Supporting Information for

Small Molecule Inhibition of SAMHD1 dNTPase by Tetramer Destabilization

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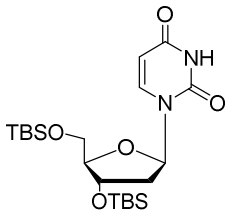
I. Materials and reagents.

Deoxynucleotide triphosphates (dNTPs) were obtained from Roche, 2'-deoxyuridine 5'-triphosphate tetraammonium salt ([5-^{3}H] dUTP) was from Moravek Biochemicals, 2'-deoxyguanosine-5'-[α-thio] triphosphate lithium salt (dGTPαS) was from ChemCyte, C18-reversed phase thin layer chromatography (TLC) plates were purchased from Macherey-Nagel. Guanosine-5'-triphosphate, 2'-deoxyguanosine, and tripolyphosphate were from Sigma-Aldrich. All reagents for chemical syntheses were purchased from Alfa Aesar or Sigma-Aldrich. Dry dimethylformamide (DMF), tetrahydrofuran (THF), and acetonitrile

were purchased from EMD Chemicals and used without further purification. Methanol and *tert*-butyl alcohol were purchased from Macron Fine Chemicals and TCI, respectively. Dowex 50WX8-200 ion exchange resin was purchased from Sigma-Aldrich. Iodoxybenzoic acid (IBX) was prepared by the method of Sputore[1]. Sodium hydride was used as a 60% dispersion in oil. Thin layer chromatography (TLC) plastic-back sheets (20 × 20; silica gel 60 F_{254}) were purchased from EMD Chemicals. Silica gel, Grade 62 (60-200 mesh) used for column chromatography was purchased from Macron. HPLC experiments were carried out using a Varian ProStar 210, equipped with a semi-preparative column (21.4 × 150 mm SP 15/20 Nucleogel SAX, Macherey-Nagel or 21.20 × 250 mm Phenomenex Luna 5u C18 columns) and a Shimadzu SPD-10A VP UV-vis detector. Eluates were detected at λ = 260 nm. Proton (^{1}H), carbon (^{13}C), and phosphorus (^{31}P) NMR spectra were measured on a Varian VNMRS-500 NMR spectrometer. Chemical shifts (δ) are reported in parts per million (ppm) relative to internal residual $CHCl_3$ in $CDCl_3$ (δ 7.26, ^{1}H) or HDO in D_2O (δ 4.80, ^{1}H), internal $CDCl_3$ (δ 77.0, ^{13}C) or external 85% H_3PO_4 (δ 0.00, ^{31}P). NMR FIDs were processed using MestReNova v9.0.0. Low resolution mass spectrometry for chemical synthesis was performed on a Finnigan LCQ Deca XP Max spectrometer equipped with an ESI source operated in negative ion mode. HRMS data were obtained at the UC Riverside (Dr. Ron New) high resolution mass spectrometry facility. Microwave-assisted synthesis was performed using a Milestone Ethos Synth Microwave Labstation. Compound IUPAC names were assigned using MarvinSketch 6.1.5.

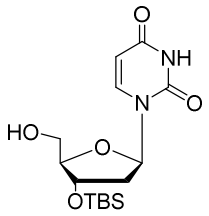
II. Chemical Synthesis



2

1•[(*2R*,*5R*)•4•[(*tert*•butyldimethylsilyl)oxy]•5•{[(*tert*•butyldimethylsilyl)oxy]methyl}oxolan•2•yl]•1,2,3,4•tetrahydropyrimidine•2,4•dione (2)[2,3]

To a solution of 2'-deoxyuridine (0.4 g, 1.75 mmol) and imidazole (0.6 g, 8.75 mmol) in 4 mL of dry DMF was added *t*-butyldimethylsilyl chloride (660 mg, 4.4 mmol). The reaction mixture was stirred for 4 h at rt. The reaction mixture was quenched by addition of saturated $NaHCO_3$, and the product was extracted with CH_2Cl_2 (3×15 ml). The organic layers were washed with brine, dried over Na_2SO_4, filtered, and the solvent was evaporated. The white crystalline product (774 mg, 97%) was used in the next step without further purification. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.97 (1H, br, N*H*), 7.90 (1H, d, *J* = 8.2 Hz, H-6), 6.28 (1H, t, *J* = 6.2 Hz, H-1'), 5.67 (1H, d, *J* = 8.2 Hz, H-5), 4.41 (1H, dt, *J* = 6.5 Hz, *J* = 4.0 Hz, H-3'), 3.93-3.91 (1H, m, H-4'), 3.91-3.75 (2H, m, H-5'), 2.32 (1H, ddd, *J* = 13.5 Hz, *J* = 6.2 Hz, *J* = 4.2 Hz, H-2'), 2.06 (1H, dt, *J* = 13.5 Hz, *J* = 6.3 Hz, H-2'), 0.92 (9H, s, C(C*H*$_3$)$_3$), 0.89 (9H, s, C(C*H*$_3$)$_3$), 0.10 (3H, s, Si(C*H*$_3$)), 0.01 (3H, s, Si(C*H*$_3$)), 0.08 (3H, s, Si(C*H*$_3$)), 0.07 (3H, s, Si(C*H*$_3$)).

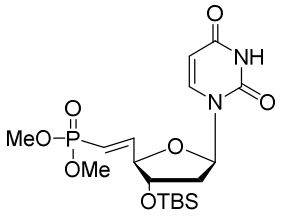


3

1•[(*2R,5R*)•4•[(*tert*•butyldimethylsilyl)oxy]•5•(hydroxymethyl)oxolan•2•yl]•1,2,3,4-tetrahydropyrimidine•2,4•dione (3)[3,4]

A solution of **2** (0.775 g, 1.7 mmol) in 8 mL of THF was cooled to 0°C, and a solution of trichloroacetic acid (5.56 g, 34 mmol) in 10 mL of H_2O was added. The reaction mixture was stirred for 1 h at 0°C. R eaction progress was monitored by TLC (EtOAc:Hexanes = 2:1).

After 1 h the reaction mixture was quenched with solid Na_2CO_3, and 20 mL of water was added. The product was extracted with CH_2Cl_2 (3×10 mL). The combined organic layers were washed with brine and dried over Na_2SO_4. After filtration, the solvent was removed, and the residue was purified by silica gel column chromatography (EtOAc:Hexanes, 1:1 → 3:1). The product was isolated as a white crystalline solid (344 mg, 60%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.05 (1H, br, N*H*), 7.62 (1H, d, *J* = 8.1 Hz, H-6), 6.16 (1H, t, *J* = 6.6 Hz, H-1'), 5.73 (1H, d, *J* = 8.1 Hz, H-5), 4.49 (1H, dt, *J* = 6.2 Hz, *J* = 4.0 Hz, H-3'), 3.96-3.75 (3H, m, H-4', H-5'), 2.35-2.24 (2H, m, H-2'), 2.12 (1H, br, O*H*), 0.90 (9H, s, C(C*H*$_3$)$_3$), 0.09 (6H, s, Si(C*H*$_3$)$_2$).



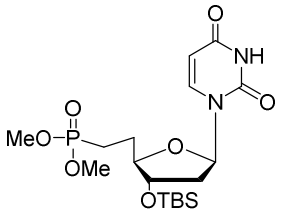
5

Dimethyl [(E)•2•[(*2R,5R*)•3•[(*tert*•butyldimethylsilyl)oxy]•5•(2,4•dioxo•1,2,3,4•tetrahydropyrimidin•1•yl)oxolan•2•yl]ethenyl]phosphonate (5)[3]

Alcohol **3** (120 mg, 0.35 mmol) was dissolved in 4 mL of CH_3CN, and freshly prepared IBX (108 mg, 0.385 mmol) was added. The reaction mixture was refluxed for 1 h. Product formation was monitored by TLC (EtOAc:Hexanes = 2:1). Upon completion of the reaction, the mixture was cooled to 0°C, filtered and washed with cold acetonitrile. The solvent was removed, and the residue was co-evaporated with dry THF and then dried under high vacuum. Aldehyde **4** was used in the following reaction without further purification.

To a suspension of NaH (26 mg, 0.65 mmol) in dry THF (2 mL) at 0°C was added a solution of tetramethyl methylenebisphosphonate in 4 mL of dry THF. The reaction mixture was stirred at 0°C for 10 min. The resulting suspension was then added dropwise to a solution of aldehyde **4** in 4 mL of dry THF at 0°C. The reaction mixture was stirred for 3 h and allowed to slowly warm up to rt. It was then quenched with saturated NH_4Cl and extracted with EtOAc (3×10 mL). The combined organic layers were washed with brine and dried over Na_2SO_4. The residue was purified by silica gel column chromatography (1% MeOH in EtOAc), giving 124 mg of **5** as a colorless oil (79% over two steps). ^{31}P NMR (202 MHz, $CDCl_3$): δ 19.68 (s); ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.66 (1H, bs, N*H*), 7.31 (1H, d, *J* = 8.1 Hz, H-6), 6.83 (1H, ddd, *J* = 22.2 Hz, 17.1 Hz, 5 Hz, H-6'), 6.27 (1H, t, *J* = 6.5 Hz, H-1'), 5.94 (1H, ddd, *J* = 19.4 Hz, 17.1 Hz, 1.7 Hz, H-5'), 5.76 (1H, dd, *J* = 8.1 Hz, 1.7 Hz, H-5), 4.36-4.33 (1H, m, H-3'), 4.24-4.21 (1H, m, H-4'), 3.73 (3H, d, *J* = 11.1 Hz, P(O)(OC*H*$_3$)), 3.73 (3H, d, *J* = 11.2 Hz, P(O)(OC*H*$_3$)), 2.31 (1H, ddd, *J* = 13.4 Hz, 6.3 Hz, 4.7 Hz, H-2'), 2.12 (1H, dt, *J* = 13.4 Hz, 6.6 Hz, H-2'), 0.87 (9H, s, C(C*H*$_3$)$_3$), 0.06 (6H, s, Si(C*H*$_3$)$_2$); ^{13}C NMR (126 MHz, $CDCl_3$): δ 163.1 (C-4), 150.2 (C-2), 148.5 (d, *J* = 5.7 Hz, C-5'), 139.3 (C-6), 117.7 (d, *J* = 189.3 Hz, C-6'), 103.0 (C-5), 86.1 (d, *J* = 22.4 Hz, C-4'), 85.3 (C-1'), 74.7 (d, *J* = 1.9 Hz, C-3'), 52.5 (d, *J* = 9.0 Hz,

P(O)(O*C*H$_3$)), 52.5 (d, *J* = 8.9 Hz, P(O)(O*C*H$_3$)), 40.1 (C-2'), 25.6 (C(*C*H$_3$)$_3$), 17.8 (*C*(CH$_3$)$_3$), -4.8 (Si(*C*H$_3$)), -4.9 (Si(*C*H$_3$)).

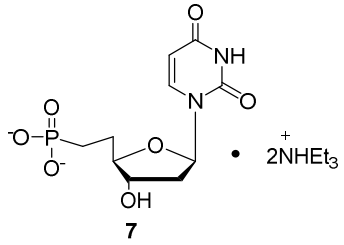


6

Dimethyl {2•[(*2R,5R*)•3•[(*tert*•butyldimethylsilyl)oxy]•5•(2,4•dioxo•1,2,3,4•tetrahydropyrimidin•1•yl)oxolan•2•yl]ethyl}phosphonate (6)[3]

To a solution of compound **5** (40 mg, 0.09 mmol) in 4 mL of methanol was added Pd/C (4 mg, 10 wt %), and the reaction mixture stirred under H_2 (1 atm) for 1 h. It was then filtered through a celite pad, and the methanol was evaporated. The residue was dried under vacuum, yielding 38 mg of compound **5** (94%) as a colorless oil, which was used without further purification. HRMS (ESI/APCI): calcd for $C_{18}H_{34}N_2O_7PSi$, $[M-H]^-$ 449.1867; found 449.1871 m/z. ^{31}P NMR (202 MHz, $CDCl_3$): δ 33.70 (s); ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.59 (1H, br, N*H*), 7.31 (1H, d, *J* = 8.1 Hz, H-6), 6.15 (1H, t, *J* = 6.5 Hz, H-1'), 5.74 (1H, d, *J* = 8.1 Hz, H-5), 4.05 (1H, dt, *J* = 6.7 Hz, 4.4 Hz, H-3'), 3.79-3.73 (1H, m, H-4'), 3.73 (6H, d, *J* = 10.8 Hz, P(O)(OC*H$_3$*)$_2$), 2.28 (1H, ddd, *J* = 13.6 Hz, 6.5 Hz, 4.4 Hz, H-2'), 2.06 (1H, dt, *J* = 13.6 Hz, 6.6 Hz, H-2'), 2.00-1.74 (4H, m, H-5', H-6'), 0.86 (9H, s, C(C*H$_3$*)$_3$), 0.05 (3H, s, Si(C*H$_3$*)), 0.05 (3H, s, Si(C*H$_3$*)). ^{13}C NMR (126 MHz, $CDCl_3$): δ 163.3 (C-4), 150.2 (C-2), 139.4 (C-6), 102.7 (C-5), 86.0 (d, *J* = 16.7 Hz, C-4'), 85.0 (C-1'), 74.5 (C-3'), 52.5 (d, *J* = 6.3 Hz, P(O)(O*C*H$_3$)), 52.4 (d, *J* =

6.3 Hz, P(O)(O*C*H_3)), 40.6 (C-2'), 26.4 (d, *J* = 4.7 Hz, C-5'), 25.6 (C(*C*H_3)$_3$), 21.2 (d, *J* = 143.4 Hz, C-6')), 17.8 (*C*(CH_3)$_3$), -4.6 (Si(*C*H_3)), -4.9 (Si(*C*H_3)).



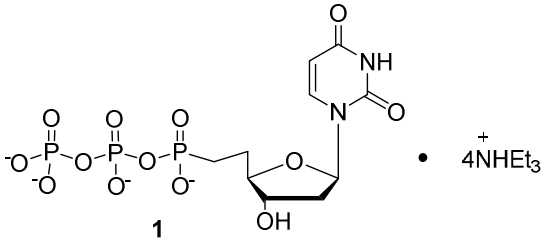
7

{2•[(*2R,5R*)•5•(2,4•dioxo•1,2,3,4•tetrahydropyrimidin•1•yl)•3•hydroxyoxolan•2•yl]ethyl}phosphonate (TEA salt) (7)[5]

Compound **6** (17 mg, 0.038 mmol) was dissolved in 500 μL of dry acetonitrile, and BTMS (bromotrimethylsilane; 11 μL, 0.08 mmol) was added. The reaction mixture was microwaved at 60°C for 7 min. Volatiles were removed under reduced pressure, and the residue treated with water. After stirring for 30 min, water was evaporated, the reaction mixture dried under vacuum and the resulting residue used in the following step without further purification.

A sample of compound **7** was isolated using two-step HPLC (first pass, SAX using a 0→50% linear gradient, A = water, B = 0.5 M TEAB, pH 7.5, 20 min, 8 mL/min; second pass, RP HPLC on the C-18 column at 8 mL/min using 0.1 M TEAB, 5% CH_3CN, pH 7.0 under isocratic conditions). HRMS (ESI/APCI): calcd for $C_{10}H_{14}N_2O_7P^-$, $[M-H]^-$ 305.0533; found 305.0548 m/z. ^{31}P NMR (202 MHz, D_2O): δ 20.92; ^1H NMR (500 MHz, D_2O): δ 7.71 (1H, d, *J* = 7.8 Hz, H-6), 6.30 (1H, t, *J* = 6.9 Hz, H-1'), 5.87 (1H, d, *J* = 7.8 Hz, H-5), 4.35 (1H, dt, *J* = 6.9 Hz, *J* = 3.7 Hz, H-3'), 3.97 (1H, td, *J* = 6.9 Hz, *J* = 3.7 Hz, H-4'), 2.39-2.28 (2H, m, H-2'), 1.93-1.84 (2H, m, H-5'/6'), 1.61-1.40 (2H, m, H-5'/6'). ^{13}C NMR (126 MHz, D_2O): δ 163.6 (C-4), 157.6 (C-2),

141.4 (C-6), 103.4 (C-5), 88.4 (d, *J* = 17.5 Hz, C-4'), 85.6 (C-1'), 73.9 (C-3'), 39.0 (C-2'), 29.1 (d, *J* = 3.4 Hz, C-5'), 26.0 (d, *J* = 130.5 Hz, C-6').



1

({[({2•[(*2R*,*5R*)•5•(2,4•dioxo•1,2,3,4•tetrahydropyrimidin•1•yl)•3•hydroxyoxolan•2•yl]ethyl}(hydroxy)phosphoryl)oxy](hydroxy)phosphoryl}oxy)phosphonate (TEA salt) (1).

Compound **7** was dissolved in 1 mL of a 1:1 mixture of *t*-BuOH and H_2O. To this solution was added distilled morpholine (10 μL, 0.114 mmol), and the reaction mixture was stirred at rt for 10 min, then set to reflux. A solution of DCC (25 mg, 0.114 mmol) in 0.5 mL of *t*-BuOH was added over a period of 1.5 h, and the reaction mixture was then refluxed for 5 h. Volatiles were removed under reduced pressure, the residue taken up in water, and the resulting suspension filtered. The aqueous solution was extracted twice with ether, and then re-evaporated under reduced pressure. The resulting residue (**8**) was dried using the oil pump and used in the next step without further purification.

A solution of tetrasodium pyrophosphate (30 mg, 0.114 mmol) in 1 mL water was passed through DOWEX H^+. The solvent was removed and the residue dissolved in 1 mL 50% aqueous ethanol. Tributylamine (34 μL, 0.171 mmol, 1.5 eq) was added and the

resulting solution stirred at rt for 1 h. The solvent was removed, and the residue was co-evaporated with ethanol, and the resulting glassy solid was dried using an oil pump. The product was dissolved in 1 mL of dry DMSO.

The solution of tributylammonium pyrophosphate in DMSO was added dropwise to a solution of morpholidate **8** in 1 mL dry DMSO dropwise. The reaction was stirred for 24 h at rt. The product **1** was isolated using two-step preparative HPLC: first pass, SAX ion exchange column, 0 → 100% linear gradient, A = water, B = 0.5 M TEAB, pH = 7.5, 25 min, 8 mL/min; second pass, RP HPLC on a C-18 preparative column using 0.1 M TEAB, 5% CH_3CN, pH = 7, 8 mL/min under isocratic conditions. The fractions containing **1** were combined and lyophilized, giving the product as a white crystalline solid (4.1 mg, 25% over three steps). HRMS (ESI/APCI): calcd for $C_{10}H_{16}N_2O_{13}P_3^-$, $[M-H]^-$ 464.9871; found 464.9872 m/z. ^{31}P NMR (202 MHz, D_2O): δ 18.63 (1P, d, *J* = 25.8 Hz, P_α), -10.67 (1P, d, *J* = 16.4 Hz, P_γ), -23.32 (1P, dd, *J* = 25.8, 16.4, P_β); ^1H NMR (500 MHz, D_2O): δ 7.79 (1H, d, *J* = 8.1 Hz, H-6), 6.29 (1H, t, *J* = 6.9 Hz, H-1'), 5.95 (1H, d, *J* = 8.1 Hz, H-5), 4.42-4.39 (1H, m, H-3'), 4.06-4.03 (1H, m, H-4'), 2.43-2.34 (2H, m, H-2'), 2.06-1.83 (4H, m, H-5', H-6'). ^{13}C NMR (126 MHz, D_2O): δ 166.7 (C-4), 152.1 (C-2), 142.3 (C-6), 102.9 (C-5), 87.6 (d, *J* = 19.3 Hz, C-4'), 85.6 (C-1'), 73.6 (C-3'), 38.4 (C-2'), 27.4 (d, *J* = 4.2 Hz, C-5'), 24.5 (d, *J* = 138.1 Hz, C-6').

III. SAMHD1 cloning and overexpression.

Full-length SAMHD1 (1881 bp) was expressed as a N-terminal His_{10}-PPS fusion in *Escherichia coli* BL21-DE3 cells (Novagen) as previously described[6]. Protein concentration was determined by absorbance measurements at 280 nm using the calculated (Protparam

tool, ExPASy) molar extinction coefficients for full-length human SAMHD1 monomer (ε = 76,500 M^{-1} cm^{-1}). Protein yields were typically 20 mg/L of bacterial culture. Purified protein was stored at -80°C in small portions. Experiments were initiated by thawing a single (20 µL) aliquot, which was stored at -20°C, and then used over the course of three days before being discarded.

IV. Mass spectrometry analysis of $H_2{}^{18}O$ incorporation into product.

Mass spectrometry was performed on a TSQ Vantage TripleStage Quadrupole mass spectrometer (Thermo Fisher Scientific). Instrument settings were optimized in negative ion mode for dG and PPP_i by infusing standard solutions. The SAMHD1 enzymatic reactions were carried out at 22°C with 2 µM SAMHD1 and 1 mM dGTP in a buffer consisting of 50 mM ammonium acetate (pH 7.5), 50 mM KCl, and 5 mM $MgCl_2$ in either standard ddH_2O or 97% ^{18}O H_2O (Cambridge Isotope Laboratories). A control was prepared in ^{18}O H_2O containing 1 mM dG and 1 mM PPPi to ensure there was no exchange of ^{18}O in the products. Reactions were allowed to proceed for 45 minutes and reaction completion was verified by TLC. The solution was run through a 30 kDa MWCO Microcon Centrifugal Filter (EMD Millipore) to remove the protein and the membrane was washed with 2 reaction volumes. This was dried in a speedvac at room temperature and resuspended in 5 reaction volumes 50:50 methanol:water with 0.5% acetic acid immediately prior to analysis. This solution was infused at a rate of 20 µL/min until the Total Ion Chromatogram stabilized. The spectra were obtained in parent ion mode at m/z 266 or 268 for dG or ^{18}O-dG and m/z 257 or 259 for PPP_i or ^{18}O-PPP_i[7,8]. In all cases only one of the oxygen isotopic forms was detectable above background. Fragment ion spectra were also obtained for dG.

V. Steady-state kinetic measurements.

Standard reaction conditions for steady-state kinetic measurements were 5 mM GTP (activator), 50 mM Tris-HCl (pH 7.5), 50 mM KCl, 5 mM $MgCl_2$, and 0.5 mM TCEP in a 12 µL total reaction volume at 22 °C. Concentrations of the [5-^{3}H] dUTP substrate were varied in the range 0.01 to 5 mM and standard reactions were initiated by the addition of SAMHD1. Two microliter samples were removed at indicated times and quenched by spotting onto a C18-reversed phase thin layer chromatography (TLC) plate. The TLC plate was developed in 50 mM KH_2PO_4 (pH 4.0) to separate substrate [dUTP (R_f = 0.97] from products [dU (R_f = 0.54)]. Plates were exposed to a tritium sensitive screen for 5 h and then scanned on a Typhoon phosphoimager (GE Healthcare) and the counts present in the substrate and product were quantified using the program Quantity One (Bio-Rad). The amount of product formed at each timepoint was calculated from the ratio (cpm dN product)/(cpm dNTP substrate + cpm dN product) × (intial [dNTP]). Initial rates of product formation were obtained from the slopes of linear plots of [dN] versus time at reaction extents of less than 20%.

Inhibition by 1. To investigate the mechanism of inhibition by **1**, we used fixed concentrations of GTP (5 mM) activator and [^{3}H] dUTP (1 mM) substrate while varying the concentration of **1** in the range of 50 µM to 2 mM. The reaction rates (µM/s) were plotted versus dUTP substrate concentration. The inhibition constant (K_i) was determined by globally fitting the entire data set to a competitive inhibition model (eq 1).

$$v = \frac{V_{\max [S]}}{K_m\left(1 + \frac{[I]}{K_i}\right) + [S]} \qquad (1)$$

Inhibition by 1 at the A2 or catalytic sites of SAMHD1. Distinguishing between competitive binding by **1** at either the A2 or catalytic sites was achieved by addition of **1** before or after A1 site activator (GTP). Competitive binding by **1** at the catalytic site was examined by pre-assembling a reaction mixture containing SAMHD1 (0.5 μM), GTP (5 mM) activator and dUTP (1 mM) substrate, which were allowed to proceed for 2.5 minutes. At this time, varying concentrations of **1** (0.5 to 4 mM) were added to the reaction mixture and dUTP substrate hydrolysis was monitored over time. Alternatively, inhibition caused by binding of **1** to the A2 activator site was probed by pre-incubation of SAMHD1 (0.5 μM), GTP (5 mM) activator and varying concentrations of **1** (50 μM to 2 mM) prior to initiation with dUTP (1 mM) substrate. In both cases, the inhibited reaction rates (μM/s) were normalized to rates obtained in the absence of inhibitor and plotted versus concentration of **1** (eq 2,3). The data was fitted using nonlinear least squares optimization to obtain true inhibition constants (K_i) for the A2 and catalytic binding sites.

$$\frac{v_i}{v_0} = \frac{1}{1 + \frac{[I]}{K_i^{app}}} \qquad (2)$$

$$K_i^{app} = K_i\left(1 + \frac{S}{K_m}\right) \qquad (3)$$

Dilution-jump kinetic measurements. In the standard assay SAMHD1 (10 μM) was incubated for 15 seconds with varying concentrations of GTP (0.5 mM), dUTP(1 mM), and/or **1** (5 mM) prior to a 100-fold dilution into standard reaction buffer containing [5-^{3}H] dUTP (1 mM). Time points were then quenched on a TLC plate and product formation was quantified as described above. Plots of [dU] against time were fitted to the equation [dU] = A[1 - exp(-$k_{inact}t$)] + vt, where A is the burst amplitude (μM), k_{inact} is the rate constant

for the burst decay (s^{-1}), and *v* is the linear steady-state rate (μM/s). Variations of the standard assay were performed as indicated in the text.

Dilution-jump glutaraldehyde crosslinking. The standard dilution-jump procedure described above was performed except that samples from the diluted reactions were taken at various times and mixed with 50 mM glutaraldehyde. Samples were incubated at 22°C for 15 min, quenched by addition of 1 M Tris, pH 7.5 and loaded on to a 4-12% Bis-Tris denaturing polyacrylamide gel. Protein samples were visualized by silver staining. Briefly, gels were fixed overnight in a 50 mL solution containing 50% methanol, 12% acetic acid and 0.02% paraformaldehyde. Gels were washed three times for 20 min in 20% ethanol, followed by a 5 min incubation in sensitizing solution containing 0.02% sodium thiosulfate. Sensitizing solution was removed by washing twice with 50 mL of H_2O and immediately transferred to an equivalent volume of staining solution containing 0.2% silver nitrate and 0.03% paraformaldehyde. Finally, gels were developed in a 6% sodium carbonate, 0.02% paraformaldehyde (50 mL) solution and incubated for 2-5 min. Developer was quenched by direct addition of an equivalent volume of 50% methanol and 10% acetic acid. Gels were imaged immediately using an Epson V750 flat-bed scanner.

VI. Supplementary References

(1) Frigerio, M.; Santagostino, M.; Sputore, S. *Journal of Organic Chemistry* **1999**, *64*, 4537.

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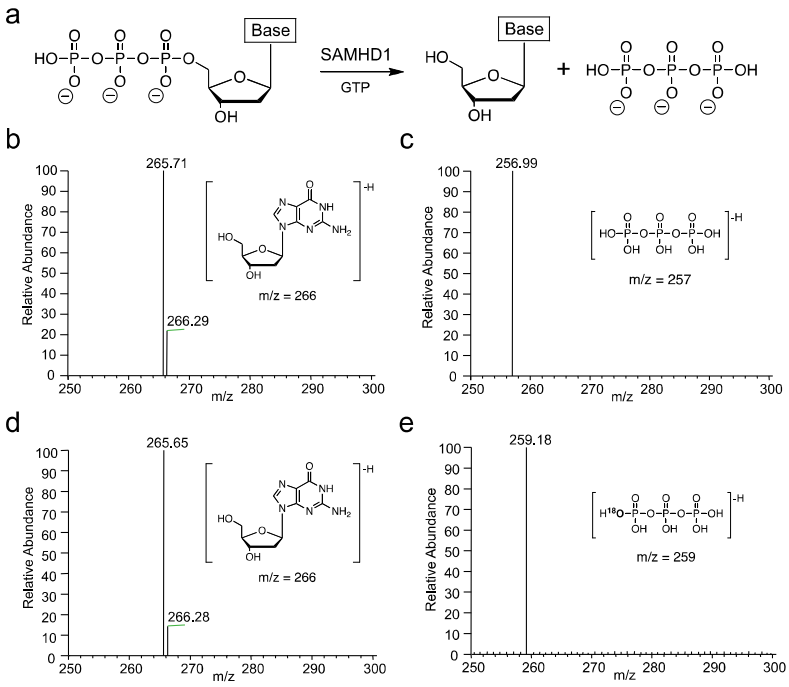
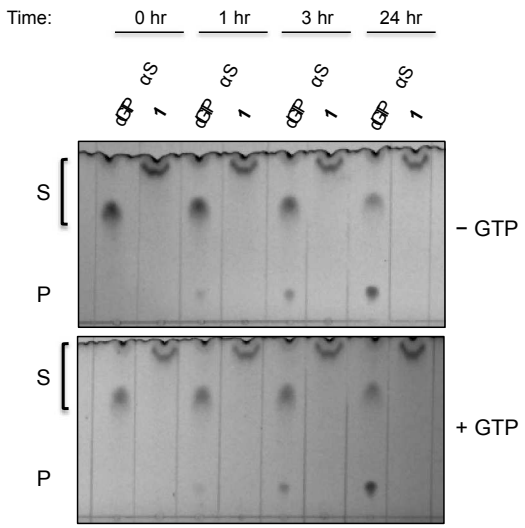
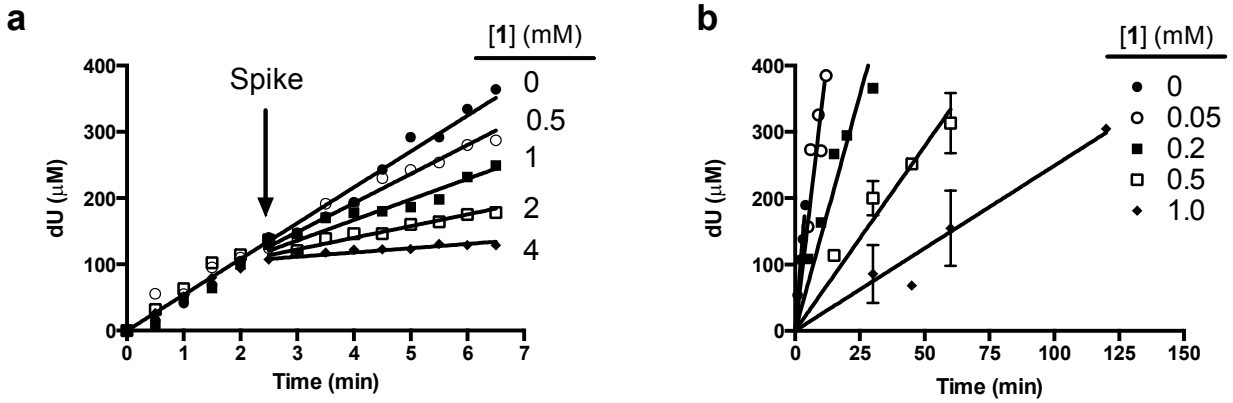
VII. Supplementary Figures



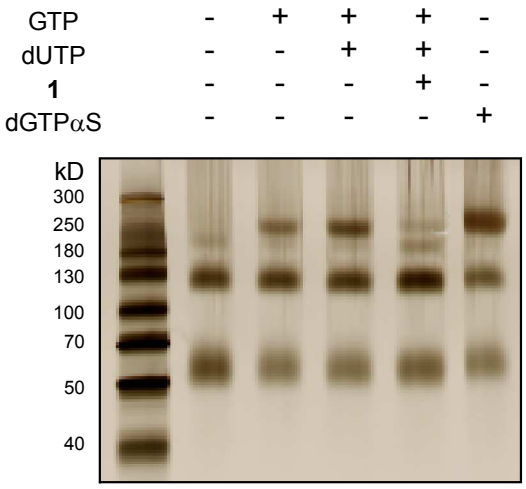
Supplementary Figure 1. Mass spectrometry of dGTP hydrolysis products. (**a**) The hydrolysis reaction of deoxynucleotide triphosphates catalyzed by SAMHD1. Following complete hydrolysis of dGTP by SAMHD1 in [^{16}O] or [^{18}O] H_2O, the reaction products dG and PPP_i were isolated and analyzed electrospray mass spectrometry in negative ion mode. The singly charged molecular ions of dG and PPP_i are shown from reactions in (**b, c**) [^{16}O] H_2O or (**d, e**) [^{18}O] H_2O, respectively. No mass shift occurred when dG and PPP_i were incubated with SAMHD1 in [^{18}O] H_2O, indicating that this is not due to spontaneous ^{18}O exchange in the products.



Supplementary Figure 2. Reactivity of 1 and dGTPαS with SAMHD1 (0.5 μM) in the presence and absence of GTP. Reactant and products were separated using C18-reversed-phase TLC with detection by fluorescence quenching (UV_{254nm}). Reaction progress was monitored at 0, 1, 3 and 24 hr by spotting two microliter samples plates. The concentration of **1** and dGTPαS were 1 mM and the GTP activator concentration was 50 μM.

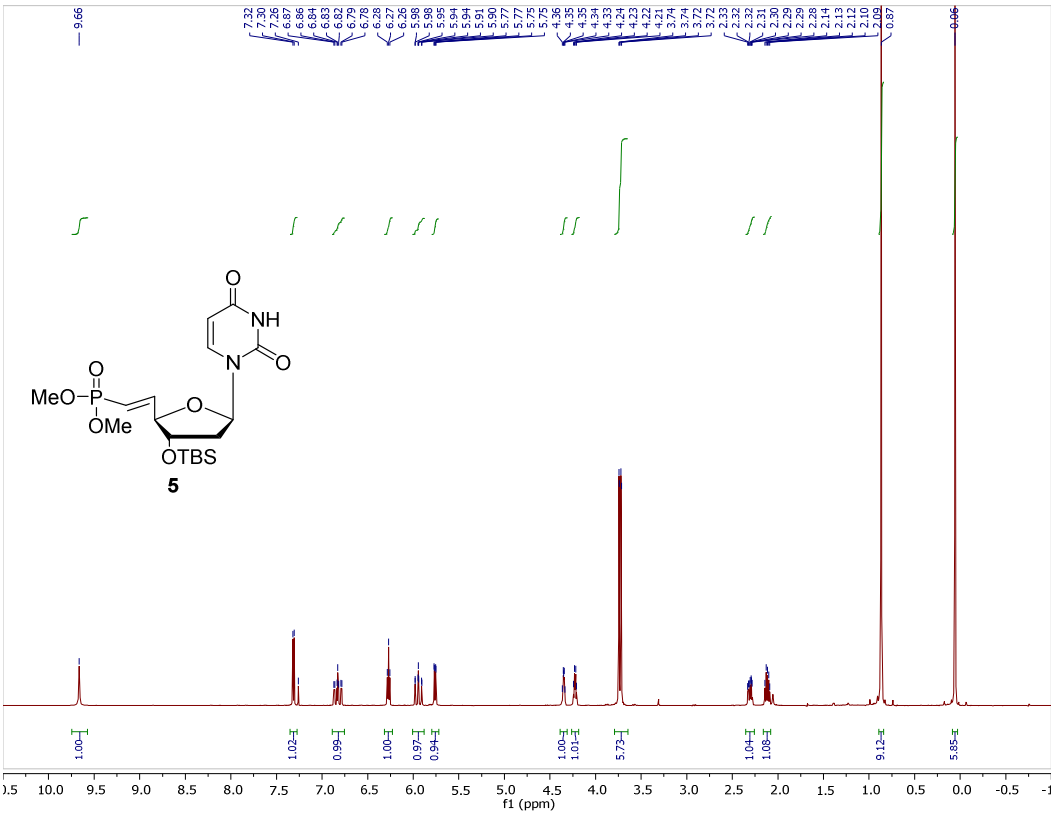


Supplementary Figure 3. Order-of-Addition experiments to distinguish between binding of 1 to A2 or catalytic sites. (**a**) A reaction containing SAMHD1 (0.5 μM), A1 site activator GTP (5 mM) and [5-^{3}H] dUTP (1 mM) substrate was allowed to proceed for 2.5 minutes prior to spiking the reaction with varying concentrations of 1 and dUTP consumption was followed over time. (**b**) Alternatively, GTP (5 mM) and 1 (0.05 – 1 mM) were pre-incubated (5 min) with SAMHD1 (0.5 μM) prior to initiation with [5-^{3}H] dUTP (1 mM) substrate. Substrate hydrolysis was evaluated using the C18-RP TLC kinetic assay.

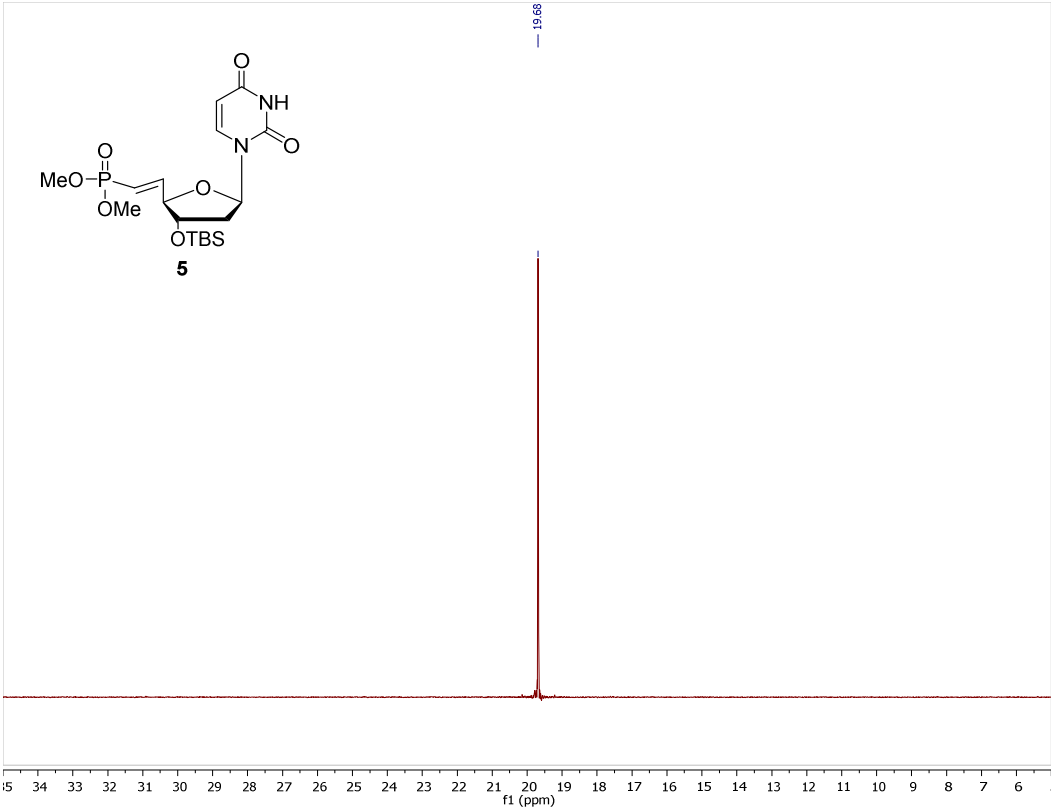


Supplementary Figure 4. dGTPαS induces tetramer formation but 1 does not. Standard control reactions contained SAMHD1 (0.5 μM) alone, specific A1 site GTP (5 mM) activator or a combination of GTP and dUTP (1 mM) substrate. Contrasting effects on tetramer formation were evaluated by addition of **1** (2 mM) or dGTPαS (2 mM). Reactions were initiated by addition of SAMHD1 and followed by immediate chemical cross-linking with 50 mM glutaraldehyde. Separation of cross-linked species was performed by denaturing polyacrylamide gel electrophoresis and visualized by silver staining.

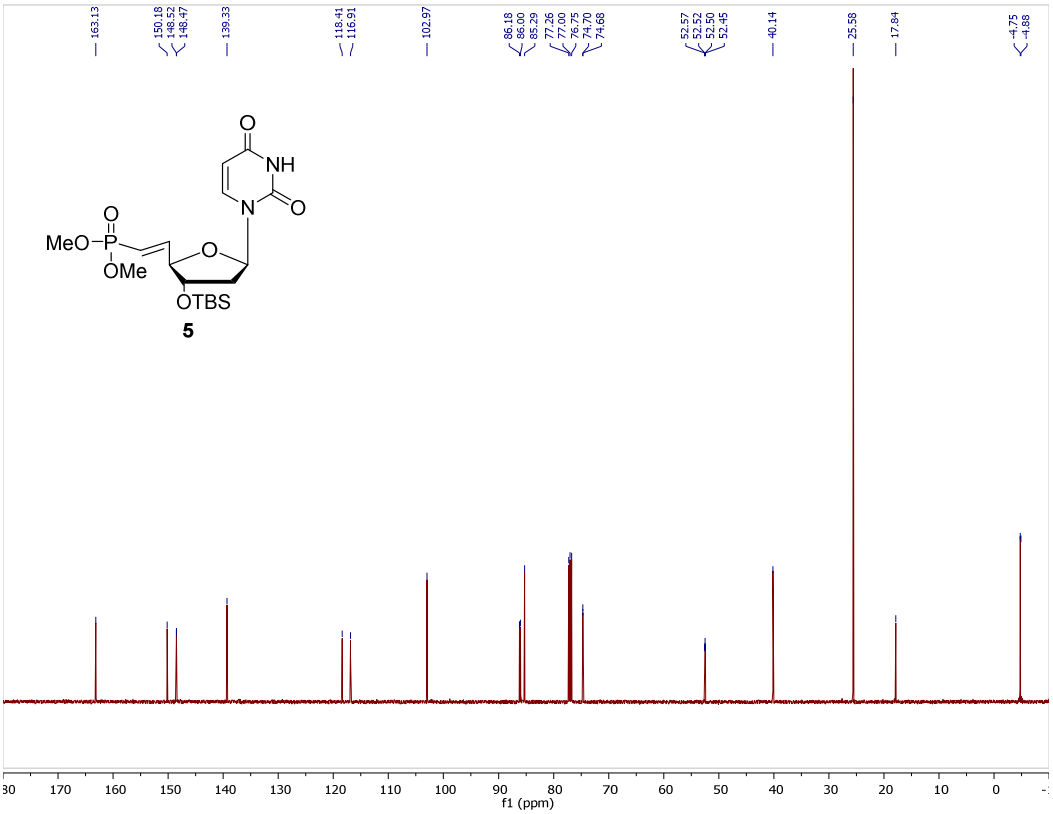
Characterization of Compounds 5, 6, 7, 1



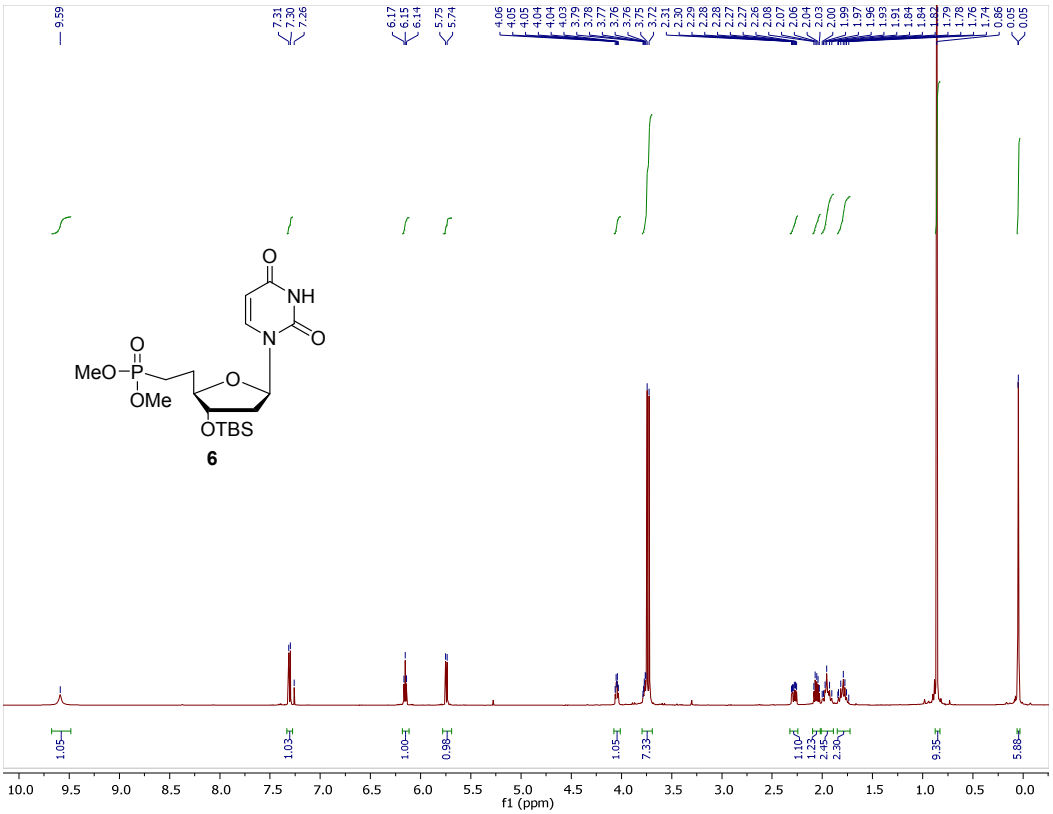
Supplementary Figure 5. 500 MHz ^{1}H NMR spectrum of **5** in $CDCl_3$.



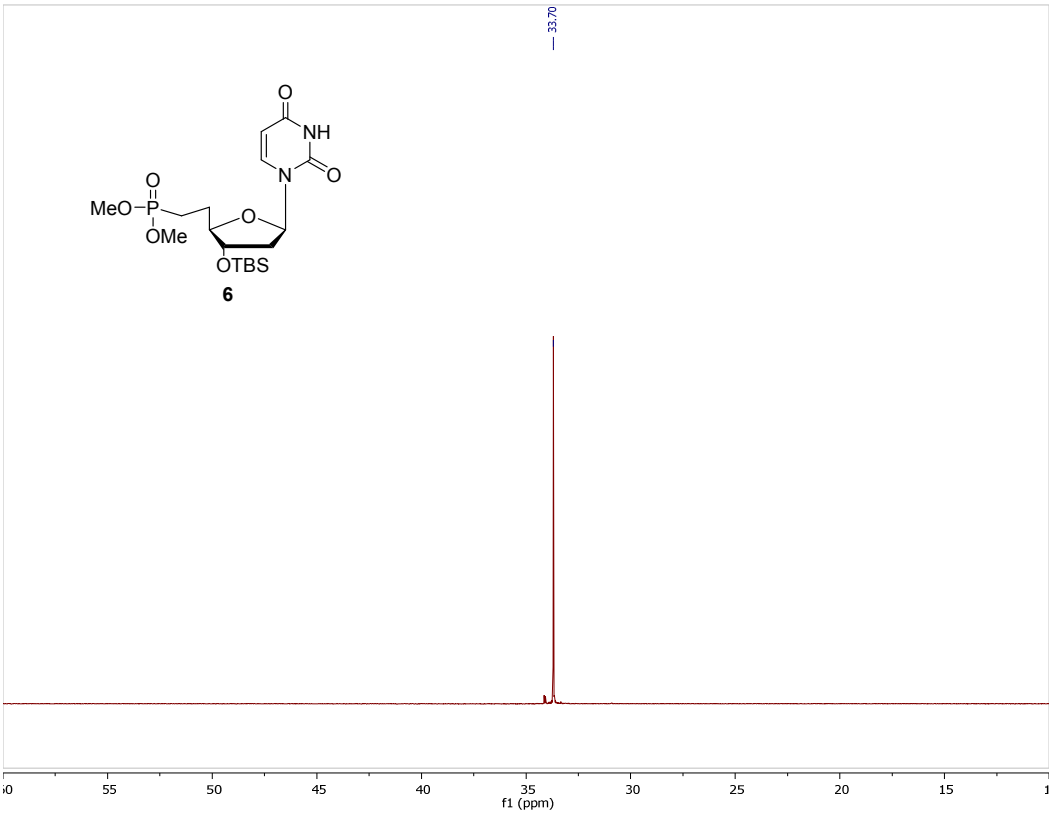
Supplementary Figure 6. 202 MHz ^{31}P NMR spectrum of **5** in $CDCl_3$.



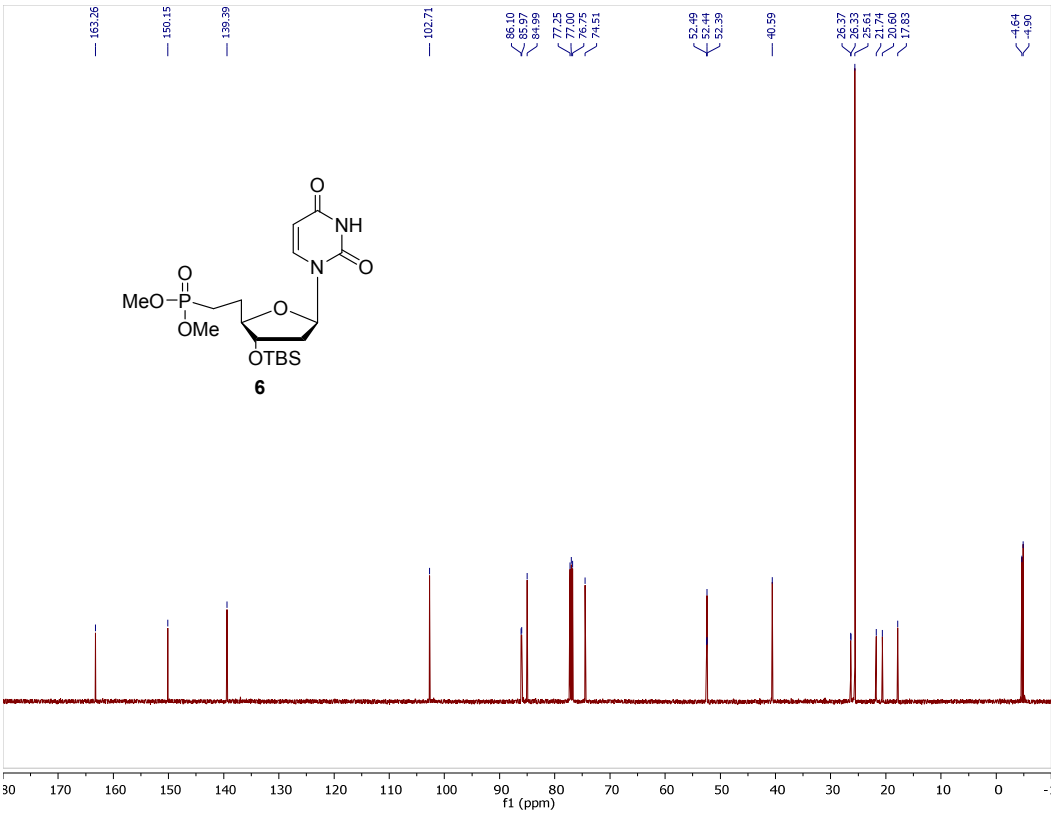
Supplementary Figure 7. 126 MHz ^{13}C NMR spectrum of **5** in $CDCl_3$.



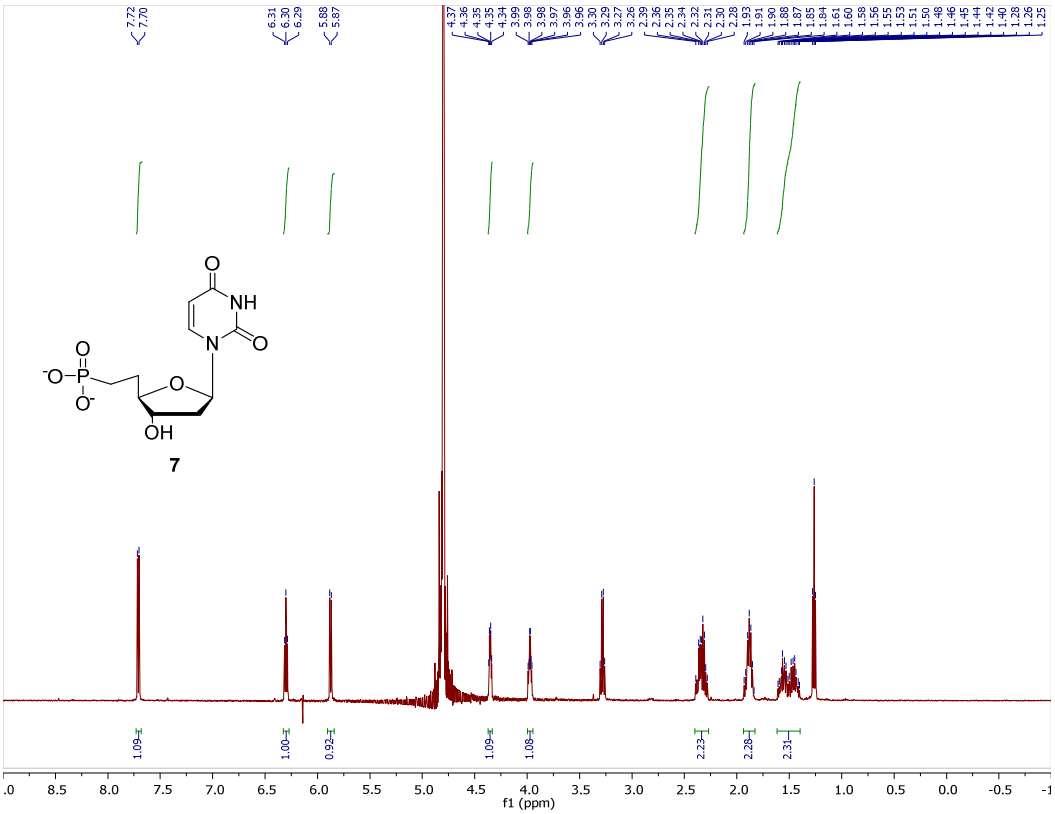
Supplementary Figure 8. 500 MHz ^{1}H NMR spectrum of **6** in $CDCl_3$.



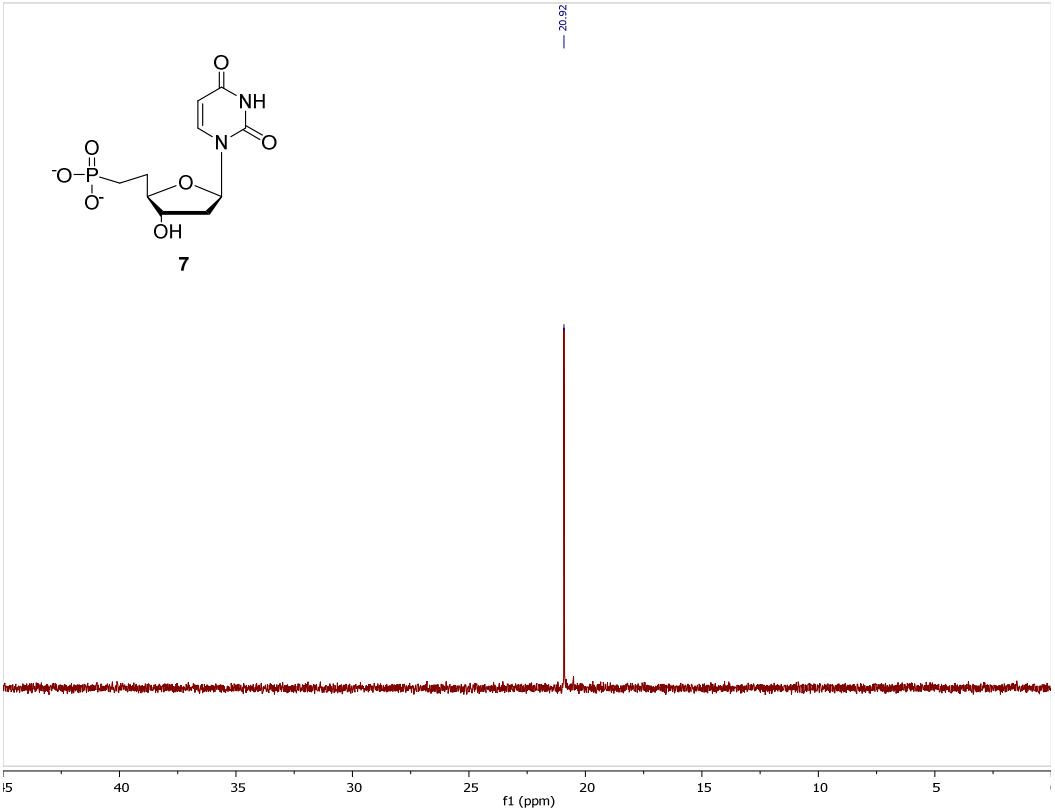
Supplementary Figure 9. 202 MHz ^{31}P NMR spectrum of **6** in $CDCl_3$.



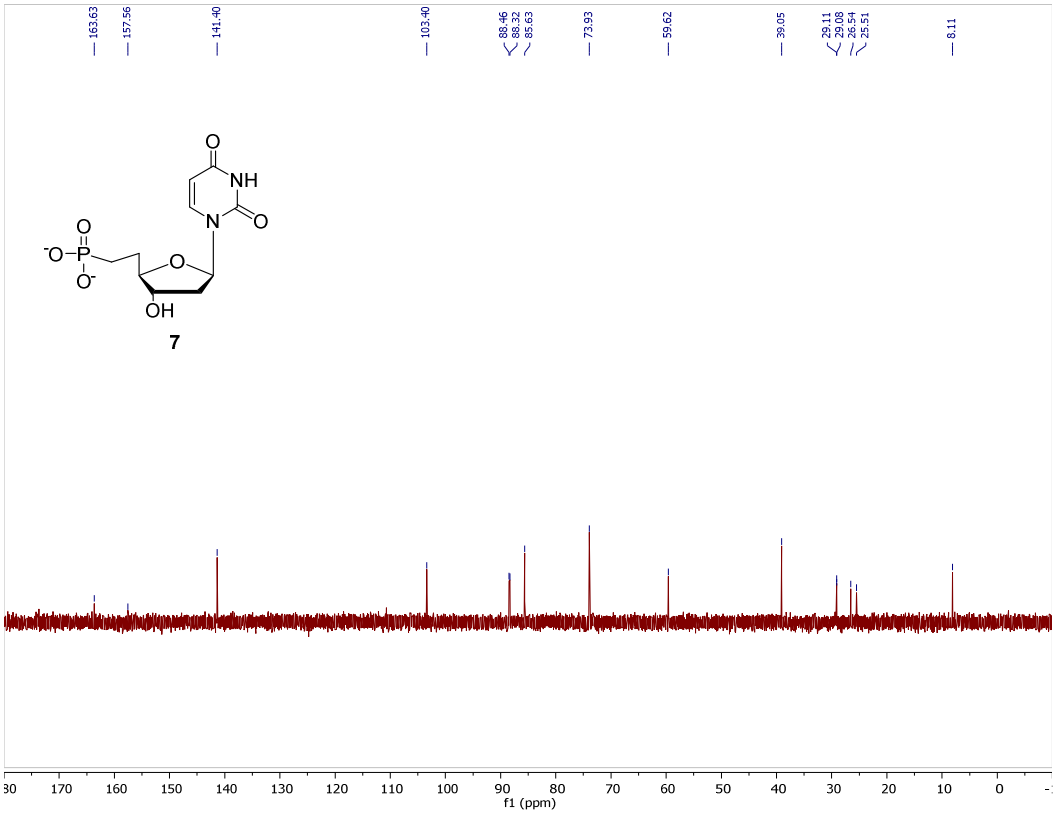
Supplementary Figure 10. 126 MHz ^{13}C NMR spectrum of **6** in $CDCl_3$.



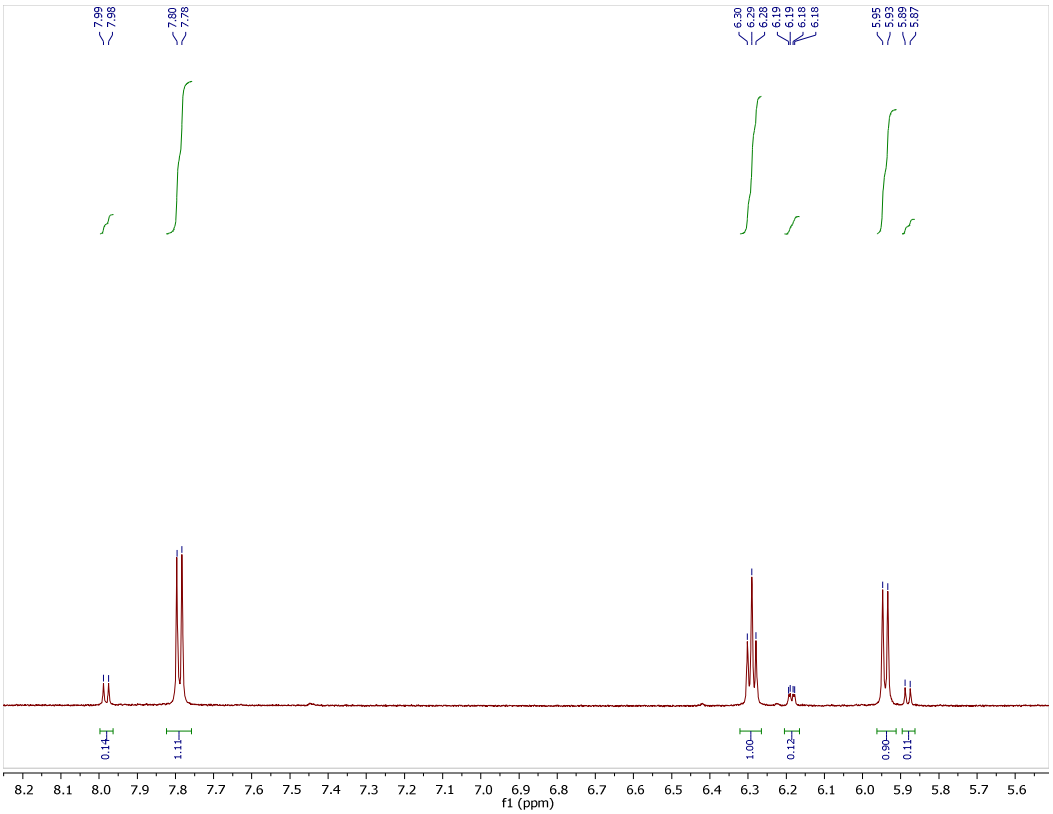
Supplementary Figure 11. 500 MHz ^{1}H NMR spectrum of **7** in D_2O.



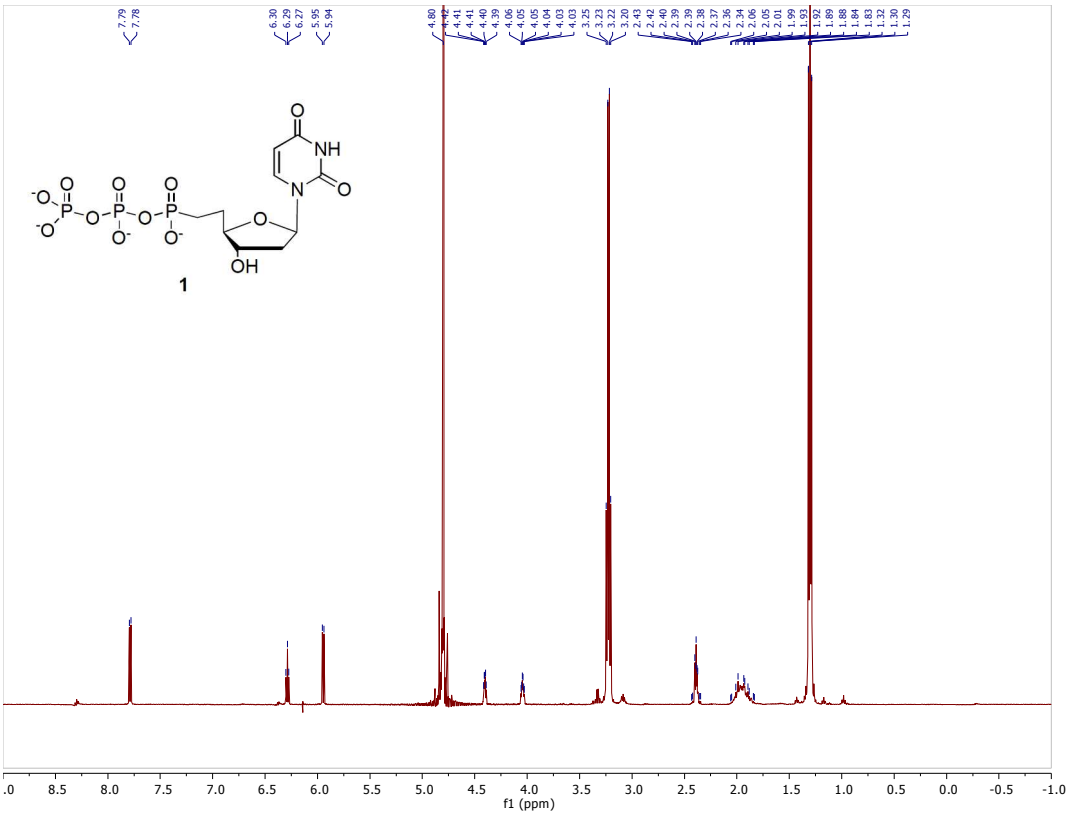
Supplementary Figure 12. **202 MHz ^{31}P NMR spectrum of 7 in D_2O.**



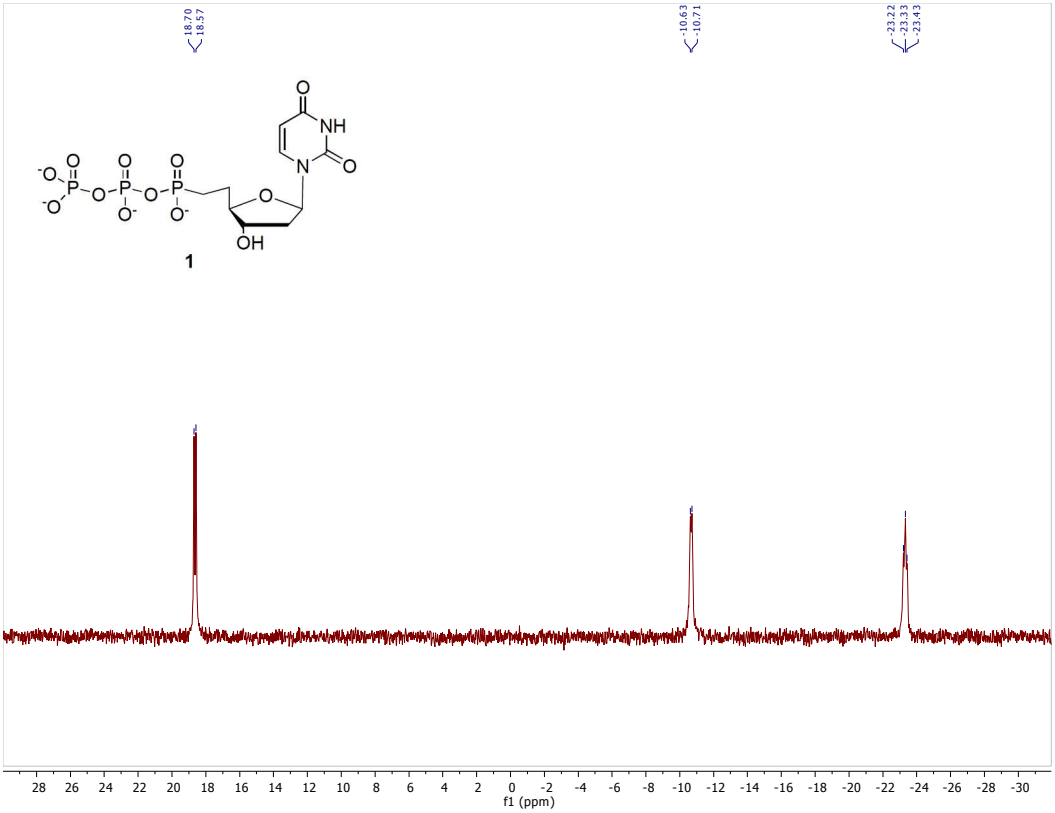
Supplementary Figure 13. 126 MHz ^{13}C NMR spectrum of **7** in D_2O.



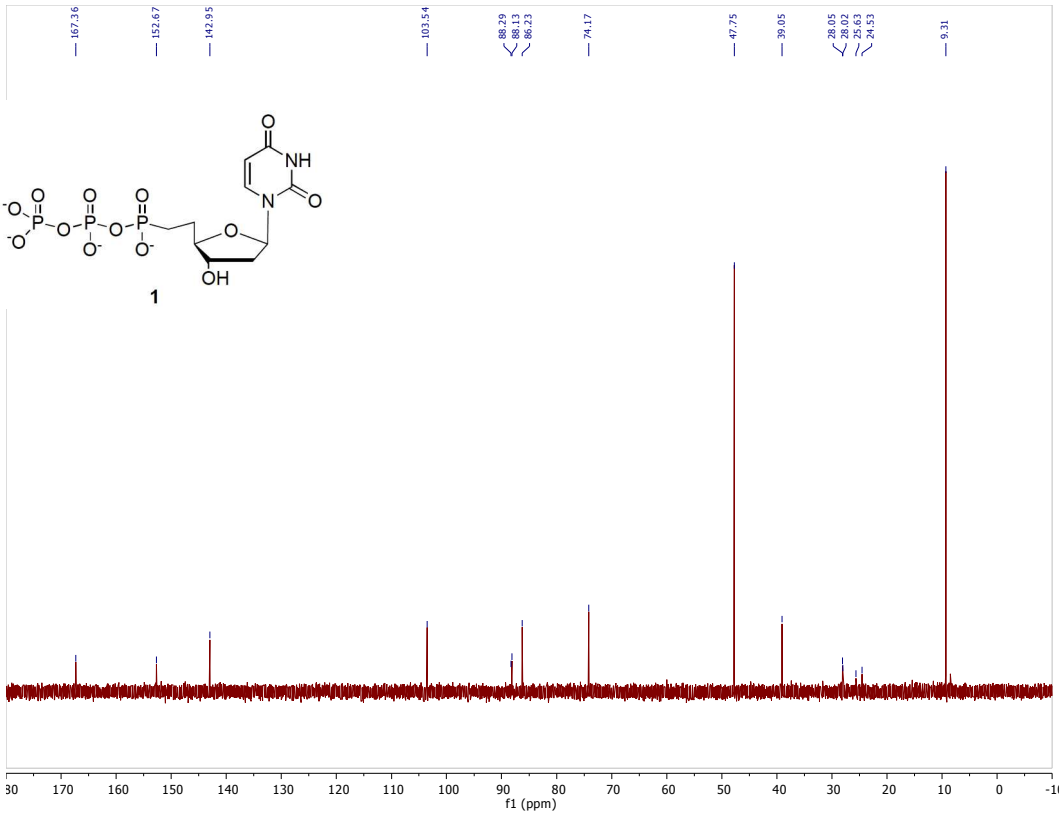
Supplementary Figure 14. Part of the 500 MHz ^{1}H NMR spectrum of α- and β- anomers of **1** in D_2O (after SAX purification).



Supplementary Figure 15. 500 MHz ^{1}H NMR spectrum of **1** in D_2O.

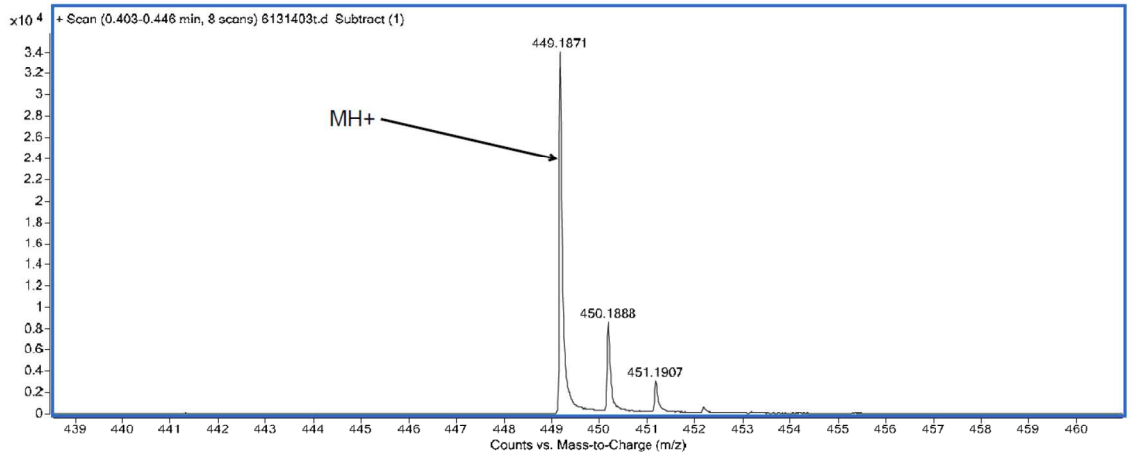


Supplementary Figure 16. 202 MHz ^{31}P NMR spectrum of **1** in D_2O.



Supplementary Figure 17. 126 MHz ^{13}C NMR spectrum of **1** in D_2O

High-resolution MS of compound **6**

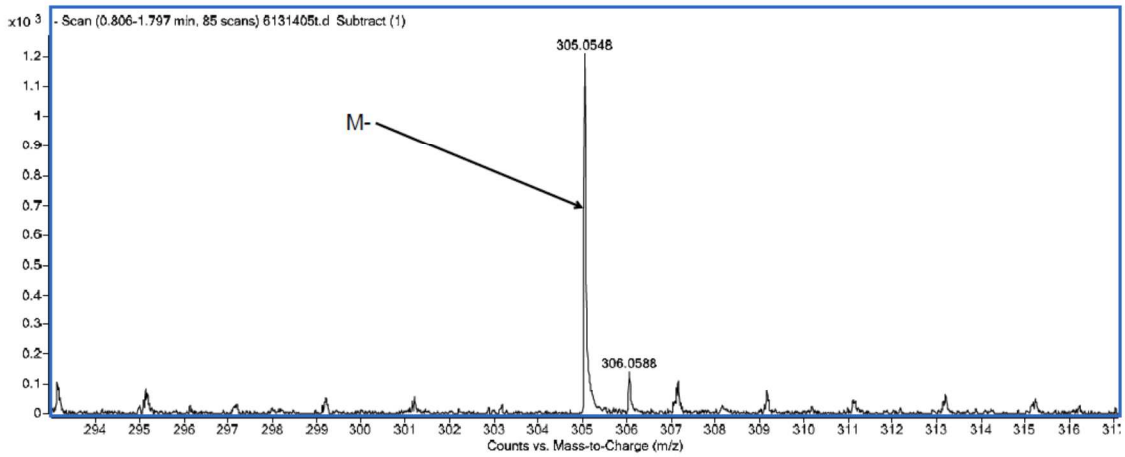


Measured Mass	449.1871	
Element	**Low Limit**	**High Limit**
C	10	25
H	25	45
N	0	3
O	5	9
P	0	1
Si	0	1

Formula	Calculated Mass	mDaError	ppmError	RDB
C18 H34 N2 O7 Si P	449.1867	0.4	0.8	4.5
C22 H31 N O7 Si	449.1864	0.7	1.5	9

Supplementary Figure 18. HRMS (ESI/APCI) of compound **6.**

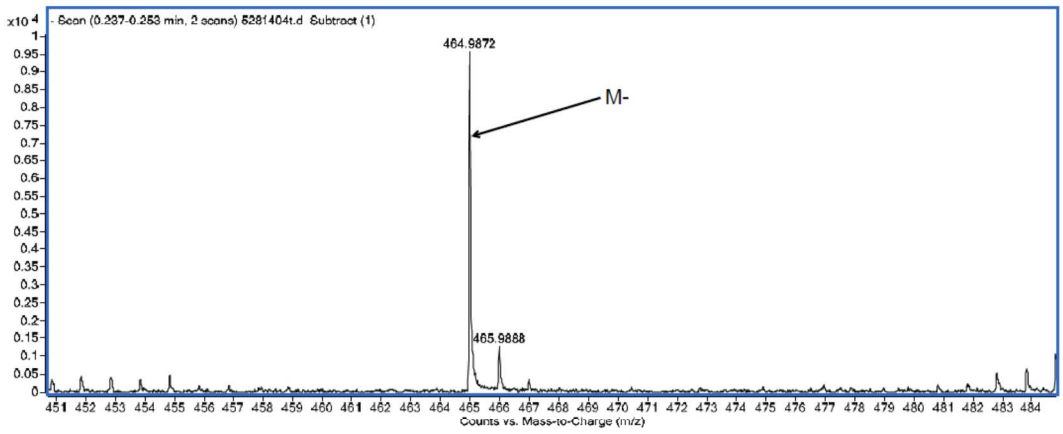
High-resolution MS of compound **7**



Measured Mass	305.0548			
Element	Low Limit	High Limit		
C	5	15		
H	5	25		
N	0	3		
O	5	9		
P	0	1		
Formula	Calculated Mass	mDaError	ppmError	RDB
C10 H14 N2 O7 P	305.0533	1.5	4.9	5.5
C14 H11 N O7	305.0530	1.8	5.9	10
C15 H14 O5 P	305.0573	-2.5	-8.3	9.5

Supplementary Figure 19. HRMS (ESI/APCI) of compound **7.**

High-resolution MS of compound **1**



Measured Mass 464.9872

Element	Low Limit	High Limit
C	5	15
H	5	25
N	0	3
O	11	15
P	0	4

Formula	Calculated Mass	mDaError	ppmError	RDB
C10 H16 N2 O13 P3	464.9871	0.1	0.3	5.5
C6 H19 N3 O13 P4	464.9874	-0.2	-0.4	1
C14 H13 N O13 P2	464.9868	0.4	0.9	10
C8 H21 O14 P4	464.9887	-1.5	-3.3	0.5

Supplementary Figure 20. HRMS (ESI/APCI) of compound **1.**