50% MeOH, 30% tBuOH, 20% H_2O (82 mL, 100 mM). Activated palladium on carbon (1.3 g, 60 wt%) was added and flushed with a hydrogen balloon three times. The reaction was continuously sparged with H_2 (1 atm). After 3 h, TLC (3% MeOH in DCM, UV active and stained with PAA) confirmed the starting material (R_f = 0.8) converted to the product (R_f = 0.02). The crude mixture was filtered through Celite and concentrated to yield 1.7 g (86%) of the 3'-amine precursor to **3**. ^{1}H NMR (400 MHz, CD_3OD) δ 7.87 (s, 1H), 6.18 (dd, *J* = 2, 6.8 Hz, 1H), 3.86 (dd, *J* = 2, 10 Hz, 1H), 3.77 (dd, *J* = 3.2, 10 Hz, 1H), 3.70 (quint, *J* = 3.2 Hz, 1H), 3.54 (q, *J* = 6.8 Hz, 1H), 2.26 (m, 1H), 2.20 (m, 1H), 1.88 (s, 3H).

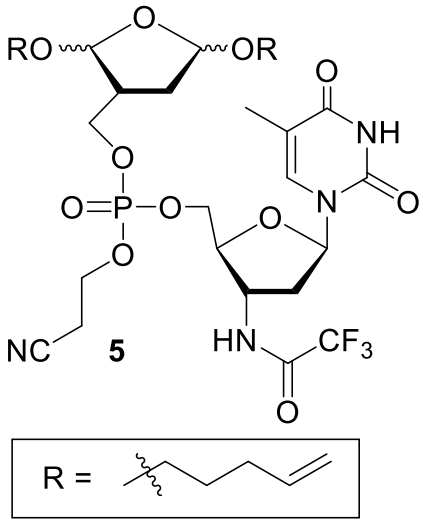


The crude mixture (1.72 g, 7.12 mmol) was dissolved in THF (25 mL, 280 mM). Triethylamine (14 mL, 106.8 mmol, 15 eq) and ethyl trifluoroacetate (8.4 mL, 71.3 mmol, 10 eq) were added to the flask to make a final concentration of 100 mM. The reaction was stirred for 4 h at 25 ^{o}C. TLC (3% MeOH in DCM, R_f = 0.5, UV active and stained by PAA) confirmed conversion to product. The reaction was concentrated and purified by column chromatography (1% MeOH in DCM to yield 2.29 g (93%) of **3**. ^{1}H NMR (400 MHz, CD_3OD) δ 7.85 (s, 1H), 6.27 (t, *J* = 6.4 Hz, 1H), 4.60 (q, *J* = 6.4 Hz, 1H), 3.97 (quint, *J* = 2.8 Hz, 1H), 3.85 (dd, *J* = 2.8, 12 Hz, 1H), 3.73 (dd, *J* = 2.8, 12 Hz, 1H), 2.40 (m, 2H), 1.89 (s, 3H).

^{13}C NMR (400 MHz, CD_3OD) δ 166.2, 159.4, 158.9, 158.6, 152.2, 138.0, 118.6, 115.8, 111.5, 85.9, 85.6, 62.3, 50.7, 37.9, 12.3, 9.09.

ESI-TOF m/z calculated for $C_{12}H_{14}F_3N_3O_5$ (M + H) − 338.0886, 338.3413 observed.

Preparation of 5



Phosphoramidite **4** (101 mg, 0.38 mmol, 1.2 eq) and **3** (172 mg, 0.32 mmol, 1 eq) were azeotropically dried together with toluene (2 x 1 mL). S-Ethyl tetrazole/MeCN (250 mM, 1.5 mL, 0.38 mmol, 1.2 eq) was added to the flask. After 4 h, TLC (1:1 EtOAc/Hex, R_f = 0.6, UV active and stained with PAA) indicated that the majority of **55** was consumed. tBuOOH (500 mM, 200 μL, 1 mmol, 3 eq) was added and the reaction was stirred for 15-20 min. The reaction was concentrated under

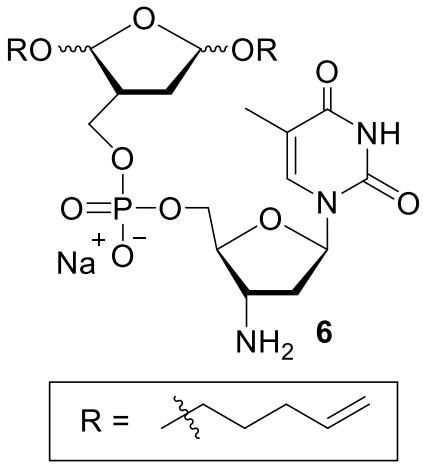
vacuum and column chromatography (2:1 EtOAc/Hex) yielded 131 mg (56 %) of **5.** ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.36 (d, *J* = 10.1 Hz, 1H), 8.86 (d, *J* = 5 Hz, 1H), 7.46 (d, *J* = 2 Hz, 1H), 6.38 (q, *J* = 5 Hz, 1H), 5.74 (dsextet, *J* = 2, 4 Hz, 2H), 5.10-4.90 (m, 6H), 4.51 (s, 1H), 4.34 (s, 2H), 4.25 (q, *J* = 6 Hz, 2H), 4.16 (s, 2H), 4.06 (m, 1H), 3.63 (sextet, *J* = 2 Hz, 2H), 3.33 (m, 2H), 2.75 (q, *J* = 6 Hz, 3H), 2.44 (q, *J* = 8 Hz, 2H), 2.28 (m, 1H), 2.15 (m, 1H), 2.01 (d, *J* = 4 Hz, 4H), 1.96 (s, 3H), 1.58 (q, *J* = 4 Hz, 4H).

^{13}C NMR (400 MHz, CD_3OD) δ 166.23, 159.79, 152.15, 141.21, 139.40, 137.73, 129.01, 115.25, 113.27, 112.05, 106.87, 105.52, 104.85, 85.22, 84.82, 68.26, 67.38, 64.33, 61.54, 50.55, 48.65, 45.97, 37.24, 33.76, 31.45, 30.10, 27.94, 20.10, 15.31, 12.63.

^{31}P NMR (400 MHz, $CDCl_3$) δ -2.14.

ESI-TOF m/z calculated for $C_{30}H_{42}F_3N_4O_{11}P$ (M + H) – 723.2540.2717, 723.2303 observed.

Preparation of 6



Compound **5** (115 mg, 0.15 mmol) was dissolved in concentrated aqueous ammonia (1.5 mL). The reaction was stirred at 0 °C for 4 h. After 4 h, TLC (20% MeOH in DCM, R_f = 0.2, UV active and stained with $KMnO_4$) confirmed the disappearance of starting material. The reaction was concentrated to yield 85 mg (100%) of **6**. ^{1}H NMR (400 MHz, CD_3OD) δ 6.34 (t, *J* = 5.8 Hz, 1H), 5.78 (m, 2H), 5.10-4.98 (m, 6H), 4.13 (s, 3H), 3.88 (m, 2H), 3.66 (m, 2H), 3.40 (q, *J* = 5.8 Hz, 2H), 2.81 (s, 2H), 2.68 (t, *J* = 7 Hz, 1H), 2.49 (m, 1H), 2.44 (m, 2H), 2.30 (m, 1H), 2.06 (t, *J* = 7 Hz, 4H), 1.93 (s, 3H), 1.60 (quint, *J* = 7 Hz, 4H).

^{13}C NMR (400 MHz, CD_3OD): δ 164.92, 150.84, 138.03, 137.99, 136.57, 116.47, 113.84, 110.75, 105.95, 104.39, 103.54, 67.30, 66.66, 50.97, 35.82, 35.58, 30.04, 30.02, 28.72, 15.82, 14.19, 11.28.

^{31}P NMR (400 MHz, CD_3OD) δ 0.07.

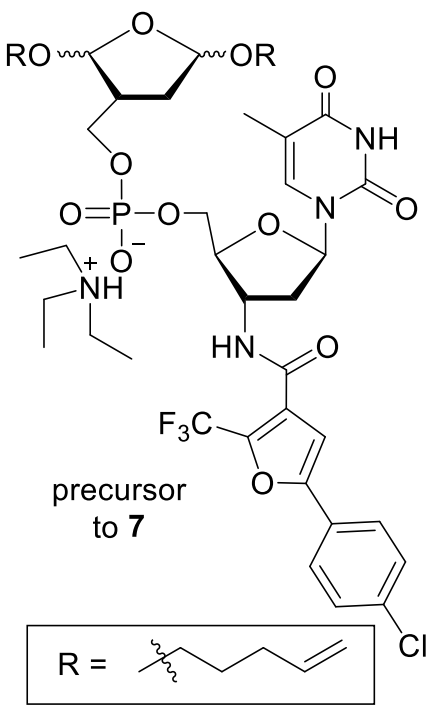
ESI-TOF m/z calculated for $C_{25}H_{39}N_3O_{10}P$ (M - H) – 572.2379, 572.2399 observed.

General Procedure for the preparation of first-generation library

Amine scaffold **6** (100 nmol) was azeotropically dried with carboxylic acid (140 nmol, 1.4 eq) in pyridine (1 x 15 μL) using a Speed Vac concentrator in a 384-well microtiter plate (VWR). To each well, activating solution (5 μL; 28 mM HBTU and 28 mM HOBt in DMF), DIPEA (2 μL), and DMF (3 μL) were added. The final concentrations during reaction were: [**6**] = 10 mM, [acid] = 14 mM, [HBTU] = 14 mM, [HOBt] = 14 mM, 20% DIPEA in DMF. The well plate was shaken at 25 °C overnight. Some wells were analyzed by ESI-MS to confirm coupling efficiency. The solutions were concentrated to dryness using a Speed Vac concentrator and the well plate was covered and stored at -80 °C.

Immediately before an assay, the amide was thawed, dissolved in DMF (4 μL, 25 mM). An aliquot (2 μL, 50 nmol) was mixed with NBS (8 μL, 15 mM, 2.4 eq, 40% H_2O in MeCN) at 0 °C for 9 min. The concentrations during reaction were: [SM] = 5 mM, [NBS] = 12 mM, 20% DMF, 30% H_2O in MeCN. After 9 min, $Na_2S_2O_3$ (5 μL, 200 mM) was added and reaction quenched on ice for 10 min. Samples were concentrated with a Speed Vac concentrator. Some samples were analyzed ESI-MS to confirm product formation.

Preparation of 7



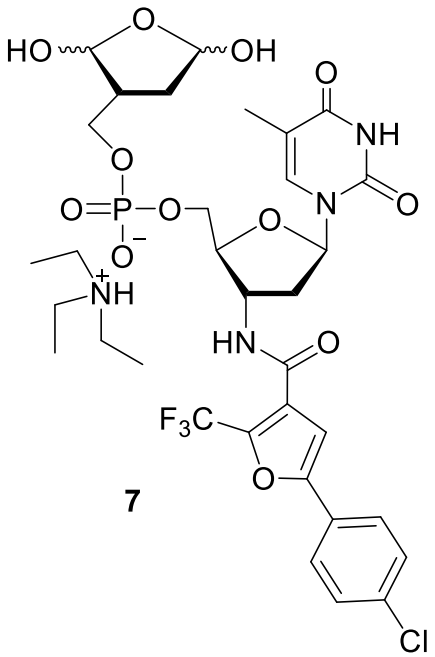
Scaffold **6** (17 mg, 0.03 mmol) and 5-(4-chlorophenyl)-3-(trifluoromethyl) furan-2-carboxylic acid (11 mg, 0.04 mmol, 1.4 eq) were azeotropically dried together with pyridine (2 x 1 mL). HBTU (14.0 mg, 0.037 mmol, 1.4 eq) and HOBt (5 mg, 0.037 mmol, 1.4 eq) were added to the starting materials. The contents were dissolved in DMF (1.04 mL) and DIPEA (260 µL). The reaction was stirred at 25 ^{o}C overnight. TLC (20% MeOH in DCM, R_f = 0.3, UV active and stained with PAA) suggested **57** was consumed. The reaction was concentrated and purified by column chromatography (5% MeOH in DCM) to yield 21.2 mg (96%) of the precursor to **7**. ^{1}H NMR (400 MHz, CD_3OD) δ 7.76 (d, *J* = 6.8 Hz, 1H), 7.70 (d, *J* = 7.6 Hz, 2H), 7.59 (d, *J* = 6.8 Hz, 2H), 7. 50 (d, *J* = 7.6 Hz, 1H), 7.35 (quint, *J* = 6.8 Hz, 4H), 6.37 (t, *J* = 3.6 Hz, 1H), 5.78 (m, 2H), 5.00 (m, 6H), 4.68 (m, 1H), 4.15 (d, *J* = 1.6 Hz, 1H), 4.10 (m, 2H), 3.90 (m, 2H), 3.66 (dd, *J* = 1.6, 5.2 Hz, 2H), 3.21 (q, *J* = 8 Hz, 6H), 2.49 (m, 1H), 2.40 (d, *J* = 1.6 Hz, 2H), 2.30 (m, 1H), 2.07 (d, *J* = 6.4 Hz, 4H), 1.97 (s, 3H), 1.90 (m, 1H), 1.60 (quint, *J* = 6.4 Hz, 4H), 1.32 (t, *J* = 8 Hz, 9H).

^{13}C NMR (400 MHz, $CDCl_3$) δ 169.9., 169.7, 163.8, 150.2, 136.2, 134.9, 128.6, 123.9, 116.6, 111.43, 111.40, 98.45, 98.42, 96.1, 87.3, 87.2, 86.2, 86.1, 80.9, 80.8, 67.3, 66.3, 66.2, 62.5, 62.45, 62.43, 42.9, 42.8, 42.7, 36.1, 32.3, 32.2, 21.1, 19.72, 19.67, 12.4.

^{31}P NMR (400 MHz, CD_3OD) δ -2.4, -3.0.

ESI-TOF m/z calculated for $C_{37}H_{44}ClF_3N_3O_{12}P$ (M - H) − 844.2303, 844.2221 observed.

ε_{273} (50% MeCN in H_2O) = 2.0×10^4 $M^{-1}cm^{-1}$



7

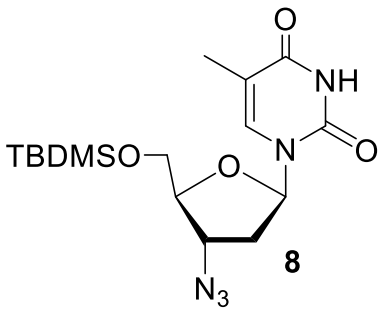
The pentenoyl protected inhibitor was stored as a 25 mM stock solution in DMF at -20 °C. An aliquot of the starting material (2 µL, 50 nmol) was placed in a microtiter well plate and cooled to 4 °C. Cold H_2O (3 µL) was added to the well. NBS in MeCN (25 mM, 5 µL) was added and the well plate was shaken at 4 °C for 9 min. After 9 min, the reaction was quenched by an equal volume of $Na_2S_2O_3$ (200 mM in H_2O, 10 µL) and the plate was shaken for additional 5-10 min. The reaction was concentrated by speed vacuum, redissolved in 1:1 MeCN/H_2O and used directly.

Reaction Conditions: [SM] = 5 mM, [NBS] = 12.5 mM, 30% H_2O, 20% DMF, 50% MeCN

Quenching Conditions: [SM] = 2.5 mM, [NBS] = 6.25 mM, [Na_2S2O_3] = 100 mM, 65% H_2O, 10% DMF, 25% MeCN.

ESI-MS m/z calculated for $C_{27}H_{27}ClF_3N_3O_{12}P$ (M - H) – 708.09, 708.20 observed.

Preparation of 8[9]



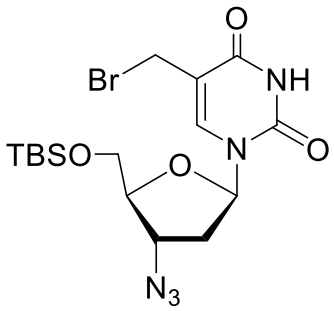
8

AZT (587 mg, 2.19 mmol, 1 eq) was azeotropically dried twice with pyridine (2 x 1 mL). Imidazole (604 mg, 8.76 mmol, 4 eq) and TBDMSCl (672 mg, 4.38 mmol, 2 eq) were added and dissolved in DMF (4.5 mL, 500 mM). The reaction was heated to 50° C overnight. When confirmed complete by TLC (5% MeOH in DCM, R_f = 0.5, UV active and stained with PAA), the reaction was cooled and diluted with

EtOAc (10 mL). The organic layer was washed with sat. NH4Cl (3 x 15 mL) and brine (2 x 15 mL). The combined aqueous layers were extracted using EtOAc (2 x 50 mL). The combined organic layers were dried over Na_2SO_4 and concentrated under vacuum. The residue was purified by column chromatography (2:1 Hex/EtOAc) yielding 825 mg (95%) of **8**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.72 (s, 1H), 7.43 (s, 1H), 6.22 (t, *J* = 6.8 Hz, 1H), 4.24 (q, *J* = 3.2 Hz, 1H), 3.96 (d, *J* = 3.2 Hz, 1H), 3.94 (dd, *J* = 2.8, 14 Hz, 1H), 3.79 (dd, *J* = 2.8, 14 Hz, 1H), 2.44 (m, 1H), 2.22 (m, 1H), 1.91 (s, 3H), 0.93 (s, 9H), 0.13 (s, 6H).

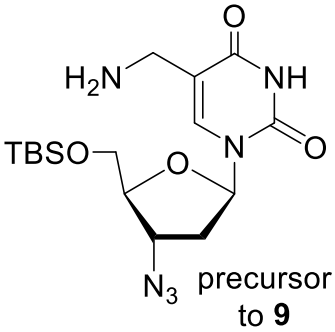
^{13}C NMR (400 MHz, $CDCl_3$) δ 134.9, 111.0, 84.5, 84.4, 62.9, 60.5, 37.9, 25.9, 25.7, 25.6, 18.3, 12.5, -5.4, -5.5.

Preparation of 9



Compound **8** (982.8 mg, 2.58 mmol, 1 eq) was mixed with NBS (366 mg, 2.06 mmol, 0.8 eq) and dissolved in distilled benzene (13 mL, 200 mM). The mixture was sparged with Ar for 20 min. The reaction was activated by sun lamp. The reaction was stirred for 1 h while the mixture turned red. When TLC (1:1 Hex/EtOAc, UV active and stained with iodine) showed a mixture of starting material and one new spot, a second aliquot of 0.8 eq NBS was added to the reaction. After a total of 2 h and 2 x 0.8 eq NBS added, the SM was consumed to yield one major spot. The mixture was immediately filtered through a glass frit and diluted with DCM (10 mL) and H_2O (20 mL). The aqueous layer was extracted with DCM (2 x 20 mL) and the combined organic layers were washed with sat. bicarbonate solution (1 x 40 mL), brine (1 x 40 mL), dried over Na_2SO_4, and concentrated under vacuum to give 853.6 mg (72%) a yellow crude residue. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.67 (s, 1H), 7.84 (s, 1H), 6.17 (t, *J* = 6.4 Hz,

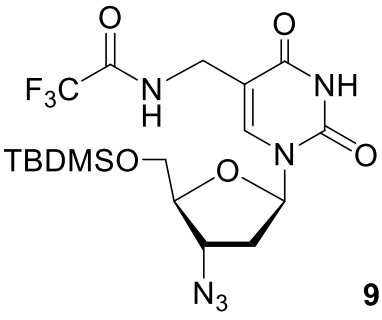
1H), 4.24 (m, 2H), 4.20 (m, 1H), 3.98 (m, 1H), 3.96 (dd, *J* = 2.8, 11.6 Hz, 1H), 3.80 (dd, *J* = 2.8, 11.6 Hz, 1H), 2.50 (m, 1H), 2.25 (m, 1H), 0.94 (s, 9H), 0.14 (s, 6H).



The crude bromide (853.2 mg, 1.85 mmol) was dissolved in EtOH (13 mL). Concentrated aqueous ammonia (23 mL, 15.7 M, 47.1 mmol, 25 eq) was added to the flask. The reaction was stirred at 25 °C for 1.5 h. After 1.5 h, TLC (10% MeOH in DCM) indicated the formation of product (R_f = 0.3). The reaction was concentrated and purified by column chromatography (DCM -> 3% MeOH in DCM) to yield 339 mg (43%) of the amine. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.78 (s, 1H), 6.06 (t, *J* = 6.4 Hz, 1H), 4.22 (m, 1H), 3.88 (q, *J* = 4.4 Hz, 1H), 3.82 (d, *J* = 4.4 Hz, 1H), 3.78 (d, *J* = 4.4 Hz, 1H), 3.73 (s, 2H), 2.35 (t, *J* = 6.4 Hz, 2H), 0.85 (s, 9H), 0.05 (s, 6H).

^{13}C NMR (400 MHz, $CDCl_3$) δ 164.3, 150.3, 140.2, 109.2, 85.1, 84.6, 62.9, 60.6, 50.1, 37.3, 25.8, 22.4, 18.3, -5.4, -5.5.

ESI-TOF m/z calculated for $C_{16}H_{28}N_6O_4Si$ (M + H) – 397.1941 calculated, 397.2012 observed.



9

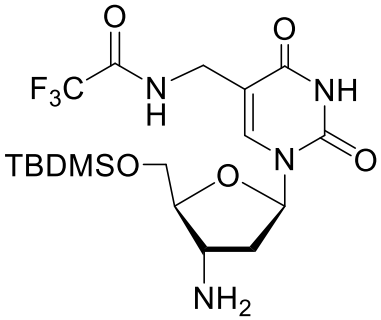
The second intermediate (313.5 mg, 0.791 mmol) was dissolved in THF (5.2 mL, 280 mM). Triethylamine (1.6 mL, 11.8 mmol, 15 eq) and ethyl trifluoroacetate (0.95 mL, 7.91 mmol, 10 eq) were added to the flask to make the final concentration 100 mM. The reaction was stirred for 4 h at 25 °C. TLC (3% MeOH in DCM, R_f = 0.5, UV active and stained by PAA) confirmed conversion to product. The reaction was concentrated and carried further to the next step without further purification, yielding 500 mg of **66** (91%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.17 (s, 1H), 7.91 (s, 1H), 7.80 (s, 1H), 6.13 (t, *J* =

6.4 Hz, 1H), 4.23 (q, *J* = 4 Hz, 1H), 4.20 (s, 2H), 4.00 (q, *J* = 4 Hz, 1H), 3.91 (dd, *J* = 3.6, 11.6 Hz, 1H), 3.84 (dd, *J* = 3.6, 11.6 Hz, 1H), 2.49 (m, 1H), 2.24 (m, 1H), 0.91 (s, 9H), 0.13 (s, 6H).

^{13}C NMR (400 MHz, $CDCl_3$) δ 162.9, 161.3, 160.9, 149.9, 138.7, 117.8, 114.9, 109.2, 84.8, 84.6, 60.8, 59.9, 53.2, 49.2, 45.5, 37.4, 35.7, 7.9.

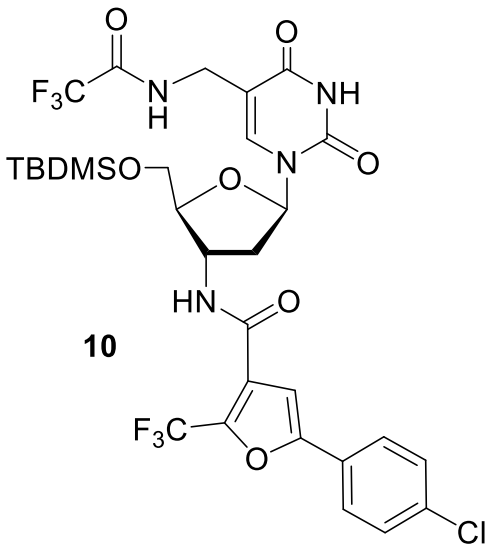
ESI-TOF m/z calculated for $C_{18}H_{27}F_3N_6O_5Pi$ (M + H) – 493.1764, 493.1716 observed.

Preparation of 10



precursor to **10**

Compound **9** (22.5 mg, 0.05 mmol) was dissolved in 50% MeOH, 20% H_2O, 30% tBuOH (1 mL). Activated Pd/C (14.1 mg, 60 wt%) was added to the solution. The reaction was continuously sparged with H_2 (1 atm). After 1 h, TLC (5% MeOH in DCM, UV active and stained with PAA, R_f = 0.35) suggested the reaction was complete. The mixture was passed through a Celite column and concentrated to 17 mg (82%) of crude amine, which was carried forward without further purification. ^{1}H NMR (400 MHz, CD_3OD) δ 7.83 (s, 1H), 6.18 (t, *J* = 6.4 Hz, 1H), 4.12 (s, 2H), 3.93 (dd, *J* = 4, 11.2 Hz, 1H), 3.88 (dd, *J* = 4, 11.2 Hz, 1H), 3.77 (dt, *J* = 1.2, 4 Hz, 1H), 3.50 (q, *J* = 6.4 Hz, 1H), 2.25 (m, 2H), 0.93 (s, 9H), 0.12 (d, *J* = 1.2 Hz, 6H).



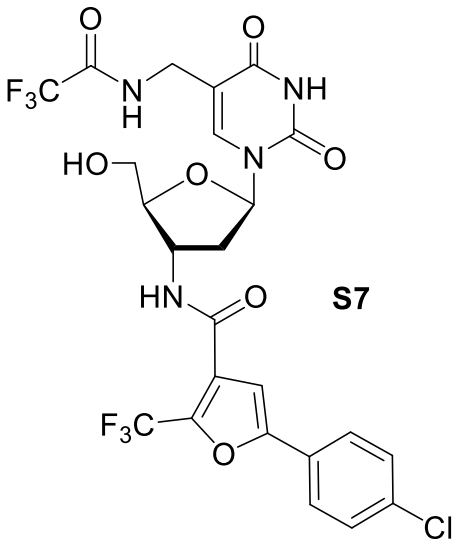
10

Crude amine (17 mg, 0.033 mmol) and 5-(4-chlorophenyl)-3-(trifluoromethyl) furan-2-carboxylic acid (14.2 mg, 0.05 mmol, 1.4 eq) were azeotropically dried together with pyridine (2 x 1 mL). HBTU (17.8 mg, 0.05 mmol, 1.4 eq) and HOBt (6.3 mg, 0.05 mmol, 1.4 eq) were added to the starting materials. The contents were dissolved in DMF (528 µL) and DIPEA (132 µL). The reaction was stirred at 25 °C overnight. TLC (10% MeOH in DCM, R_f = 0.4, UV active and stained with PAA) suggested the starting material was gone. The reaction was concentrated and purified by column chromatography (1:1 Hex/EtOAc -> 5% MeOH, 1:1 Hex/EtOAc) to yield 18.1 mg (75%) of **10**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.23 (s, 1H), 8.10 (d, *J* = 6.4 Hz, 1H), 7.86 (s, 1H), 7.75 (d, *J* = 8.2 Hz, 2H), 7.63 (d, *J* = 8.2 Hz, 2H), 6.09 (t, *J* = 6.4 Hz, 1H), 4.59 (t, *J* = 4 Hz, 1H), 4.12 (t, *J* = 4 Hz, 2H), 3.88 (m, 2H), 2.43 (m, 1H), 2.25 (m, 1H), 0.82 (s, 9H), 0.04 (s, 6H).

^{13}C NMR (400 MHz, $CDCl_3$) δ 162.7, 160.8, 150.1, 134.9, 129.0, 126.5, 125.8, 125.6, 120.1, 117.3, 110.7, 108.9, 105.9, 85.6, 54.7, 38.4, 36.5, 31.4, 25.7, 18.2, 16.9, -5.6. -5.7.

ESI-TOF m/z calculated for $C_{30}H_{33}ClF_6N_4O_7Si$ (M + H) – 739.1711, 739.1784 observed.

Preparation of S7

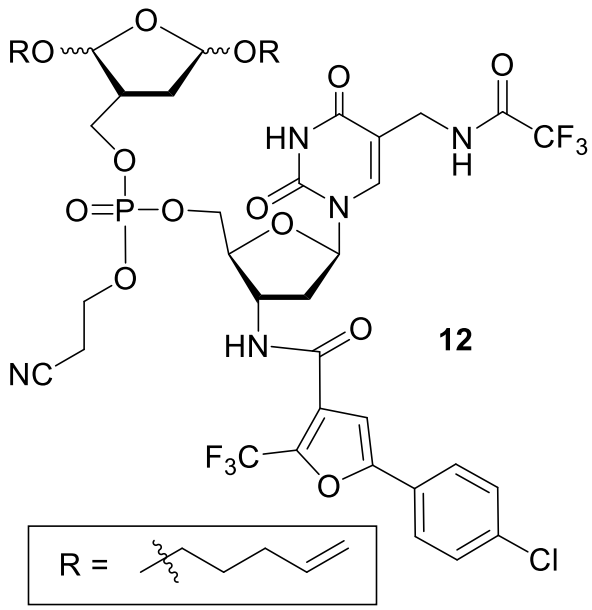


Compound **10** (135 mg, 0.19 mmol) was dissolved in THF (3.6 mL). TEA•3HF (74 mg, 0.46 mmol, 0.08 mL, 2.5 eq) was added to the flask. The reaction was stirred at 25 °C overnight. TLC (5% MeOH in DCM, R_f = 0.5, UV active and stained with PAA) confirmed conversion to product. The reaction was concentrated and purified by column chromatography (DCM -> 2% MeOH) to yield 100 mg (86%) of **S7**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.28 (t, *J* = 6.9, 1H), 7.79 (m, 3H), 7.74 (dd, *J* = 1.6, 6.9 Hz, 1H), 7.57 (dd, *J* = 1.6, 8 Hz, 1H), 7.23 (d, *J* = 8 Hz, 1H), 7.19 (m, 2H), 6.06 (q, *J* = 4.4 Hz, 1H), 4.54 (t, *J* = 7.6 Hz, 1H), 4.32 (d, *J* = 7.6 Hz, 1H), 4.21 (dd, *J* = 5.6, 14.8 Hz, 1H), 4.15 (dd, *J* = 5.6, 14.8 Hz, 1H), 3.84 (d, *J* = 10.8 Hz, 1H), 3.74 (d, *J* = 10.8 Hz, 1H), 2.30 (m, 2H).

^{13}C NMR (400 MHz, $CDCl_3$) δ 163.2, 160.8, 157.2, 153.0, 149.9, 134.1, 128.6, 127.4, 126.1, 124.4, 123.9, 120.2, 117.9, 110.9, 108.6, 107.4, 83.9, 50.1, 46.1, 8.31, 8.29, 8.26, 8.24.

ESI-TOF m/z calculated for $C_{24}H_{19}ClF_3N_4O_7$ (M + H) − 625.0846, 625.0844 observed.

Preparation of 12



Compounds **S7** (61 mg, 0.08 mmol, 1.2 eq) and **4** (32 mg, 0.07 mmol, 1 eq) were azeotropically dried with toluene (2 x 0.5 mL). S-Ethyl tetrazole/MeCN (250 mM, 1 mL, 0.24 mmol, 1.2 eq) was added to the reaction flask. After 3 h, TLC (2:1 EtOAc/Hex, R_f = 0.5, UV active and stained with PAA) indicated that the majority of **S7** was consumed. tBuOOH (500 mM, 18 mg, 40 µL, 0.20 mmol, 3 eq) was added for 15-20 min. The reaction was concentrated and column chromatography (2:1 EtOAc/Hex) yielded 61 mg (90 %) of **12**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.87 (d, *J* = 5.9, 1H), 7.56 (d, *J* = 8.4 Hz, 2H), 7.35 (d, *J* = 8.4 Hz, 2H), 7.07 (d, *J* = 3.6 Hz, 1H), 6.22 (t, *J* = 5.9, 1H), 5.78 (m, 2H), 4.99 (m, 6H), 4.75 (m, 1H), 4.40 (m, 3H), 4.30 (m, 4H), 4.28 (m, 2H), 3.88 (m, 2H), 3.61 (m, 2H), 3.38 (m, 2H), 2.78 (quint, *J* = 3.6 Hz, 2H), 2.63 (m, 1H), 2.52 (m, 2H), 2.45 (m, 1H), 2.08 (m, 4H), 1.88 (m, 1H), 1.62 (m, 4H).

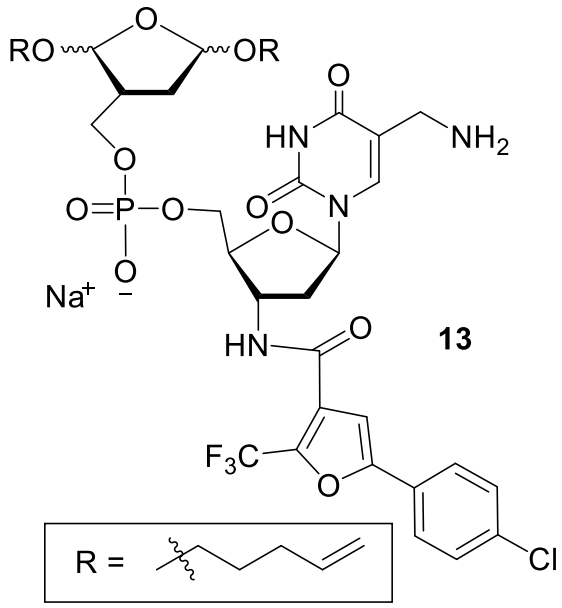
^{13}C NMR (400 MHz, $CDCl_3$) δ 137.9, 135.1, 129.0, 126.5, 125.6, 114.6, 114.5, 66.6, 65.8, 30.1, 30.0, 28.6, 269, 26.1, 14.6.

^{31}P NMR (400 MHz, $CDCl_3$) δ 7.8, -0.9, -1.0, -2.57, -2.62, -2.63, -2.65, -2.67, -2.69, -2.71, -3.06, -3.1.

^{19}F NMR (300 MHz, $CDCl_3$) δ -64.02, -64.03, -64.1, -78.12, -78.13 -78.2.

ESI-TOF m/z calculated for $C_{42}H_{47}ClF_6N_5O_{13}P$ (M + H) − 1010.2501, 1010.2183 observed.

Preparation of 13



Compound **12** (8 mg, 0.008 mmol) was dissolved in concentrated aqueous ammonia (160 μL). The reaction was capped and stirred at 25 °C. After 5 h, TLC (15% MeOH in DCM, R_f = 0.1, UV active and stained with PAA) confirmed the disappearance of starting material and formation of a new spot. The reaction was concentrated and purified by column chromatography (5% MeOH in DCM -> 20% MeOH in DCM) to yield 9.3 mg (73%) of **13**. The product was passed through a Dowex (Na+) column. ^{1}H NMR (400 MHz, CD_3OD) δ 8.37 (s, 1H), 7.76 (d, *J* = 11.6 Hz, 2H), 7.68 (q, *J* = 5.2 Hz, 1H), 7.50 (d, *J* = 11.6 Hz, 2H), 7.24 (m, 3H), 6.28 (t, *J* = 5.2 Hz, 1H), 5.80 (m, 2H), 4.95 (m, 6H), 4.78 (q, *J* = 8.6 Hz, 1H), 4.26 (m, 2H), 4.08 (m, 2H), 3.83 (m, 4H), 3.63 (m, 2H), 2.53 (q, *J* = 8.6 Hz, 2H), 2.47 (m, 2H), 2.09 (m, 4H), 1.60 (m, 4H).

^{13}C NMR (400 MHz, CD_3OD) δ 166.3, 164.8, 139.4, 137.9, 136.3, 130.3, 128.4, 128.3, 127.0, 115.0, 114.98, 111.3, 108.1, 106.0, 68.5, 68.2, 62.2, 55.7, 43.7, 36.8, 35.1, 31.5, 31.4, 31.39, 31.35, 30.03, 30.00, 19.2, 18.6, 17.2, 13.0, 12.5.

^{31}P NMR (400 MHz, CD_3CN) δ -2.3.

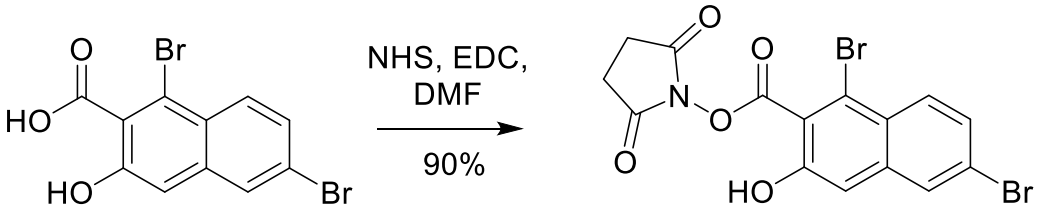
ESI-TOF m/z calculated for $C_{37}H_{45}ClF_3N_4O_{12}P$ (M - H) − 859.2412, 859.2277 observed.

General Procedure for the preparation of second-generation library

Amine scaffold **13** (100 nmol) was azeotropically dried with carboxylic acid (120 nmol, 1.2 eq) in pyridine (1 x 15 μL) using a Speed Vac concentrator in a 384-well microtiter plate

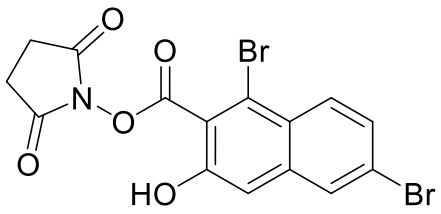
(VWR). To each well, activating solution (5 μL; 24 mM HBTU and 24 mM HOBt in DMF), DIPEA (2 μL), and DMF (3 μL) were added. The final concentrations during reaction were: [**13**] = 10 mM, [acid] = 12 mM, [HBTU] = 12 mM, [HOBt] = 12 mM, 20% DIPEA in DMF. The well plate was shaken at 25 °C overnight. Some wells were analyzed by ESI-MS to confirm coupling efficiency. The solutions were concentrated to dryness using a Speed Vac concentrator and the well plate was covered and stored at -80 °C.

Immediately before an assay, the amide was thawed, dissolved in DMF (4 μL, 25 mM). An aliquot (2 μL, 50 nmol) was mixed with NBS (8 μL, 15 mM, 2.4 eq, 40% H_2O in MeCN) at 0 °C for 9 min. The concentrations during reaction were: [SM] = 5 mM, [NBS] = 12 mM, 20% DMF, 30% H_2O in MeCN. After 9 min, $Na_2S_2O_3$ (5 μL, 200 mM) was added and reaction quenched on ice for 10 min. Samples were concentrated to dryness with a Speed Vac concentrator. Some samples were analyzed ESI-MS to confirm product formation.



S8

Preparation of S8



S8

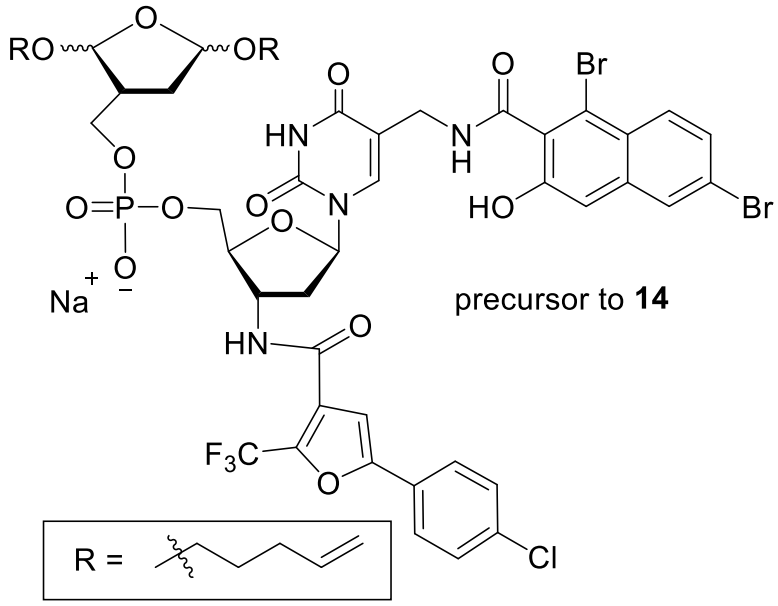
Commercially available 1,6-dibromo-3-hydroxy-2-naphthoic acid (102 mg, 0.3 mmol) was azeotropically dried in pyridine (2 x 1 mL). NHS (54 mg, 0.45 mmol, 1.5 eq), and EDC (86 mg, 0.45 mmol, 1.5 eq) were added to the flask and the reagents were dissolved in DMF and stirred overnight at 25 °C. After 16 h, TLC (2% MeOH in DCM, R_f = 0.8, UV active) suggested the SM was completely converted so the reaction was

concentrated. The product was purified by column chromatography (DCM -> 1% MeOH in DCM) to yield 119 mg (90%) of NHS ester (**S8)**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.79 (s, 1H), 8.60 (s, 1H), 8.09 (d, J = 8.8 Hz, 1H), 8.01 (s, 1H), 7.75 (d, J = 8.8 Hz, 1H), 2.97 (s, 4H).

^{13}C NMR (400 MHz, $CDCl_3$) δ 169.6, 162.5, 153.4, 134.6, 133.6, 131.2, 130.6, 128.2, 127.7, 118.3, 115.0, 107.2, 53.4, 53.1, 36.4, 31.4, 29.6.

ESI-TOF m/z calculated for $C_{15}H_9NO_5$ (M + H) − 441.8847, 441.8807 observed.

Preparation of 14



Scaffold **13** (14 mg, 0.016 mmol) and **15** (11 mg, 0.024 mmol, 1.5 eq) were azeotropically dried together with pyridine (2 x 1 mL). The contents were dissolved in DMF (350 µL) and DIPEA (5 µL). The reaction was stirred at 25 °C overnight. TLC (20% MeOH in DCM) indicated formation of product (R_f = 0.3, UV active and stained with PAA). The reaction was concentrated and purified by column chromatography (2:1 Hex/EtOAc -> 1:1 Hex/EtOAc -> 1:1 Hex/EtOAc, 5% MeOH) to yield 12.2 mg (45%) of the precursor to **14**. The product was passed through a Dowex (Na+) column. ^{1}H NMR (400 MHz, CD_3OD) δ 8.38 (s, 2H), 8.02 (s, 3H), 7.98 (d, J = 7.2 Hz, 2H), 7.74 (d, J = 8.8 Hz, 1H), 7.61 (d, J = 7.2, 2H), 7.48 (d, J = 8.8 Hz, 1H), 7.24 (m, 1H), 6.30 (s, 1H), 5.82 (m, 2H), 4.99 (m, 6H), 4.73 (s, 1H), 4.44 (s, 1H), 4.22 (s, 1H), 4.1 (m, 1H), 3.73 (m, 3H), 3.66 (m,

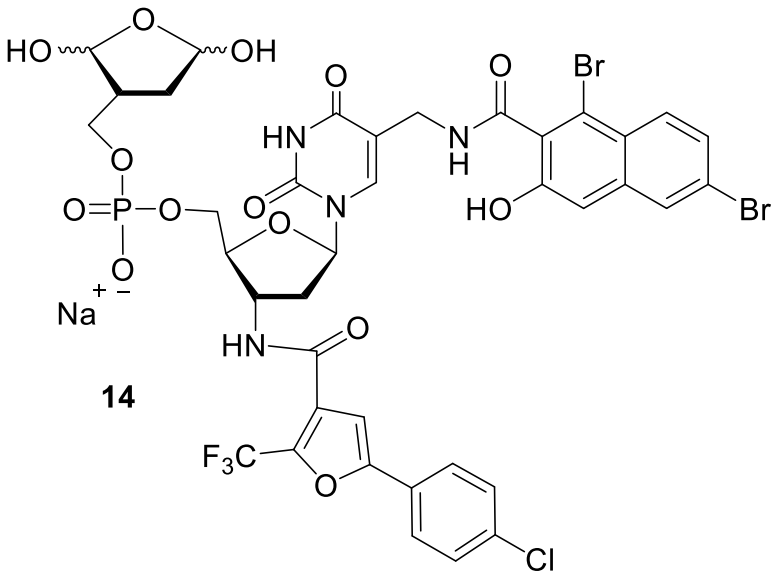
2H), 3.41 (m, 2H), 2.53 (m, 1H), 2.48 (m, 2H), 2.12 (q, *J* = 7.2 Hz, 4H), 2.05 (m, 2H), 1.63 (q, *J* = 7.2 Hz, 4H).

^{13}C NMR (600 MHz, CD_3OD) δ 173.8, 172.4, 169.0, 163.8, 161.7, 155.7, 154.3, 143.8, 139.6, 138.1, 135.0, 133.9, 132.2, 131.2, 130.7, 129.8, 128.5, 127.1, 126.7, 125.7, 122.4, 116.3, 113.7, 106.5, 106.1, 105.6, 104.5, 85.6, 83.6, 45.4, 35.3, 31.7, 30.1, 29.4, 28.7, 24.8, 22.3, 13.0.

^{31}P NMR (400 MHz, CD_3OD) δ 4.8, 1.7, -0.1, -0.2.

ESI-TOF m/z calculated for $C_{48}H_{49}ClF_3N_4O_{14}P$ (M - H) − 1185.0990, 1185.0579 observed.

ε_{275} (50% MeCN in H_2O) = 1.96 × 10^4 $M^{-1}cm^{-1}$, ε_{370} = 3.81 × 10^3 $M^{-1}cm^{-1}$



14

The starting material was stored as a 25 mM stock solution in DMF at -20 °C. An aliquot of the starting material (2 μL, 50 nmol) was placed in a microtiter well plate and cooled to 4 °C. Cold H_2O (3 μL) and NBS in MeCN (25 mM, 5 μL) was added and the well plate was shaken at 4 °C for 9 min. After 9 min, the reaction was quenched by an equal volume of $Na_2S_2O_3$ (200 mM in H_2O) and well plate was shaken for additional 5-10 min. The reaction was concentrated by speed vacuum, redissolved in 1:1 MeCN/H_2O and used directly.

Reaction Conditions: [SM] = 5 mM, [NBS] = 12.5 mM, 30% H_2O, 20% DMF, 50% MeCN

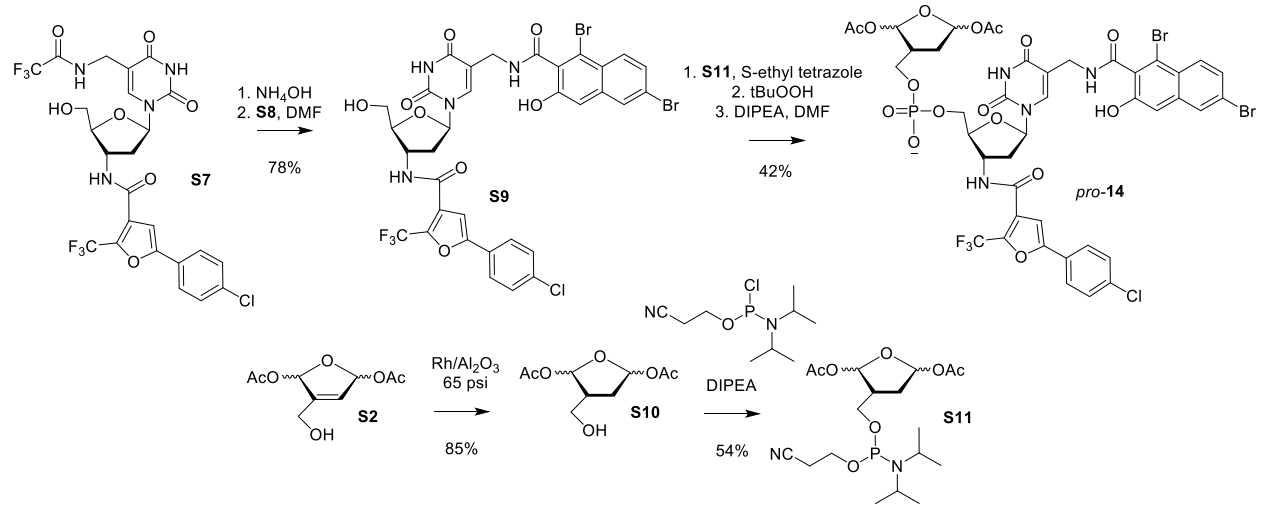
Quenching Conditions: [SM] = 2.5 mM, [NBS] = 6.25 mM, [Na_2S2O_3] = 100 mM, 65% H_2O,

10% DMF, 25% MeCN.

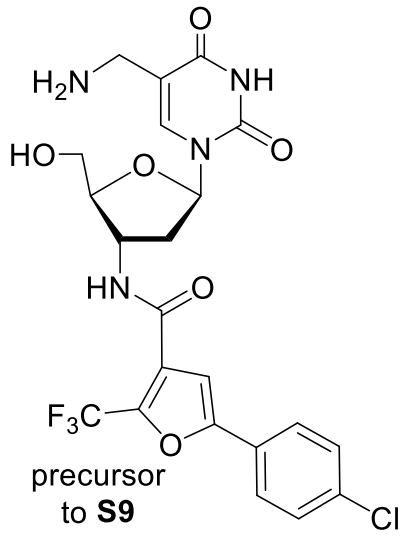
^{1}H NMR (800 MHz, CD_3CN) δ 9.31 (s, 1H), 8.46 (s, 1H), 8.00 (m, 1H), 7.91 (d, J = 8 Hz, 1H), 7.75 (d, J = 8 Hz, 2H), 7.71 (s, 1H), 7.68 (m, 1H), 7.64 (m, 1H), 7.53 (d, J = 8 Hz, 2H), 7.50 (m, 1H), 7.37 (s, 1H), 7.32 (m, 2H), 7.11 (s, H), 6.32 (t, J = 8 Hz, 1H), 4.93 (d, J = 8 Hz, 1H), 4.78 (m, 1H), 4.67 (m, 1H), 4.48 (s, 1H), 4.37 (s, 1H), 4.22 (m, 1H), 4.17 (m, 1H), 4.05 (m, 1H), 3.91 (s, 1H), 3.91 (s, 1H), 3.80 (s, 1H), 3.69 (m, 3H), 3.59 (m, 2H), 3.43 (s, 1H), 2.95 (s, 2H), 2.47 (m, 3H), 2.28 (m, 3H).

ESI-TOF m/z calculated for $C_{48}H_{49}ClF_3N_4O_{14}P$ (M + H) – 1049.9738, 1049.9699 observed.

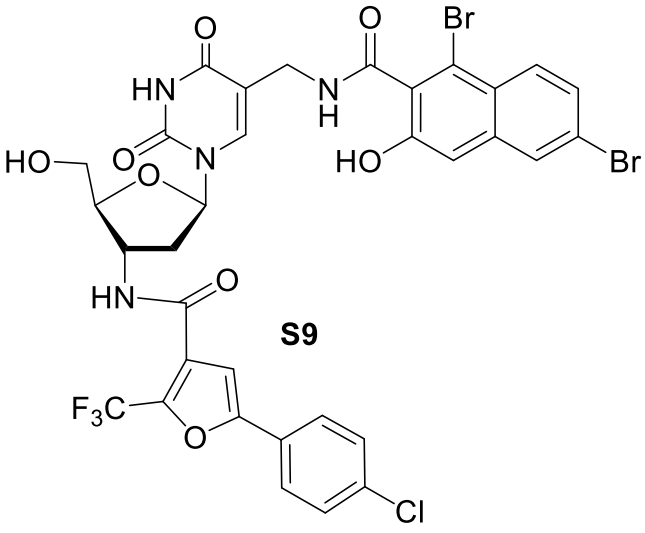
Scheme S2. Synthesis of *pro*-14



Preparation of S9



Compound **S7** (50 mg, 0.08 mmol) was dissolved in concentrated aqueous ammonia (0.8 mL). The reaction was capped and stirred at 25 °C. After 5 h, TLC (5% MeOH in DCM, R_f = 0.2, UV active) confirmed the disappearance of starting material and formation of a new, more polar spot. The reaction was concentrated to yield 45 mg of crude amide.

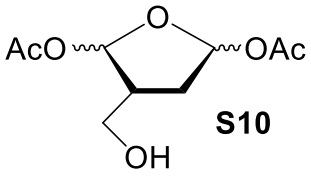


The crude amide (45 mg, 0.08 mmol) and NHS ester **S8** (36 mg, 0.09 mmol. 1.1 eq) were azeotropically dried together in pyridine (2 x 0.5 mL). The contents were dissolved in DMF (1.6 mL). The reaction was stirred at 25 °C overnight. TLC (1:1 EtOAc/Hex, 2% MeOH, UV active, R_f = 0.2) indicated the starting materials were converted to product. The reaction was concentrated and purified by column chromatography (2:1 Hex/EtOAc -> 1:1 Hex/EtOAc -> 1:1 Hex/EtOAc -> 2% MeOH -> 1:1 Hex/EtOAc, 5% MeOH) to yield 53 mg (78%) of **S9**. ^{1}H NMR (400 MHz, CD_3OD) δ 8.56 (s, 3H), 8.34 (s, 1H), 8.26 (s, 1H), 8.04 (m, 3H), 7.94 (d, *J* = 8 Hz, 1H), 7.86 (d, *J* = 8 Hz, 1H), 7.21 (s, 1H), 6.30 (t, *J* = 6.8 Hz, 1H), 4.69 (m, 1H), 4.34 (d, *J* = 3.2 Hz, 2H), 4.03 (d, *J* = 2.8 Hz, 1H), 3.87 (dd, *J* = 3.2, 13.9 Hz, 1H), 3.77 (dd, *J* = 2.8, 13.9 Hz, 1H), 2.45 (m, 2H).

^{13}C NMR (600 MHz, CD_3OD) δ 199.3, 193.6, 193.1, 163.4, 161.7, 159.9, 157.7, 150.4, 142.3, 141.9, 140.0, 139.2, 138.9, 135.1, 129.0, 128.9, 126.7, 125.9, 125.2, 121.1, 119.6, 118.0, 117.7, 116.9, 110.3, 109.3, 107.3, 105.9, 85.7, 81.5, 64.6, 57.4, 50.3, 41.4, 37.5, 36.4, 36.0, 29.3, 25.1, 7.8.

ESI-TOF m/z calculated for $C_{33}H_{24}Br_2ClF_3N_4O_8$ (M + H) – 854.9602, 854.9636 observed.

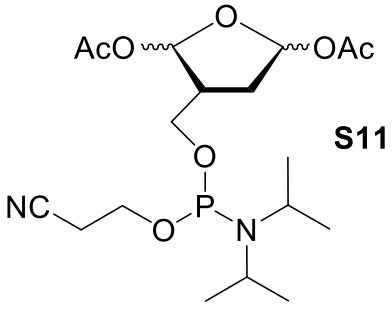
Preparation of S10[10]



S10

Compound **S2** (163 mg, 0.886 mmol, 1 eq) was dissolved in EtOAc (16 mL, 50 mM). Rhodium on alumina catalyst (85.2 mg) was added to the pressure bottle equipped with a regulator. The vial was pressurized with H2 to 70 psi, purged three times, and stirred at 25 °C for 2-4 h. After venting the pressure bottle, TLC (4:6 Hex/EtOAc, R_f = 0.3, stained with PAA) showed the starting material was no longer present. When complete, the reaction mixture was passed through celite to remove the Rh catalyst. The filtrate was concentrated under vacuum to a pale, yellow residue, which was purified by column chromatography (1:1 DCM/EtOAc) resulting in 67 mg (41%) of a mixture of diastereomers (**S10**). ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.44 (s, 1H), 6.34 (d, *J* = 1.0 Hz, 1H), 3.86 – 3.75 (m, 1H), 3.69 (dt, *J* = 5.5, 10.9 Hz, 1H), 2.56 – 2.43 (m, 2H), 2.05 (s, 3H), 2.04 (s, 3H), 1.90 (t, *J* = 5.5 Hz, 1H), 1.82 (dd, *J* = 1.0, 5.5 Hz, 1H).

Preparation of S11[10]



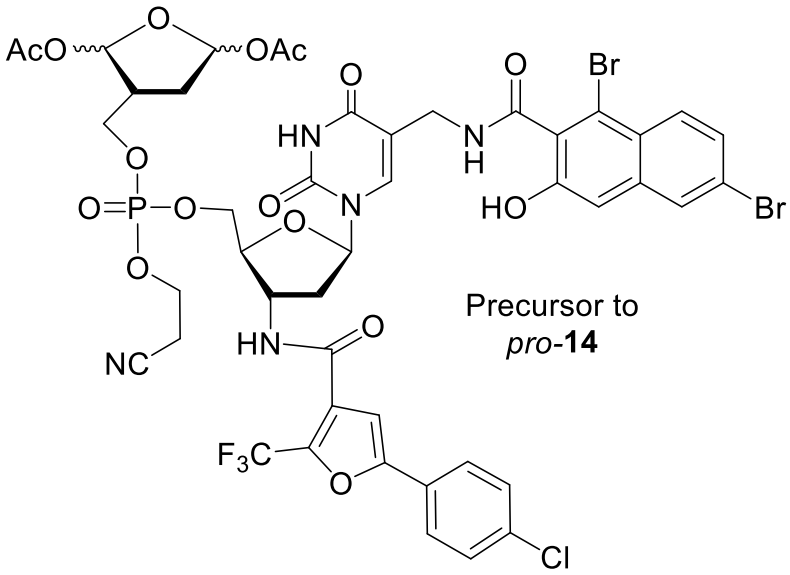
S11

Compound **S10** (67 mg, 0.28 mmol) was azeotropically dried with pyridine (2 x 0.5 mL). The flask was cooled to 0 °C. DIPEA (0.2 mL, 144 mg, 1.12 mmol, 4 eq) was added to the cold starting material and the reactants were dissolved in DCM (1.2 mL, 200 mM). 2-Cyanoethyl-N, N-diisopropylchlorophosphoramidite (75

μL, 80 mg, 0.34 mmol. 1.2 eq) was added and the cold mixture stirred with periodic monitoring by TLC (7:1 Hex/EtOAc, R_f = 0.2, stained with PAA). After 2 h, TLC showed complete conversion to the phosphoramidite. The reaction was diluted with EtOAc (10 mL). The organic layer was washed with saturated bicarbonate solution (2 x15 mL) and the aqueous layers were extracted with EtOAc (2 x 20 mL). The combined organic layers were washed with brine (1 x 25 mL) and dried over Na_2SO_4. The organic layer was concentrated under vacuum and purified by column chromatography (3:1 hexanes/ EtOAc) yielding 85 mg (75%) of **S11**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.31 (m, 1H), 4.28 (m, 1H), 3.78 (m, 3H), 3.55 (m, 3H), 2.79 (m, 1H), 2.60 (m, 2H), 2.44 (m, 1H), 2.04 (m, 6H), 1.81 (m, 1H), 1.14 (m, 12H).

^{31}P NMR (400 MHz, $CDCl_3$) δ 149.7, 149.5, 148.3, 148.1, 13.2.

Preparation of *pro*-14



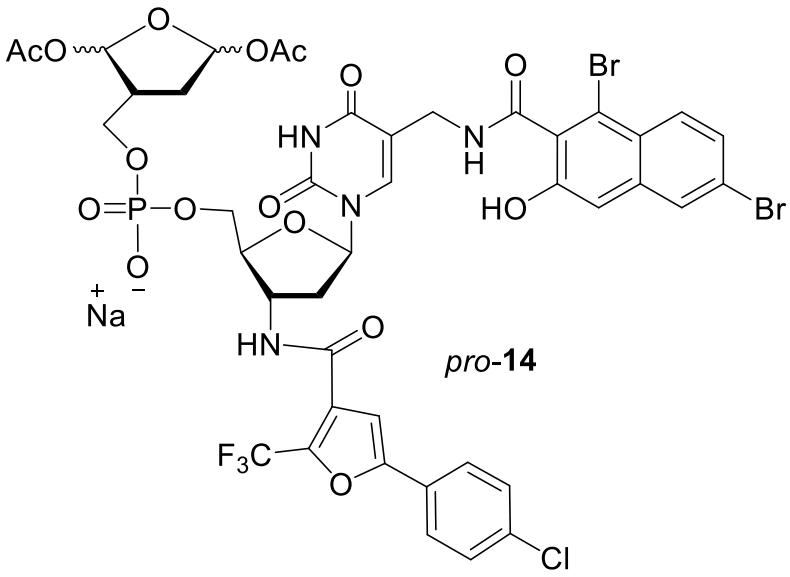
Compounds **S9** (30 mg, 0.035 mmol) and **S11** (35 mg, 0.042 mmol, 1.2 eq) were azeotropically dried together in pyridine (2 x 1 mL). S-ethyl tetrazole/MeCN (250 mM, 0.25 mL, 0.053 mmol, 1.5 eq) was added to the reaction flask. After 4 h, TLC (1:1 EtOAc/Hex) suggested the majority of **S9** was consumed. tBuOOH (0.5 M, 10 mg, 25 μL, 0.11 mmol, 3 eq) was added for 20 min. The reaction was concentrated and purified by column chromatography (1:1 Hex/EtOAc -> 1:1 Hex/EtOAc, 5% MeOH) to yield 25 mg (60%) of the phosphate triester precursor to *pro*-**14**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.16 (m, 1H), 7.87 (s, 1H),

7.78 (d, *J* = 8.8 Hz, 1H), 7.50 (s, 1H), 7.29 (d, *J* = 8.8 Hz, 1H), 6.95 (s, 1H), 6.75 (m, 1H), 6.36 (quint, *J* = 3.2 Hz, 1H), 6.20 (s, 1H), 6.15 (m, 1H), 4.74 (q, *J* = 5.6 Hz, 1H), 4.31 (m, 4H), 4.23 (m, 6H), 3.82 (d, *J* = 5.6 Hz, 1H), 3.51 (m, 1H), 2.77 (m, 4H), 2.53 (m, 1H), 2.08 (s, 3H), 2.03 (s, 3H), 2.00 (m, 1H), 1.87 (m, 1H).

^{13}C NMR (400 MHz, $CDCl_3$) δ 169.8, 100.8, 98.2, 60.2, 50.5, 32.2, 25.2, 20.9, 20.83, 20.77, 19.8, 19.7, 14.6, 14.0.

^{31}P NMR (400 MHz, $CDCl_3$) δ 17.3, 14.3, 9.2, 9.1, 8.1, 7.9, 7.8, 7.75, 7.70, 7.6, 7.5, -0.5, -0.9, -1.4, -1.9, -2.0, -2.5, -2.6, -2.7, -2.8.

ESI-TOF m/z calculated for $C_{45}H_{40}Br_2ClF_3N_5O_{16}P$ (M + H) – 1188.0215, 1188.0200 observed.



pro-**14**

The intermediate phosphate triester (25 mg, 0.023 mmol) was dissolved in DMF (0.5 mL) and DIPEA (0.5 mL). The reaction was stirred at 25 °C overnight. The following morning, TLC (5% MeOH in DCM, R_f = 0.2, UV active) indicated product formation. The reaction was concentrated and purified by column chromatography (2% MeOH in DCM -> 5% MeOH in DCM) and then passed through a TEA^+ Dowex and then Na^+ Dowex column to yield 12.4 mg (50%) of *pro*-**14**. A portion (~30%) of the product exists at the triethylammonium salt, even after the Na^+ ion exchange column. ^{1}H NMR (800 MHz, CD_3OD) δ

8.53 (s, 1H), 8.12 (s, 1H), 8.02 (s, 1H), 7.92 (d, *J* = 8.8 Hz, 1H), 7.78 (d, *J* = 8.5 Hz, 2H), 7.56 (d, *J* = 8.8 Hz, 1H), 7.53 (d, *J* = 8.5 Hz, 2H), 7.24 (s, 1H), 6.30 (quint, *J* = 5.4 Hz, 1H), 6.25 (m, 1H), 4.73 (t, *J* = 6 Hz, 1H), 4.61 (q, *J* = 6 Hz, 1H), 4.49 (d, *J* = 14 Hz, 1H), 4.42 (dd, *J* = 3.6, 14 Hz, 1H), 4.24 (m, 3H), 4.14 (m, 1H), 4.05 (t, *J* = 7 Hz, 1H), 3.06 (q, *J* = 8 Hz, 1H), 2.62 (m, 2H), 2.48 (q, *J* = 6 Hz, 2 H), 2.05 (m, 3H), 1.99 (s, 3H), 1.92 (s, 1H), 1.84 (m, 1H), 1.32 (t, *J* = 8 Hz, 3H).

^{13}C NMR (800 MHz, $CDCl_3$) δ 175.7, 170.4, 170.1, 168.6, 163.8, 161.7, 157.5, 154.3, 150.7, 139.5, 138.6, 135.0, 133.9, 131.3, 130.8, 129.0, 128.4, 127.1, 126.5, 125.7, 125.3, 119.6, 118.3, 115.0, 111.1, 106.2, 101.1, 100.0, 98.6, 96.7, 85.7, 83.3, 65.0, 63.4, 59.7, 50.2, 43.2, 37.1, 35.4, 32.0, 26.7, 24.7, 22.6, 19.7, 19.6, 19.5, 14.2.

^{31}P NMR (400 MHz, CD_3OD) δ 6.4, 4.4, 4.3, 4.0, 3.9, 1.6, 1.5, 1.2, -0.26, -0.29, -0.4, -0.5.

ESI-TOF m/z calculated for $C_{42}H_{37}Br_2ClF_3N_4O_{16}P$ (M - H) – 1132.9950, 1133.0007 observed.

Radiolabeling and Preparation of Oligonucleotide Complexes

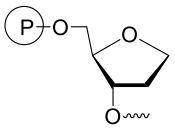
Oligonucleotides were 5'-^{32}P labeled by T4 polynucleotide kinase and γ-^{32}P-ATP. Ternary complexes were hybridized by mixing ^{32}P-labeled oligonucleotides with the appropriate template and flanking strand in a 1:2.5:5 ratio in phosphate buffered saline (10 mM sodium phosphate, 100 mM NaCl, pH 7.3), heating to 95 ^{o}C, and slowly cooling to 25 ^{o}C.

Ternary complexes containing fluorophore labeled oligonucleotides were prepared by annealing the fluorophore-labeled strand with the appropriate quencher-labeled template and flanking strand in a 1:2:3 ratio. All oligonucleotides used to prepare ternary complexes are described in Table S1.

Table S1. DNA substrates for assays.

Pol β and Klenow (**TC1**)	5'- d(TCA CCC TCG TAC GAC TC TTT TTT TTT TGC F) - 3' 3'- d(AGT GGG AGC ATG CTG AG__AAA AAA AAA ACG Q) - 5'
Pol θ (**P2**)	5'- d(TTT TTT TAG GTT T) -3'
Pol η (**D3**)	5'- d(TCA CCC TCG TAC GAC TC) -3' 3'- d(AGT GGG AGC ATG GTC AGG ACC T) -3'
Pol λ (**TC4**)	5'- d(ACC ATG GGA CGT GCT G ACT CCA CTA GAT ACA CTT) -3' 3'- d(TGG TAC CCT GCA CGA CTC TGA GGT GAT CTA TGT GGA) -5'
Fluorescence Anisotropy (**TC5**)	5'- d(TAA TGG CTA ACG CTT pFCC GTA ATG CAG TCT) -3' 3'- d(ATT ACC GAT TGC GAA__AGG CAT TAC GTC AGA Fl) -5'

X = dRP, F = TAMRA, Q = BHQ, pF=



Fl= dichloro-diphenyl-fluorescein (SIMA-HEX)

General Procedure for Library Screening

A 1 mM stock solution of each inhibitor is prepared using 50% MeCN in H_2O. A solution of Pol β (100 nM) was preincubated with library compounds (25 μM) in 1X reaction buffer (total volume: 50 μL; 50 mM HEPES buffer pH = 7.4, 5 mM MgCl2, 0.2 mM EDTA, 50 mM KCl, 0.01 % Tween 20, 0.01 mg/mL BSA, and 4% glycerol by volume) in a 384-well plate at 25 ^{o}C for 30 min. In control experiments, an equal volume of a control solution (containing all coupling and deprotection reagents but lacking inhibitor) was added to keep the percentage of solvents and reagents consistent. An aliquot (3 μL) was diluted with a 2X solution (15 μL) containing **TC1** (100 nM, Table S1) and dTTP (200 μM) in 1X reaction buffer (total volume: 30 μL) in a different 384-well plate. The final reaction mixture contained 10 nM Pol β, 5 μM inhibitor, 50 nM DNA, 100 μM dTTP, 1X reaction buffer, and 0.25% MeCN. The solution in each well was mixed thoroughly, and the fluorescence measurements were collected immediately.

Time-dependent irreversible kinetics of Pol β

A 1 mM stock solution of each inhibitor is prepared using 50% MeCN in H_2O. A solution of Pol β (50 nM) was preincubated with **14** (0, 100, 250, 400, 500, 750 nM) in 1X reaction buffer (total volume: 50 μL; 50 mM HEPES buffer pH = 7.4, 5 mM MgCl2, 0.2 mM EDTA, 50 mM KCl, 0.01 % Tween 20, 0.01 mg/mL BSA, and 4% glycerol by volume) in a 384-well plate at 25 °C for various preincubation times (2, 5, 10, 15, 20 min). In control experiments, an equal volume of a control solution (containing all coupling and deprotection reagents but lacking inhibitor) was added to keep the percentage of solvents and reagents consistent. The volume of inhibitor solution added was unchanged across experiments that used various inhibitor concentrations. To achieve various inhibitor concentrations, the stock solution of inhibitor (1 mM) was diluted appropriately for the desired conditions.

An aliquot (3 μL) was diluted with a 2X solution (15 μL) containing **TC1** (100 nM, Table S1) and dTTP (200 μM) in 1X reaction buffer (total volume: 30 μL) in a different 384-well plate. The final reaction mixture contained 5 nM Pol β, **14** (0, 10, 25, 40, 50, 75 nM), 50 nM DNA, 100 μM dTTP, 1X reaction buffer, and 0.25% MeCN. The solution in each well was mixed thoroughly, and the fluorescence measurements were collected immediately.

The data were fit to a single exponential growth equation (1) that follows a plateau. The plateau was important because the strand displacement assay exhibited an induction period in which several nucleotides of the fluorescently labelled DNA were displaced before the fluorescently labeled oligonucleotide was released into solution. This induction time was determined by inspection and typically varied between 10 and 15 min. The data were fit beginning at the time when a growth in fluorescence was observed.[12,13]

$$Y = F_0 + (F_1 - F_0) \times (1\text{-}e^{-kt}) \quad (1)$$

Y is the fluorescence intensity, F_0 is the fluorescence value at time 0, F_1 is the fluorescence value at time ∞, k is the rate constant, and t is time. Rate constants are extracted for each experiment and relative rates are determined using equation (2).

$$k_{rel} = k_{inhibitor}/k_{polβ} \quad (2)$$

Where $k_{inhibitor}$ is the rate constant for experiments containing inhibitor and $k_{polβ}$ is the rate constant for control experiments lacking inhibitor.

This procedure was also used to measure strand displacement activity of Klenow exo$^-$ with minor changes: (1) the concentration of **14** during preincubation was 0.5 or 10 μM, and (2) the samples were preincubated for 20 min.

Primer Extension Assay (Pol θ)[14]

A solution of 10X **14** (5 μM or 100 μM, 2 μL) was mixed with a 40X solution of Pol θ (200 nM, 5 μL) in 1X reaction buffer (20 mM Tris•HCl pH 7.5, 100 mM NaCl, 5 mM $MnCl_2$, 0.5 mM TCEP, 10% glycerol, 0.01% NP-40, 0.1 mg/mL BSA) in a 384 microtiter well plate. This 10X preincubation mixture (50 nM Pol θ, 0.5 or 10 μM **14**) was incubated at 25 °C for 20 min. An aliquot of the 10X preincubation mixture (2 μL) containing Pol θ and **14** was added to a new well and mixed with 1X buffer (8 μL) and 2X cocktail solution (10 μL) containing DNA substrate **P2** (1 μM, Table S1), dNTPs (0.8 mM each), and 2X SYBR Gold in 1X buffer (Table 1). The final reaction mixture (20 μL) contained 5 nM Pol θ, 0.05 or 1 μM **14**, 500 nM **P2**, 0.4 mM dNTPs, 1X Sybr Gold in 1X reaction buffer. Fluorescence data was collected for 80-120 min on a Varian Cary Eclipse fluorescence spectrophotometer.

Primer Extension Assays (Pol η and Pol λ)[15]

A 10X working solution of **14** (5 or 100 μM) was prepared in 1:1 MeCN/H_2O. A 10X preincubation mixture was prepared by mixing a 50X working solution of polymerase (250 nM) with an aliquot of the inhibitor (5 or 100 μM, 10X) in 1X reaction buffer (50 mM Tris•HCl, 50 mM NaCl, 5 mM MgCl2, 5 mM DTT, 0.1 mg/mL BSA, 10% glycerol, pH 7.5) (see Table 19 for volumes). The concentration of the preincubation mixture was 50 nM polymerase (10X) and 0, 0.5 or 10 μM **14** (1X).

Table S2. 10X preincubation mixture for primer extension assays.

	Pol η (volume added, μL)	Pol λ (volume added, μL)
50X Polymerase (250 nM)	2	3
10X **14** (5 or 100 μM) (or 1:1 MeCN/H_2O for control)	1	1.5
20X reaction buffer	1	1.5
H_2O	6	9
Total[1]	10	15

[1]Preincubated at 25 °C for 20 min.

The samples containing 50 nM polymerase and **14** (0, 0.5, or 10 μM) were preincubated at 25 °C for 20 min. After preincubation, an aliquot (2 μL, pol η or 3 μL, pol λ) was diluted with 10X ternary complex (pol η: 2 μL, 500 nM **D3** or pol λ: 3 μL, 100 nM **TC4**), 10X dNTPs (5 mM; 2 μL, pol η or 3 μL, pol λ), and 1X reaction buffer (14 μL, pol η or 21 μL, pol λ) (see Table 20 for volumes). While the samples were incubated at 37 °C, aliquots (pol η: 0, 2, 5, 10, 15, 20 min, 3 μL or pol λ: 0, 5, 15, 20, 30 min, 5 μL) were removed and quenched by the addition of 95% formamide, 20 mM EDTA loading buffer (10 μL). Aliquots were heated at 95 °C for 5 min, spun down, and loaded onto a 20% denaturing PAGE and run for 4 h at 55 watts. The gel was exposed in a radiography cassette, which was scanned using a Phosphorimager.

Table S3. Reaction mixture for primer extension assays.

	Pol η (volume added, μL)	Pol λ (volume added, μL)
10X preincubation	2	3
10X DNA	2 (500 nM **D3**)	3 (100 nM **TC4**)
10X dNTPs (5 mM)	2	3
1X reaction buffer	14	21
Total[1]	20	30

[1]Incubated at 37 °C, taking time points between 0-30 min.

General Procedure for Dialysis

Pol β (100 nM, total volume 200 μL) was preincubated in the absence or presence of **14** (e.g. 750 nM) in 1X reaction buffer (50 mM HEPES buffer pH = 7.5, 5 mM $MgCl_2$, 0.2 mM EDTA, 50 mM KCl, 0.01 % Tween 20, 0.01 mg/mL BSA, and 4% glycerol) at 25 °C for 20 min. The strand displacement activity of each sample was immediately measured by mixing an aliquot of each sample (3 μL, 100 nM Pol β, ± 750 nM **14**) with a 2X solution (15 μL) containing **TC1** (100 nM) and dTTP (200 μM) in 1X reaction buffer (total volume: 30 μL). The final concentrations during kinetics were 10 nM Pol β, 750 nM **14,** 50 nM **TC1**, and 100 μM dTTP.

The remaining sample (197 μL) was dialyzed in a 3.50K MW cassette in reaction buffer (1 L, buffer exchanged after 12 h) containing 50 mM HEPES buffer (pH = 7.4, 5 mM $MgCl_2$, 4 mM DTT) for 24 h. The volume of the solution in the cassette was marked and no considerable volume change was observed after dialysis. The remaining strand displacement activity of the enzyme was measured as previously described. An aliquot (3 μL) was mixed with a 2X solution (15 μL) containing **TC1** (100 nM) and dTTP (200 μM) in 1X reaction buffer (total volume: 30 μL).

This method was also used to analyze the effect of pH on the inhibitory activity of 14 with one minor change: the dialysis buffer contained 50 mM HEPES buffer (pH = 8.0, 5 mM $MgCl_2$, 4 mM DTT).

UPLC MS/MS Analysis of Pol β modification by covalent inhibitors

A solution of Pol β (25 μL, 20 μM, 500 pmol) was mixed with **14** (5 μL, 30 μM, 100X), H_2O (420 μL) and 10X reaction buffer (50 μL, 500 mM HEPES buffer, pH = 7.4, 50 mM $MgCl_2$, 20 mM DTT) and incubated at 25 °C for 30 min. The reaction mixture was concentrated by centrifugation using an Amicon 3K centrifugal filter. To prevent the loss of protein, the centrifugal was blocked with Pol β prior to addition of the sample. Blocking was conducted by adding Pol β (500 μL, 0.5 μM), followed by centrifugation (13,000 g, 25 min, 4 °C) and removal of the supernatant. Following blocking of the membrane filter, the sample (500 μL, 1 μM Pol β ± 300 nM **14**) was added to the Amicon centrifugal filter, and centrifugation was carried out (13,000 g, 25 min, 4 °C). The sample was then washed twice with 500 μL of 1X reaction buffer can concentrated by centrifugation in the Amicon filter to 50 μL (10 μM Pol β). Digestion buffer (25 μL, 500 mM Tris•HCl pH 7.4), 10X trypsin (25 μL, 400 μM), and H_2O (150 μL) were added to yield a final mixture of 2 μM Pol β and 40 μM trypsin (1:20 ratio) in 1X digestion buffer (total volume, 250 μL, 50 mM Tris•HCl, pH 7.4).

The digestion sample was incubated at 37 °C overnight. A portion (100 μL) of the digestion mixture was spun down (16,000 g, 10 min, 4 °C). The sample (10 μL) was injected onto and analyzed by UPLC-MS/MS using an ACQUITY UPLC HSS T3 Column (100 Å, 1.8 μm, 2.1 mm x 100 mm). The flow rate was 0.3 mL/min running a gradient from 85:5:10 water: acetonitrile: 1% formic acid to 50:40:10 water: acetonitrile: 1% formic acid over 35 min. Analysis was conducted using BioPharmaLynx with tolerance set to 30 ppm and allowing for 4 missed cleavages.

Fluorescence Anisotropy[16]

Anisotropy measurements were conducted using a solution of dichloro-diphenyl-fluorescein-labeled **TC5** (2.5 nM, Table S1) and Pol β (varying concentrations) in reaction buffer (50 mM HEPES, pH 7.5, 20 mM KCl, 1 mM EDTA, and 1 mM β-mercaptoethanol). Samples also contained 10% storage buffer (20 mM Tris•HCl, pH 7, 300 mM NaCl, 10% glycerol, 5 mM BME) by volume.

In a typical experiment, a sample (300 μL) was prepared by mixing Pol β (30 μL, 1 μM) in storage buffer with 10X reaction buffer (30 μL), **TC5** (30 μL), a solution 50% MeCN in H_2O containing or lacking **14** (200 μM, 3 μL) and H_2O (207 μL). These samples, termed solutions **A** and **A'** (**A** did not contain **14** and **A'** contained 2 μM **14**), contained 250 pM **TC5**, 100 nM Pol β, ± 2 μM **14**. Samples containing various concentrations of Pol β were prepared by serial dilution with solution **B** and **B'**. Solution **B** (10 mL) was prepared by mixing H_2O (7.85 mL), with 10X reaction buffer (1 mL), 10X storage buffer (1 mL), **TC5** (50 nM, 50 μL), and a solution of 50% MeCN in H_2O containing or lacking **14** (200 μM, 100 μL). Similarly, solution **B** did not contain **14** and was used exclusively to dilute solution **A**, whereas solution **B'** contained 2 μM **14** and was used to dilute solution **A'.** By mixing equal volumes of **A** or **A'** (150 μL) with **B** or **B**' (150 μL) respectively, the concentration of Pol β decreased to 50 nM, while the concentration of DNA and **14** remain unchanged. An aliquot (150 μL) of this new solution was then mixed with solution **B** or **B'** (150 μL) to prepare a new solution containing 25 nM Pol β. Serial dilutions were repeated such that samples contained Pol β concentrations of 100 nM, 50 nM, 25 nM, 12.5 nM, 6.25 nM, 3.13 nM, 1.56 nM, 0.78 nM, 0.39 nM, and 0.2 nM.

Samples were incubated at 25 °C for 1 h and fluorescence anisotropy (A) was measured using a portion (125 μL) of each sample with a PMT voltage of 800 mV, 8 nm slit width, 535 nm excitation and 556 nm emission. Fluorescence anisotropy was measured for **TC5** in the absence of enzyme (A_0), and the change in anisotropy ($A-A_0$) was calculated for each sample and plotted against the concentration of Pol β. Each fluorescence anisotropy measurement was collected in triplicate.

Growing conditions for different cell lines

Mouse embryonic fibroblast cells were grown in DMEM with high glucose supplemented with 9% FBS at 34 °C in a 10% CO_2 humidified incubator.

HeLa cells were grown in DMEM with high glucose supplemented with 9% FBS and 1% antibiotic antimycotic solution (penicillin, streptomycin, and amphotericin B). HeLa cells were grown at 37 °C 5% CO_2 in a humidified incubator.

Clonogenic assay for cell survival

Approximately 2 x 10^5 HeLa cells were plated in each well of a 6-well culture plate (well size; 35 mm x 18 mm) in DMEM containing 10% FBS (1 mL) and kept in a humidified incubator at 37 °C with 5% CO_2. After overnight incubation, cells were subjected to either the vector (50% MeCN in H_2O) or treatment (100X *pro*-**14**, 10 μL; in 50% MeCN in H_2O and/or 100X DNA damaging agent (e.g. 20 mM MMS, 200 μM BLM; 10 μL; in DMEM-FBS medium). For alkylation experiments, cells were incubated with MMS (0 or 0.2 mM), with or without *pro*-**14** (5, 25 μM) at 37 °C with 5% CO_2 for 1 or 2 h. After treatment, the medium was removed, and the cells were washed with 1X PBS (2 x 1 mL). The cells were trypsinized with 0.25 w/v Trypsin-EDTA (1 mL in each well, 5 min incubation at 37 °C), washed with DMEM-FBS (10 mL) to quench the trypsin cleavage, and spun down (3,000 RCF x 5 min). The medium was removed, and

the cells were resuspended with fresh DMEM-FBS (10 mL). The single cell suspensions were collected and counted using a TC20 Automated Cell Counter (BIO-RAD).

Stock solutions of single cell suspensions were prepared for all untreated and treated cells. For example, 100 cells/mL stock solution of untreated cells were prepared; 500 cells/mL stock solutions of treated cells were prepared. The concentration of stock solution for each sample was determined based on expected toxicity of the treatment (i.e. higher concentrations for more toxic conditions). The appropriate number of cells for each experiment were seeded in each well of a 6-well plate (well size; 35 mm x 18 mm) in 3 mL of DMEM-FBS medium. The cells were grown in a humidified incubator at 37 ^{o}C with 5% CO_2 for 14 days. No significant change in media volume was observed after two weeks due to evaporation. After 14 days, the growth medium was discarded, and the attached cells were treated with 0.2% w/v crystal violet solution. The excess dye was washed with water. The plates were dried and scanned with an HP Scanjet 3970 and colonies were counted using ImageJ (FIJI).

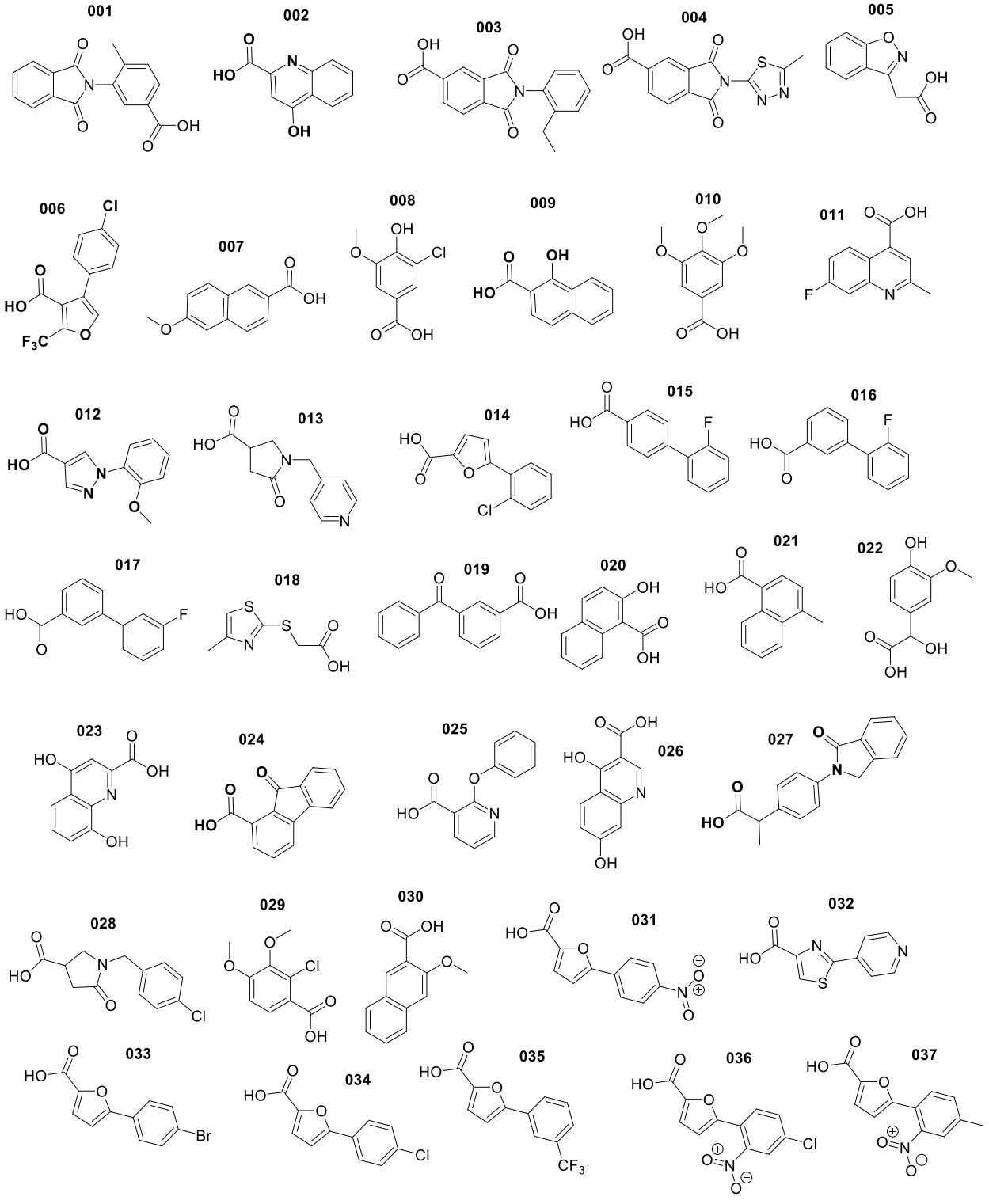
Plating efficiencies (PE) and survival fractions (SF) were calculated as follows: PE = number of colonies/number of cells seeded; SF = $PE/PE_{control}$.

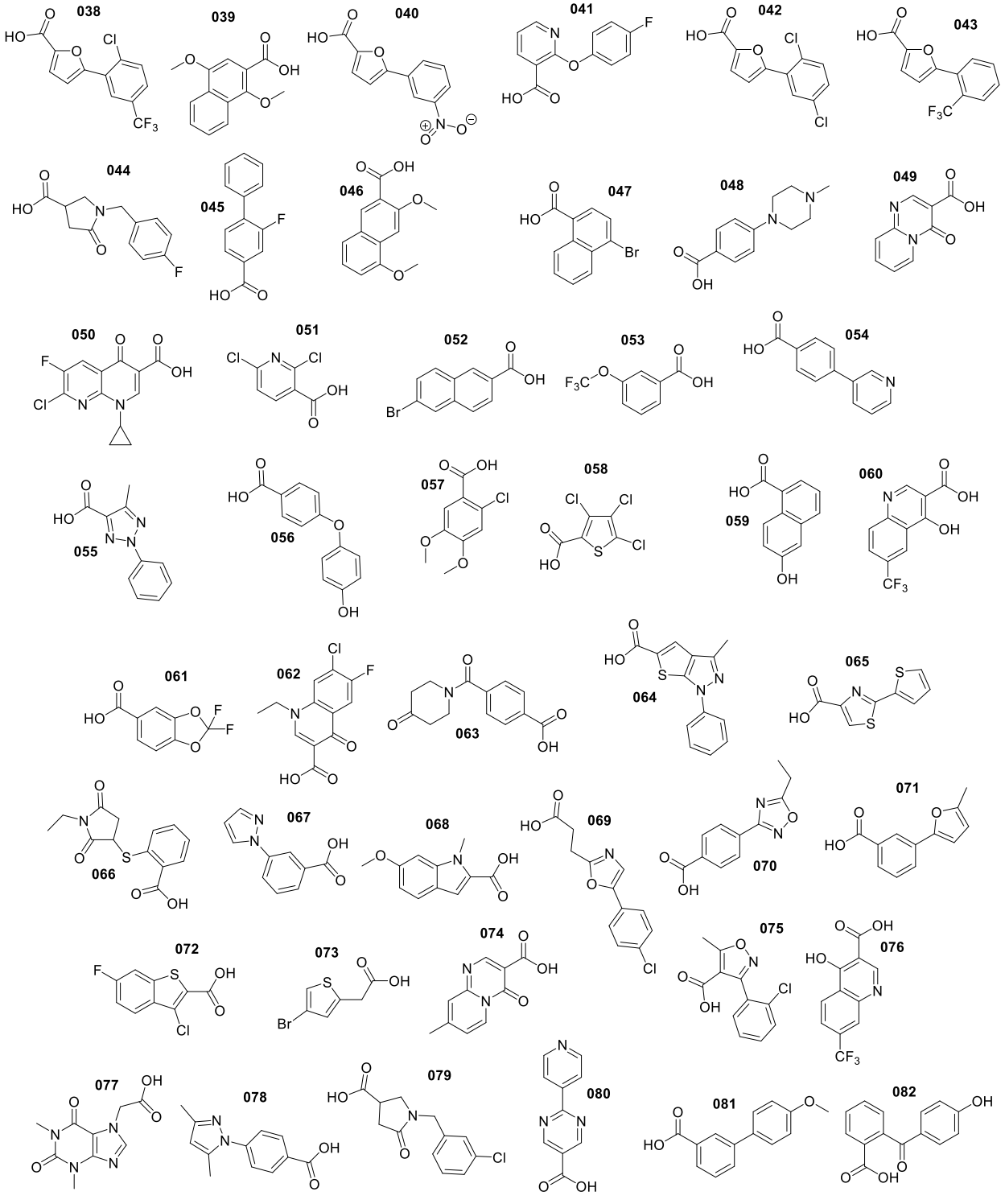
Cell Viability Assays for MEFs.[17,18]

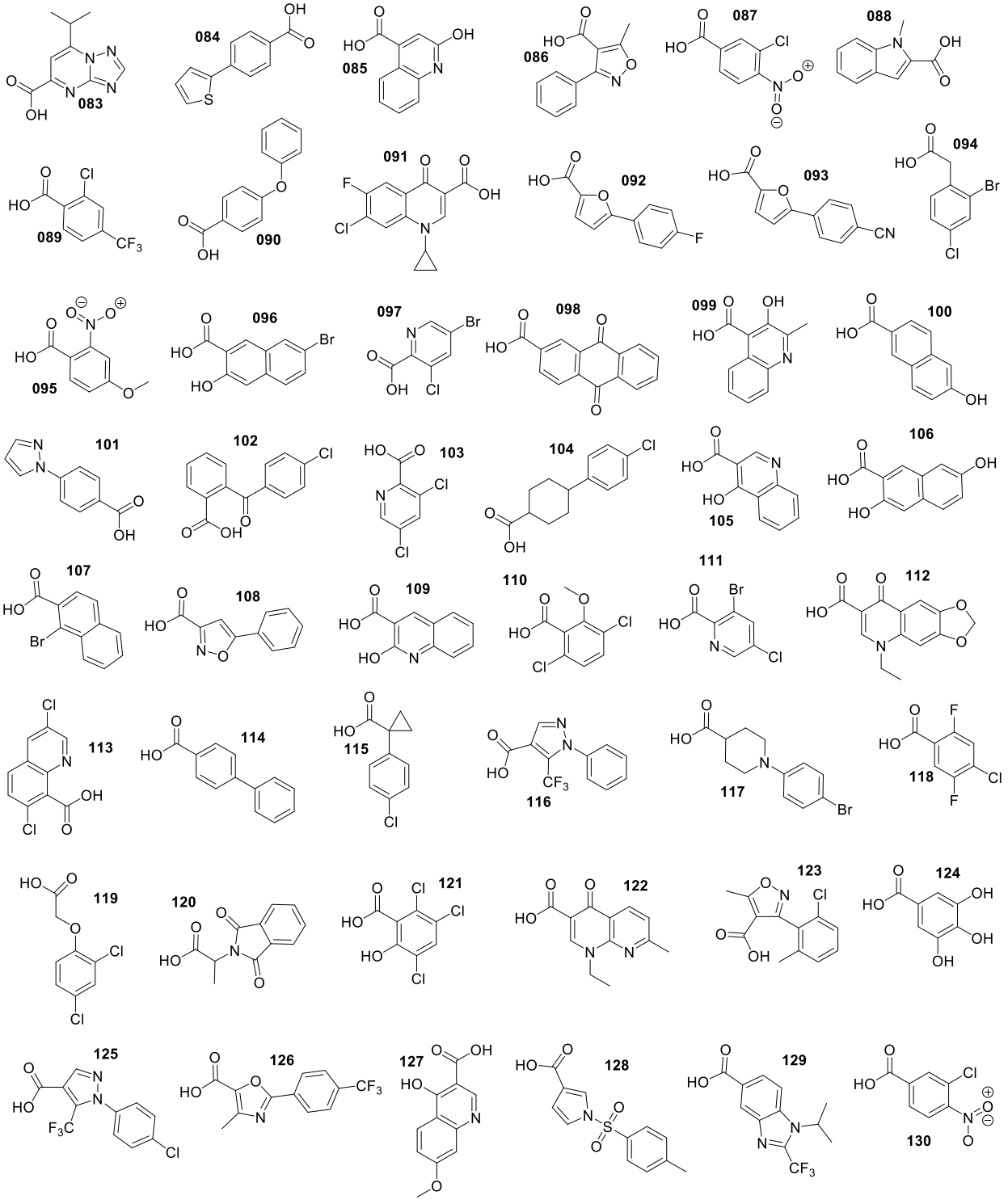
Mouse embryonic fibroblasts (Pol β WT, Pol β-/-, Pol λ WT, Pol λ-/-, Pol β-/λ-) were seeded at a density of ~0.3 x 10^6 cells/well in 6-well dishes. The following day, cells were exposed for 1 h to a range of MMS concentrations (0, 0.1, 0.2, 0.5, 1.0, 1.5 mM) in growth medium in the presence of absence of *pro*-**14** (0, 5, 15, 25 μM). Control wells were treated with an equal volume of vector (50% MeCN in H_2O). Cells were washed with 1X PBS and fresh medium was added. Dishes were incubated for 5 days at 34 ^{o}C in a 10% CO_2 incubator until untreated control cells were ~80% confluent. Cells (triplicate wells for each treatment concentration were counted by a

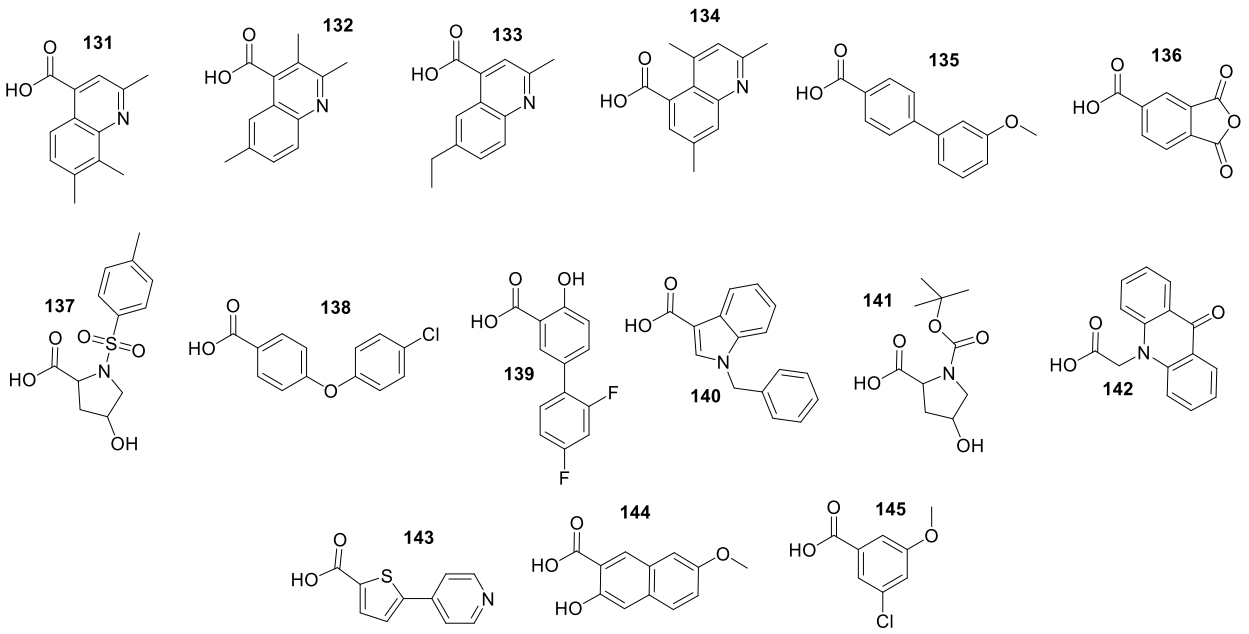
cell lysis procedure (described previously), and the results were expressed as the surviving fraction of cells in drug-treated wells relative to control wells.

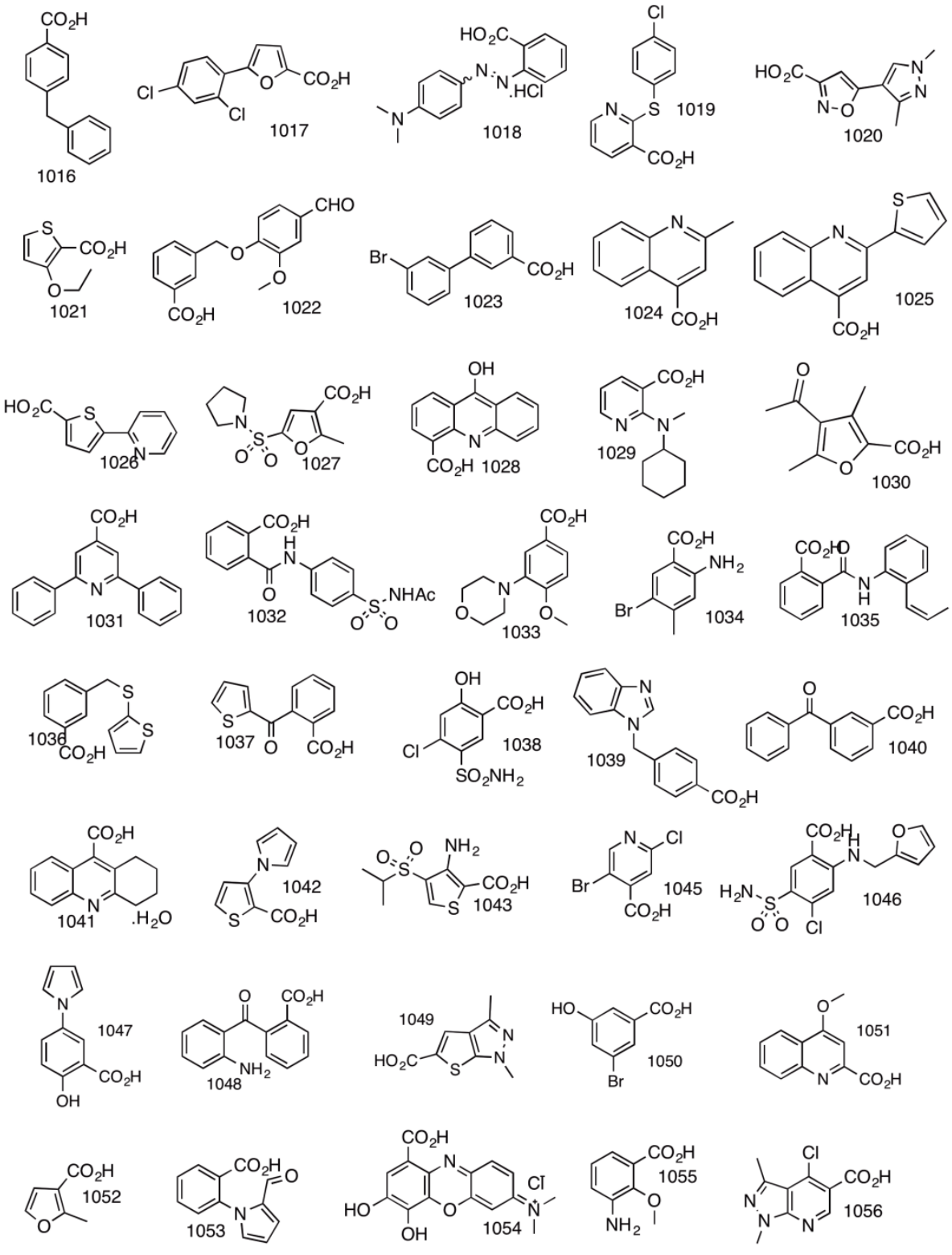
Chart S1. Carboxylic acids used to prepare inhibitor candidates.

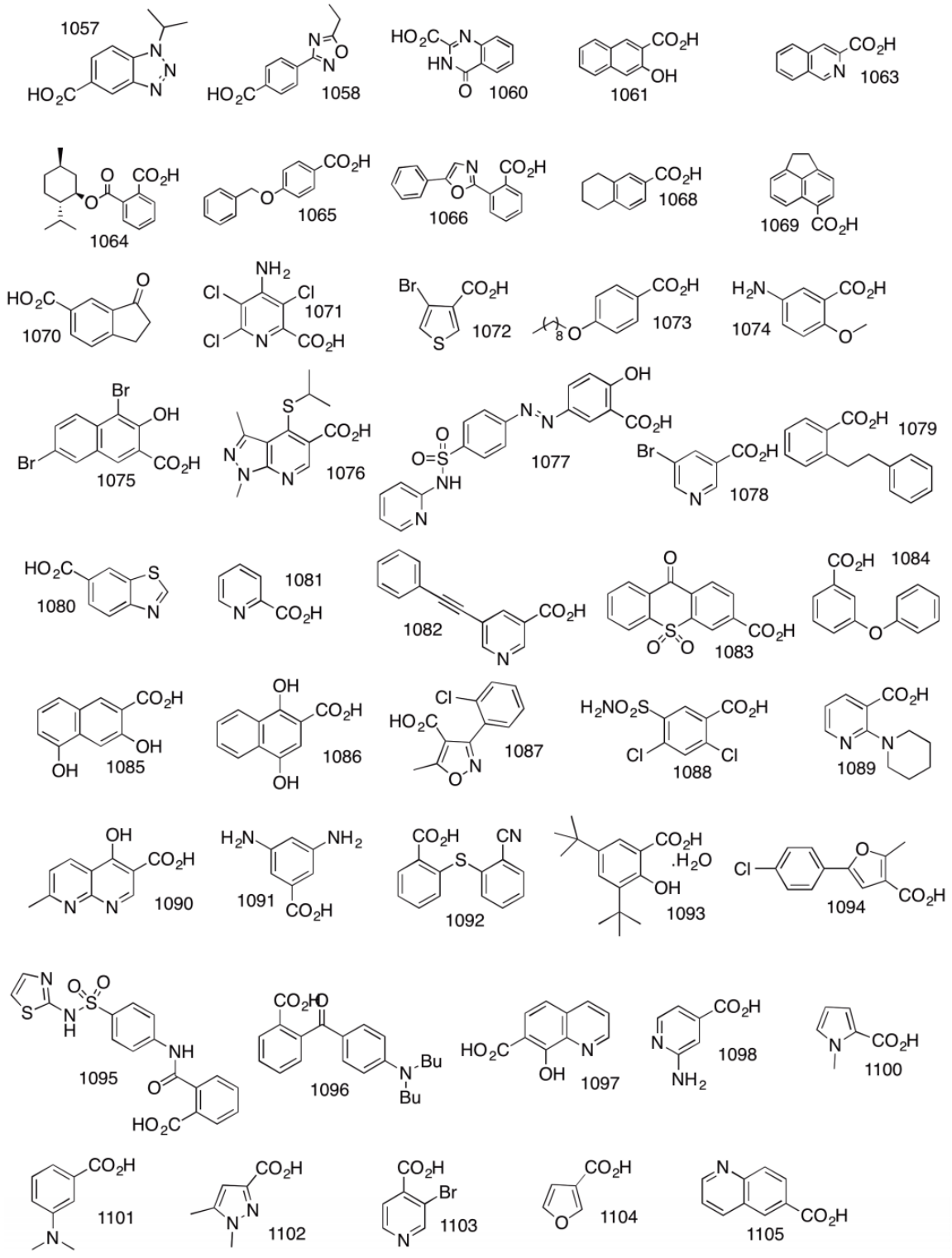


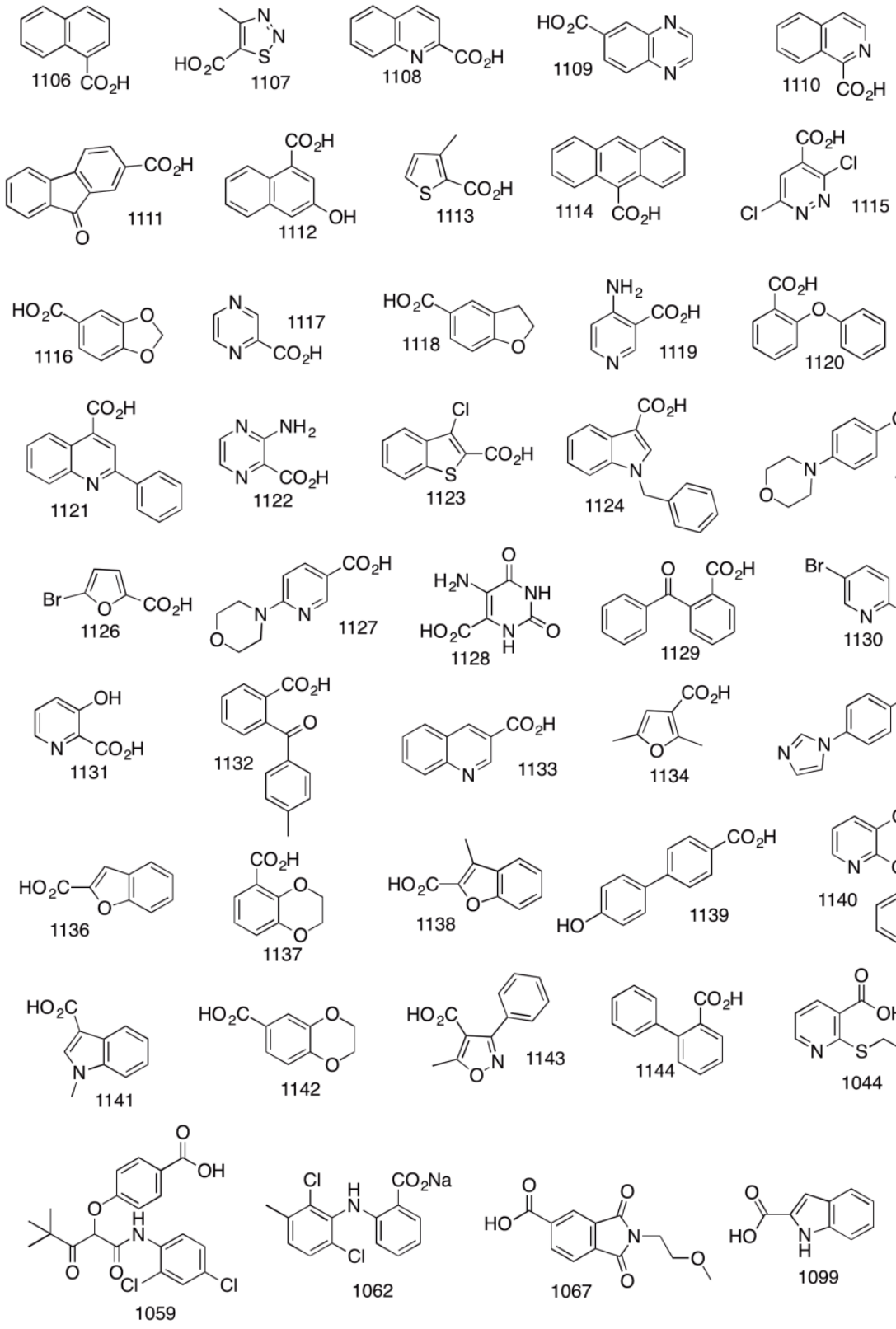

038
039
040
041
042
043
044
045
046
047
048
049
050
051
052
053
054
055
056
057
058
059
060
061
062
063
064
065
066
067
068
069
070
071
072
073
074
075
076
077
078
079
080
081
082

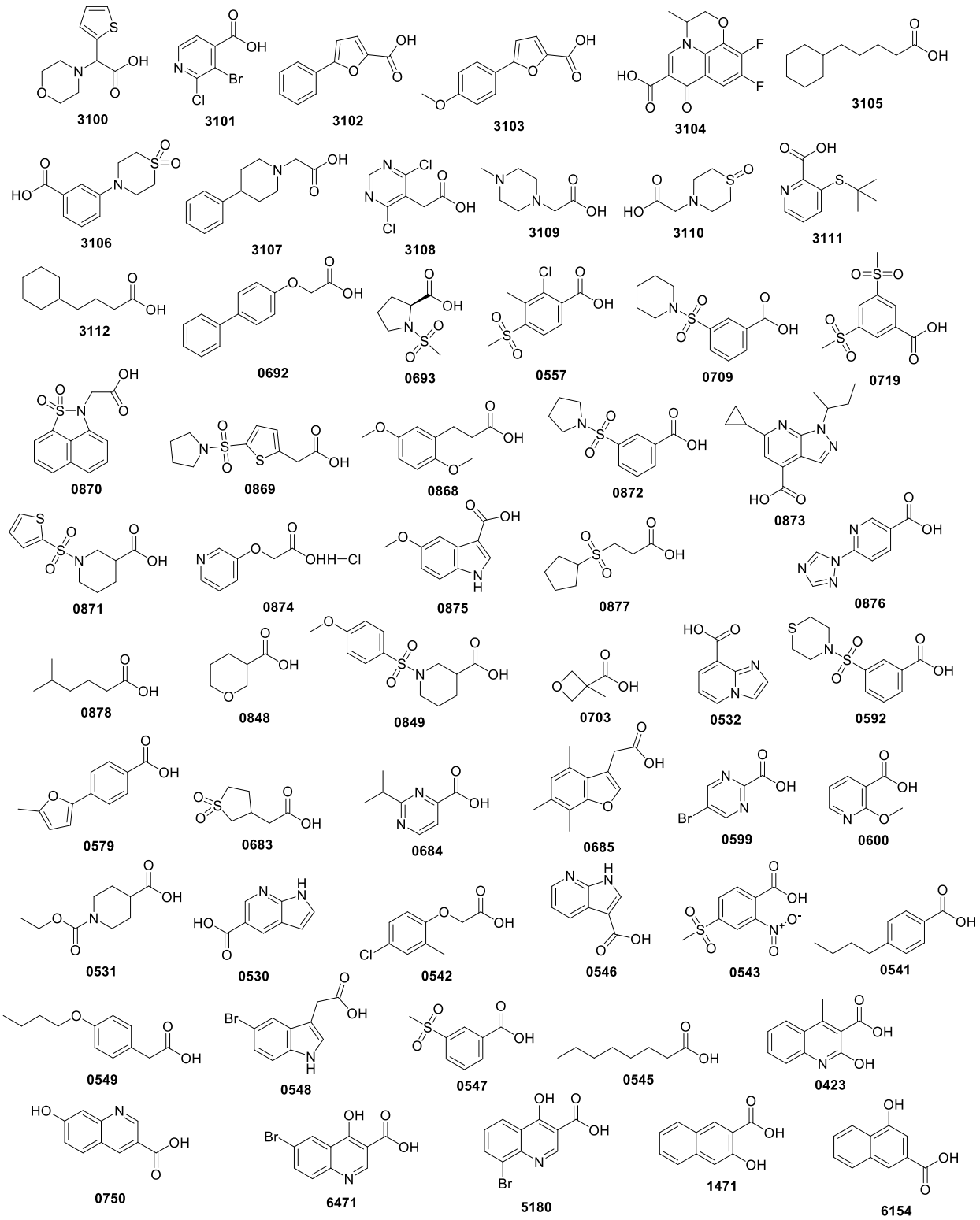

083
084
085
086
087
088
089
090
091
092
093
094
095
096
097
098
099
100
101
102
103
104
105
106
107
108
109
110
111
112
113
114
115
116
117
118
119
120
121
122
123
124
125
126
127
128
129
130


131
132
133
134
135
136
137
138
139
140
141
142
143
144
145

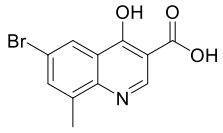

1016
1017
1018
1019
1020
1021
1022
1023
1024
1025
1026
1027
1028
1029
1030
1031
1032
1033
1034
1035
1036
1037
1038
1039
1040
1041
1042
1043
1045
1046
1047
1048
1049
1050
1051
1052
1053
1054
1055
1056

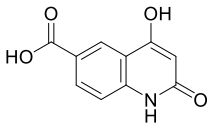

1057
1058
1060
1061
1063
1064
1065
1066
1068
1069
1070
1071
1072
1073
1074
1075
1076
1077
1078
1079
1080
1081
1082
1083
1084
1085
1086
1087
1088
1089
1090
1091
1092
1093
1094
1095
1096
1097
1098
1100
1101
1102
1103
1104
1105

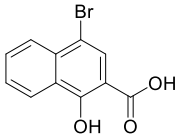

1106
1107
1108
1109
1110
1111
1112
1113
1114
1115
1116
1117
1118
1119
1120
1121
1122
1123
1124
1125
1126
1127
1128
1129
1130
1131
1132
1133
1134
1135
1136
1137
1138
1139
1140
1141
1142
1143
1144
1044
1059
1062
1067
1099

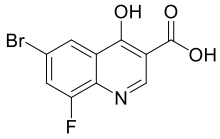

3100
3101
3102
3103
3104
3105
3106
3107
3108
3109
3110
3111
3112
0692
0693
0557
0709
0719
0870
0869
0868
0872
0873
0871
0874
0875
0877
0876
0878
0848
0849
0703
0532
0592
0579
0683
0684
0685
0599
0600
0531
0530
0542
0546
0543
0541
0549
0548
0547
0545
0423
0750
6471
5180
1471
6154

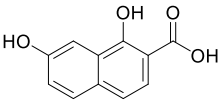
The remaining acids were only included in the second-generation library containing 375 members:

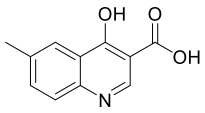

6105

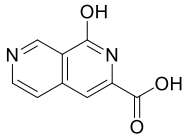

7405

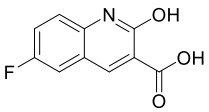

5653

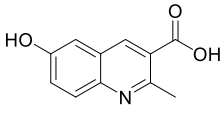

4420

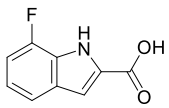

0867

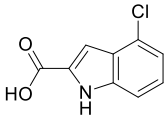

7146

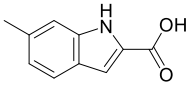

1834

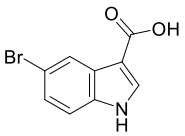

9658

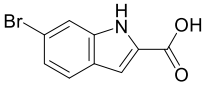

8931

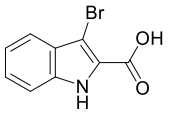

9666

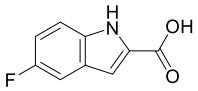

0151

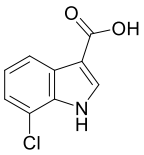

0152

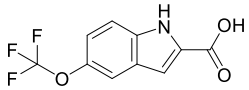

0153

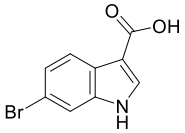

9665

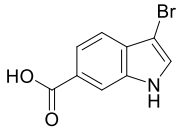

0155

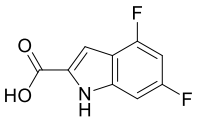

9664

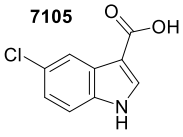

7105

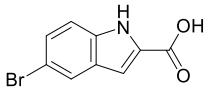

7106

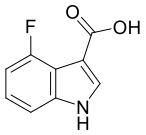

4353

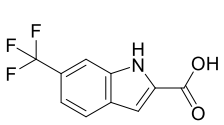

9748

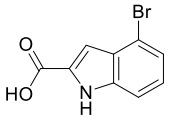

4350

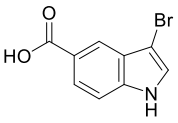

9650

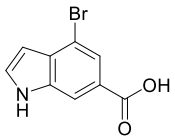

9651

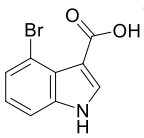

0150

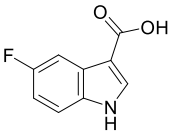

0149

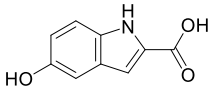

4351

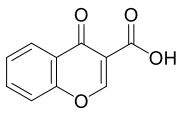

7107

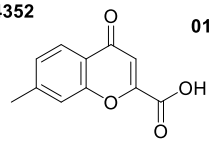

4352

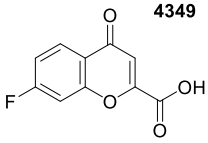

0170

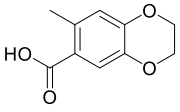

4349

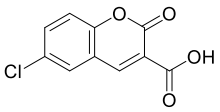

0154

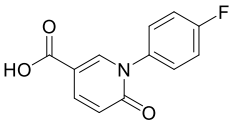

7109

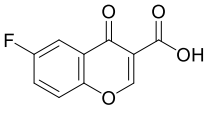

9751

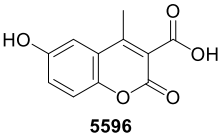

9668

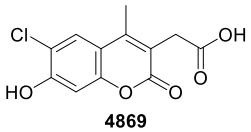

4304


5928


5597


3775


5596


4869

4870

5593

9752

5598

5594

5595

7108

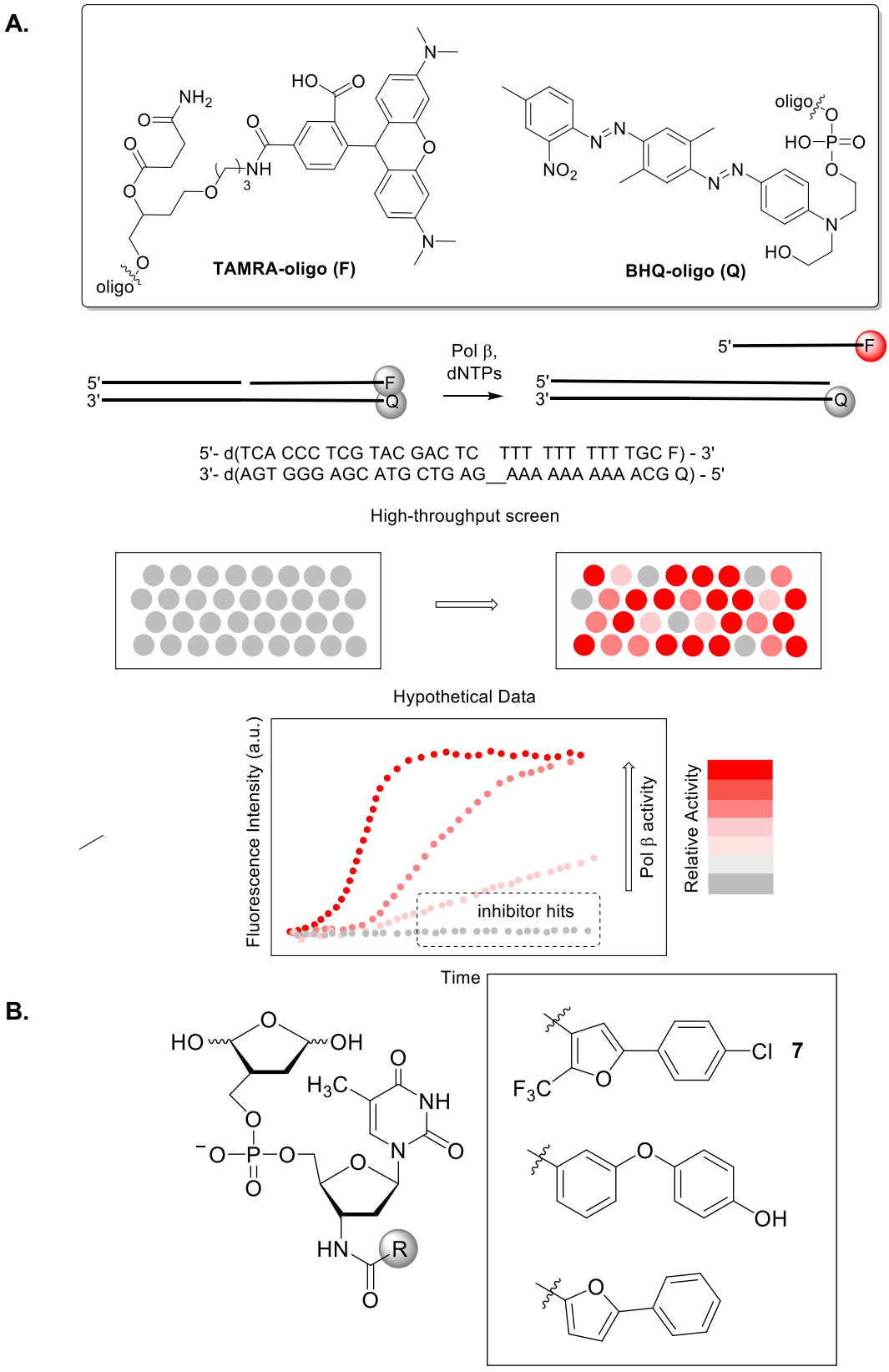


Figure S1. (a) Fluorescence-based strand displacement assay. (b) First-generation hit compounds from initial screen.

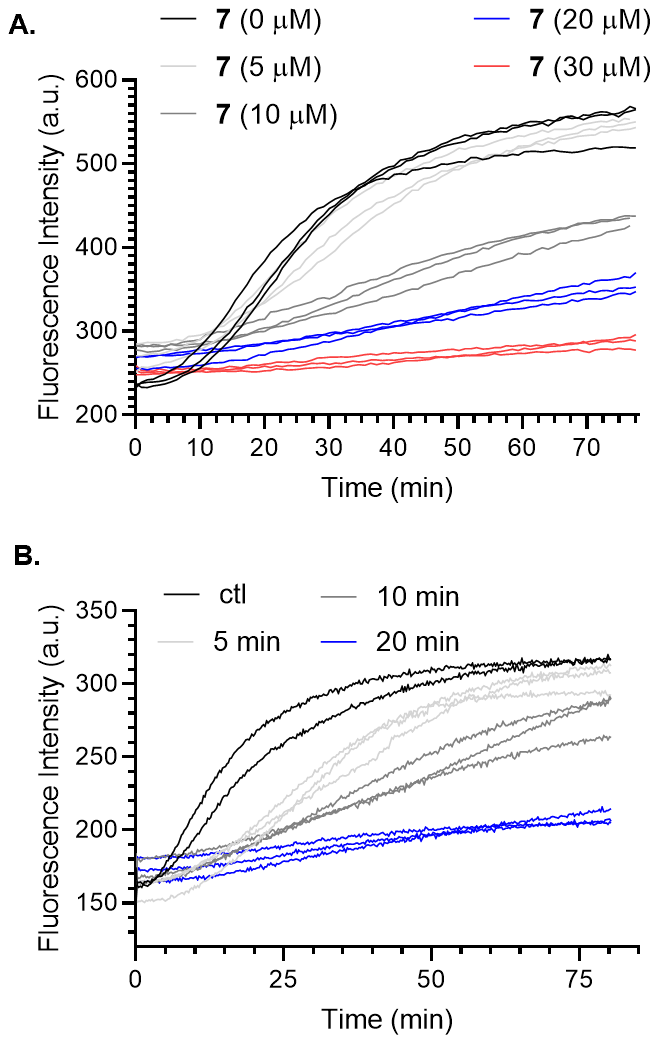


Figure S2. The inhibitory activity of first-generation inhibitor **7**. (a) Pol β strand displacement inhibition following 20 min preincubation with **7** at various concentrations (listed). (b) Effect of preincubation time on inhibitory activity of **7** at 15 μM.

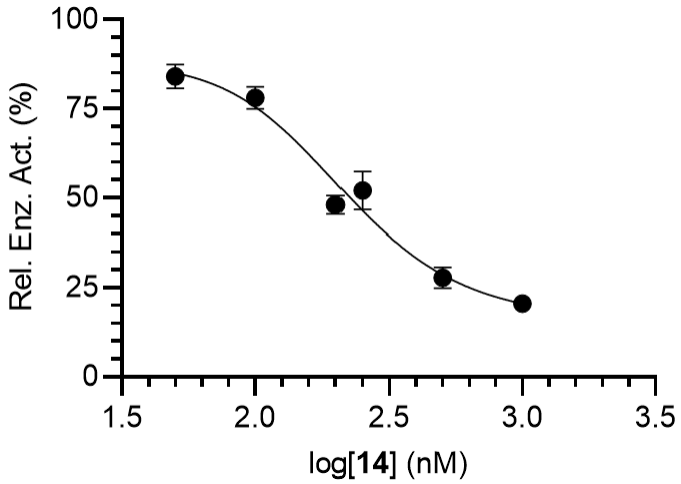


Figure S3. IC_{50} value of **14** under exact conditions used to evaluate **2**.

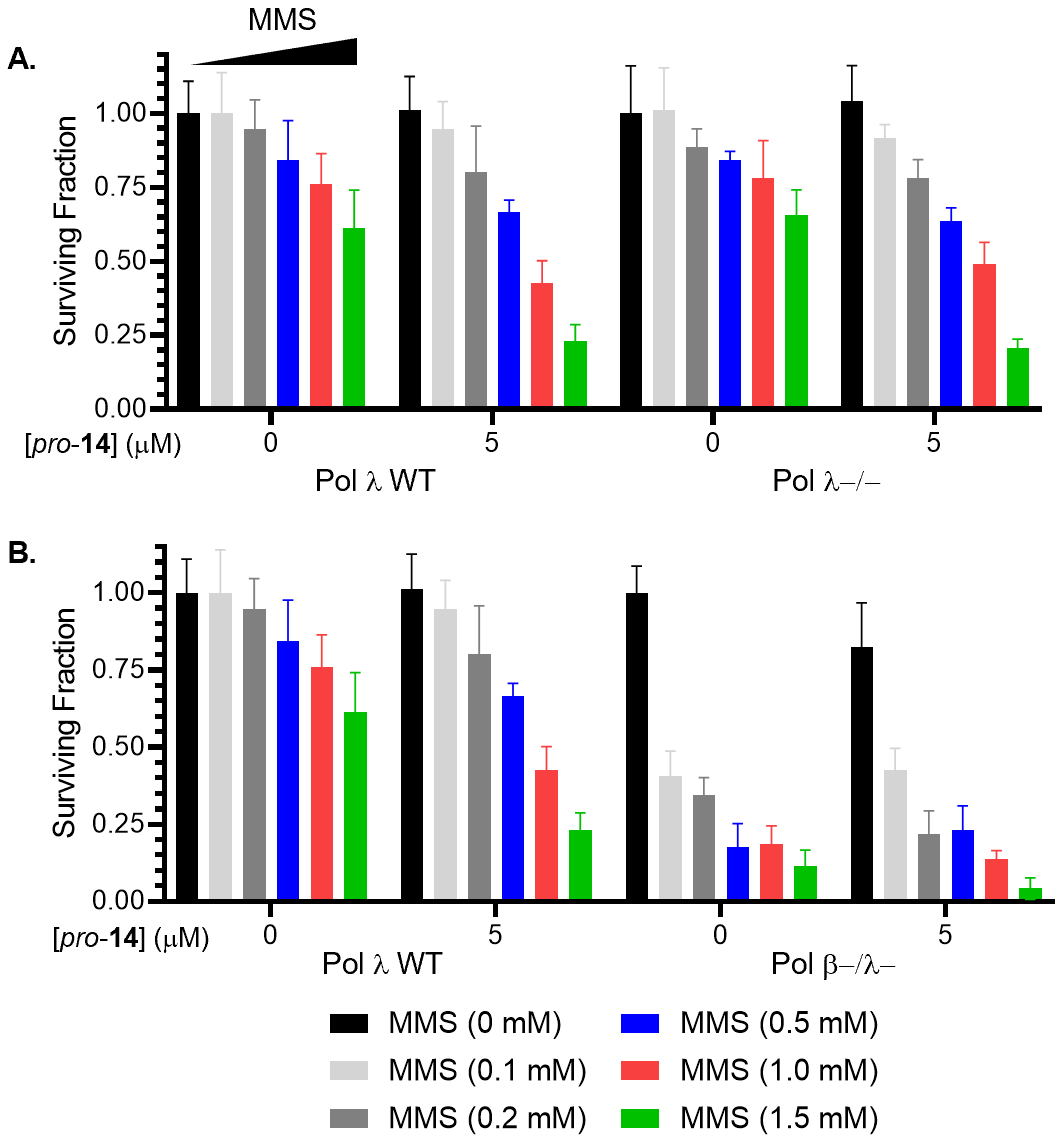


Figure S4. The effect of pro-**14** on MMS cytotoxicity in MEFs containing or lacking Pol λ. (a) The effect of pro-**14** on MMS cytotoxicity (0, 0.1, 0.2, 0.5, 1.0, 1.5 mM) in Pol λ WT and Pol λ-/- MEFs. (b) The effect of pro-**14** on MMS cytotoxicity (0, 0.1, 0.2, 0.5, 1.0, 1.5 mM) in Pol λ WT and Pol β–/λ– MEFs.

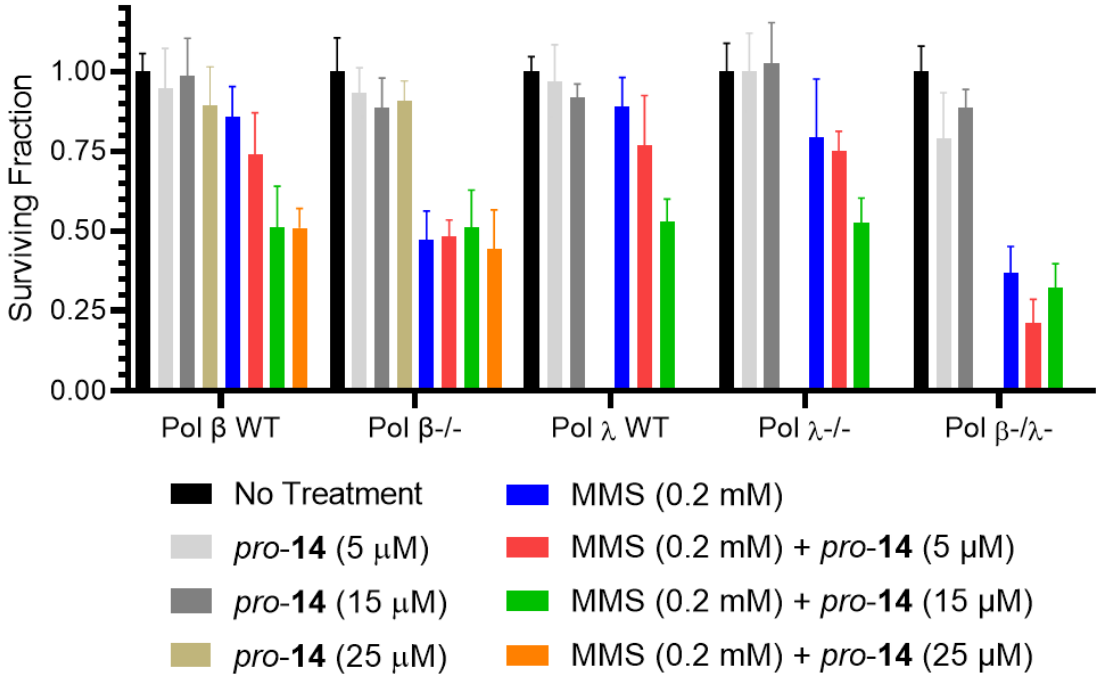


Figure S5. The effect of greater concentrations of pro-**14** (0, 5, 15, 25 μM) on MMS cytotoxicity (0, 0.1, 0.2, 0.5, 1.0, 1.5 mM) in MEFs lacking Pol β and/or Pol λ.

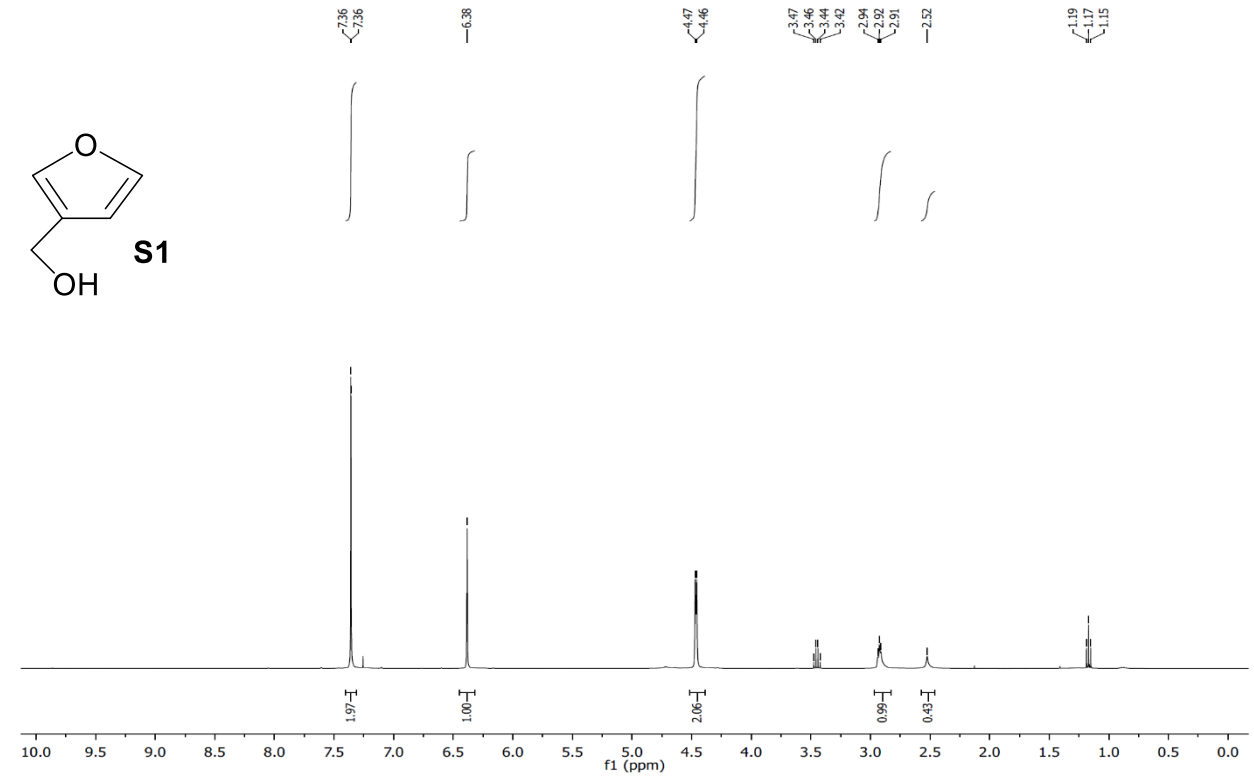


Figure S6. ^{1}H NMR spectra of **S1**

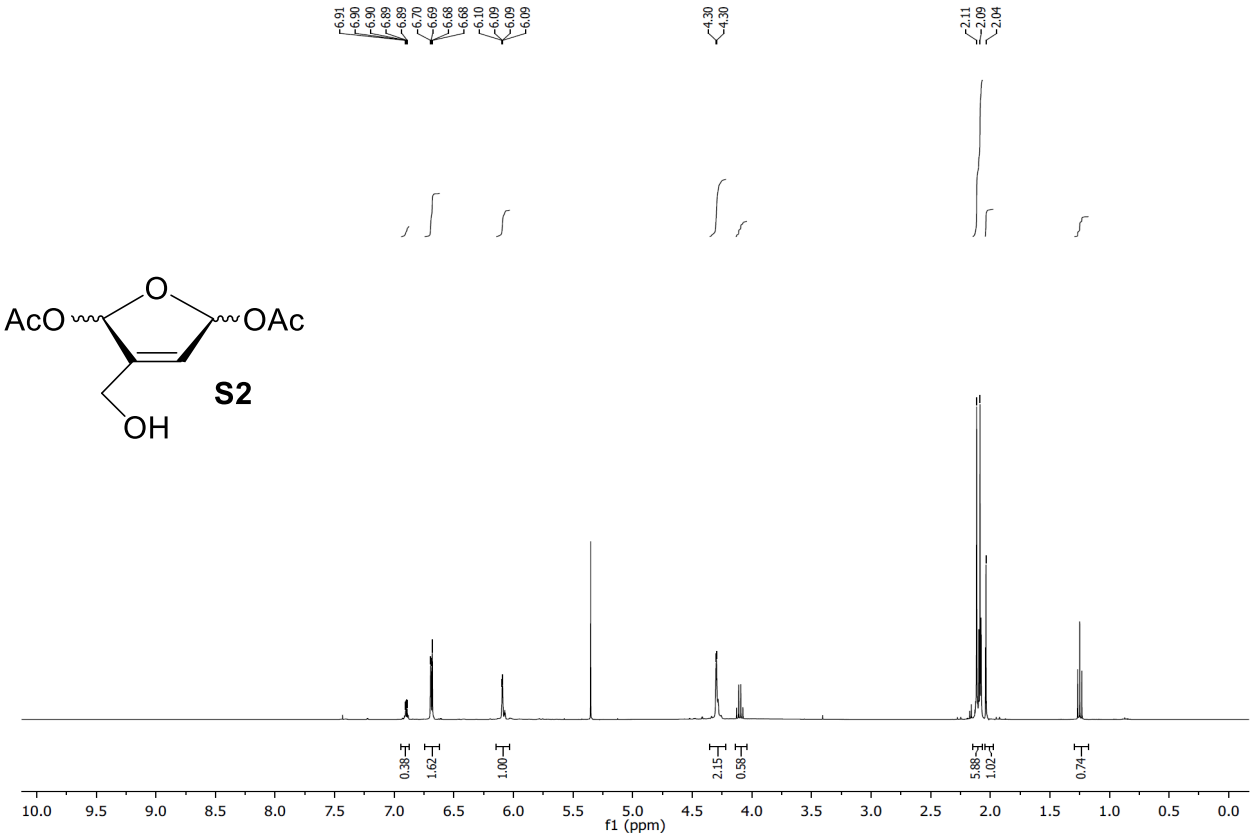


Figure S7. ^{1}H NMR spectra of **S2**

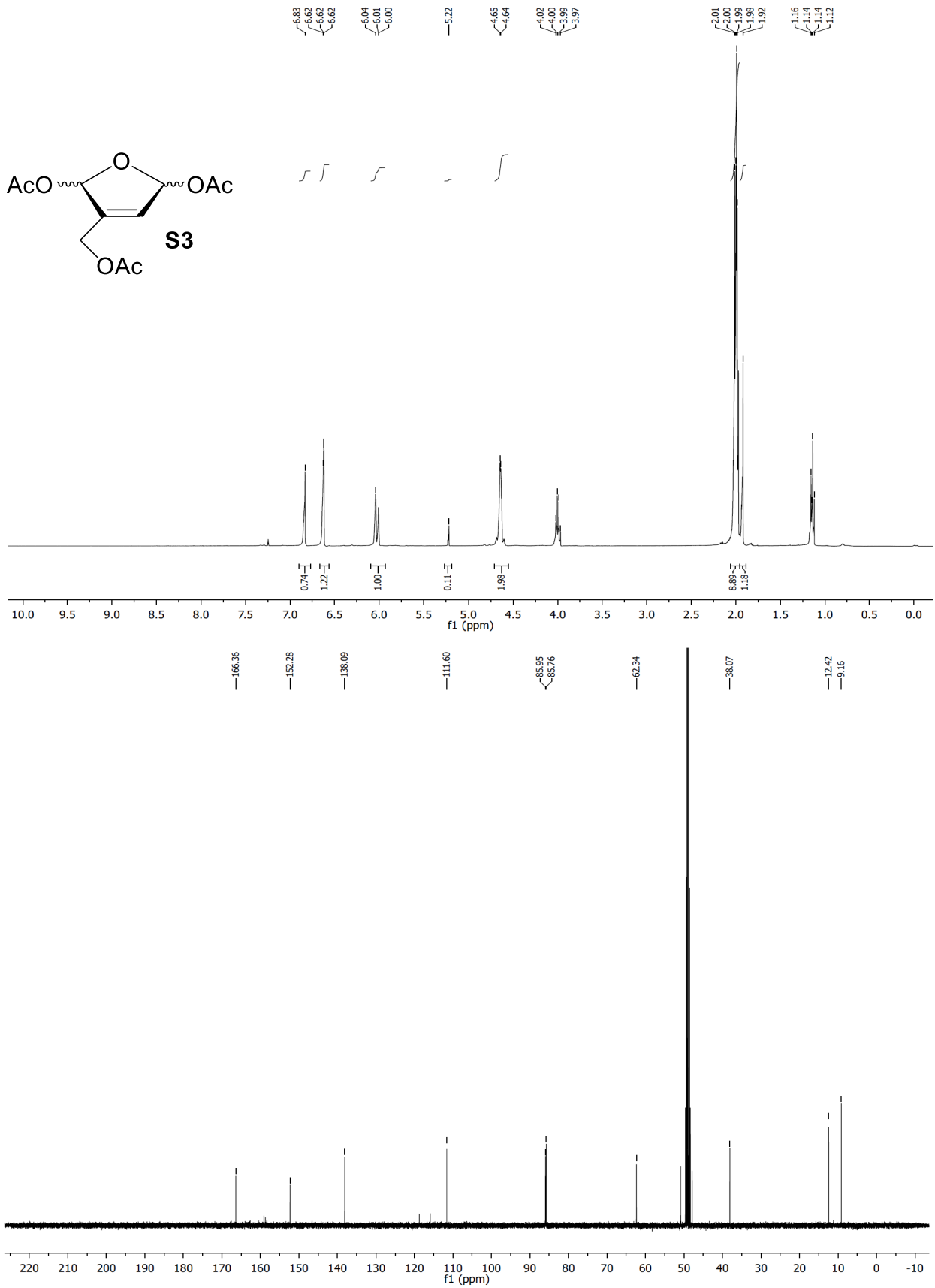


Figure S8. ^{1}H and ^{13}C NMR spectra of **S3**

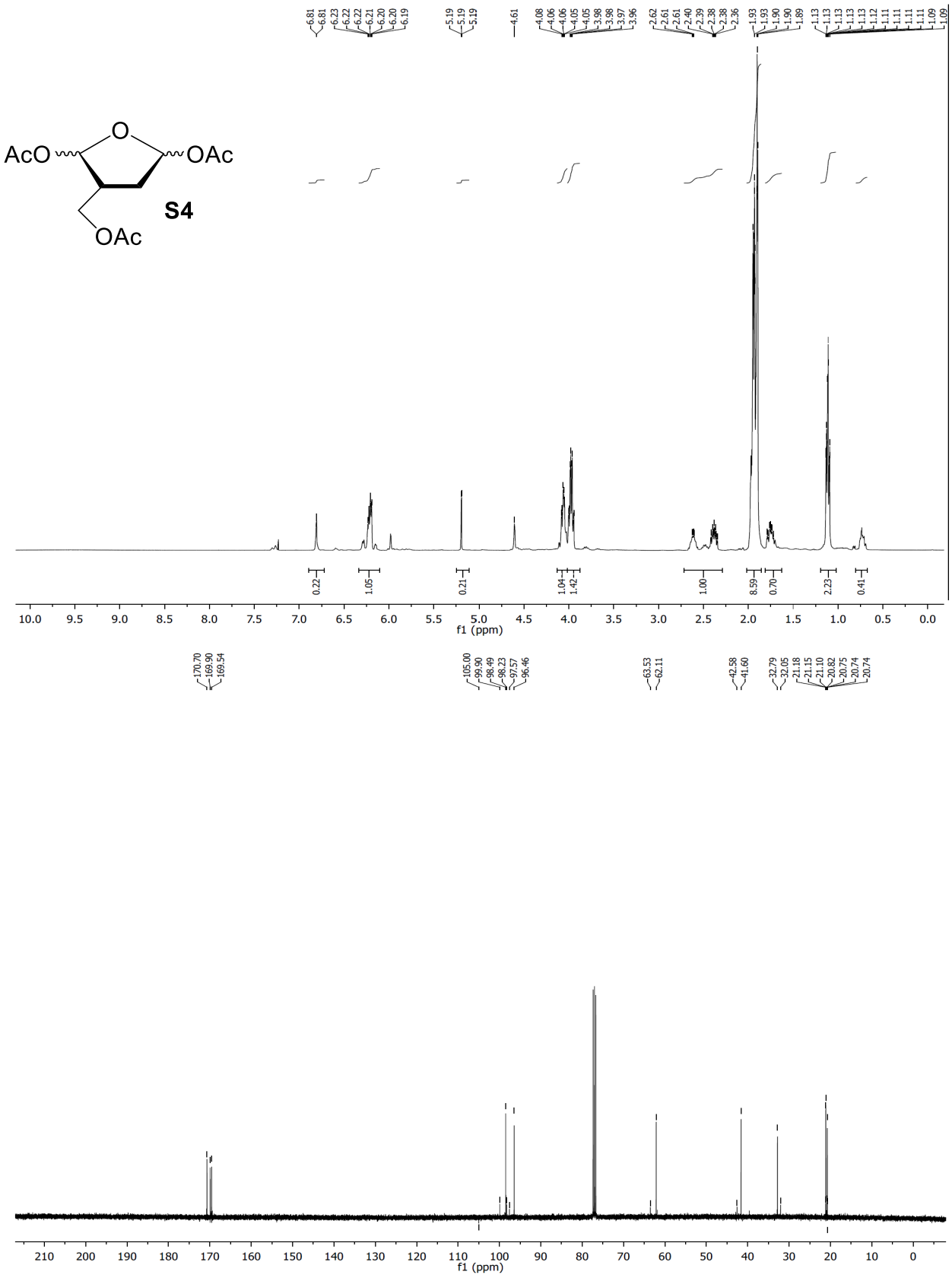


Figure S9. ^{1}H and ^{13}C NMR spectra of **S4**

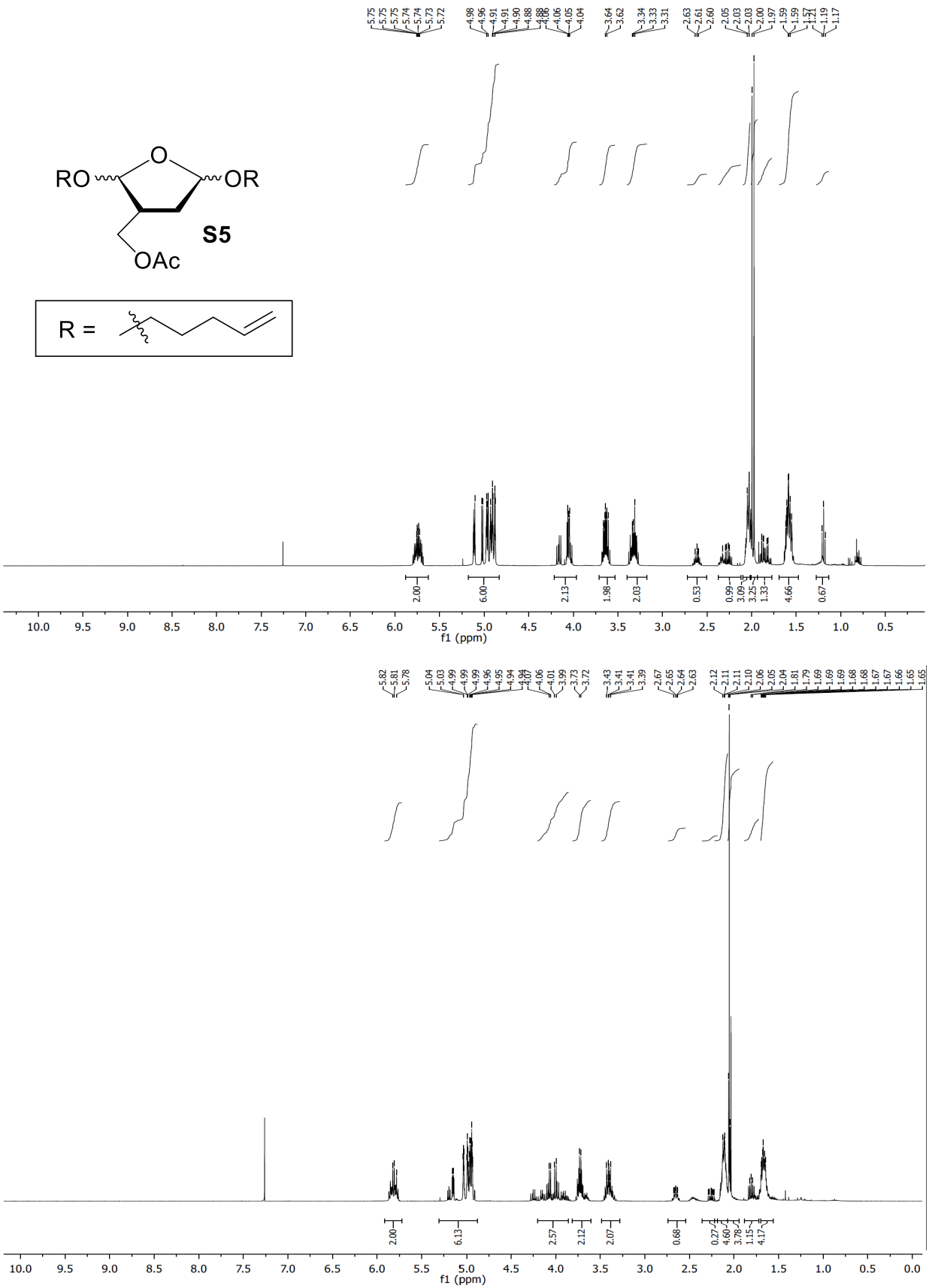


Figure S10. ^{1}H NMR spectra of **S5a** and **S5b**

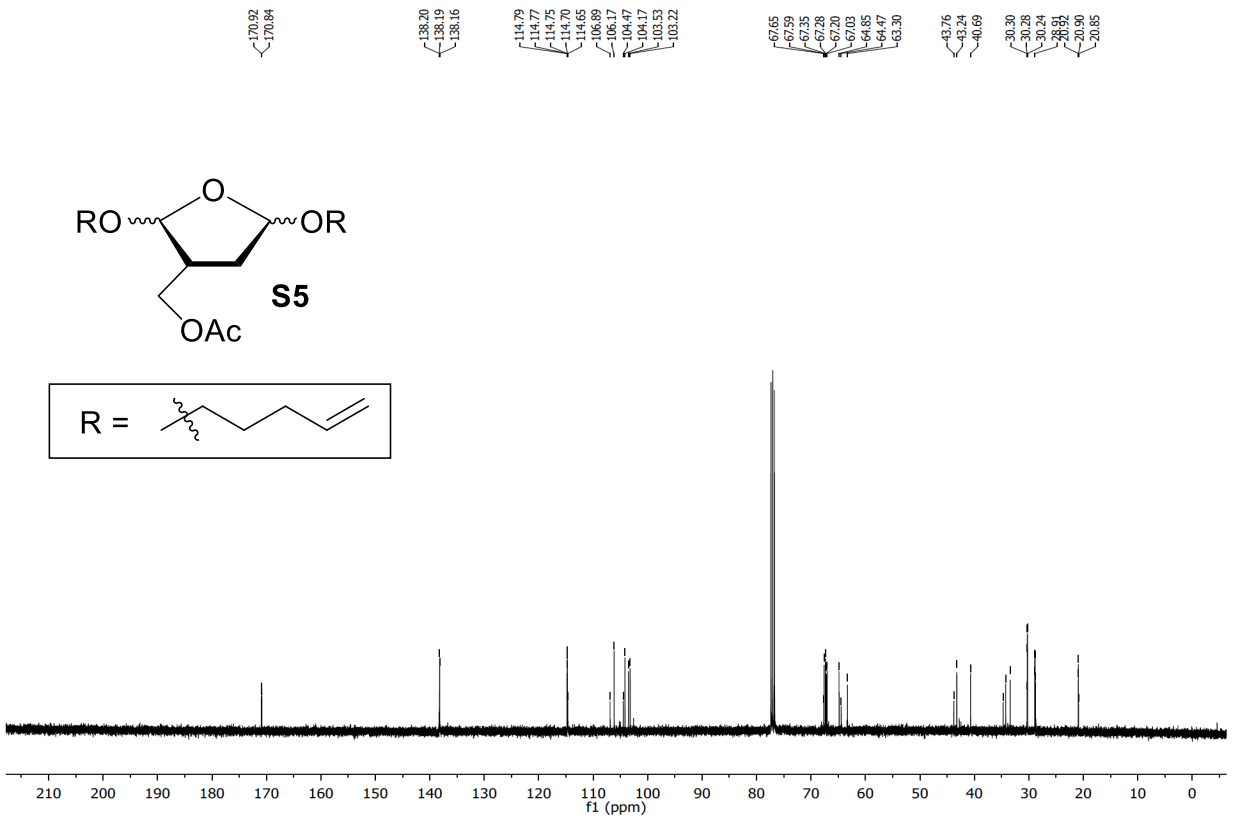


Figure S11. ^{13}C NMR spectra of **S5**

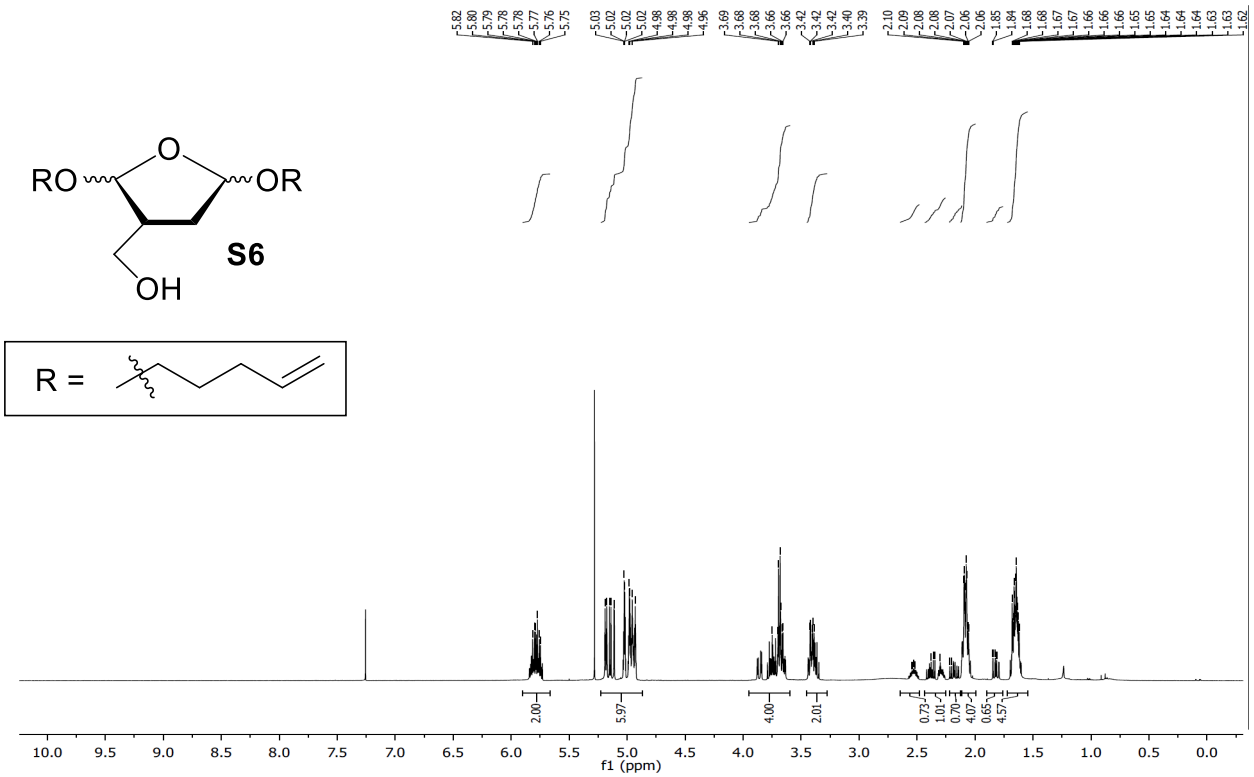


Figure S12. ^{1}H NMR spectra of **S6**

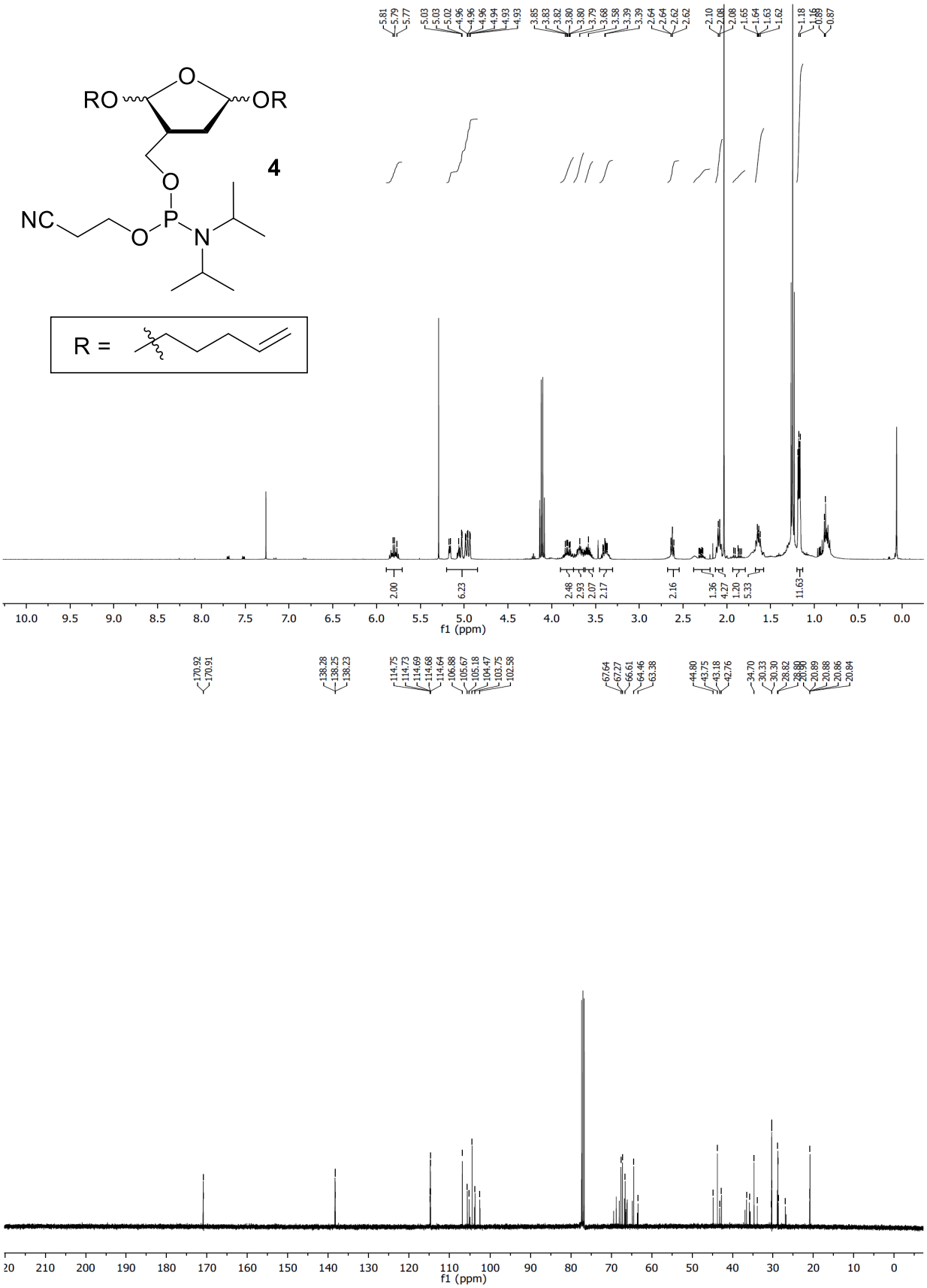


Figure S13. ^{1}H and ^{13}C NMR spectra of **4**

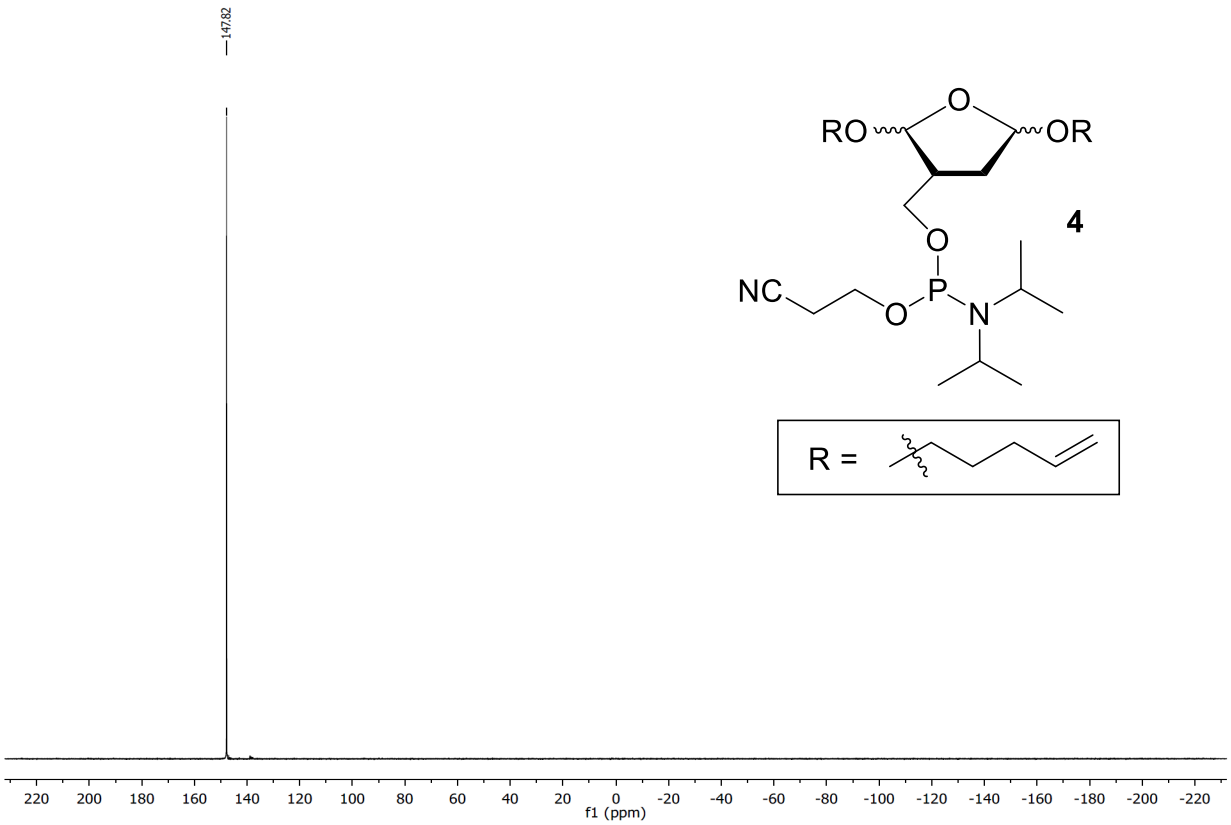


Figure S14. ^{31}P NMR spectra of **4**

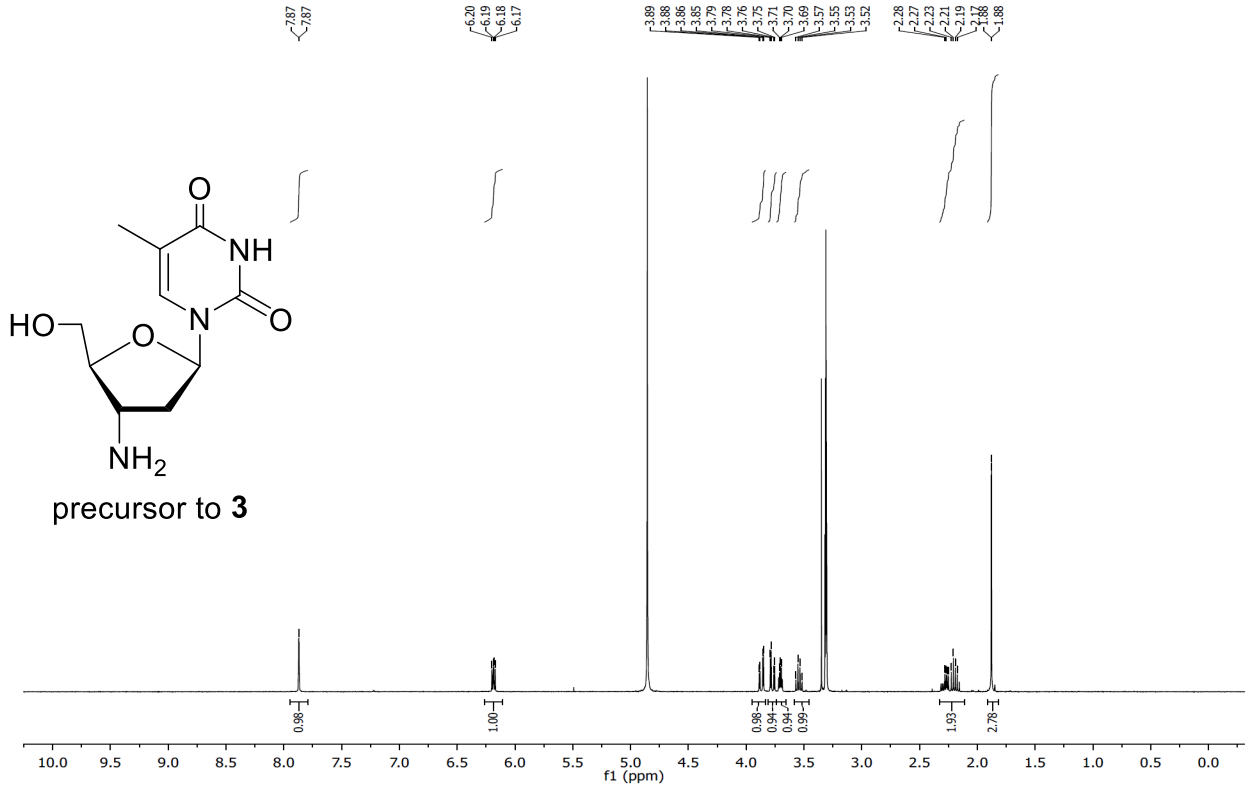


Figure S15. ^{1}H NMR spectra of precursor to **3**

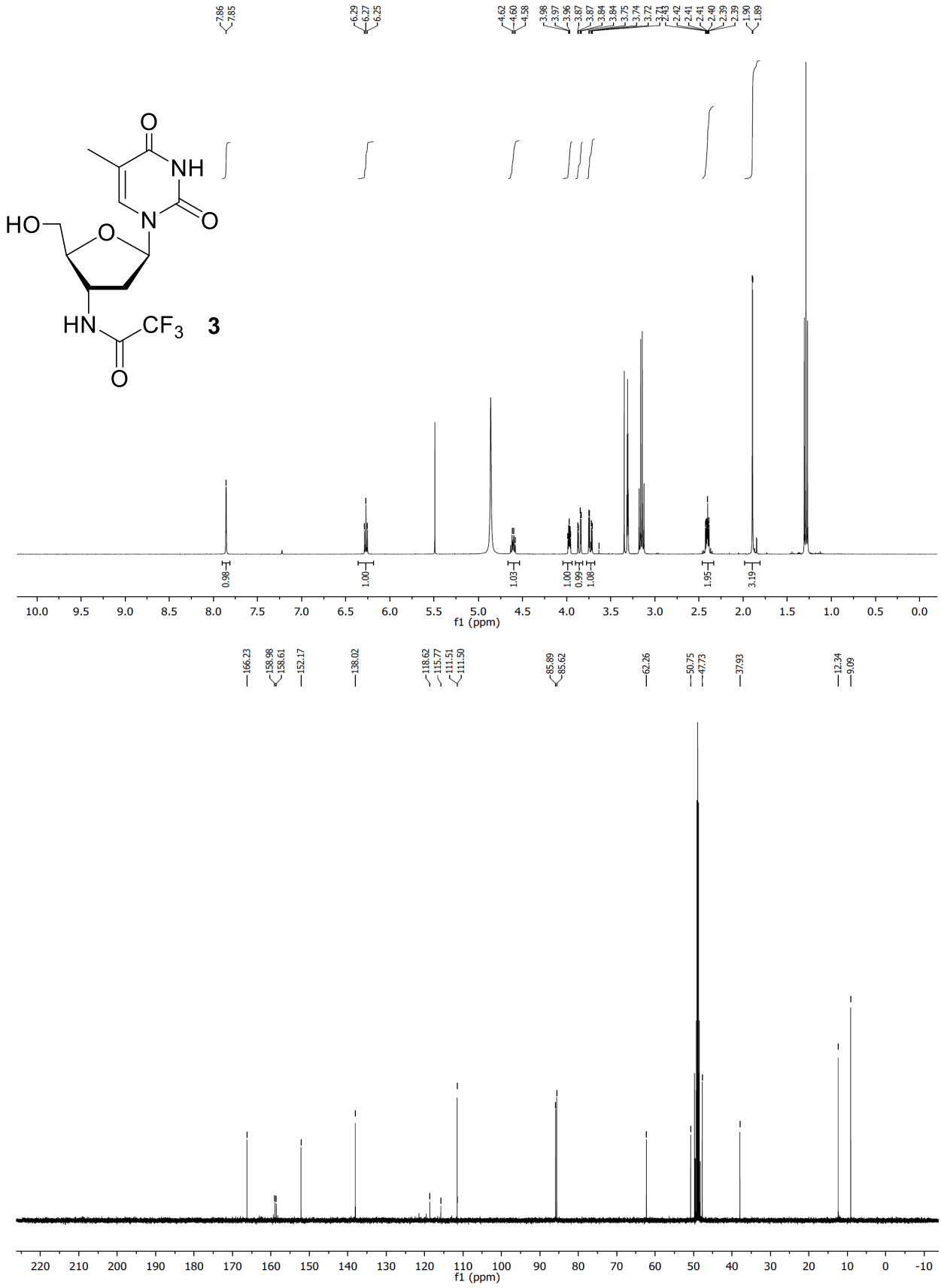


Figure S16. ^{1}H and ^{13}C NMR spectra of **3**

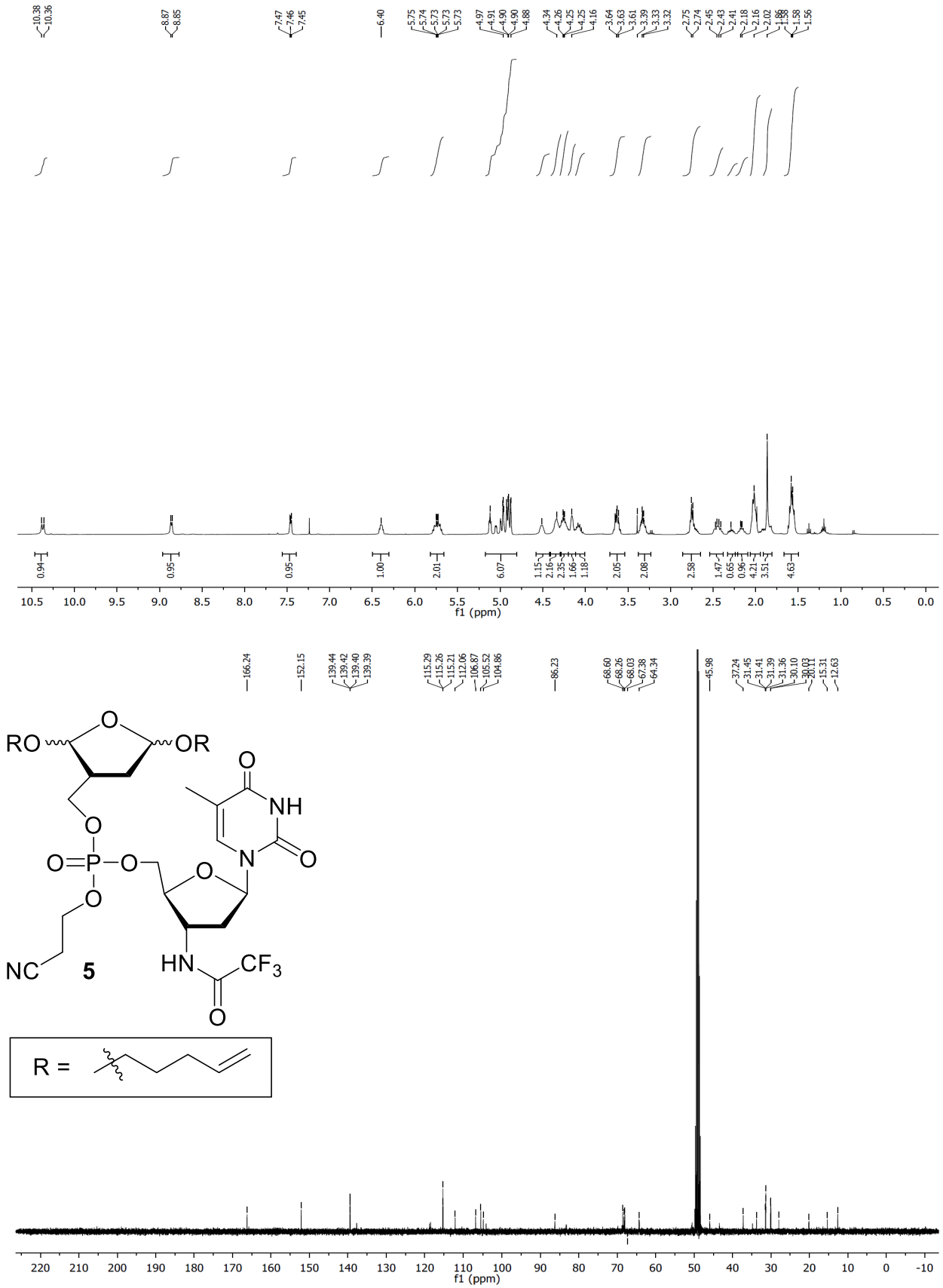


Figure S17. ^{1}H and ^{13}C NMR spectra of **5**

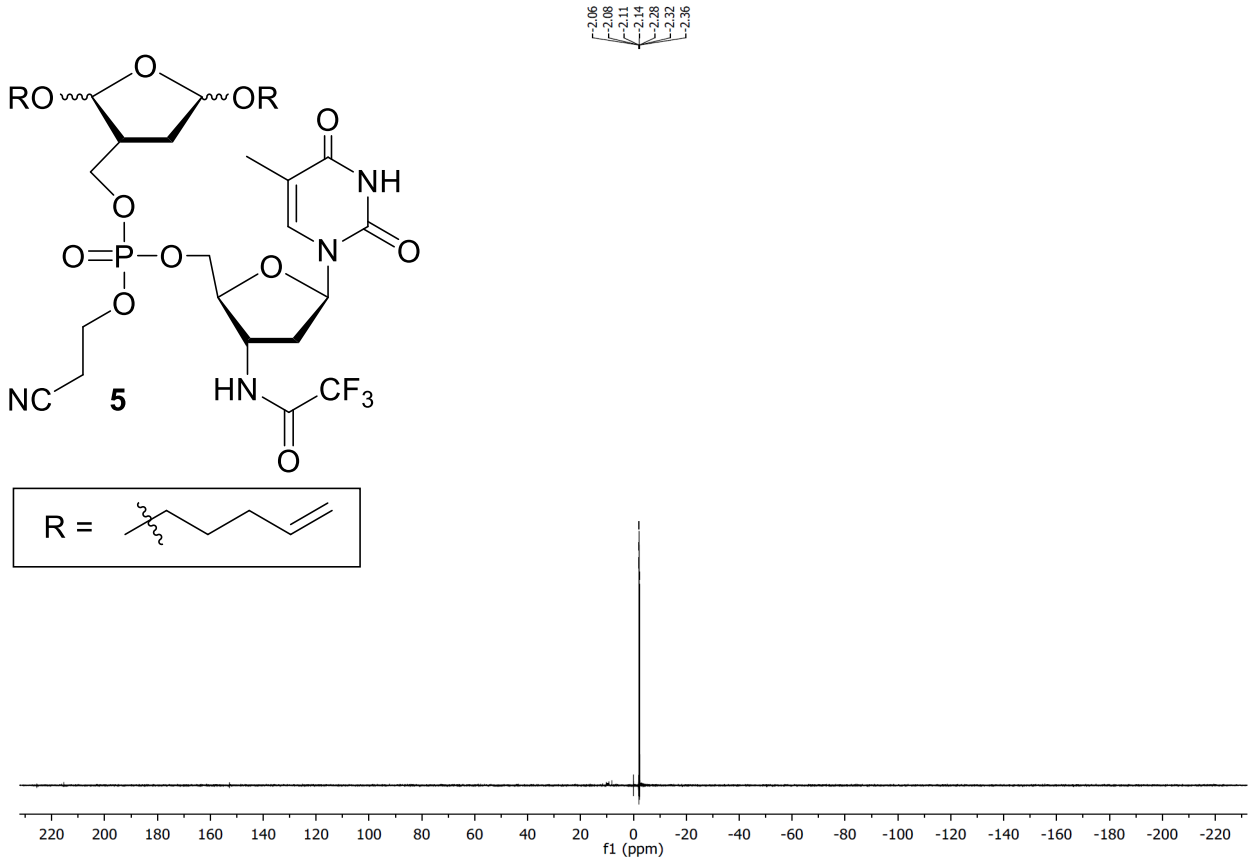


Figure S18. ^{31}P NMR spectra of **5**

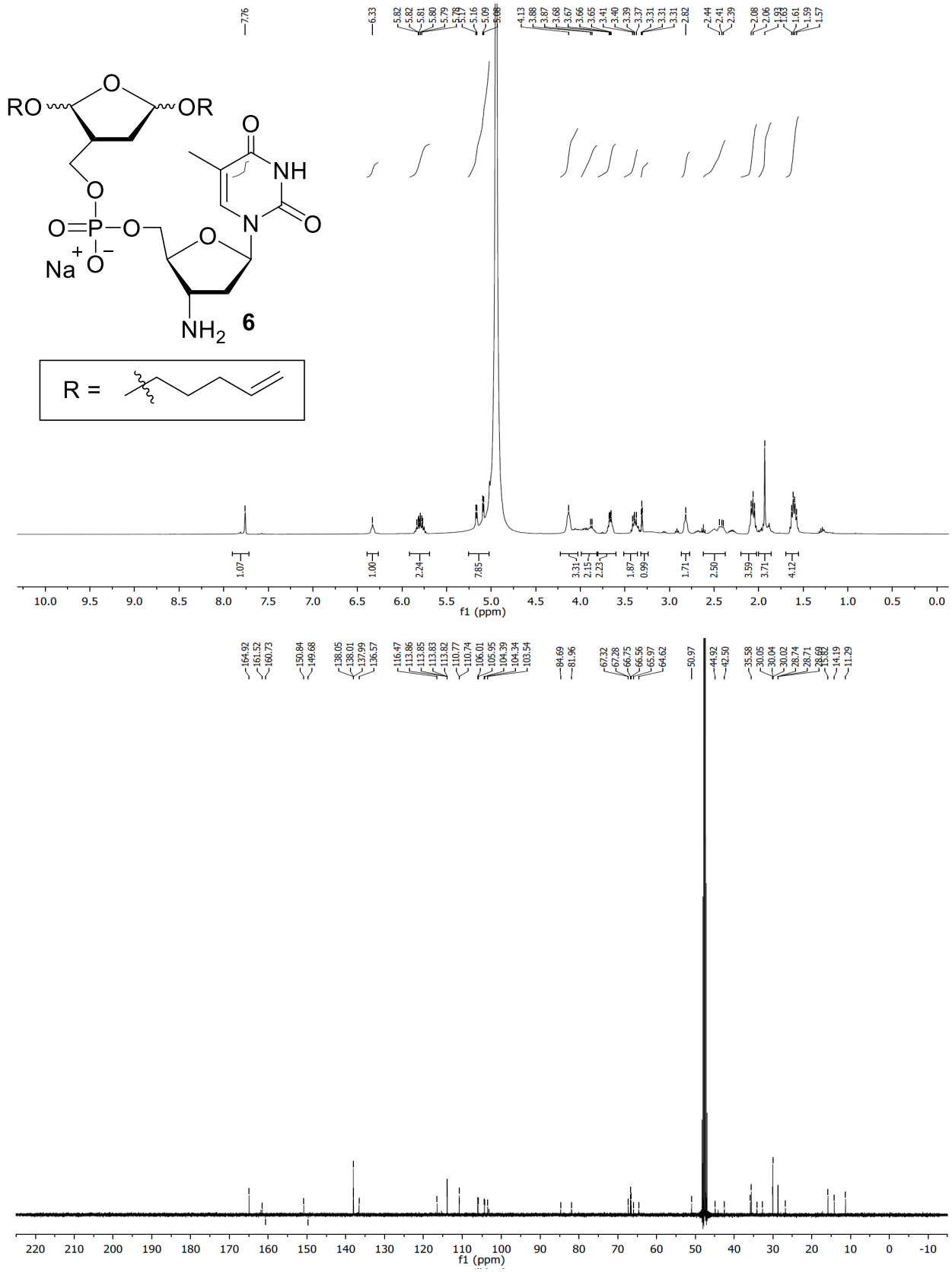


Figure S19. ^{1}H and ^{13}C NMR spectra of **6**

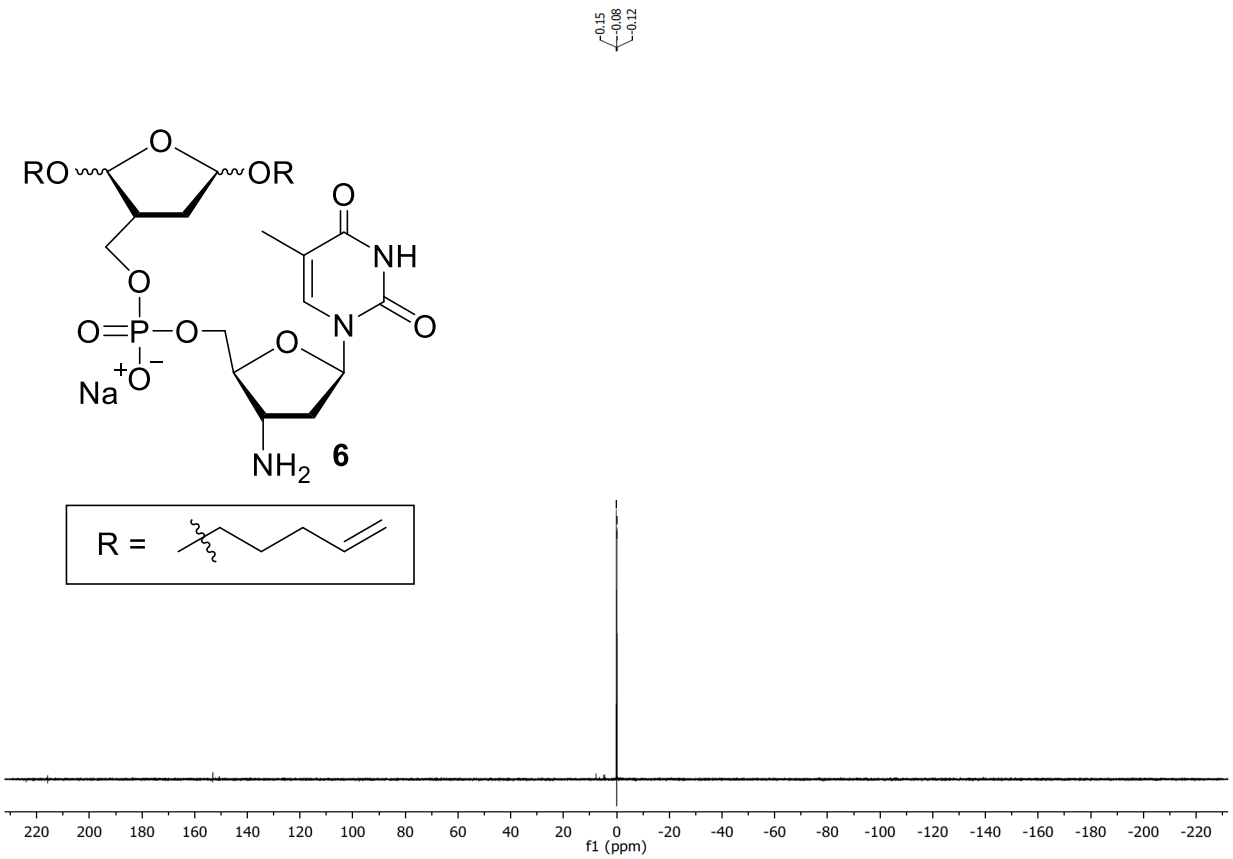


Figure S20. ^{31}P NMR spectra of **6**

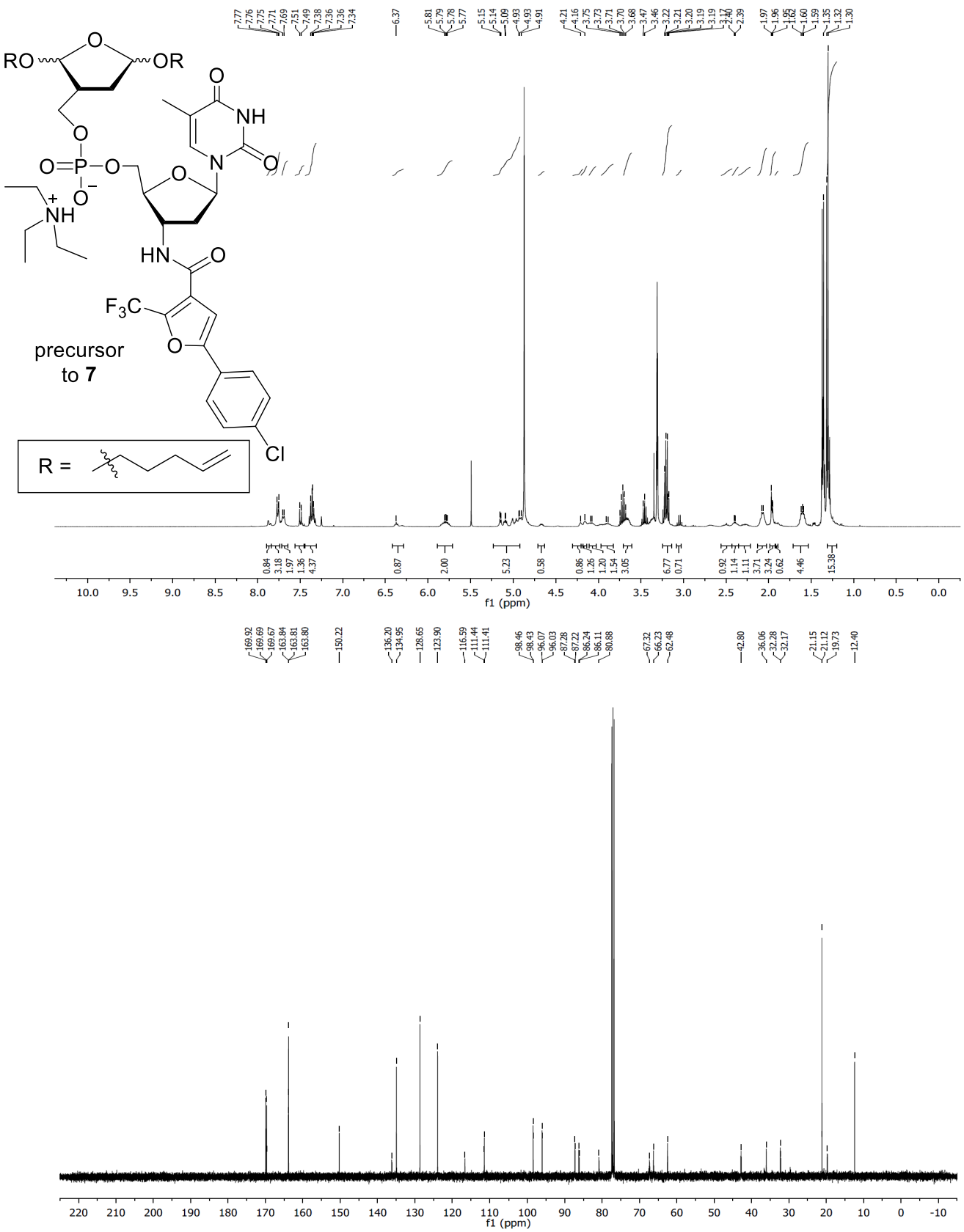


Figure S21. ^{1}H and ^{13}C NMR spectra of precursor to **7**

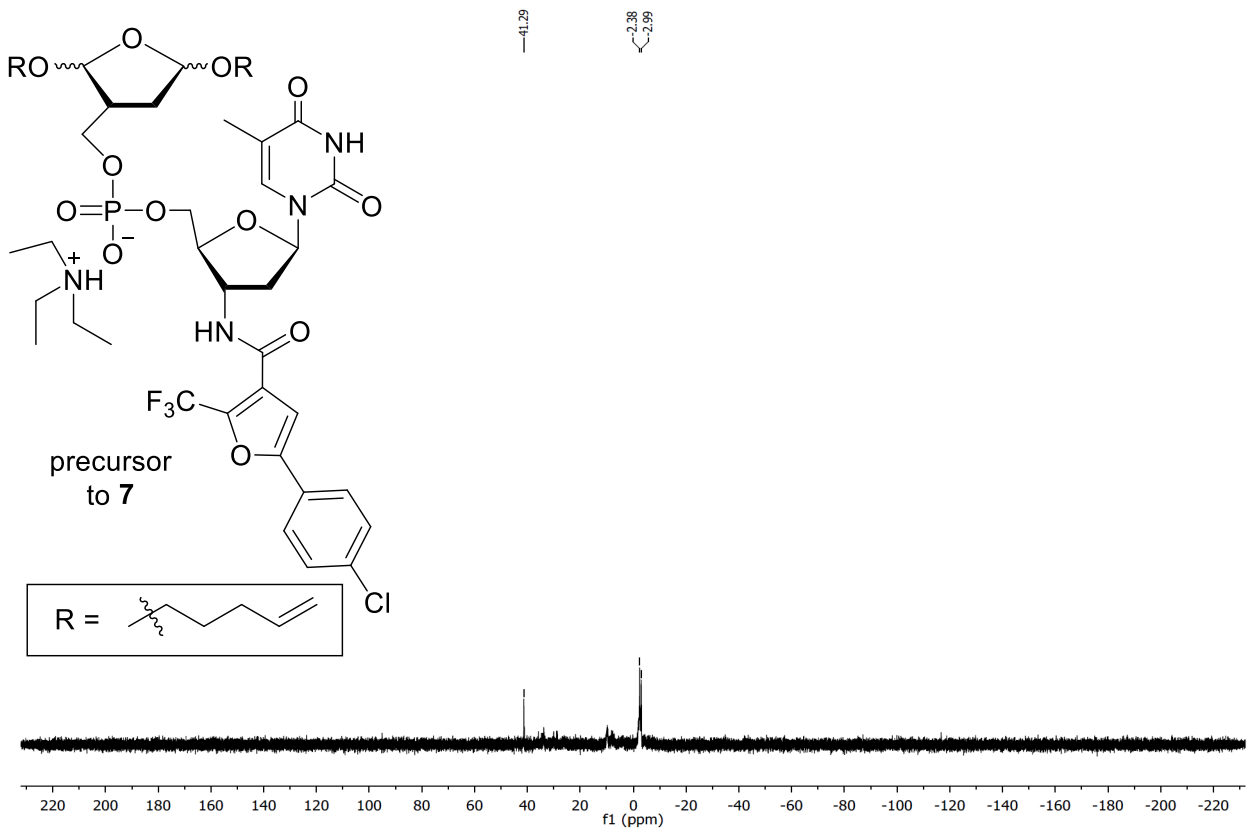


Figure S22. ^{31}P NMR spectra of precursor to **7**

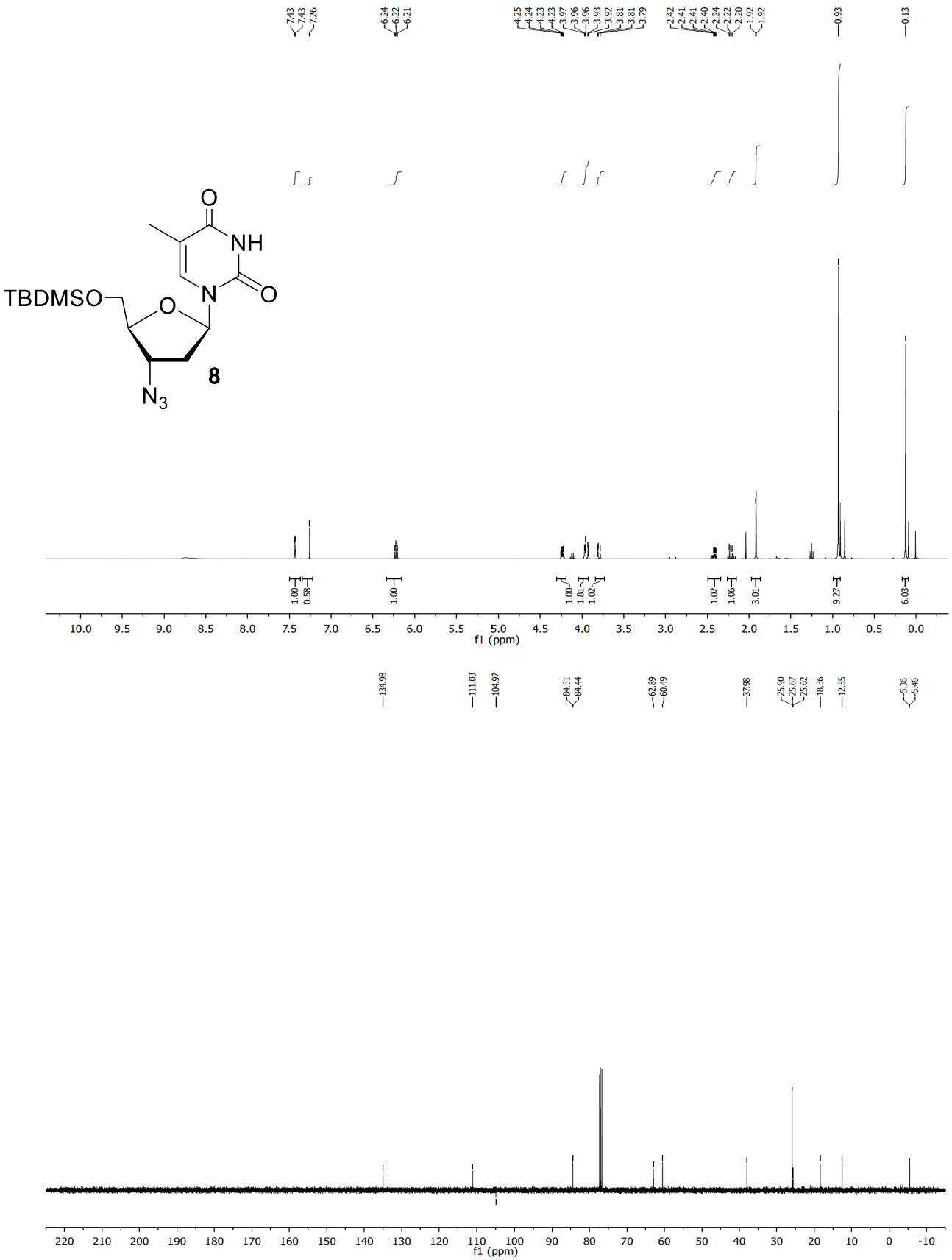


Figure S23. ^{1}H and ^{13}C NMR spectra of **8**

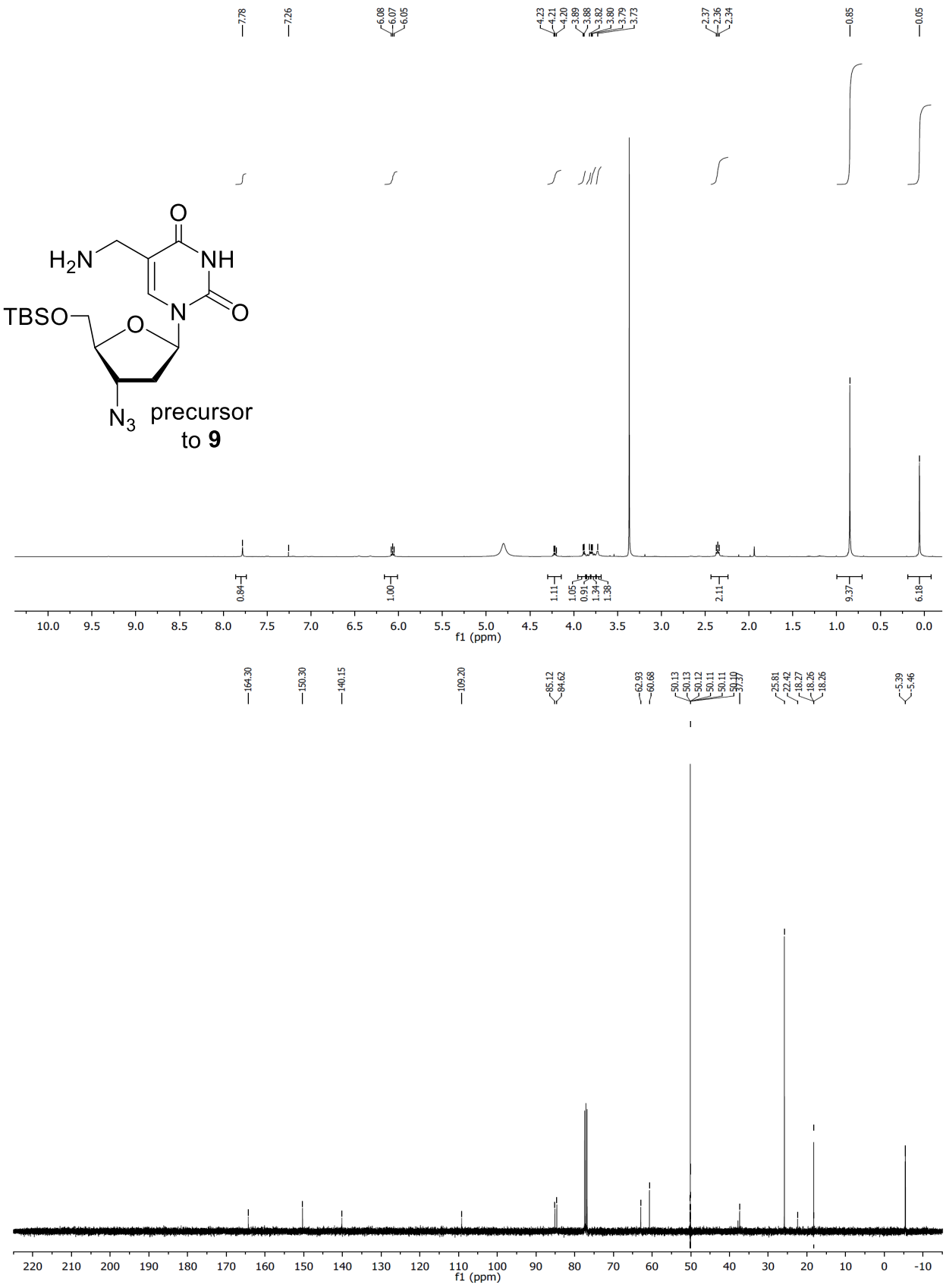


Figure S24. ^{1}H and ^{13}C NMR spectra of precursor to **9**

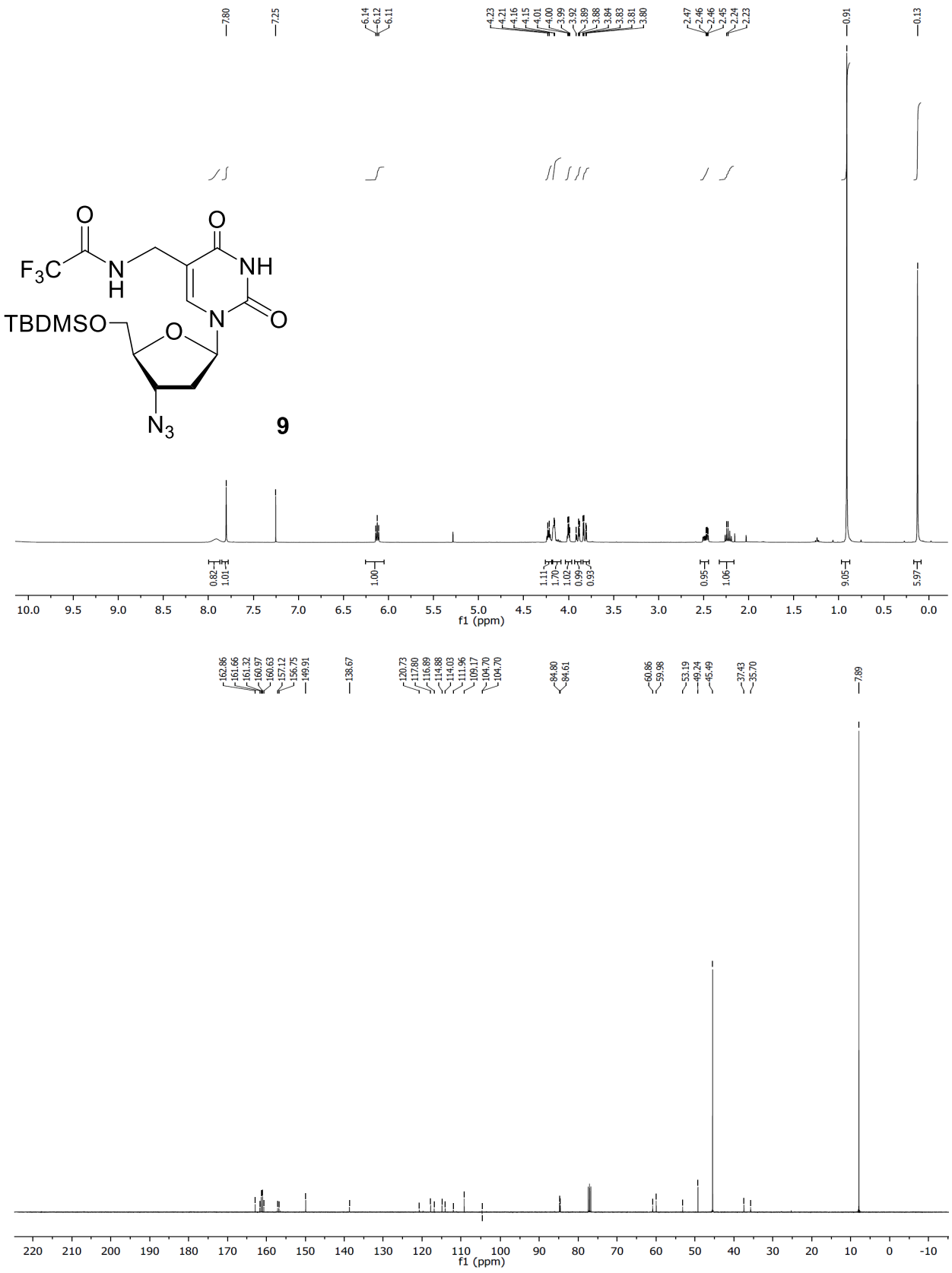


Figure S25. ^{1}H and ^{13}C NMR spectra of **9**

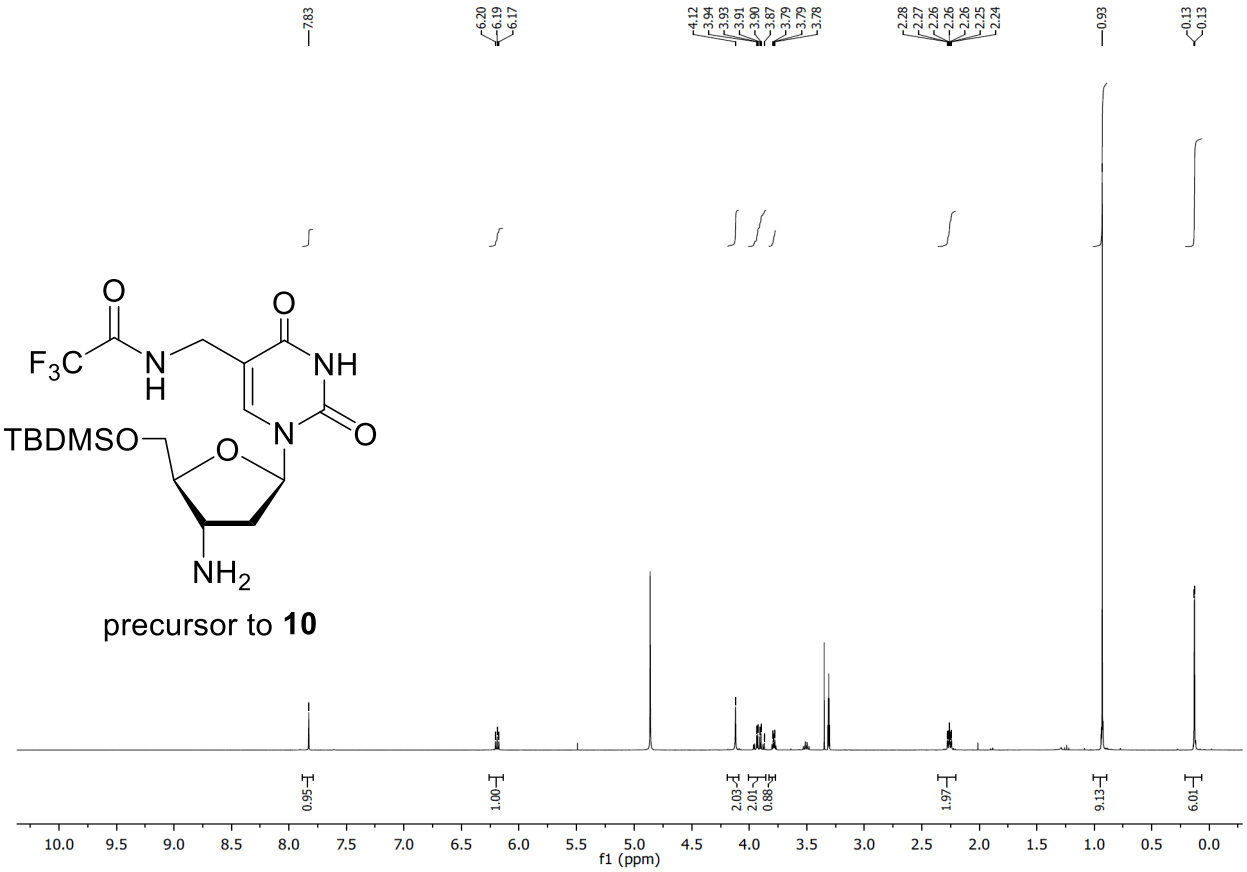


Figure S26. ^{1}H NMR spectra of precursor to **10**

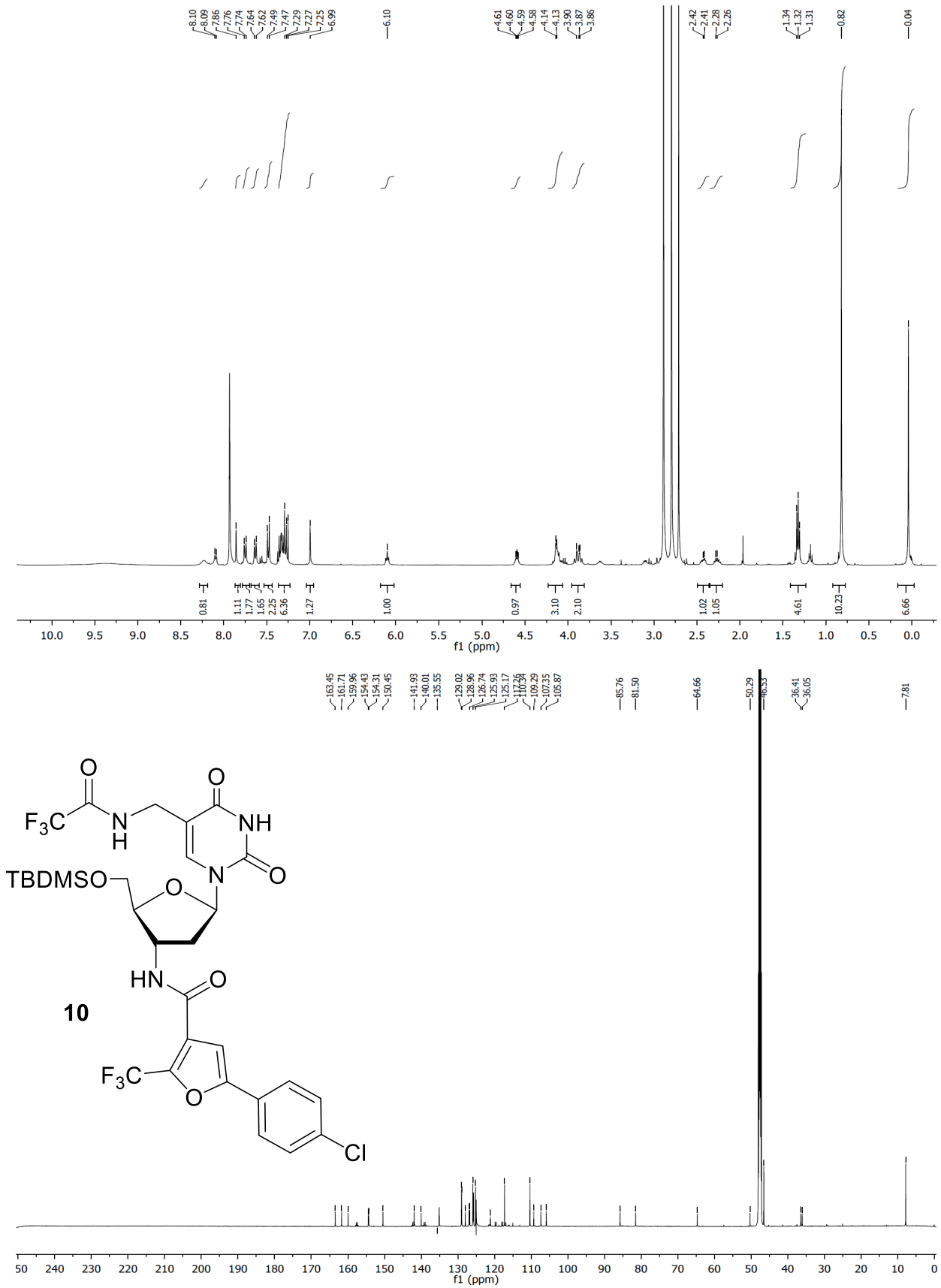


Figure S27. ^{1}H and ^{13}C NMR spectra of **10**

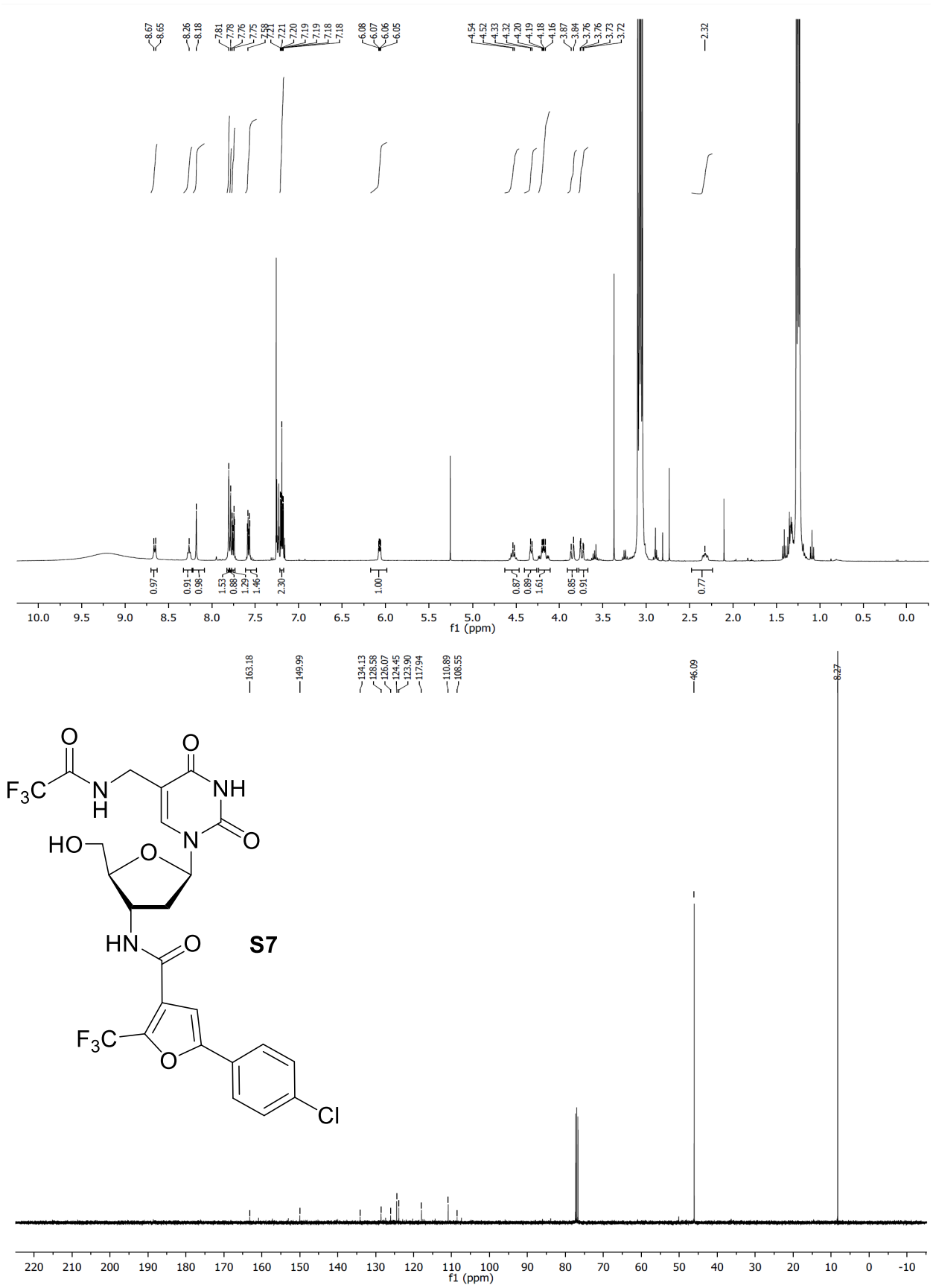


Figure S28. ^{1}H and ^{13}C NMR spectra of precursor to **S7**

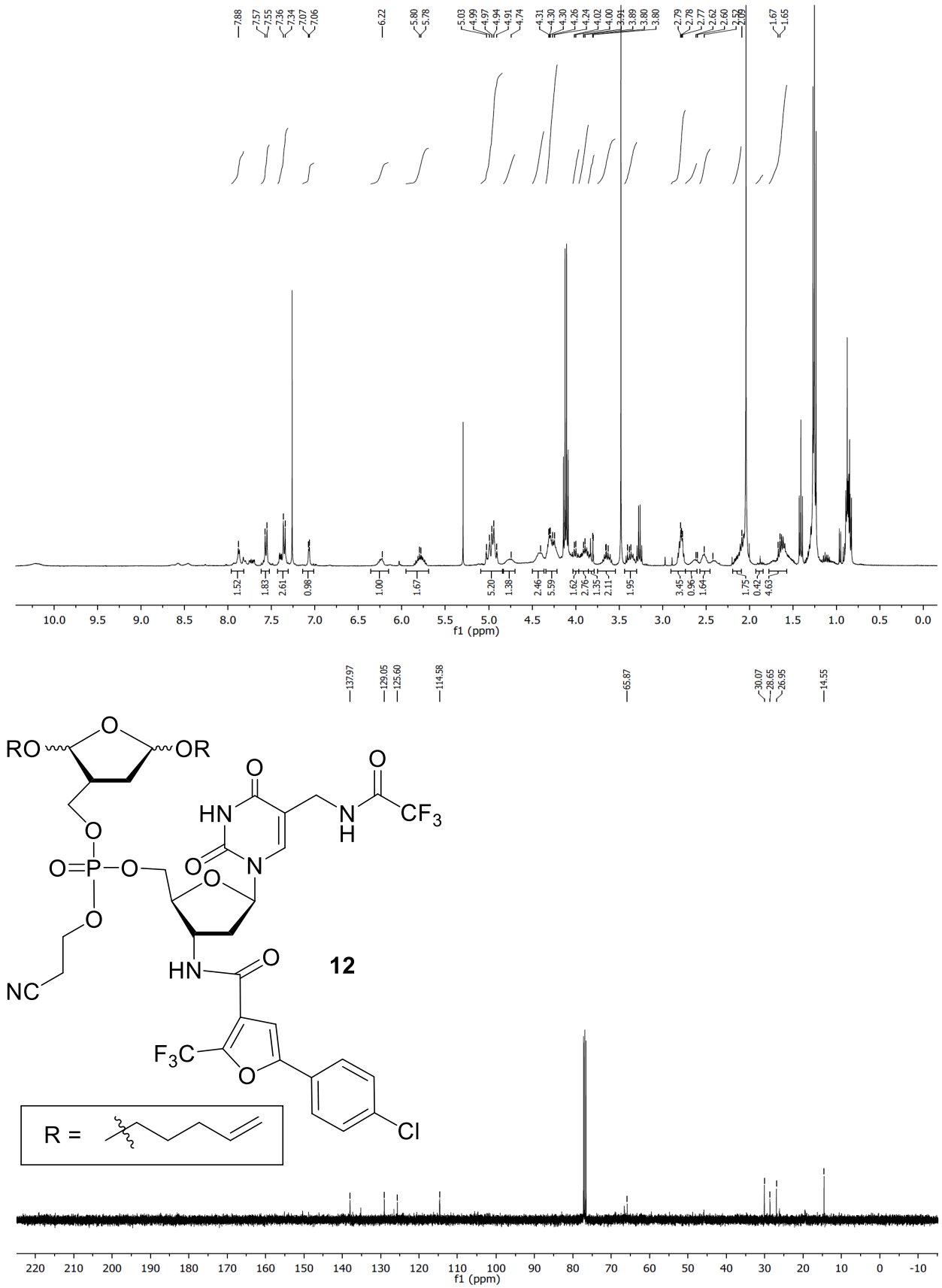
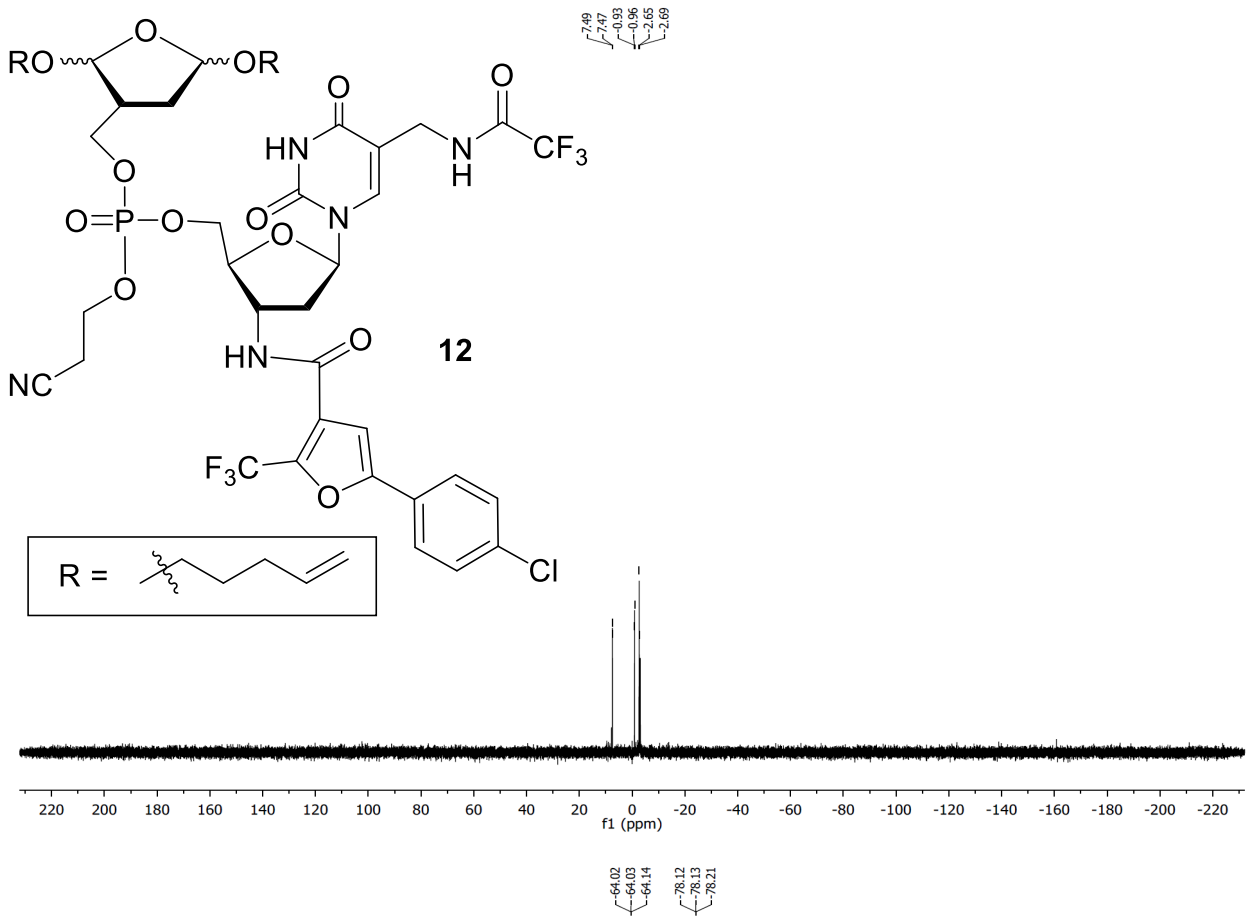


Figure S29. ^{1}H and ^{13}C NMR spectra of **12**



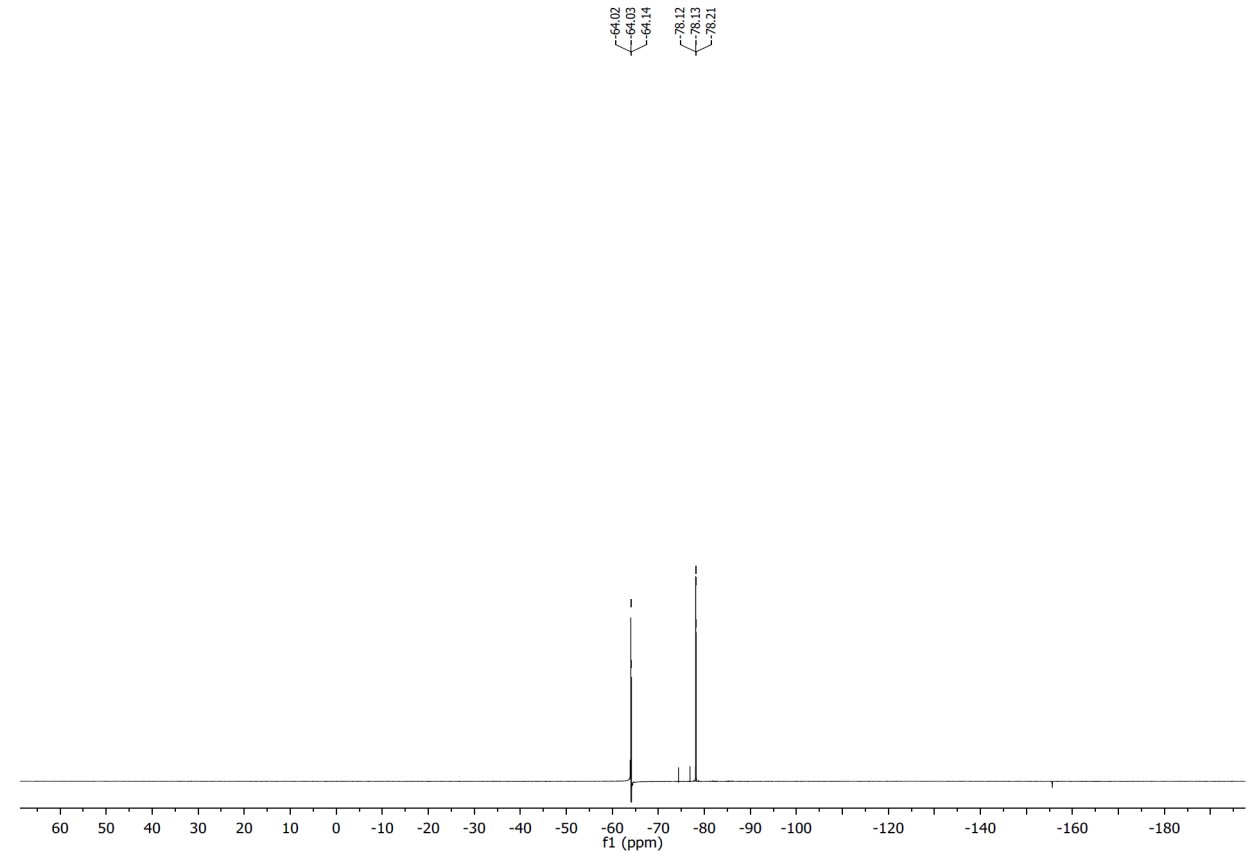


Figure S30. ^{31}P and ^{19}F NMR spectra of **12**

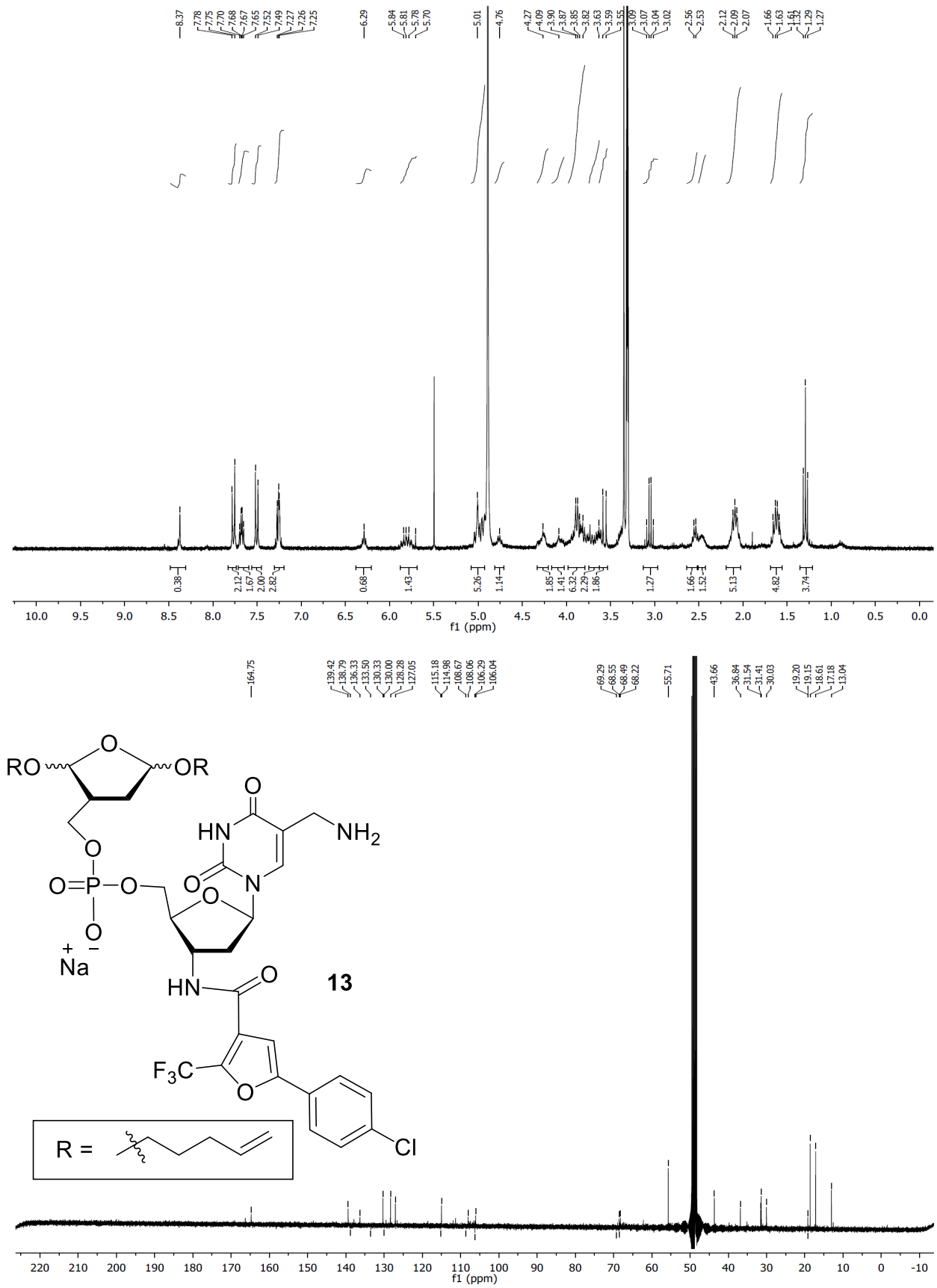


Figure S31. ^{1}H and ^{13}C NMR spectra of **13**

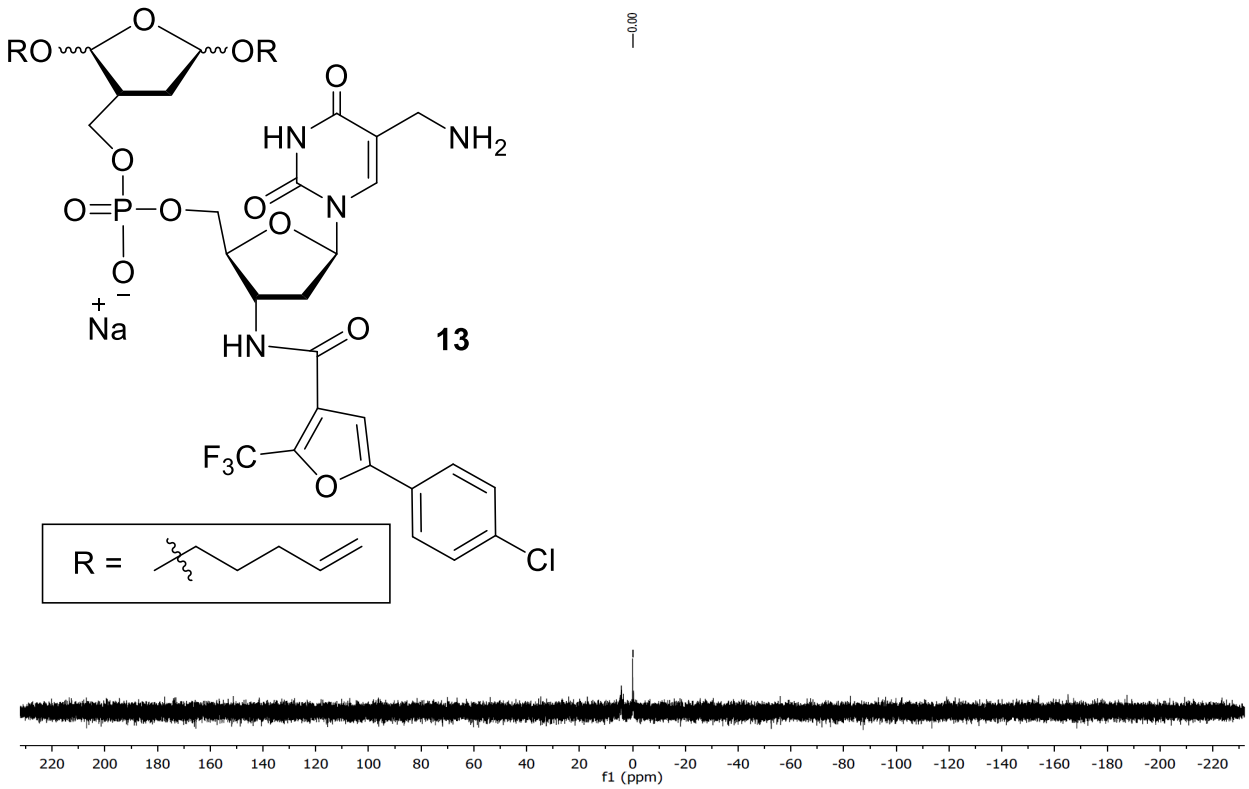


Figure S32. ^{31}P NMR spectra of **13**

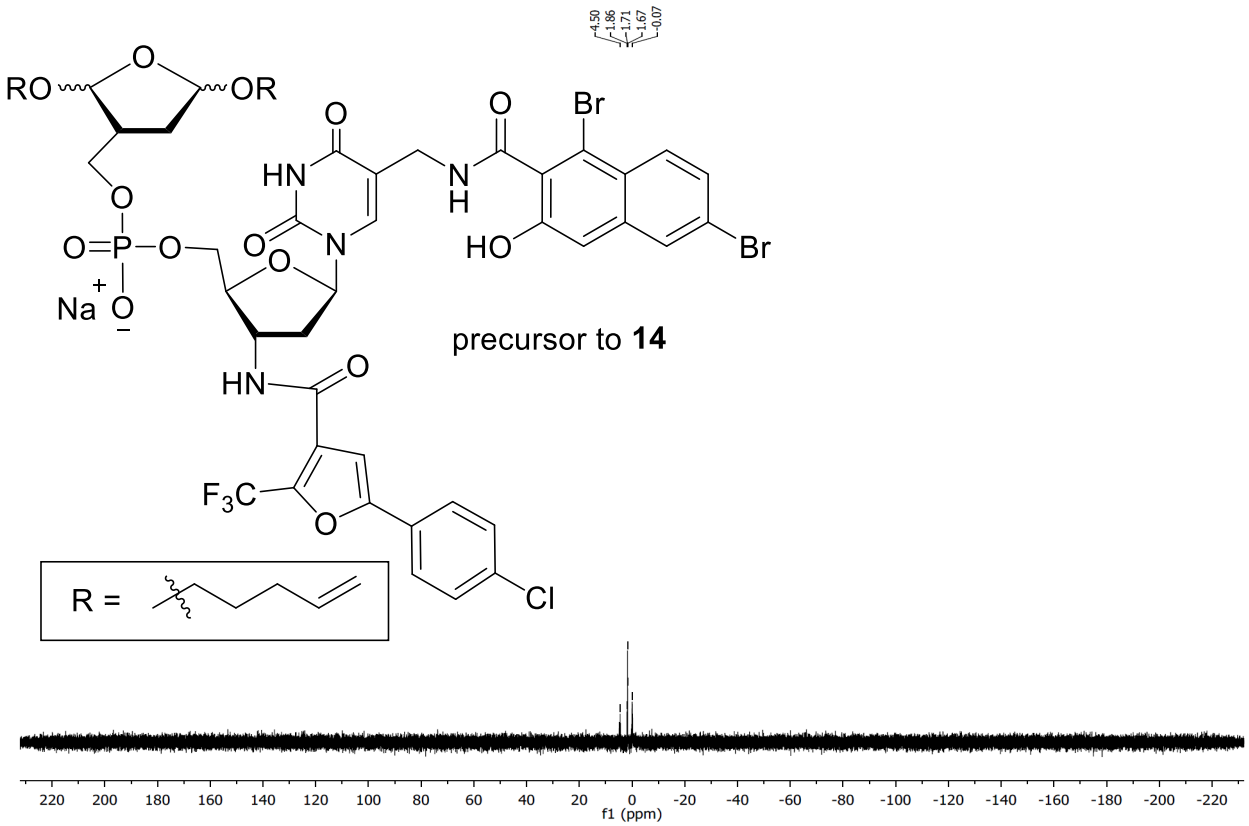


Figure S33. ^{31}P NMR spectra of precursor to **14**

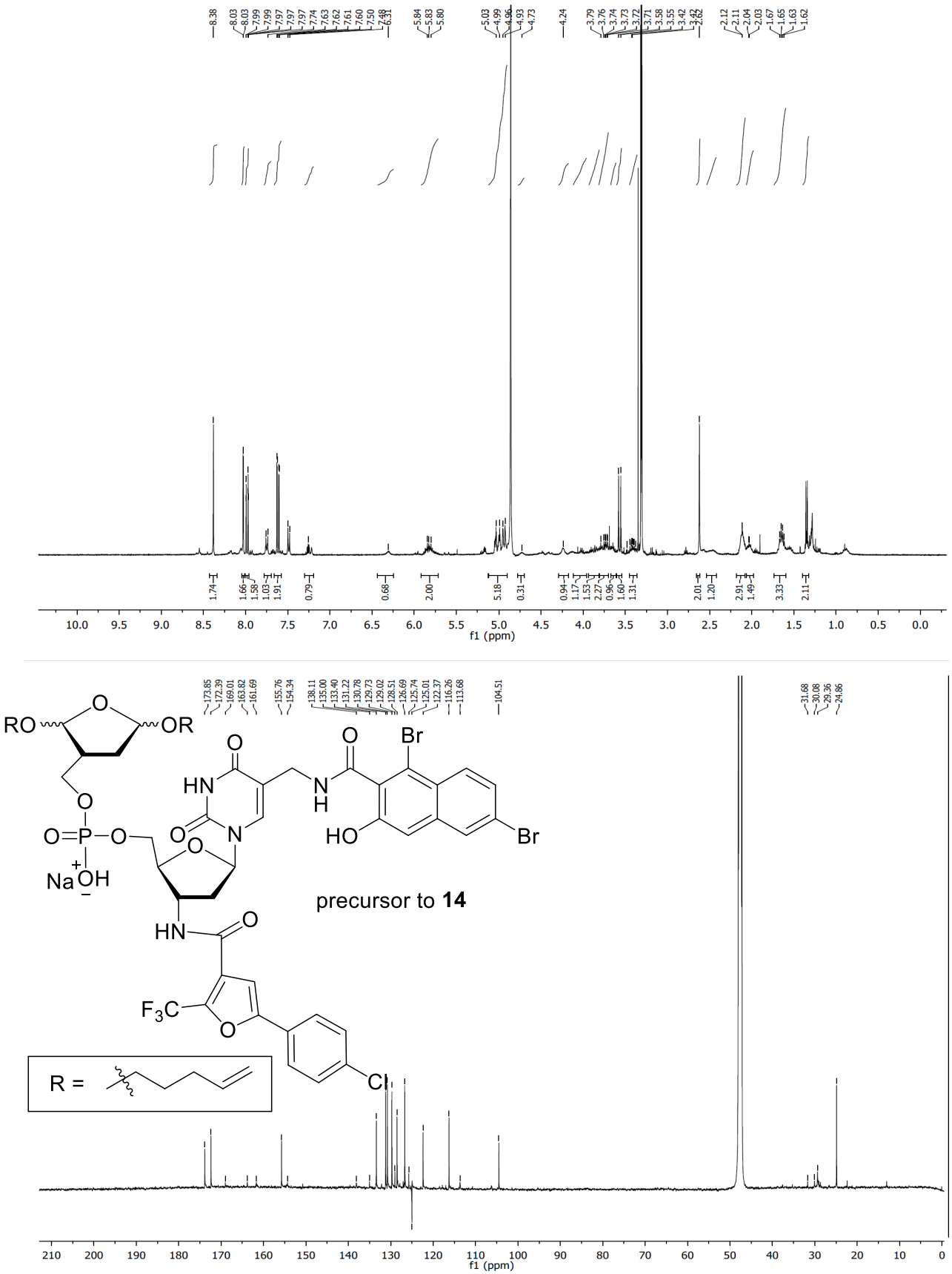


Figure S34. ^{1}H and ^{13}C NMR spectra of precursor to **14**

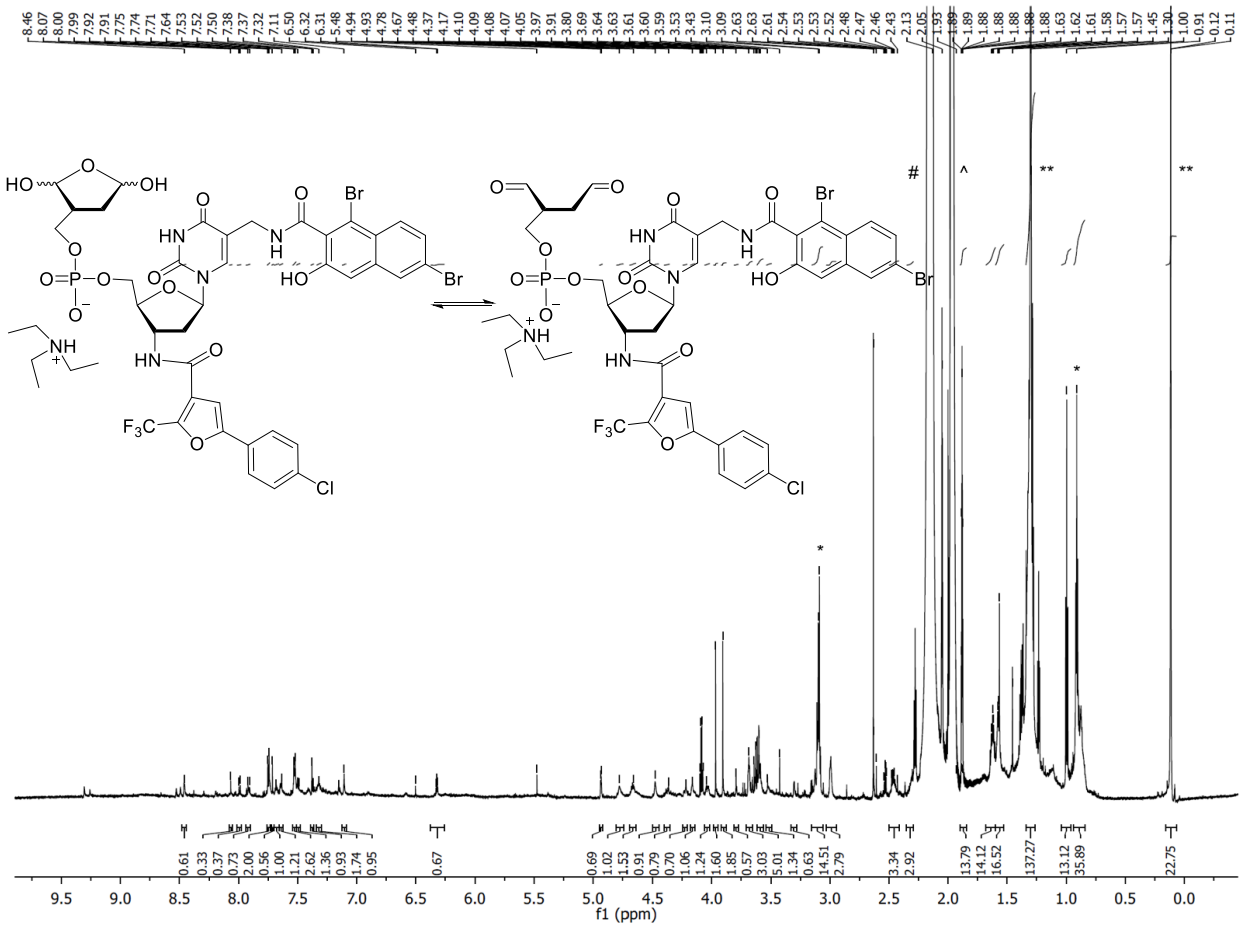


Figure S35. ^{1}H NMR spectra of **14**

^ CD_3CN, # H_2O, * triethyl ammonium salt, ** impurity from HPLC purification

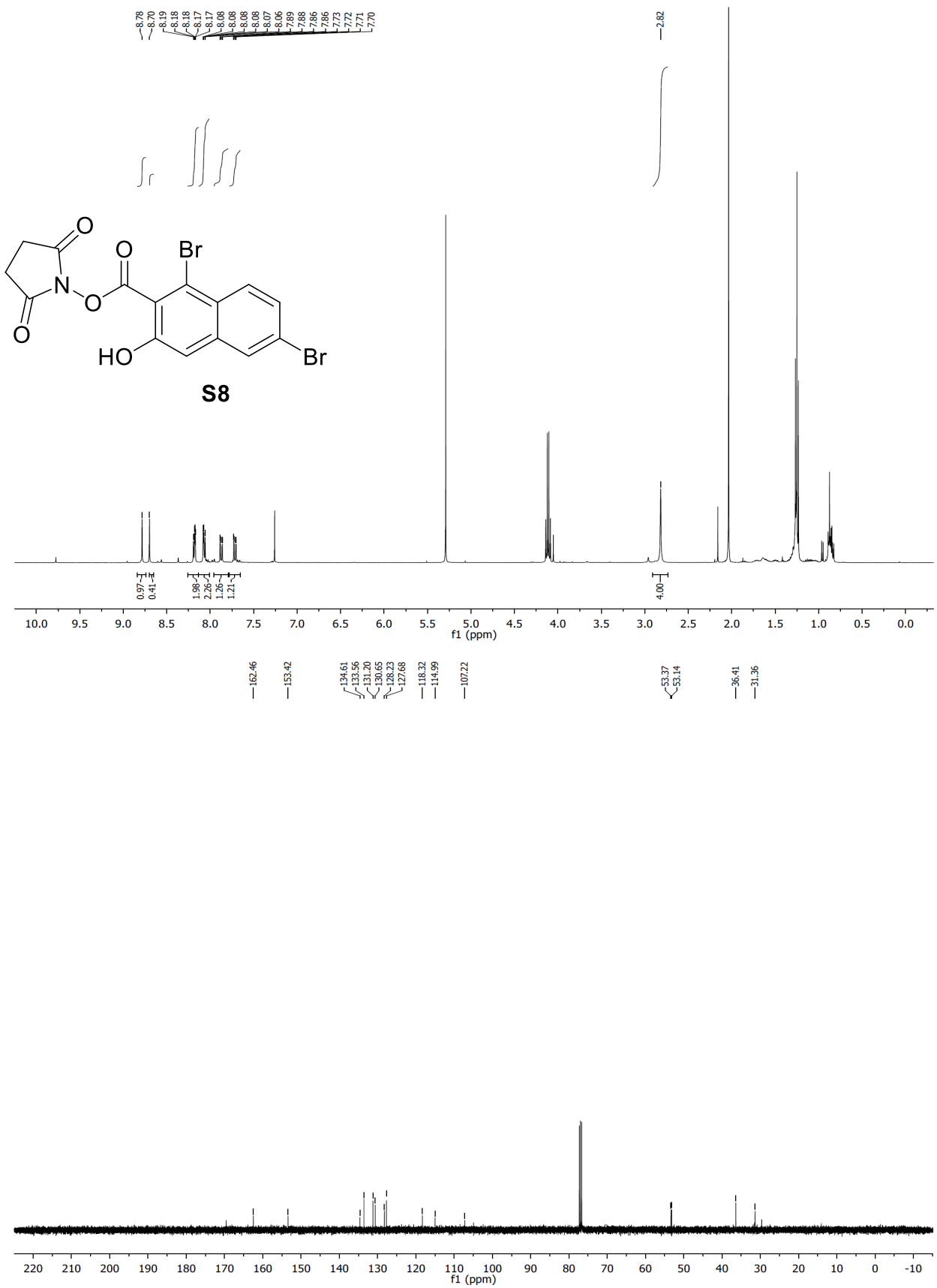


Figure S36. ^{1}H and ^{13}C NMR spectra of **S8**

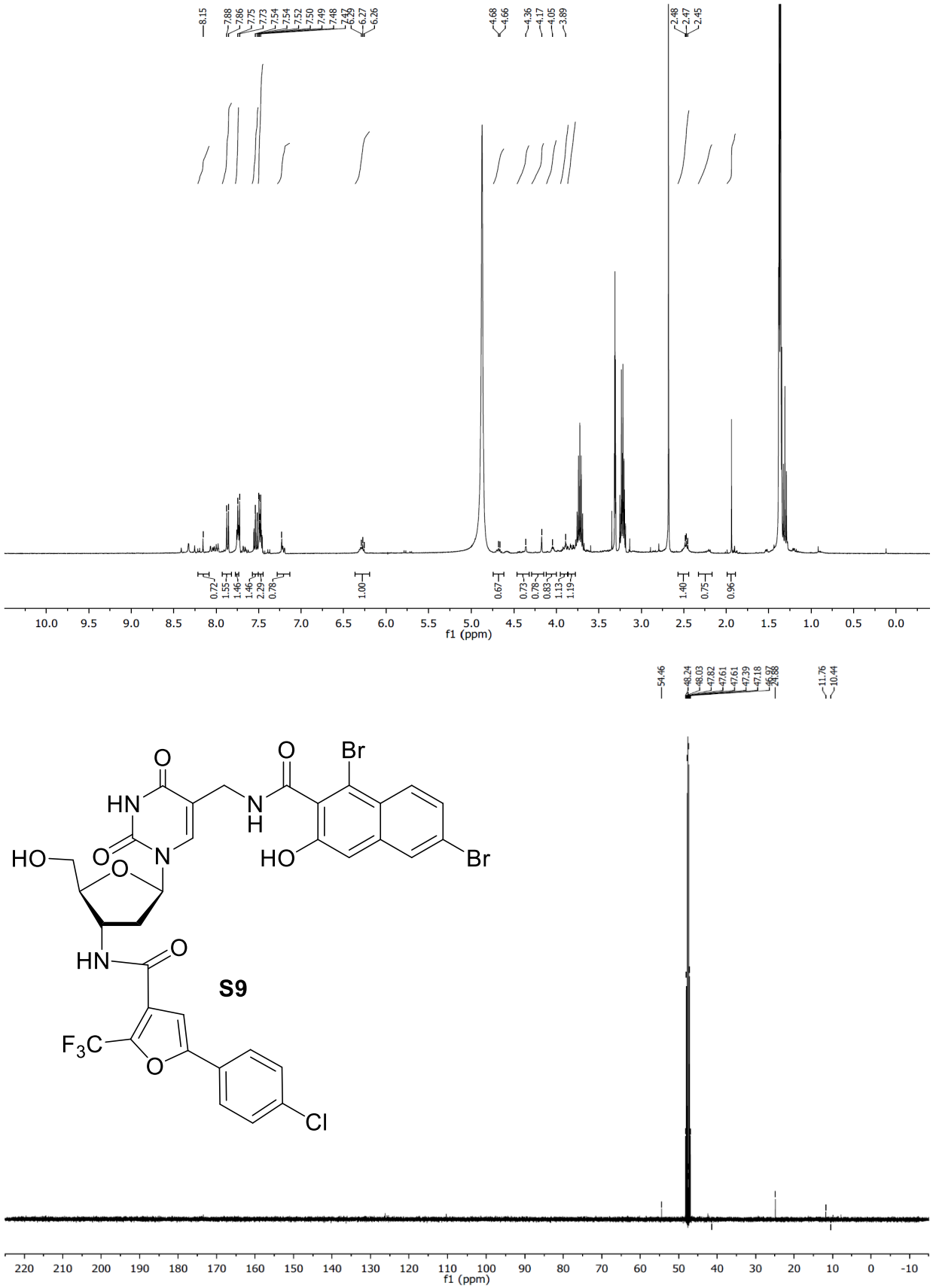


Figure S37. ^{1}H and ^{13}C NMR spectra of **S9**

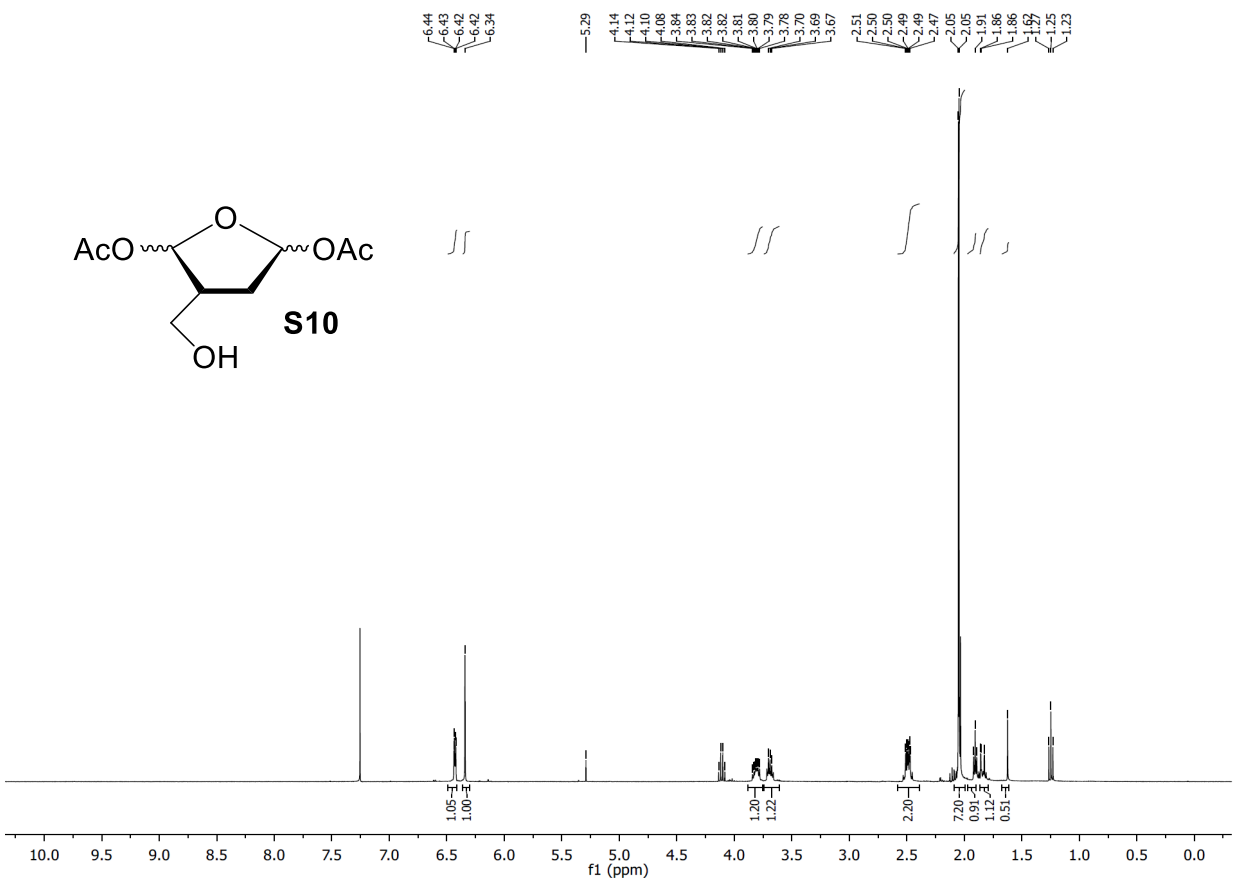


Figure S38. ^{1}H NMR spectra of **S10**

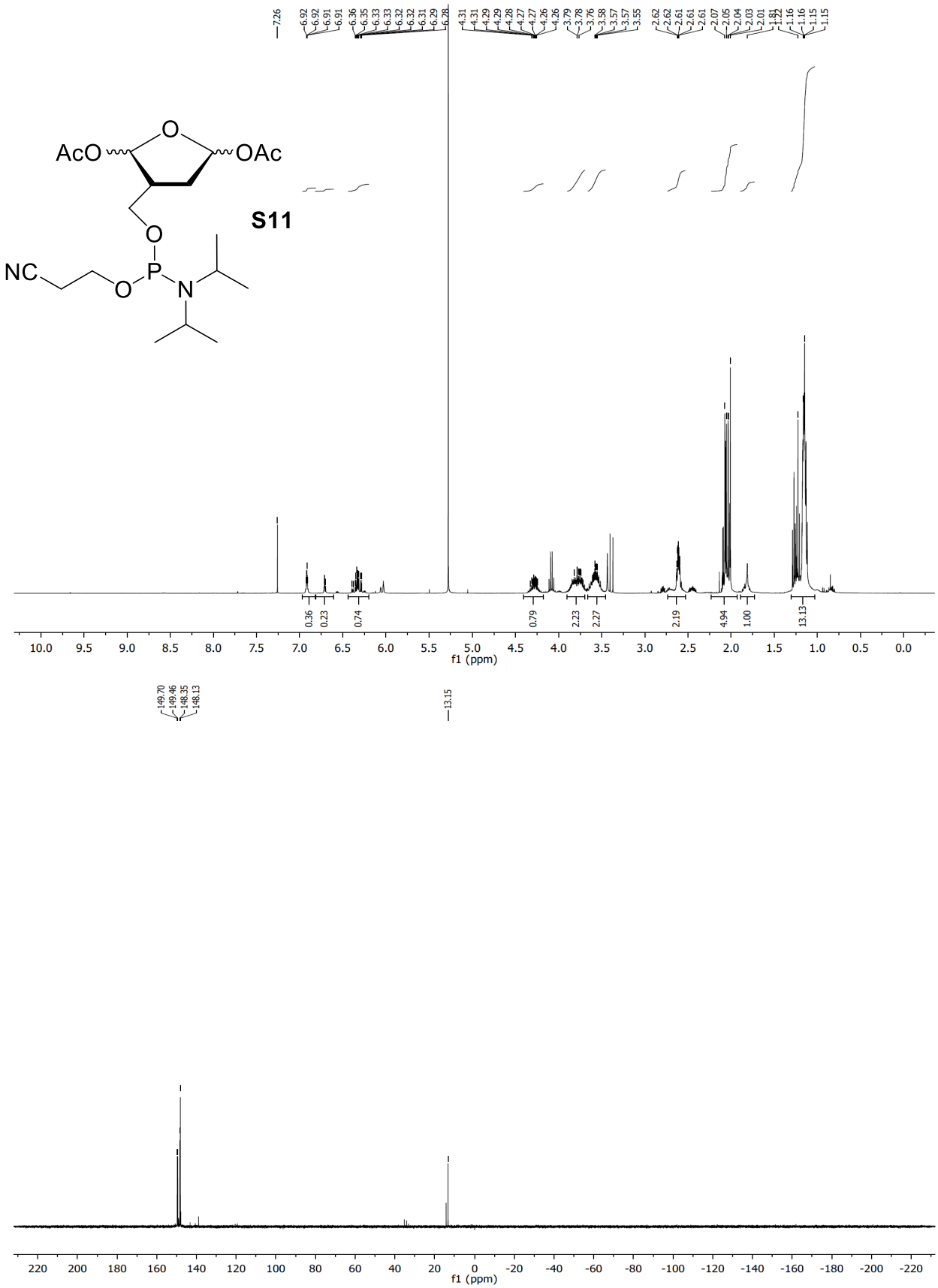


Figure S39. ^{1}H and ^{31}P NMR spectra of **S11**

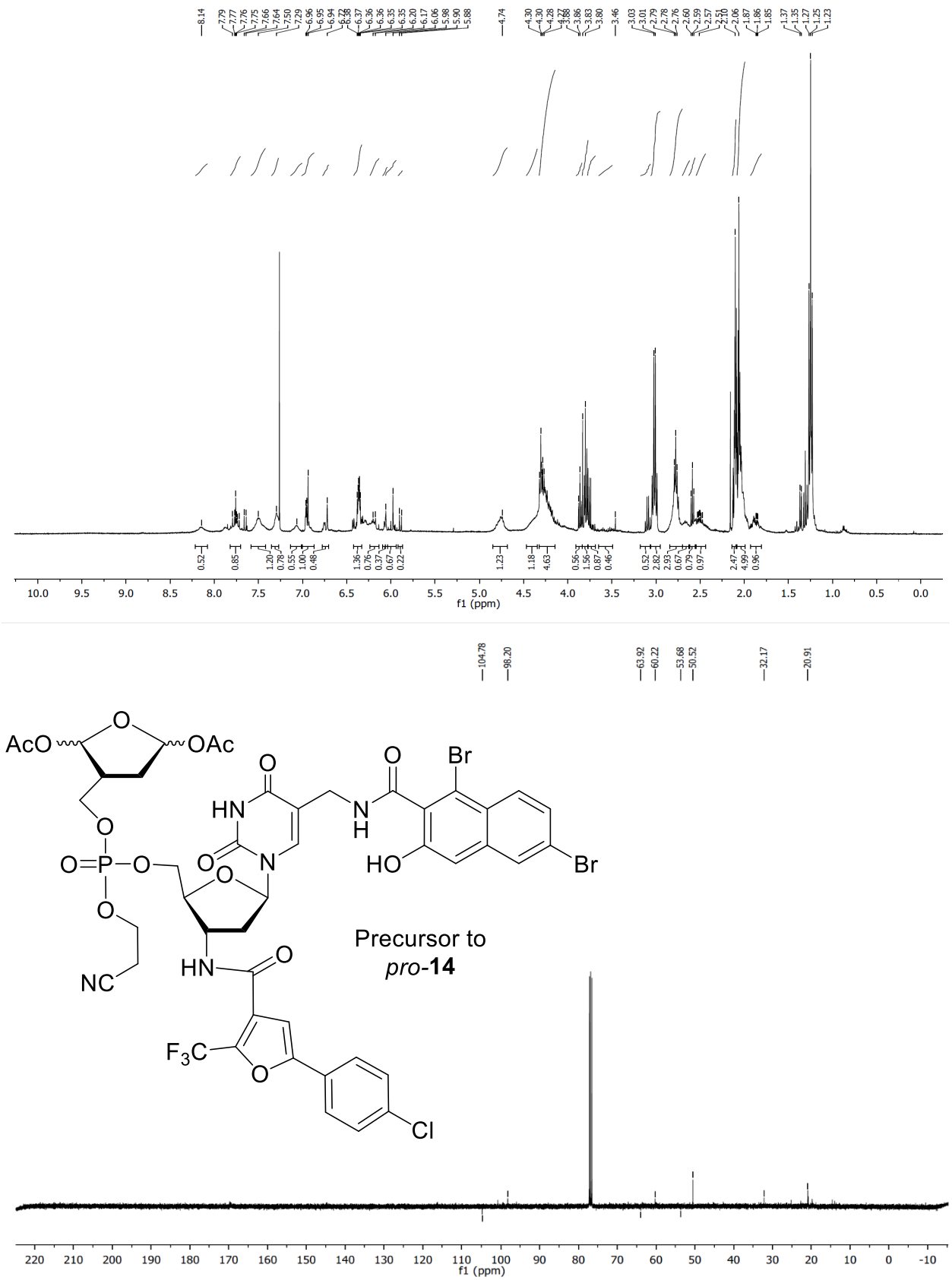


Figure S40. ^{1}H and ^{13}C NMR spectra of precursor to *pro*-**14**

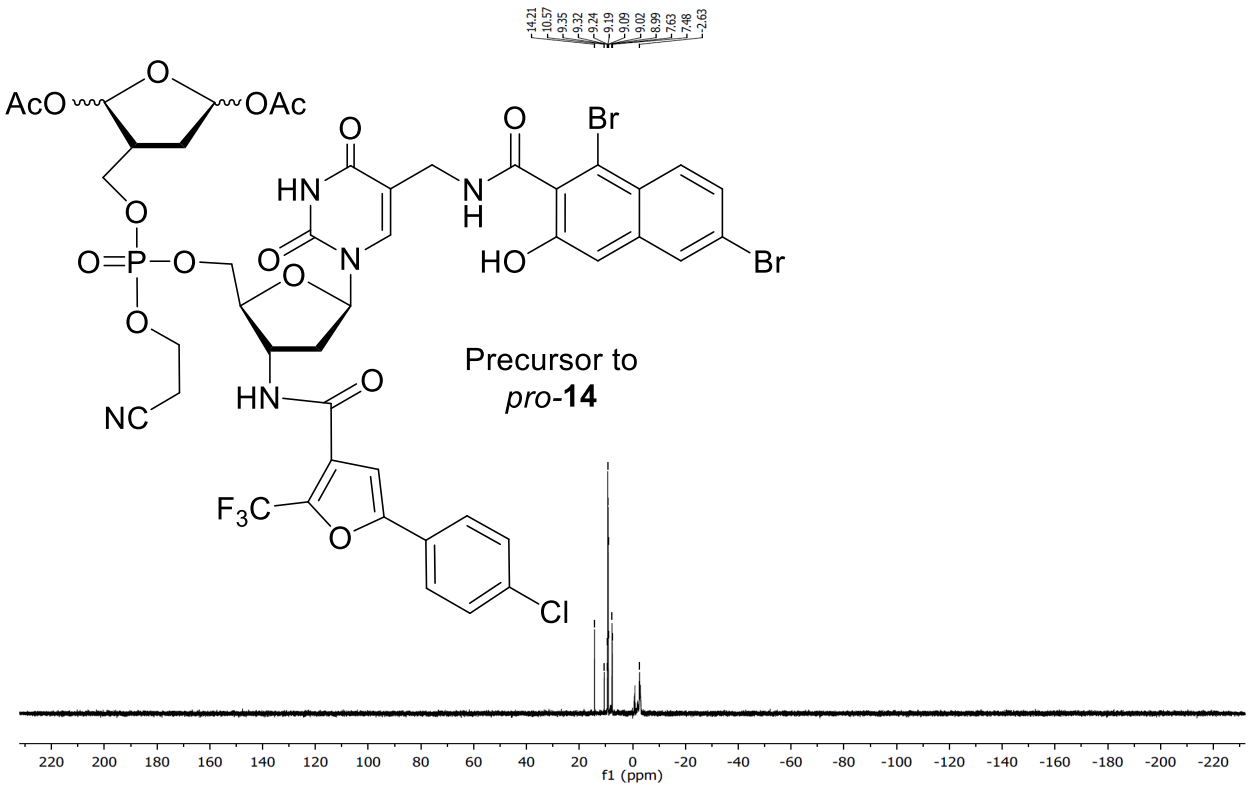


Figure S41. ^{31}P NMR spectra of precursor to *pro*-**14**

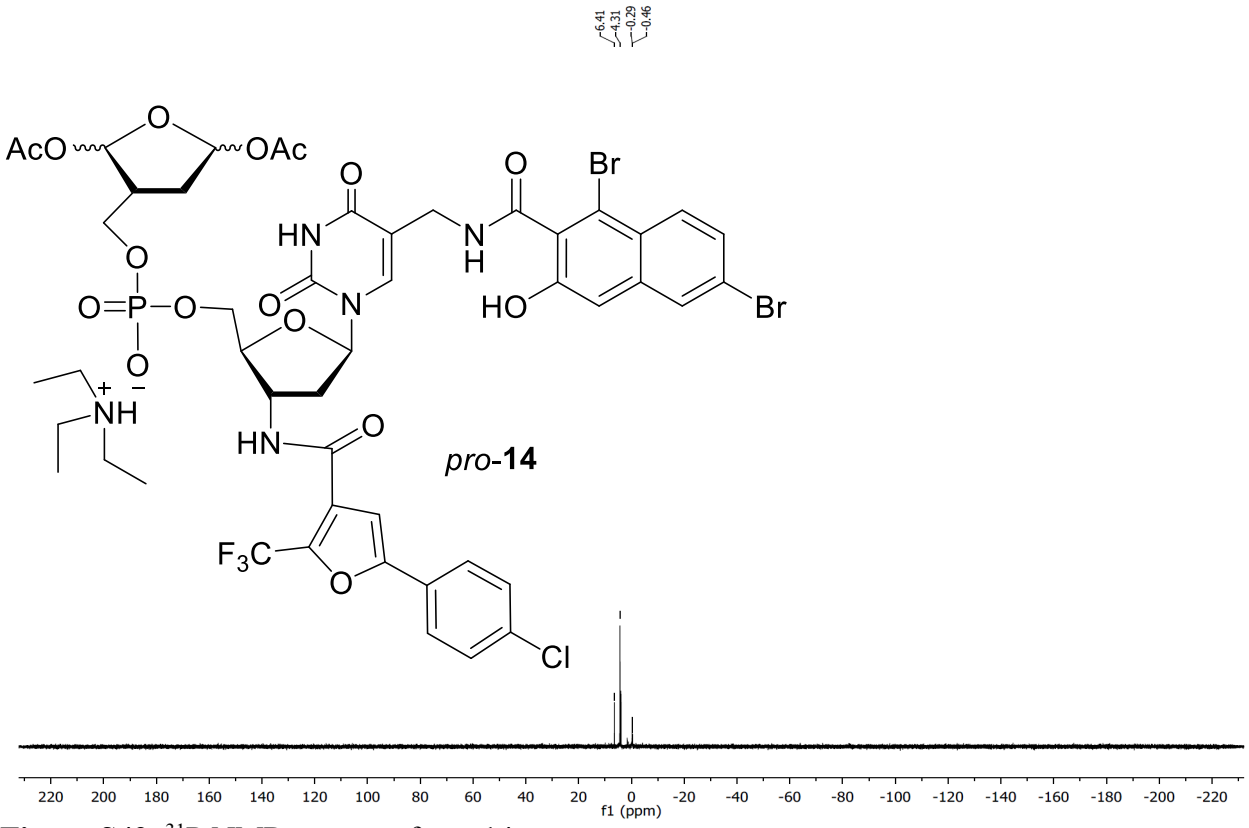


Figure S42. ^{31}P NMR spectra of *pro*-**14**

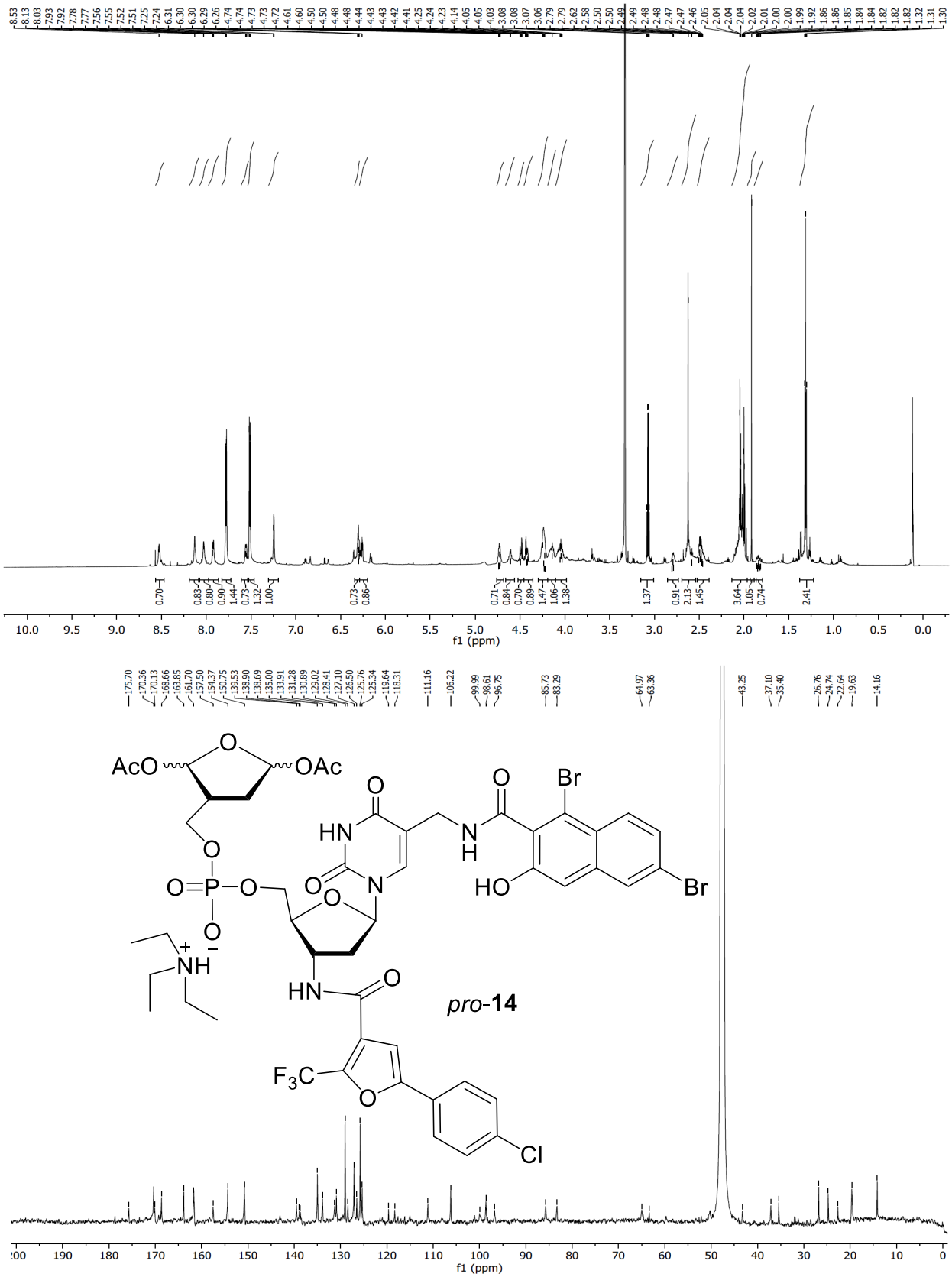


Figure S43. ^{1}H and ^{13}C NMR spectra of *pro*-**14**

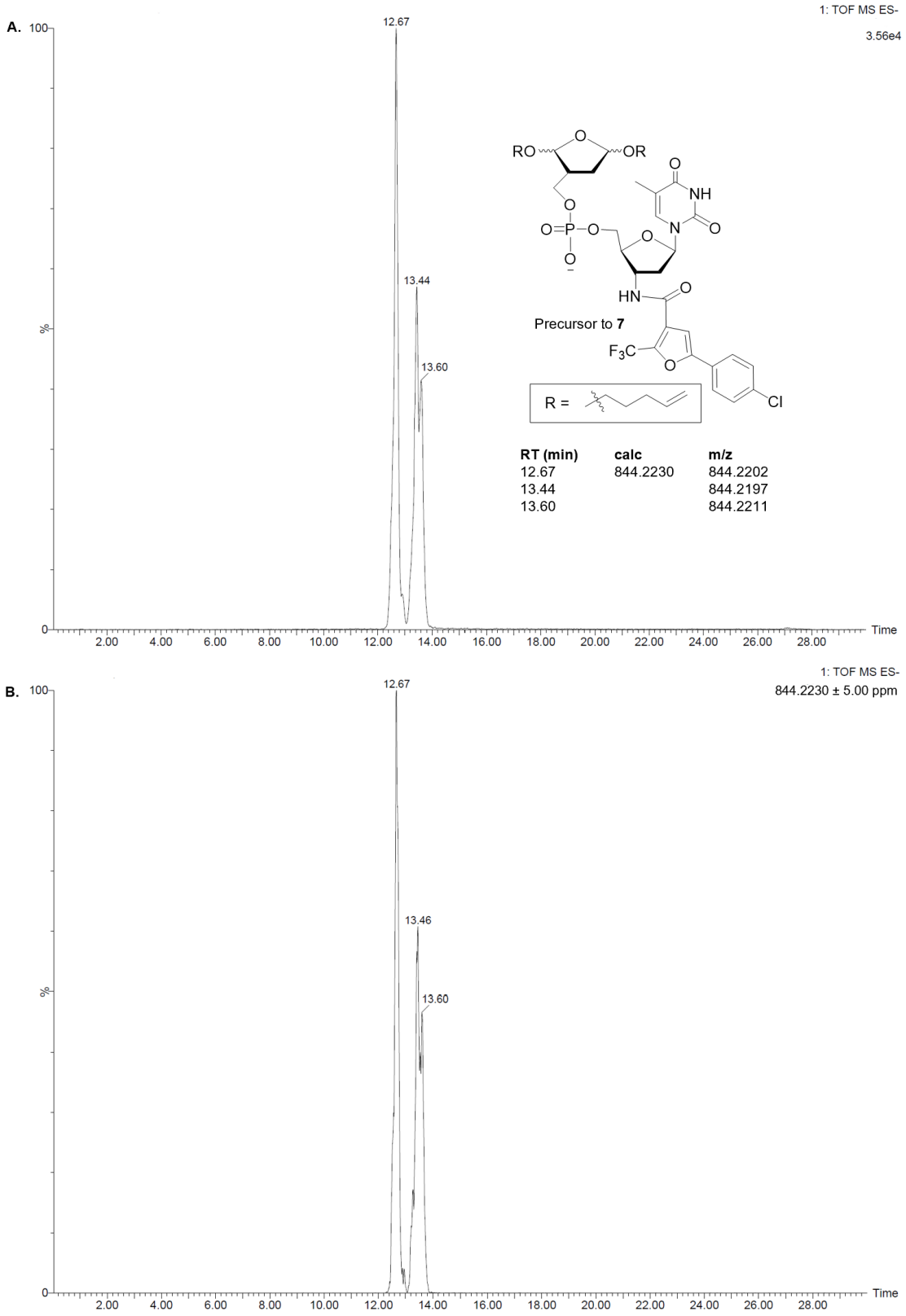


Figure S44. UPLC-MS analysis of precursor to **7**. A) Total ion chromatogram. B) Extracted ion chromatogram of the molecular ion (*m/z* = 844.2230 ± 5.00 ppm).

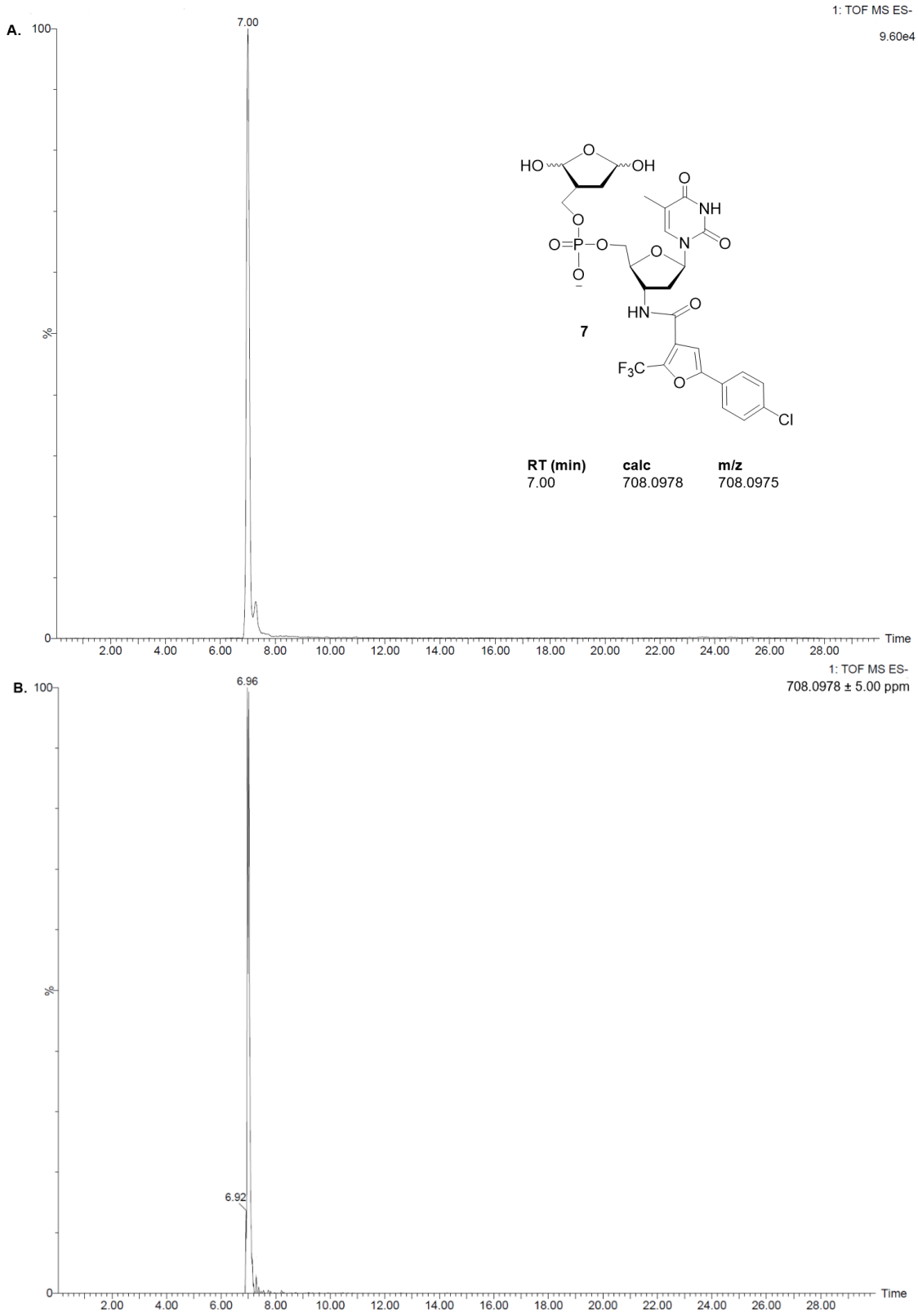


Figure S45. UPLC-MS analysis of **7**. A) Total ion chromatogram. B) Extracted ion chromatogram of the molecular ion (*m/z* = 708.0978 ± 5.00 ppm).

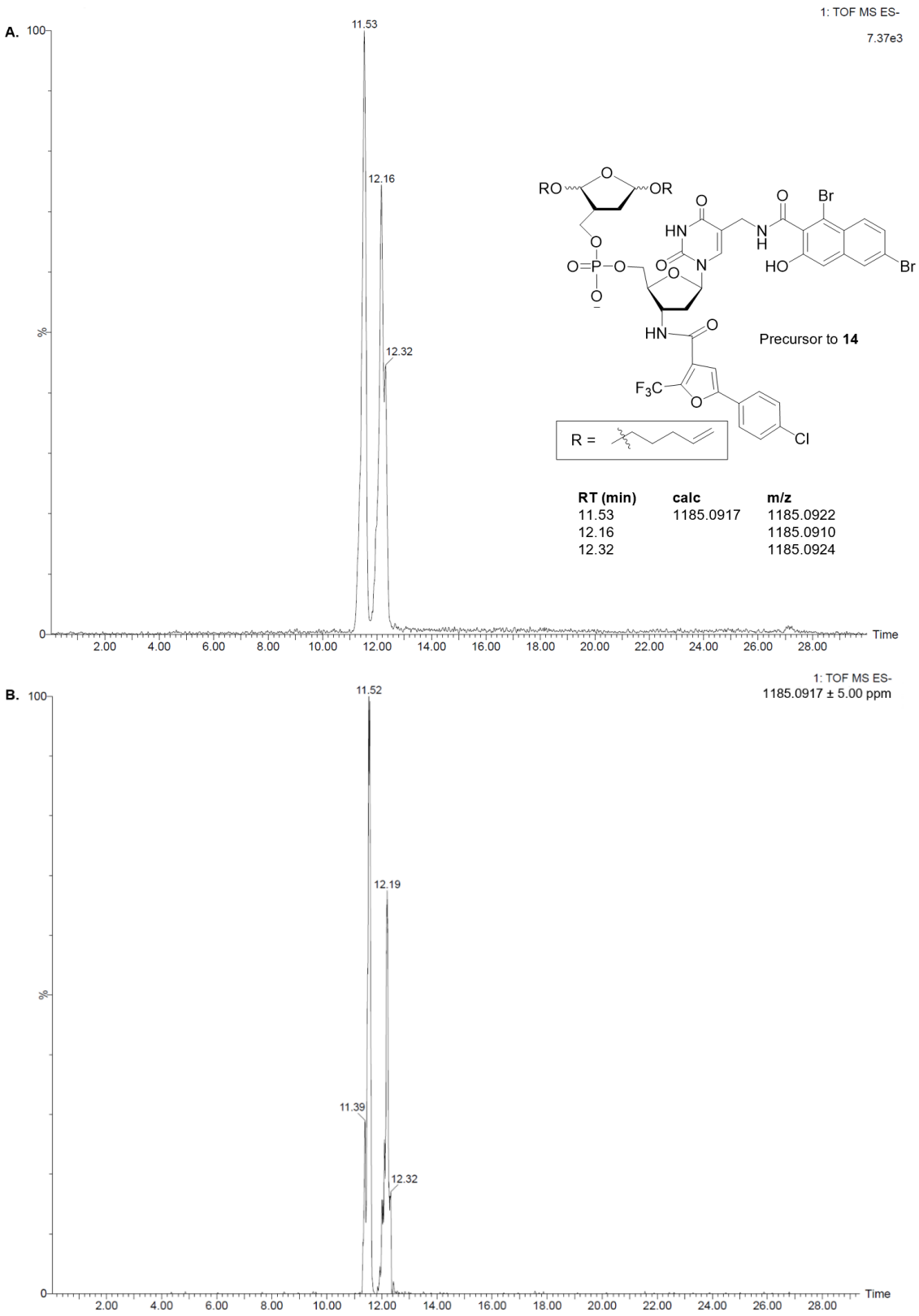


Figure S46. UPLC-MS analysis of precursor to **14**. A) Total ion chromatogram. B) Extracted ion chromatogram of the molecular ion (*m/z* = 1185.0917 ± 5.00 ppm).

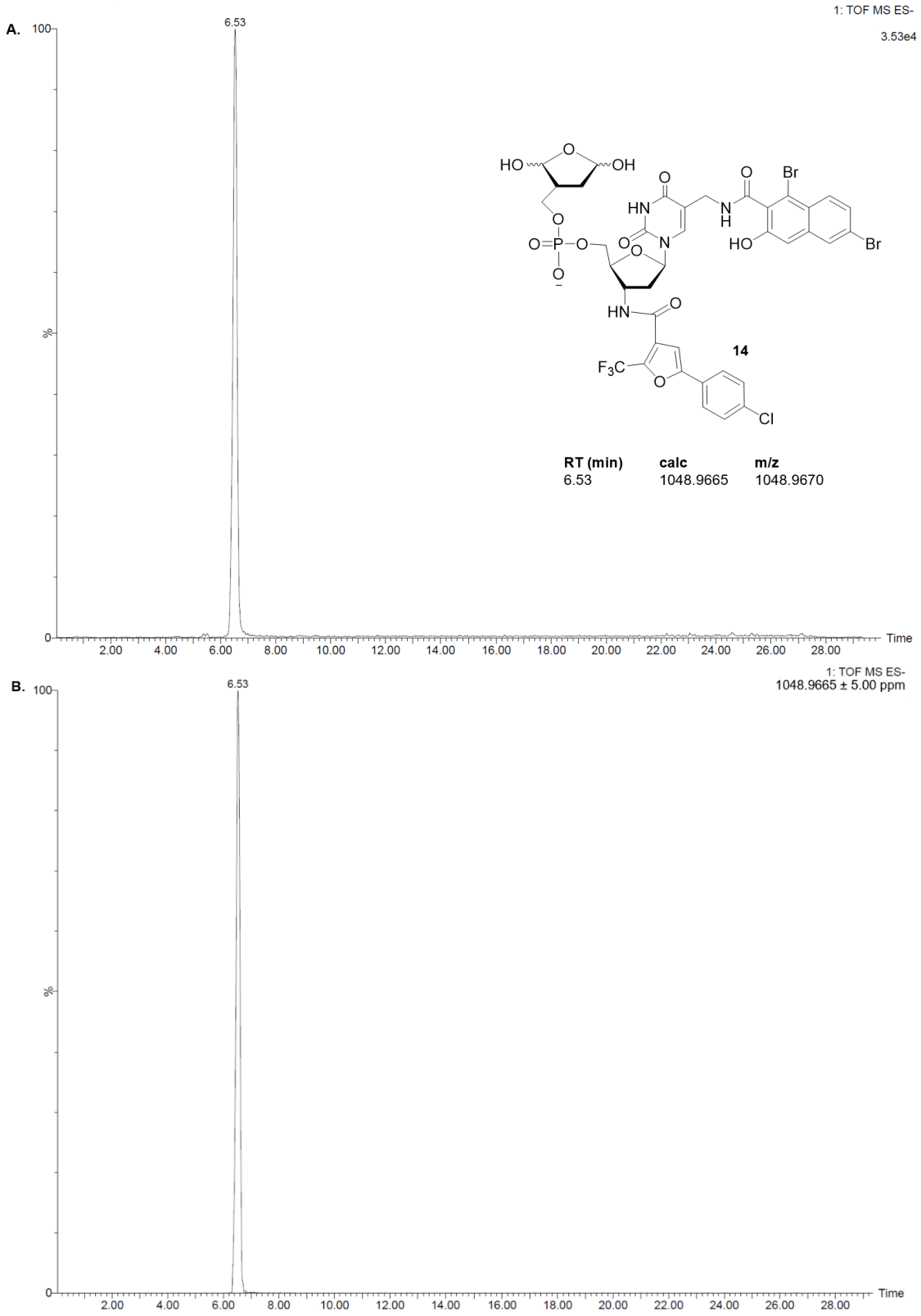


Figure S47. UPLC-MS analysis of **14**. A) Total ion chromatogram. B) Extracted ion chromatogram of the molecular ion (*m/z* = 1048.9665 ± 5.00 ppm).

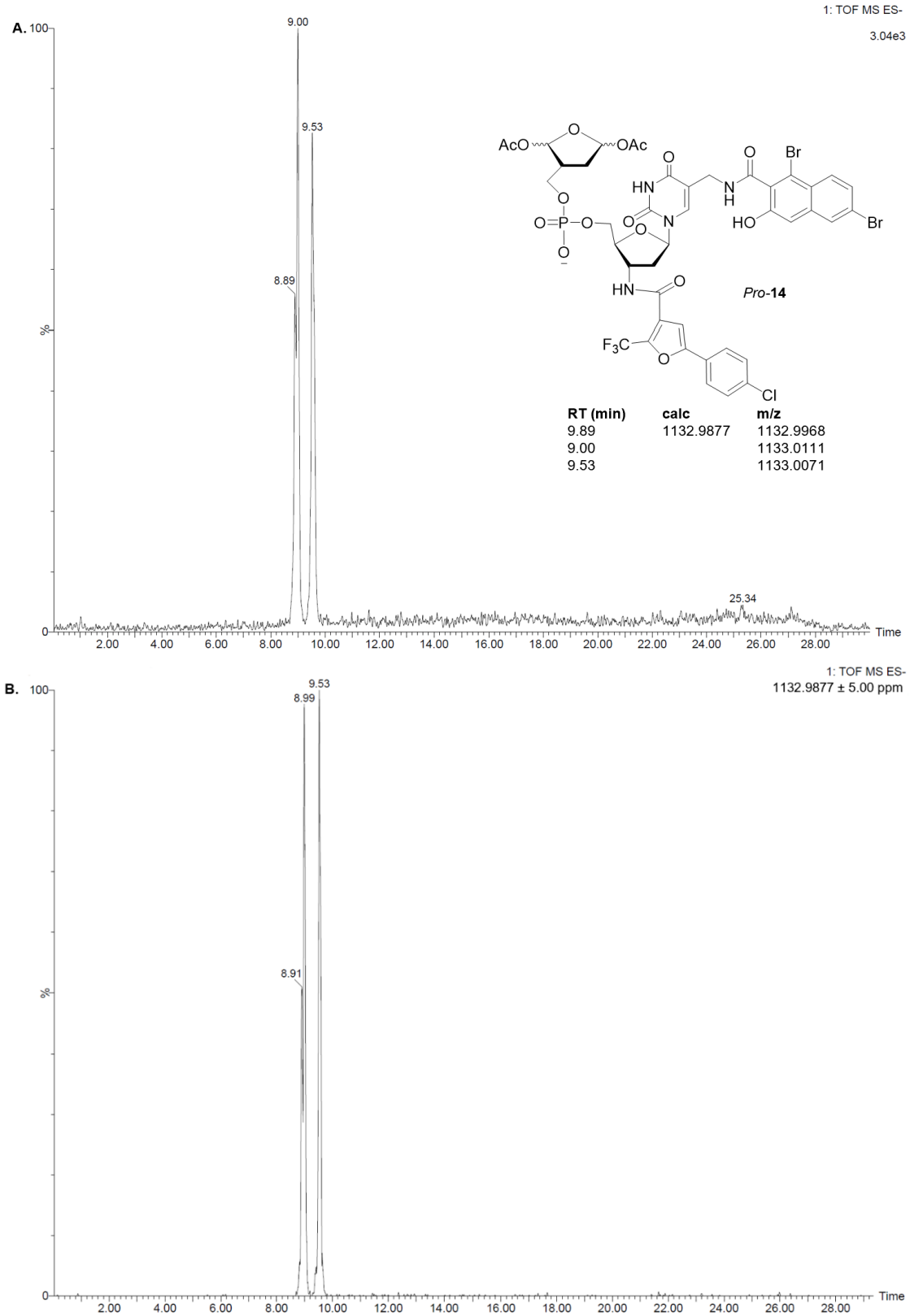
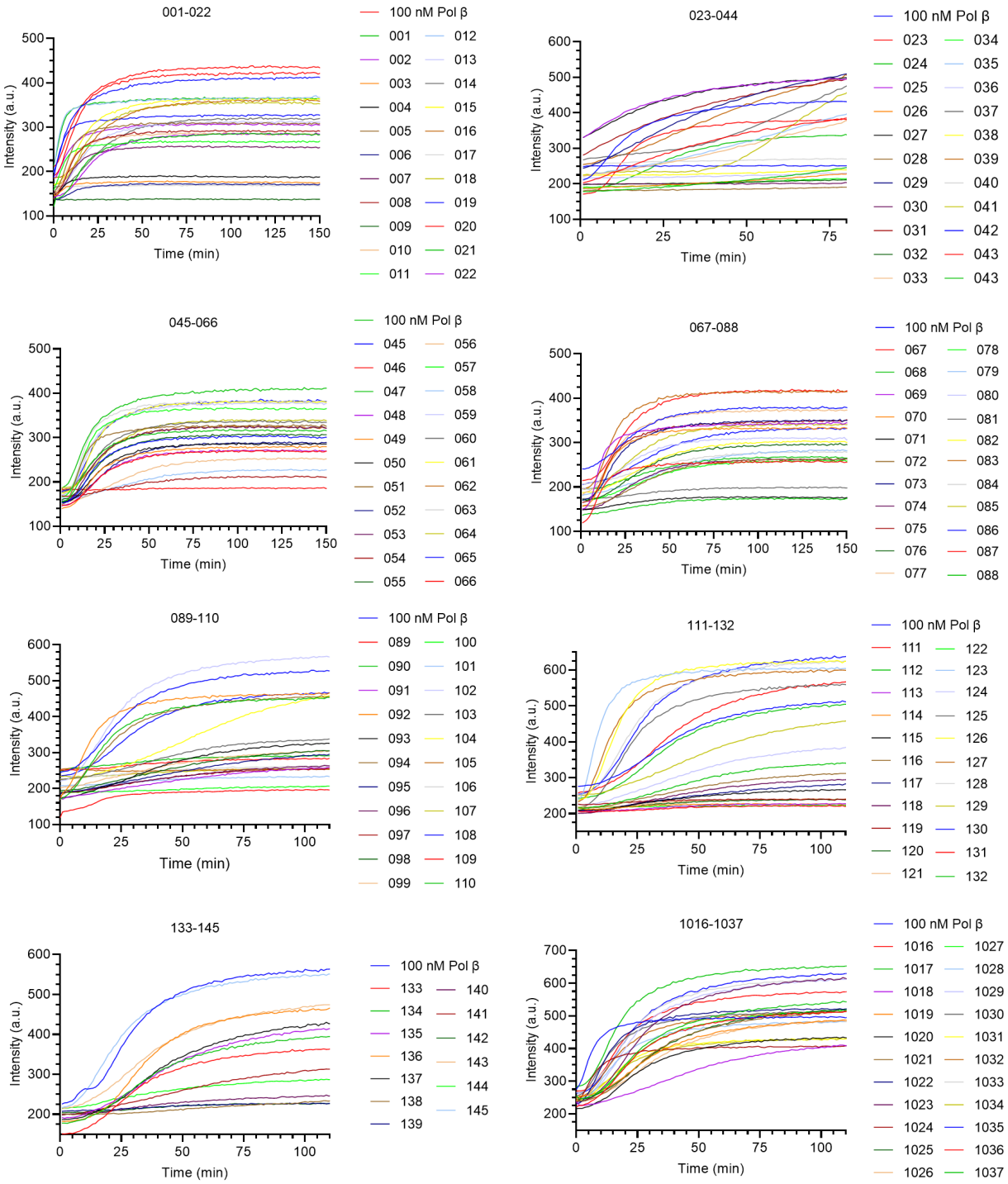


Figure S48. UPLC-MS analysis of *pro*-**14**. A) Total ion chromatogram. B) Extracted ion chromatogram of the molecular ion (*m/z* = 1132.9877 ± 5.00 ppm).


001-022
Intensity (a.u.)
Time (min)
100 nM Pol β
001 012
002 013
003 014
004 015
005 016
006 017
007 018
008 019
009 020
010 021
011 022
023-044
Intensity (a.u.)
Time (min)
100 nM Pol β
023 034
024 035
025 036
026 037
027 038
028 039
029 040
030 041
031 042
032 043
033 043
045-066
Intensity (a.u.)
Time (min)
100 nM Pol β
045 056
046 057
047 058
048 059
049 060
050 061
051 062
052 063
053 064
054 065
055 066
067-088
Intensity (a.u.)
Time (min)
100 nM Pol β
067 078
068 079
069 080
070 081
071 082
072 083
073 084
074 085
075 086
076 087
077 088
089-110
Intensity (a.u.)
Time (min)
100 nM Pol β
089 100
090 101
091 102
092 103
093 104
094 105
095 106
096 107
097 108
098 109
099 110
111-132
Intensity (a.u.)
Time (min)
100 nM Pol β
111 122
112 123
113 124
114 125
115 126
116 127
117 128
118 129
119 130
120 131
121 132
133-145
Intensity (a.u.)
Time (min)
100 nM Pol β
133 140
134 141
135 142
136 143
137 144
138 145
139
1016-1037
Intensity (a.u.)
Time (min)
100 nM Pol β
1016 1027
1017 1028
1018 1029
1019 1030
1020 1031
1021 1032
1022 1033
1023 1034
1024 1035
1025 1036
1026 1037

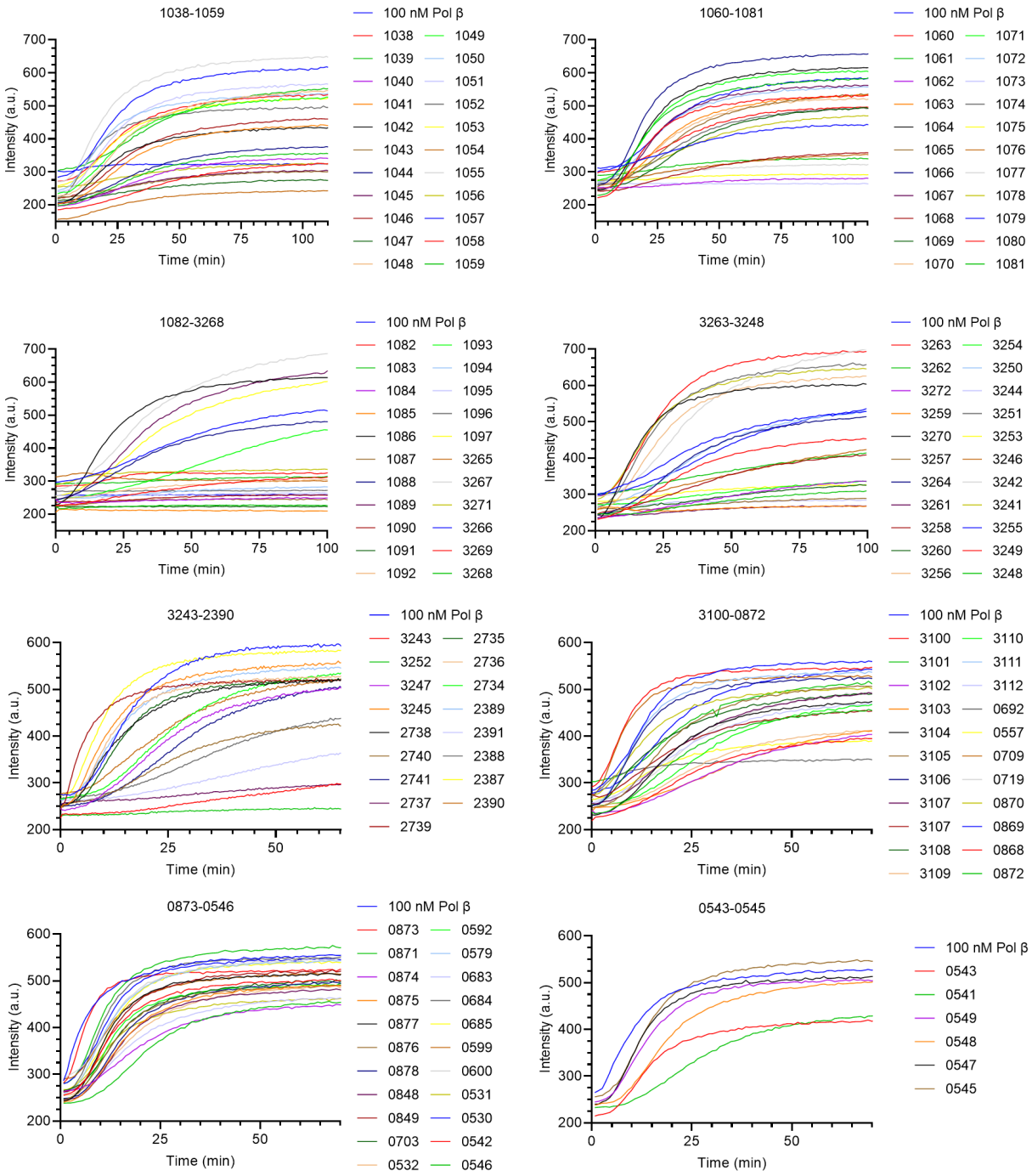
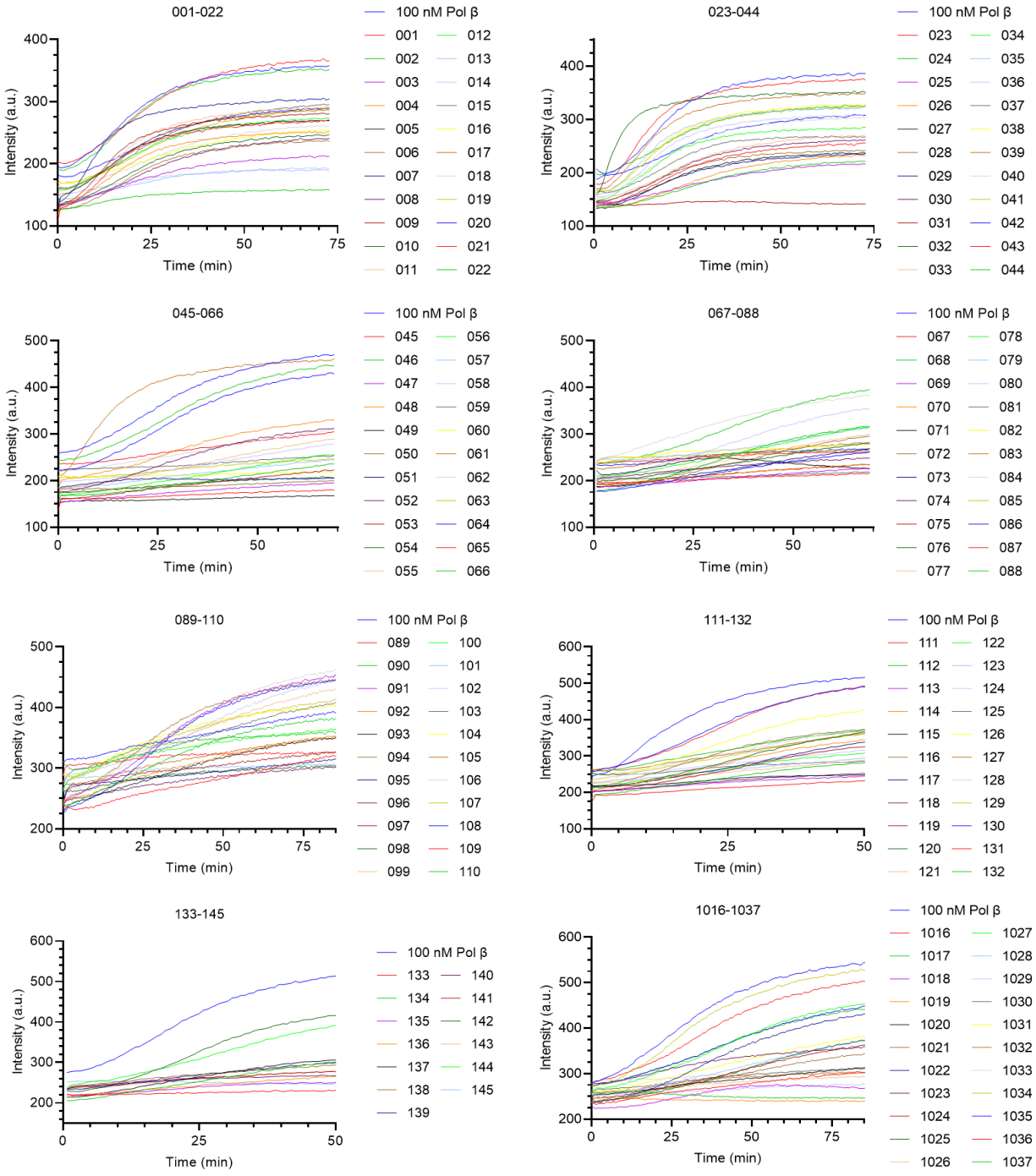


Figure S49. Primary fluorescence data of screened first-generation library (25 μM inhibitor).


001-022
100 nM Pol β
001 012
002 013
003 014
004 015
005 016
006 017
007 018
008 019
009 020
010 021
011 022
Intensity (a.u.)
Time (min)
023-044
100 nM Pol β
023 034
024 035
025 036
026 037
027 038
028 039
029 040
030 041
031 042
032 043
033 044
Intensity (a.u.)
Time (min)
045-066
100 nM Pol β
045 056
046 057
047 058
048 059
049 060
050 061
051 062
052 063
053 064
054 065
055 066
Intensity (a.u.)
Time (min)
067-088
100 nM Pol β
067 078
068 079
069 080
070 081
071 082
072 083
073 084
074 085
075 086
076 087
077 088
Intensity (a.u.)
Time (min)
089-110
100 nM Pol β
089 100
090 101
091 102
092 103
093 104
094 105
095 106
096 107
097 108
098 109
099 110
Intensity (a.u.)
Time (min)
111-132
100 nM Pol β
111 122
112 123
113 124
114 125
115 126
116 127
117 128
118 129
119 130
120 131
121 132
Intensity (a.u.)
Time (min)
133-145
100 nM Pol β
133 140
134 141
135 142
136 143
137 144
138 145
139
Intensity (a.u.)
Time (min)
1016-1037
100 nM Pol β
1016 1027
1017 1028
1018 1029
1019 1030
1020 1031
1021 1032
1022 1033
1023 1034
1024 1035
1025 1036
1026 1037
Intensity (a.u.)
Time (min)

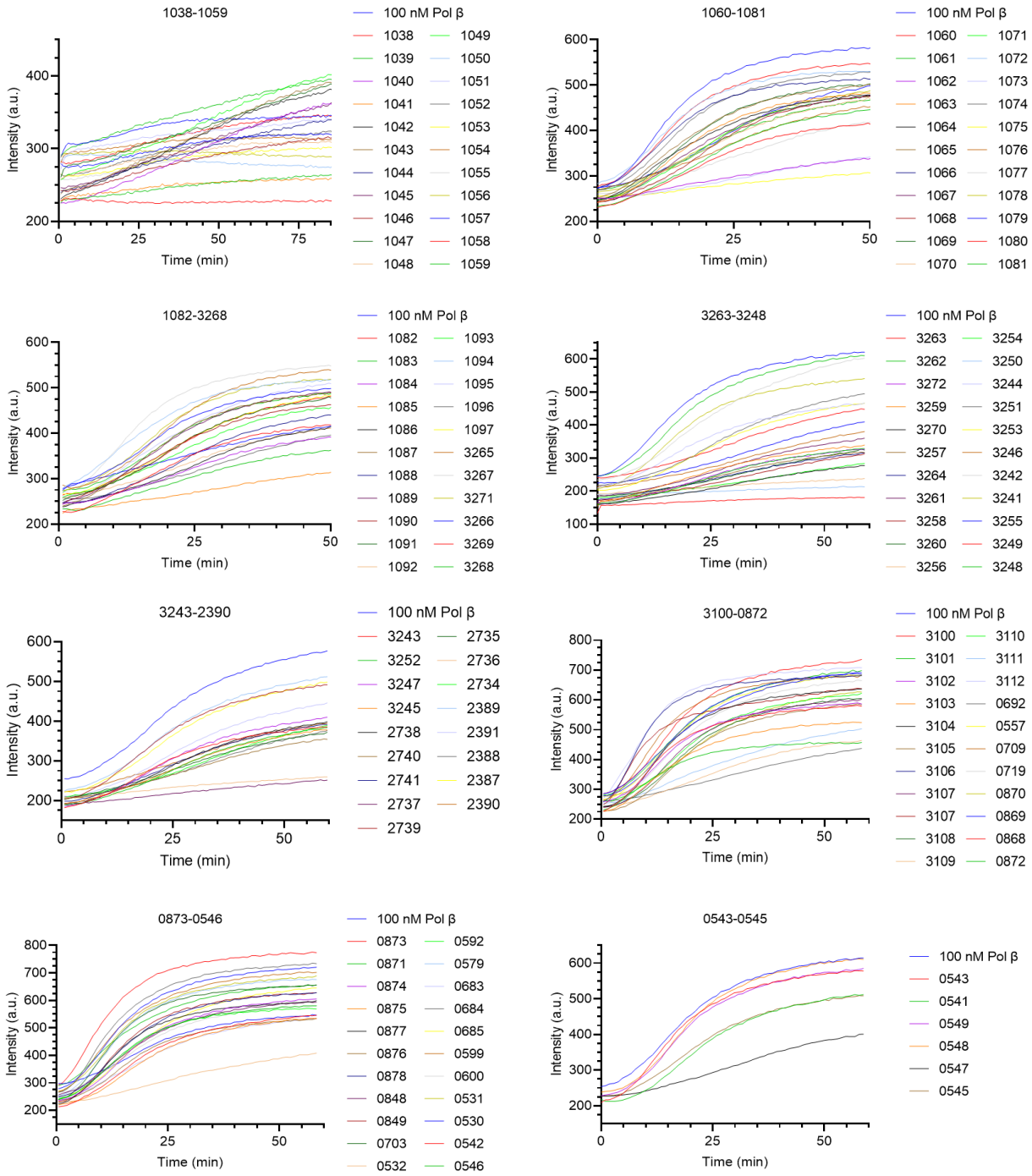
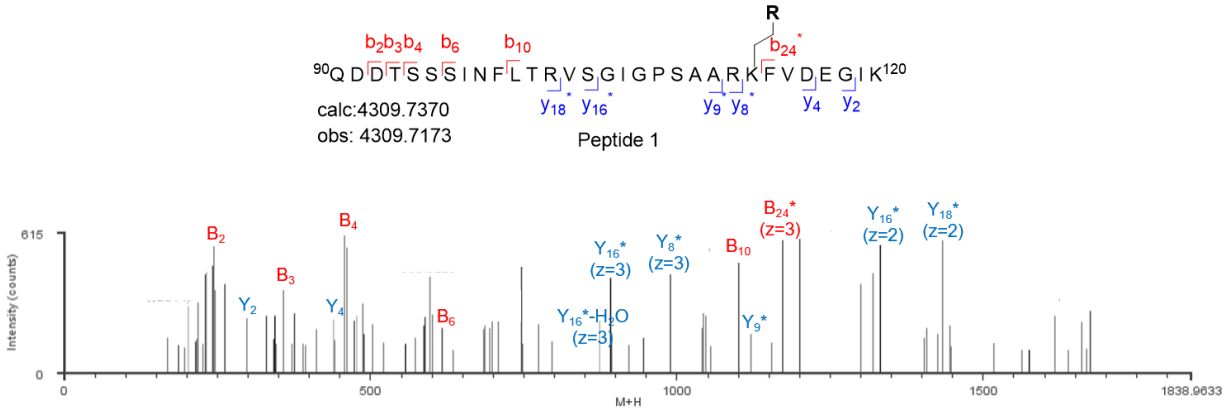
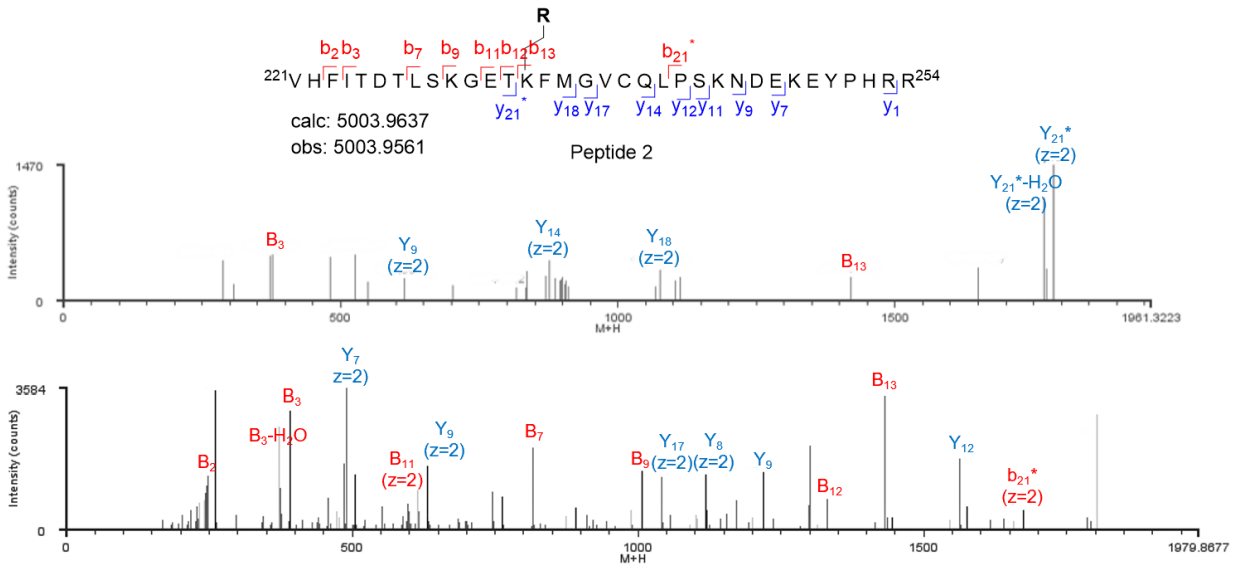


Figure S50. Primary fluorescence data of screened second-generation library (700 nM inhibitor).



Res	B	z	Calculated	Observed	Y	z	Calculated	Observed
Q	1				31			
D	2	1	244.0928	244.0848	30			
D	3	1	359.1197	359.1397	29			
T	4	1	460.1675	460.1429	28			
S	5				27			
S	6	1	634.2315	634.2309	26			
S	7				25			
I	8				24			
N	9				23			
F	10				22			
L	11				21			
T	12				20			
R	13				19			
V	14				18	2	1431.4931	1431.4858
S	15				17			
G	16				16	2	1338.4429	1338.4443
I	17				15			
G	18				14			
P	19				13			
S	20				12			
A	21				11			
A	22				10			
R	23				9	2	1061.7999	1061.8012
K*	24	3	1173.7511	1173.7426	8	3	983.7494	983.7632
F	25				7			
V	26				6			
D	27				5			
E	28				4	1	446.2610	446.2498
G	29				3			
I	30				2	1	260.1969	260.2005
K	31				1			

Figure S51. Annotated MS/MS Spectra of modified peptide 1



Res	B	z	Calculated	Observed	Y	z	Calculated	Observed
V	1				34			
H	2	1	237.1346	237.1437	33			
F	3	1	384.2030	384.2044	32			
I	4				31			
T	5				30			
D	6				29			
T	7	1	814.4094	814.4096	28			
L	8				27			
S	9	1	1014.5255	1014.5304	26			
K	10				25			
G	11				24			
E	12	1	1328.6845	1328.6821	23			
T	13	1	1429.7322	1429.9248	22			
K*	14				21	2	1788.1247	1788.1236
F	15				20			
M	16				19			
G	17				18	2	1078.0333	1078.0364
V	18				17	2	1049.5225	1049.5249
C	19				16			
Q	20				15			
L	21	2	1675.7078	1675.6978	14	2	884.4544	884.4516
P	22				13			
S	23				12	1	1558.7721	1558.7780
K	24				11			
N	25				10			
D	26				9	2	614.8011	614.8245
E	27				8	1	1114.5753	1114.5791
K	28				7	2	492.7663	492.7266

E	29				6			
Y	30				5			
P	31				4			
H	32				3			
R	33				2			
R	34				1	1	175.1190	175.1192

Figure S52. Annotated MS/MS Spectra of modified peptide 2

References

(1) Prasad, R.; Kumar, A.; Widen, S.; Casas-Finet, J.; Wilson, S. Identification of Residues in the Single-Stranded DNA-Binding Site of the 8-KDa Domain of Rate DNA Polymerase b by UV Cross-Linking. *J. Biol. Chem.* **1993**, *268*, 22746–22755.

(2) Beard, W.; Wilson, S. Purification and Domain-Mapping of Mammalian DNA Polymerase b. *Methods Enzymol.* **1995**, *262*, 98–107.

(3) Garcia-Diaz, M.; Dominguez, O.; Lopez-Fernandez, L. A.; Lera, L. T. de; Saniger, M. L.; Ruiz, J. F.; Parraga, M.; Garcia-Ortiz, M. J.; Kirchhoff, T.; Mazo, J. del; Bernad, A.; Blanco, L. DNA Polymerase Lambda (Pol Λ), a Novel Eukaryotic DNA Polymerase with a Potential Role in Meiosis. *J Mol Biol* **2000**, *301*, 851–867.

(4) Fiala, K. A.; Abdel-Gawad, W.; Suo, Z. Pre-Steady-State Kinetic Studies of the Fidelity and Mechanism of Polymerization Catalyzed by Truncated Human DNA Polymerase λ †. *Biochemistry* **2004**, *43*, 6751–6762.

(5) Xu, P.; Chen, D.; Xi, J.; Yao, Z. Short Protecting Group-free Syntheses of Camptothecin and 10-Hydroxycamptothecin Using Cascade Methodologies. *Chem. - Asian J.* **2015**, *10*, 976–981.

(6) Arian, D.; Hedayati, M.; Zhou, H.; Bilis, Z.; Chen, K.; Deweese, T. L.; Greenberg, M. M. Irreversible Inhibition of DNA Polymerase Beta by Small-Molecule Mimics of a DNA Lesion. *J. Am. Chem. Soc.* **2014**, *136*, 3176–3183.

(7) Handlon, A.; Oppenheimer, N. Thiol Reduction of 3'-Azidothymidine to 3'-Aminothymidine: Kinetics and Biomedical Implications. *Pharm Res* **1998**, *5*, 297–299.

(8) Vanheusden, V.; Munier-Lehmann, H.; Froeyen, M.; Dugue, L.; Heyerick, A.; Keukeleire, D. D.; Pochet, S.; Busson, R.; Herdewijn, P.; Calenbergh, S. V. 3′-C-Branched-Chain-Substituted Nucleosides and Nucleotides as Potent Inhibitors of Mycobacterium Tuberculosis Thymidine Monophosphate Kinase. *J. Med. Chem*. **2003**, *46*, 3811-3821.

(9) Vernekar, S. K. V.; Qiu, L.; Zhang, J.; Kankanala, J.; Li, H.; Geraghty, R. J.; Wang, Z. 5′-Silylated 3′-1,2,3-Triazolyl Thymidine Analogues as Inhibitors of West Nile Virus and Dengue Virus. *J. Med. Chem.* **2015**, *58*, 4016–4028.

(10) Paul, R.; Banerjee, S.; Greenberg, M. M. Synergistic Effects of an Irreversible DNA Polymerase Inhibitor and DNA Damaging Agents on HeLa Cells. *ACS Chem. Biol.* **2017**, *12*, 1576-1583.

(11) Guan, L.; Greenberg, M. M. Irreversible Inhibition of DNA Polymerase Beta by an Oxidized Abasic Lesion. *J. Am. Chem. Soc.* **2010**, *132*, 5004–5005.

(12) Zhang, D. Y.; Seelig, G. Dynamic DNA Nanotechnology Using Strand-Displacement Reactions. *Nat. Chem.* **2011**, *3*, 103–113.

(13) Olson, X.; Kotani, S.; Yurke, B.; Graugnard, E.; Hughes, W. L. Kinetics of DNA Strand Displacement Systems with Locked Nucleic Acids. *J. Phys. Chem. B* **2017**, *121*, 2594–2602.

(14) He, P.; Yang, W. Template and Primer Requirements for DNA Pol θ-Mediated End Joining. *Proc. Natl. Acad. Sci.* **2018**, *115*, 7747–7752.

(15) Gowda, A. S. P.; Suo, Z.; Spratt, T. E. Honokiol Inhibits DNA Polymerases β and λ and Increases Bleomycin Sensitivity of Human Cancer Cells. *Chem. Res. Toxicol.* **2017**, *30*, 715–725.

(16) Laverty, D. J.; Mortimer, I. P.; Greenberg, M. M. Mechanistic Insight through Irreversible Inhibition: DNA Polymerase θ Uses a Common Active Site for Polymerase and Lyase Activities. *J Am Chem Soc* **2018**, *140*, 9034–9037.

(17) Braithwaite, E. K.; Kedar, P. S.; Stumpo, D. J.; Bertocci, B.; Freedman, J. H.; Samson, L. D.; Wilson, S. H. DNA Polymerases β and λ Mediate Overlapping and Independent Roles in Base Excision Repair in Mouse Embryonic Fibroblasts. *PLoS ONE* **2010**, *5*, e12229.
(18) Hu, H. Y.; Horton, J. K.; Gryk, M. R.; Prasad, R.; Naron, J. M.; Sun, D. A.; Hecht, S. M.; Wilson, S. H.; Mullen, G. P. Identification of Small Molecule Synthetic Inhibitors of DNA Polymerase β by NMR Chemical Shift Mapping. *J. Biol. Chem.* **2004**, *279*, 39736–39744.