Electronic Supplementary Material (ESI) for RSC Medicinal Chemistry.

Supporting Information

PDE-stable 2'3'-cGAMP analogues, containing 5'-S phosphorothioester linkage, as STING Agonists

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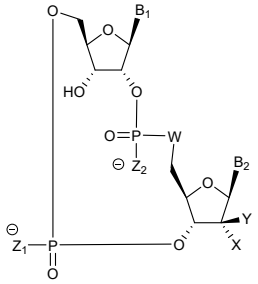
I. Biological Analysis

i. In vitro analysis of STING binding

a. Differential scanning fluorimetry data



2'3'CDNs	Linkage	B_1,B_2	Z_1,Z_2	X	Y	W	DSF ΔTm
cGAMP [1]	2',3'	G,A	O,O	OH	H	O	15.2
ADU-S100 [2]	2',3'	A,A	S,S	OH	H	O	10.4
Endo-S-cGAMP (**6**)	2',3'	G,A	O,O	OH	H	S	8.2
Endo-S-cGGMP (**7**)	2',3'	G,G	O,O	OH	H	S	3.3
Endo-S-cGA$_F$MP (**8**)	2',3'	G,A	O,O	F	H	S	11.3
Endo-S-cGA$_{F2}$MP (**9**)	2',3'	G,A	O,O	H	F	S	3.3
En-S-cGA$_H$MP (**10**)	2',3'	G,A	O,O	H	H	S	8.6

Table S1. Thermal shift assay data. 150 μM of 2'3'CDNs were incubated with 100 μM of hSTING and 1:500 (v/v) SYPRO orange at a temperature gradient of 15 to 80 °C at increment of 1 °C for 15 s.

b. Fluorescence polarization

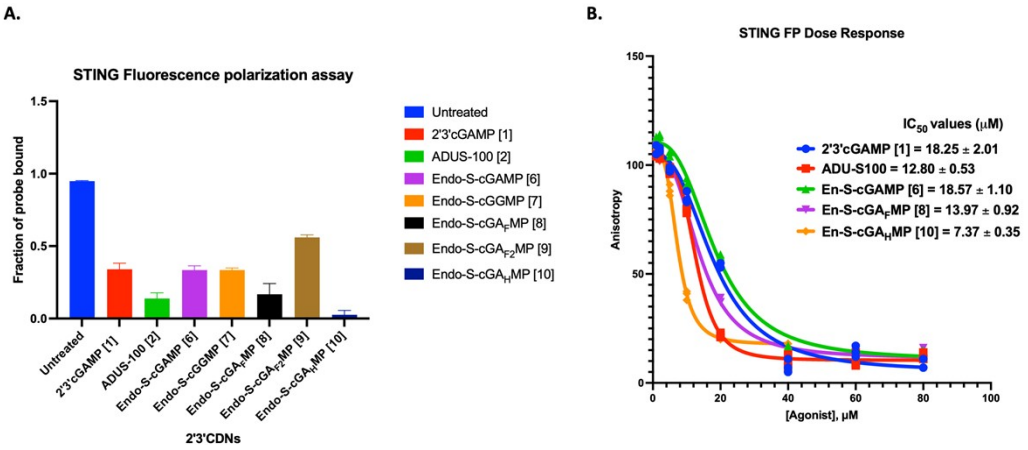


Fig S1. A) Fraction bound data generated upon incubation of 10 μM 2'3'CDNs with 20 μM STING and 50 nM of fluorescein labeled c-di-GMP probe. B) Dose dependent curves depicting anisotropy values vs ligand concentrations with IC_{50} values.

ii. Single dose IRF activation in THP1 dual cells

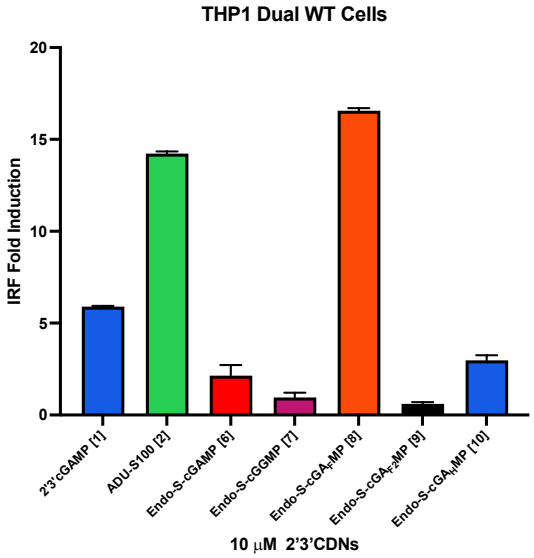


Fig S2. Single dose Quanti-Luc IRF induction assay after incubation of THP1 monocytes with 10 μM of 2'3'CDNs.

iii. Stability of 2'3'CDNs after incubating at 95 °C for 5 minutes

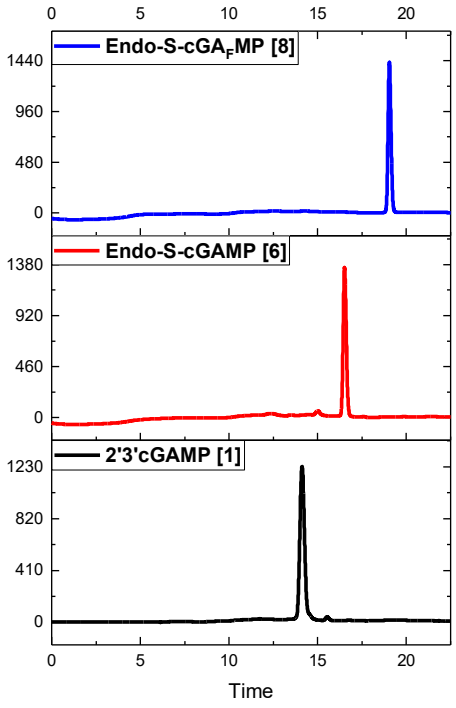


Fig S3. HPLC traces of 2'3'cGAMP, endo-S-cGAMP and endo-S-cGA$_F$MP after incubating at 95 °C for 5 minutes.

iv. Experimental procedure

Protein expression and purification

E. coli BL21 (DE3) containing plasmids of our protein of interest (hSTING-WT and Poxin) were initially cultured in 10 ml of LB media with selection antibiotics (50 μg/ml of kanamycin and chloramphenicol used for both hSTING and poxin) also added. The hSTING-WT plasmid was a gift from Prof. Pingwei Li and poxin's plasmid was a gift from Prof. Philip J. Kranzusch. After overnight growth the cultures were inoculated into 1L of terrific broth (TB) media supplemented with the same concentration of selection antibiotics as stated earlier. After the *E. coli* BL21 cultures grew to an OD of 0.6, 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to induce expression of the protein of interest. After IPTG is added, cultures were incubated at 16 °C for 12-16 hours. The culture was later centrifuged for 30 minutes at 5000 rpm and the pellets were collected. The collected pellets were resuspended in lysis buffer containing 50 mM Na_3PO_4, 300 mM NaCl, 20 mM imidazole, 5 mM β-mercaptoethanol (βME), 10% glycerol and 1 mM phenylmethylsulfonyl fluoride (PMSF) for hSTING (pH=7.4) expression and 20 mM HEPES-KOH, 20 mM imidazole, 10% glycerol, 1mM DTT and 1mM PMSF for poxin (pH=7.5). Lysis was achieved by sonication and lysed cells were centrifuged at 22000 rpm for 30 mins (at 4 ºC). The supernatant was collected and passed through a 5 ml nickel His-trap column. Washed twice with buffer containing same constituents as the lysis buffer but without PMSF and βME. The second wash and elution buffer contained 40 mM and 300 mM of imidazole respectively. Eluent were dialyzed overnight, and the purified protein concentration was determined by using UV absorbance at 280 nm wavelength for STING (extinction coefficient of hSTING is 47955 $M^{-1}cm^{-1}$ and used the Rapid Gold BCA Protein Assay Kit (from Thermo Fisher Scientific) to determine the concentration of poxin. We purchased ENPP1 stock from RnD Systems.

Thermal shift assay or DSF experiments

20 μL solutions of 100 mM Tris-HCl, pH=7.4, 150 mM NaCl,150 μM 2'3'CDNs or water (negative control), 100 μM hSTING-WT, and 1:500 (v/v) SYPRO Orange were prepared with experiments carried out in triplicates and denaturation performed using a real time PCR cycler. A gradient method from 15 to 80 ºC, holding in increment of 1 ºC for 15 seconds was used for the denaturation step.

$$\Delta Tm = T_y - T_x$$

The ΔTm values for each 2'3'CDN were calculated by subtracting the average denaturing temperature (T_x) of sample of hSTING without 2'3'CDN from the average denaturing temperature (T_y) of the hSTING-2'3'CDN complex.

Single dose hSTING fluorescence polarization assay

20 μM of hSTING-WT, 50 nM of 2'- O- (6- [Fluoresceinyl]aminohexylcarbamoyl)- cyclic diguanosine monophosphate (2'-Fluo-AHC-c-di-GMP / F-c-di-GMP purchased from Biolog) and 20 μM of synthesized 2'3'CDN analogues or controls dissolved in double deionized (DD) water was incubated for 5 mins in PBS at room temperature. Fluorescence anisotropy at $\lambda_{ex/em}$ = 485/528 nm was measured using a Gen5 microplate reader. Normalized anisotropy was calculated by subtracting the measurement of control sample without hSTING (0 μM). 98 Greiner-Bio well plates were used, and all samples/experiments were replicated in triplicates. Fraction bound was calculated from anisotropy values as shown below:

$$F_{bound} = \frac{r - r_{free}}{Q(r_{bound} - r) + (r - r_{free})}$$

Where *r* equates raw anisotropy value of 2'3'CDNs bound to 20 μM of hSTING, r_{free} is the anisotropy measurement unbound fluorophore (sample without hSTING), r_{bound} is the anisotropy value of fluorophore bound to hSTING only, Q refers to the ratio of the intensity at $\lambda_{ex/em}$ = 485/528 nm between the bound and free fluorophore.

IC_{50} determination based of hSTING fluorescence polarization assay

A mixture of 20 μM hSTING, 50 nM 2'-Fluo-AHC-c-di-GMP and different concentration of 2'3'CDNs were incubated for 5 minutes. We screened varying at 1 μM, 2 μM, 5 μM, 10 μM, 20 μM, 40 μM, 60 μM, and 80 μM of 2'3'CDNs. Fluorescence polarization was determined at $\lambda_{ex/em}$ = 485/528 nm and each concentration point was replicated in triplicates. The fluorescence anisotropy change with varying concentration was plotted using a 4-parameter dose-response model in GraphPad Prism to get the IC_{50} values.

IRF activation in THP1 dual cells

THP1 or THP1 (STING KI R232) dual reporter cells (purchased from invivogen) were cultured in RPMI media which contained 1x penicillin/streptomycin and 10 % heat inactivated foetal bovine serum (FBS) in 37 ºC, 5% carbon dioxide (incubator conditions). After successful passaging and cell growth, 5 x 10^5 cells were seeded in clear-well 96 well and left to incubate at the specified incubator conditions. After 24 hours, cells were treated in triplicates with 10 μM 2'3'CDN or water as a control and left to incubate for another 24 hours. 20 μL Cells were collected and 50 μL solution of QUANTI-Luc™ reagent (Invivogen) was added and luminescence was determined via a Biotek Cytation 5 multi-mode reader. Fold induction was gotten by dividing average raw relative light units (RLUs) values by the average RLU of cells treated.

EC_{50} determination

The activity of analogues that showed promising fold induction was further characterized by determining their EC_{50} values. Concentrations from 5 nM to 200 μM of these analogues was added to 5 x 10^5 seeded THP1 dual or KI STING R232 cells totaling a final volume of 200 μL in a clear-welled 96-well plates. Compounds were tested in triplicates. After the addition, cells were incubated for 24 hours. 20 μL of cell media is then taken and added to 50 μL solution of QUANTI-Luc™. After swirling properly, luminescence is read via a plate reader and raw triplicate measurements were analyzed and plotted via Graph Pad Prism using the dose response 4 point model.

Stability of 2'3'CDNs upon heating at 95 °C

300 μM of 2'3'CDNs (2'3'cGAMP, **6** and **8)** in 50 mM Tris and 250 mM NaCl at pH 9.5 was heated at 95 °C for 5 minutes. After incubation, the tubes were centrifuged and then 50 μL of content was subjected to HPLC analysis using a COSMOSIL C18-PAQ packed column and gradient comprising of 0–16 min: 99%–87% 0.1 M TEAA, 1–13% acetonitrile, 16–23 min: 87–10% 0.1 M TEAA, 13–90% acetonitrile 23–25 min: 10–99% 0.1 M TEAA, 90–1% acetonitrile.

ENPP1 degradation assay

Reactions with 300 μM 2'3'cGAMP or different Endo-S-2'3'CDNs is incubated at 37 ºC with 0.5 μg/ml ENPP1 (RnD Systems) in 1x buffer containing 50 mM Tris and 250 mM NaCl at pH 9.5 in solution. Reactions were monitored at 0 minutes, 30 minutes, 1 hour, 3 hour and 24 hours via HPLC runs after the enzymatic reaction is stopped by heating at 95 ºC for 5 minutes. At those time points 100 μL is pipetted from the reaction and reaction is quenched by denaturing the enzyme. Experiments were done in duplicates. Then the collected reaction is centrifuged and 50 μL of it was subjected to HPLC analysis using a COSMOSIL C18-PAQ packed column and gradient comprising of 0–16 min: 99%–87% 0.1 M TEAA, 1–13% acetonitrile, 16–23 min: 87–10% 0.1 M TEAA, 13–90% acetonitrile 23–25 min: 10–99% 0.1 M TEAA, 90–1% acetonitrile. The respective 2'3'CDN were detected by measuring absorbance at wavelength of 260 nm. The area under the curves was used to calculate the relative abundance by normalizing with reaction condition with no ENPP1.

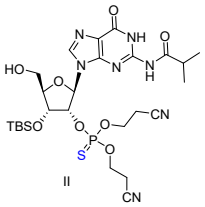
Poxin degradation assay

300 µM of 2'3'CDNs, 10 µg/ml of poxin incubated with 1X reaction buffer (50 mM HEPES–KOH pH 7.5, 35 mM KCl, and 1 mM DTT) in solution at different time points within 24 hours. Reactions were quenched by denaturing at 95 ºC for 5 minutes and 50 µL from the reaction was analyzed via HPLC. HPLC analysis conditions is as stated above.

II. Chemistry

i. General synthesis and characterization of intermediates

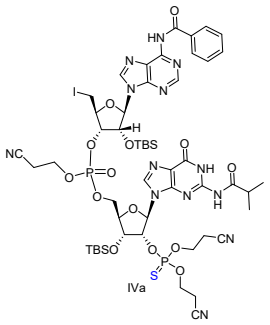
O-((2R,3R,4R,5R)-4-((tert-butyldimethylsilyl)oxy)-5-(hydroxymethyl)-2-(2-isobutyramido-6-oxo-1,6-dihydro-9H-purin-9-yl)tetrahydrofuran-3-yl) O,O-bis(2-cyanoethyl) phosphorothioate (II):



3-hydroxypropionitrile (0.40 ml, 5.5 mmol) was added to a solution of guanosine (n-ibu) 3'-tBDSilyl CED phosphoramidite (purchased from chemgenes [I], 1.06 g, 1.09 mmol) in anhydrous acetonitrile (15 ml) under argon. After stirring for 15 minutes at room temperature (RT), dicyanoimidazole (0.472 g, 4.0 mmol) was added to the reaction mixture and stirred for an extra 12 hours under argon at RT. *3H*-1,2-benzodithiole-3-one 1,1-dioxide (Beaucage reagent) was added to the reaction mixture and left to stir for an hour. The reaction was quenched with $Na_2S_2O_3$ (30 ml, 1 M) and subsequently extracted thrice using dichloromethane (50 ml). After drying with a rotary evaporator, the crude product was redissolved in DCM (20 ml) and dichloroacetic acid (DCA, 1 ml, 12.2 mmol) was added dropwise and left to stir for 12 minutes. Saturated $NaHCO_3$ (30 ml) was added to quench the reaction and the aqueous layer was extracted three times using DCM (50 ml). After concentrating under reduced pressure, the crude product was purified via column chromatography (silica gel, gradient 10-100% ethyl acetate in hexanes) to afford 0.439 g of pure II.

^{1}H NMR (500 MHz, MeOD) δ 8.34 (s, 1H), 6.23 (d, *J* = 7.6 Hz, 1H), 5.44 (ddd, *J* = 12.6, 7.6, 4.7 Hz, 1H), 4.57 (d, *J* = 4.7 Hz, 1H), 4.23 (ddt, *J* = 10.5, 8.9, 5.9 Hz, 1H), 4.18 – 4.12 (m, 2H), 4.12 – 4.05 (m, 1H), 3.94 (dddd, *J* = 10.5, 9.6, 6.7, 5.1 Hz, 1H), 3.79 (qd, *J* = 12.2, 3.1 Hz, 2H), 2.81 (td, *J* = 5.9, 0.9 Hz, 2H), 2.75 – 2.66 (m, 2H), 2.61 (ddd, *J* = 17.2, 7.3, 5.1 Hz, 1H), 2.15 (s, 1H), 1.21 (dd, *J* = 6.9, 4.1 Hz, 7H), 0.98 (s, 9H), 0.20 (d, *J* = 11.5 Hz, 6H). ^{31}P NMR (203 MHz, MeOD) δ 68.18. ^{13}C NMR (126 MHz, MeOD) δ 180.40, 156.12, 149.82, 148.81, 138.38, 119.62, 116.92, 116.76, 88.13, 84.61, 79.37, 72.64, 63.51, 63.17, 61.31, 48.12, 47.95, 47.78, 47.61, 47.44, 47.27, 47.10, 35.57, 24.91, 18.53, 18.46, 18.34, 18.27, 18.01, 17.85, 17.64, -5.64, -6.07.

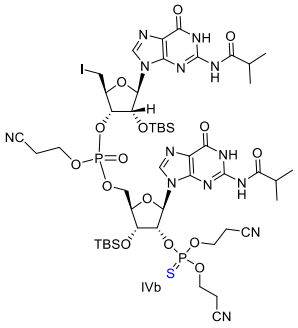
(2S,3R,4R,5R)-5-(6-benzamido-9H-purin-9-yl)-4-((tert-butyldimethylsilyl)oxy)-2-(iodomethyl)tetrahydrofuran-3-yl(((2R,3R,4R,5R)-4-((bis(2 cyanoethoxy)phosphorothioyl)oxy)-3-((tert-butyldimethylsilyl)oxy)-5-(2-isobutyramido-6-oxo-1,6-dihydro-9H-purin-9-yl)tetrahydrofuran-2-yl)methyl) (2-isocyanoethyl) phosphate (IVa):



A mixture of phosphorothioate II (0.27 g, 0.42 mmol) and adenosine (n-bz) 2'-tBDSilyl CED phosphoramidite (IIIa, 0.623 g, 0.63 mmol) was dissolved in anhydrous acetonitrile, leaving to stir at RT under argon. After 15 mins, dicyanoimidazole (0.236 g, 2 mmol) is added and the reaction is stirred at RT for 12 hrs. tBuOOH (70 % aqueous solution, 0.50 ml, 3.9 mmol) was then added and the reaction mixture was allowed to stir for an extra 10 mins. The reaction was quenched with 30 ml of $Na_2S_2O_3$ (1 M) and the aqueous layer was extracted 3 times using DCM (50 ml). After concentrating under reduced pressure, the crude product is redissolved in DCM (20 ml) and DCA (0.50 ml, 6.1 mmol) is added dropwise to the solution. After 15 mins of stirring, saturated $NaHCO_3$ (30 ml) was added to quench the reaction. The aqueous phase was extracted using DCM (50 ml, thrice). After concentrating, the crude product was purified via column chromatography with first elution gradient of 10 – 100% ethyl acetate in hexane to remove unconverted starting materials and 2-5% methanol in ethyl acetate to elute product. To the purified product (0.85 g) is added methyltriphenoxyphosphonium iodide (0.95 g, 2.10 mmol) and 2,6-lutidine (1.00 ml, 8.61 mmol) in anhydrous DMF (4 ml) under argon at RT. After stirring for an hour, $Na_2S_2O_3$ (1M, 30 ml) was added and then extracted thrice with DCM (50 ml). The concentrated crude product is then purified by column chromatography (2-5% methanol in ethyl acetate). The purified product (0.15 g) was collected as a pale-yellow solid.

^{1}H NMR (500 MHz, MeOD) δ 8.75 (d, *J* = 10.7 Hz, 1H), 8.59 (d, *J* = 38.1 Hz, 1H), 8.22 (d, *J* = 4.5 Hz, 1H), 8.09 (d, *J* = 7.7 Hz, 2H), 7.74 – 7.64 (m, 1H), 7.56 (t, *J* = 7.6 Hz, 2H), 6.23 (dd, *J* = 10.1, 7.5 Hz, 1H), 6.17 (d, *J* = 6.3 Hz, 1H), 5.60 (dt, *J* = 12.6, 6.3 Hz, 1H), 5.53 – 5.35 (m, 1H), 5.14 (d, *J* = 21.4 Hz, 1H), 4.70 (d, *J* = 5.1 Hz, 1H), 4.64 (d, *J* = 4.7 Hz, 1H), 4.56 (dt, *J* = 12.5, 5.0 Hz, 1H), 4.52 – 4.46 (m, 1H), 4.41 (s, 1H), 4.37 (q, *J* = 6.2 Hz, 1H), 4.25 (dq, *J* = 9.5, 5.0 Hz, 1H), 4.18 (dq, *J* = 10.7, 5.3 Hz, 1H), 4.09 (dd, *J* = 15.6, 8.2 Hz, 1H), 4.02 (dq, *J* = 10.3, 5.1 Hz, 1H), 3.78 (dd, *J* = 10.8, 7.0 Hz, 1H), 3.67 – 3.61 (m, 1H), 2.98 (t, *J* = 5.8 Hz, 1H), 2.89 (t, *J* = 5.8 Hz, 1H), 2.82 (q, *J* = 5.9 Hz, 2H), 2.72 (dd, *J* = 9.7, 5.1 Hz, 2H), 1.28 (s, 3H), 1.22 (ddd, *J* = 13.0, 6.9, 4.3 Hz, 7H), 0.99 (s, 9H), 0.92 – 0.82 (m, 4H), 0.78 (s, 6H), 0.72 (s, 4H), 0.25 (dd, *J* = 5.0, 2.8 Hz, 5H), -0.01 (d, *J* = 29.5 Hz, 3H), -0.21 (d, *J* = 27.6 Hz, 3H). ^{31}P NMR (203 MHz, MeOD) δ 67.92, -2.57, -2.79. ^{13}C NMR (126 MHz, MeOD) δ 180.37, 166.79, 152.06, 151.82, 150.17, 148.58, 143.91, 139.32, 133.54, 132.57, 128.40, 128.10, 124.28, 117.10, 116.92, 88.94, 88.75, 85.78, 85.00, 84.79, 83.00, 82.62, 79.54, 77.05, 72.65, 67.59, 63.70, 63.49, 63.36, 54.87, 48.13, 47.96, 47.79, 47.62, 47.45, 47.28, 47.11, 35.58, 29.34, 24.96, 24.78, 24.70, 18.99, 18.88, 18.82, 18.59, 18.45, 18.26, 17.98, 17.87, 17.67, 17.47, 17.38, 13.06, 3.31, 3.13, -5.45, -5.51, -5.81, -5.88, -5.97, -6.06, -6.52.

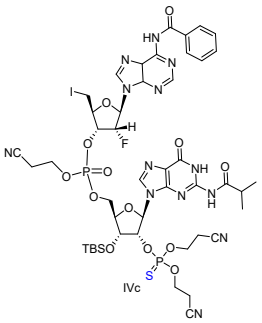
((2R,3R,4R,5R)-4-((bis(2-cyanoethoxy)phosphorothioyl)oxy)-3-((tert-butyldimethylsilyl)oxy)-5-(2-isobutyramido-6-oxo-1,6-dihydro-9H-purin-9-yl)tetrahydrofuran-2-yl)methyl ((2S,3R,4R,5R)-4-((tert-butyldimethylsilyl)oxy)-2-(iodomethyl)-5-(2-isobutyramido-6-oxo-1,6-dihydro-9H-purin-9-yl)tetrahydrofuran-3-yl) (2-isocyanoethyl) phosphate (IVb):



A mixture of phosphorothioate II (0.27 g, 0.42 mmol) and guanosine (n-ibu) 2'-tBDSilyl CED phosphoramidite (IIIb, 0.611 g, 0.63 mmol) was dissolved in anhydrous acetonitrile, leaving to stir at RT under argon following the same procedure for the synthesis of IVa.

^{1}H NMR (500 MHz, MeOD) δ 8.22 (d, *J* = 6.2 Hz, 1H), 8.18 (s, 1H), 6.25 (dd, *J* = 12.7, 7.2 Hz, 1H), 5.92 (d, *J* = 7.4 Hz, 1H), 5.72 – 5.55 (m, 1H), 5.18 (s, 1H), 5.04 (ddd, *J* = 7.9, 4.7, 1.5 Hz, 1H), 4.72 (dd, *J* = 4.8, 2.1 Hz, 1H), 4.64 (d, *J* = 4.7 Hz, 1H), 4.53 (t, *J* = 5.0 Hz, 2H), 4.47 – 4.39 (m, 3H), 4.36 – 4.28 (m, 2H), 4.29 – 4.19 (m, 2H), 4.18 – 4.09 (m, 1H), 4.07 – 3.98 (m, 1H), 3.68 (dd, *J* = 10.7, 7.7 Hz, 1H), 3.58 (dd, *J* = 10.7, 5.9 Hz, 1H), 3.51 (dd, *J* = 9.2, 6.6 Hz, 1H), 3.02 – 2.95 (m, 1H), 2.89 (t, *J* = 5.8 Hz, 1H), 2.84 (q, *J* = 6.3 Hz, 3H), 2.78 – 2.72 (m, 3H), 1.29 (d, *J* = 4.8 Hz, 2H), 1.24 (d, *J* = 6.9 Hz, 7H), 1.18 (dd, *J* = 6.9, 2.9 Hz, 4H), 0.99 (d, *J* = 0.9 Hz, 9H), 0.76 (s, 6H), 0.70 (s, 3H), 0.25 (dd, *J* = 5.9, 4.5 Hz, 6H), -0.02 (d, *J* = 37.4 Hz, 3H), -0.23 (d, *J* = 42.1 Hz, 3H). ^{31}P NMR (203 MHz, MeOD) δ 67.85, -2.55, -2.96. ^{13}C NMR (126 MHz, MeOD) δ 180.42, 180.34, 156.15, 149.67, 149.25, 148.61, 148.26, 140.01, 139.15, 139.02, 138.69, 128.86, 123.17, 121.20, 120.64, 119.90, 117.13, 116.94, 87.69, 87.22, 86.40, 85.65, 85.04, 84.63, 84.57, 83.26, 82.74, 79.80, 77.29, 76.88, 73.22, 72.96, 71.58, 71.28, 68.30, 67.19, 63.73, 63.70, 63.38, 48.14, 47.97, 47.79, 47.62, 47.45, 47.28, 47.12, 35.67, 35.59, 35.57, 34.29, 31.36, 29.32, 28.77, 24.96, 24.81, 24.65, 24.60, 22.83, 22.31, 19.64, 19.03, 18.98, 18.90, 18.83, 18.66, 18.60, 18.48, 18.42, 18.26, 18.13, 17.95, 17.86, 17.66, 17.46, 17.34, 13.05, 10.38, 3.70, 3.62, -5.45, -5.53, -5.85, -5.95, -6.07, -6.63, -6.68

(2S,3R,4R,5R)-5-(6-benzamido-4,5-dihydro-9H-purin-9-yl)-4-fluoro-2-(iodomethyl)tetrahydrofuran-3-yl (((2R,3R,4R,5R)-4-((bis(2-cyanoethoxy)phosphorothioyl)oxy)-3-((tert-butyldimethylsilyl)oxy)-5-(2-isobutyramido-6-oxo-1,6-dihydro-9H-purin-9-yl)tetrahydrofuran-2-yl)methyl) (2-isocyanoethyl) phosphate (IVc):

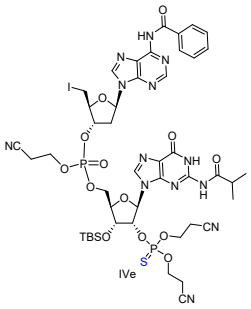


A mixture of phosphorothioate II (0.27 g, 0.42 mmol) and 5'-Dimethoxytrityl-N-benzoyl-deoxyAdenosine, 2'-fluoro-3'-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite (IIIe, 0.472 g, 0.63 mmol) was dissolved in anhydrous acetonitrile, leaving to stir at RT under argon following the same procedure for the synthesis of IVa.

^{1}H NMR (500 MHz, MeOD) δ 8.73 (d, *J* = 24.8 Hz, 1H), 8.53 (d, *J* = 37.1 Hz, 1H), 8.20 (d, *J* = 11.3 Hz, 1H), 8.15 – 8.02 (m, 2H), 7.69 – 7.63 (m, 1H), 7.57 (td, *J* = 7.6, 1.3 Hz, 2H), 6.47 (ddd, *J* = 26.9, 19.3, 2.3 Hz, 1H), 6.22 (dd, *J* = 20.0, 7.5 Hz, 1H), 5.67 – 5.42 (m, 2H), 4.66 (ddd, *J* = 20.6, 4.8, 1.4 Hz, 1H), 4.63 – 4.51

(m, 2H), 4.50 – 4.44 (m, 1H), 4.43 – 4.36 (m, 2H), 4.32 – 4.24 (m, 1H), 4.24 – 4.19 (m, 1H), 4.15 (ddt, *J* = 15.5, 10.0, 5.2 Hz, 1H), 4.08 (dtd, *J* = 12.1, 5.9, 3.5 Hz, 1H), 4.03 – 3.93 (m, 1H), 3.69 (ddd, *J* = 21.9, 11.3, 4.7 Hz, 1H), 3.56 (ddd, *J* = 27.4, 11.3, 5.5 Hz, 1H), 3.00 (td, *J* = 5.8, 1.2 Hz, 1H), 2.92 (td, *J* = 5.8, 1.2 Hz, 1H), 2.81 (dt, *J* = 11.0, 5.8 Hz, 2H), 2.75 (ddd, *J* = 10.1, 6.7, 3.2 Hz, 1H), 2.73 – 2.63 (m, 2H), 1.26 – 1.16 (m, 6H), 0.97 (t, *J* = 6.0 Hz, 9H), 0.23 (dd, *J* = 7.7, 6.2 Hz, 6H). ^{31}P NMR (203 MHz, MeOD) δ 67.92 (d, *J* = 5.1 Hz), -3.05 (d, *J* = 30.8 Hz). ^{13}C NMR (126 MHz, MeOD) δ 180.37, 166.82, 156.05, 152.17, 151.34, 150.09, 149.53, 148.63, 143.89, 143.44, 139.10, 133.52, 132.58, 128.89, 128.39, 128.12, 124.07, 120.55, 119.89, 117.08, 116.88, 90.51, 87.30, 87.05, 85.57, 84.91, 80.16, 77.47, 77.32, 76.93, 71.51, 67.86, 63.84, 63.69, 63.33, 48.12, 47.94, 47.77, 47.60, 47.43, 47.26, 47.09, 35.61, 24.91, 18.90, 18.51, 18.40, 18.34, 18.11, 17.85, 17.63, 2.54, 2.26, -5.60, -5.94, -6.00.

(2S,3S,5R)-5-(6-benzamido-9H-purin-9-yl)-2-(iodomethyl)tetrahydrofuran-3-yl (((2R,3R,4R,5R)-4-((bis(2-cyanoethoxy)phosphorothioyl)oxy)-3-((tert-butyldimethylsilyl)oxy)-5-(2-isobutyramido-6-oxo-1,6-dihydro-9H-purin-9-yl)tetrahydrofuran-2-yl)methyl) (2-isocyanoethyl) phosphate (IVe):

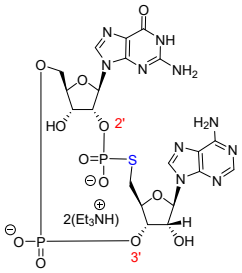


A mixture of phosphorothioate II (0.27 g, 0.42 mmol) and 5'-Dimethoxytrityl-N-benzoyl-2'-deoxyAdenosine,3'-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite (IIIg, 0.541 g, 0.63 mmol) was dissolved in anhydrous acetonitrile, leaving to stir at RT under argon following the same procedure for the synthesis of IVa.

^{1}H NMR (500 MHz, MeOD) δ 8.70 (d, *J* = 20.6 Hz, 1H), 8.52 (d, *J* = 50.7 Hz, 1H), 8.20 (s, 1H), 8.07 (dd, *J* = 7.6, 3.6 Hz, 2H), 7.64 (t, *J* = 7.4 Hz, 1H), 7.54 (t, *J* = 7.6 Hz, 2H), 6.66 – 6.39 (m, 1H), 6.22 (dd, *J* = 21.2, 7.4 Hz, 1H), 5.53 (s, 1H), 5.35 (d, *J* = 61.7 Hz, 1H), 4.69 (dd, *J* = 12.4, 4.7 Hz, 1H), 4.55 (s, 1H), 4.48 – 4.42 (m, 1H), 4.38 (dd, *J* = 14.0, 7.1 Hz, 2H), 4.26 – 4.13 (m, 2H), 4.09 (d, *J* = 6.5 Hz, 1H), 4.04 – 3.96 (m, 1H), 3.58 – 3.49 (m, 1H), 3.49 – 3.39 (m, 1H), 2.99 (t, *J* = 5.8 Hz, 1H), 2.95 – 2.89 (m, 1H), 2.84 – 2.76 (m, 3H), 2.71 (td, *J* = 7.2, 3.8 Hz, 2H), 1.28 (s, 2H), 1.21 (dd, *J* = 7.1, 2.6 Hz, 6H), 0.98 (d, *J* = 2.6 Hz, 9H), 0.88 (dt, *J* = 12.2, 7.0 Hz, 2H), 0.23 (t, *J* = 3.9 Hz, 6H). ^{31}P NMR (203 MHz, MeOD) δ 67.89, -3.18 (d, *J* = 34.7 Hz). ^{13}C NMR (126 MHz, MeOD) δ 208.78, 180.37, 166.81, 151.91, 151.62, 151.48, 149.79, 143.72, 143.52, 139.24, 139.05, 133.48, 132.62, 129.08, 128.84, 128.41, 128.12, 128.09, 124.10, 123.96, 123.17, 119.84, 117.26, 117.15, 116.94, 85.69, 85.18, 85.06, 84.88, 80.98, 77.29, 71.49, 71.31, 67.64, 63.72, 63.48, 63.35, 63.32, 54.90, 48.15, 47.98, 47.81, 47.64, 47.47, 47.30, 47.13, 36.32, 35.67, 34.29, 31.68, 31.36, 29.36, 24.97, 22.31, 19.65, 19.05, 18.99, 18.94, 18.67, 18.61, 18.51, 18.45, 18.16, 17.66, 13.07, 10.40, 3.56, 3.38, -5.45, -5.51, -5.80, -5.88.

ii. Synthesis and characterization data of analogues

Endo-S-cGAMP (Compound 6)

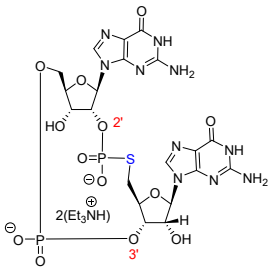


Compound 6

Ammonium hydroxide (5 ml, 30 % NH_4OH in water) was added to 5'-Iodo adenosine guanosine phosphorothioate **IVa** (52 mg, 0.038 mmol) and stirred for 24 h at RT. The solution was concentrated at reduced pressure to complete dryness under high vacuum. The crude product was redissolved in dry pyridine (2 ml) and then $Et_3N.3HF$ (0.25 µl, 1.6 mmol) at 50℃ for 6 h. After completion of reaction, 20 ml of acetone is added to precipitate the compound as a white pale solid which was collected by several rounds of centrifugation, decanting and washing. After the compound was allowed to dry 5 ml of water was added and the compound 4 was purified via HPLC using using a COSMOSIL C18-PAQ packed column and gradient comprising of 0–16 min: 99%–87% 0.1 M TEAA, 1–13% acetonitrile, 16–23 min: 87–10% 0.1 M TEAA, 13–90% acetonitrile 23–25 min: 10–99% 0.1 M TEAA, 90–1% acetonitrile. Off-white solid, 21% yield.

^{1}H NMR (800 MHz, D_2O) δ 8.29 (d, *J* = 4.0 Hz, 2H), 8.21 (s, 1H), 6.08 (d, *J* = 4.4 Hz, 1H), 6.02 (d, *J* = 8.2 Hz, 1H), 5.38 (ddd, *J* = 12.1, 8.1, 4.2 Hz, 1H), 5.01 – 4.98 (m, 2H), 4.60 (d, *J* = 4.2 Hz, 1H), 4.50 – 4.43 (m, 1H), 4.40 (d, *J* = 3.2 Hz, 1H), 4.25 – 4.17 (m, 1H), 4.13 (dd, *J* = 12.6, 3.1 Hz, 1H), 3.22 (td, *J* = 14.4, 7.5 Hz, 1H), 3.05 (td, *J* = 13.9, 13.2, 4.3 Hz, 1H). ^{31}P NMR (203 MHz, D_2O) δ 19.24, -0.92. ^{13}C NMR (201 MHz, D_2O) δ 158.83, 154.17, 153.73, 152.10, 150.78, 148.75, 142.03, 140.51, 126.87, 118.86, 87.68, 84.47, 84.25, 82.69, 77.02, 75.23, 72.40, 71.78, 66.02, 46.56, 31.86, 8.12. HRMS (ESI-) m/z calcd for $[C_{20}H_{22}N_{10}O_{12}P_2S]^{2-}$ i.e. $[M\text{-}2H]^{2-}$ 344.030734, found 344.03126. Note that M refers to fully protonated phosphate.

Endo-S-cGGMP (Compound 7)

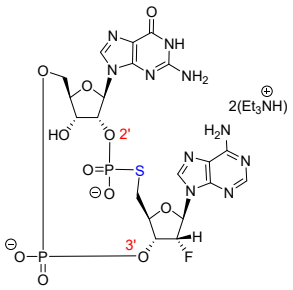


Compound 7

Ammonium hydroxide (5 ml, 30 % NH_4OH in water) was added to 5'-Iodo diguanosine phosphorothioate **IVb** (50 mg, 0.038 mmol) and stirred for 24 h at RT. The subsequent steps follow that described for compound 6. Off-white solid, 15% yield.

^{1}H NMR (800 MHz, D_2O) δ 8.24 (s, 1H), 7.88 (s, 1H), 6.03 (d, *J* = 8.0 Hz, 1H), 5.86 (d, *J* = 5.1 Hz, 1H), 5.39 (ddd, *J* = 12.1, 8.0, 4.2 Hz, 1H), 4.96 (d, *J* = 6.0 Hz, 2H), 4.43 – 4.35 (m, 2H), 4.20 (dd, *J* = 11.8, 5.4 Hz, 1H), 4.13 (d, *J* = 12.3 Hz, 1H), 3.29 (t, *J* = 6.6 Hz, 3H), 2.88 (t, *J* = 6.6 Hz, 2H). ^{31}P NMR (203 MHz, D_2O) δ 19.29, -0.99. ^{13}C NMR (201 MHz, D_2O) δ 158.75, 153.90, 153.77, 152.11, 151.59, 137.85, 127.25, 117.57, 116.29, 115.47, 100.44, 87.41, 84.30, 83.22, 76.99, 75.93, 71.87, 71.80, 66.11, 46.57, 35.18, 32.06, 15.64, 8.12. HRMS (ESI-) m/z calcd for $[C_{20}H_{22}N_{10}O_{13}P_2S]^{2-}$ i.e. $[M\text{-}2H]^{2-}$ 352.0281915, found 352.02911. Note that M refers to fully protonated phosphate.

Endo-S-cGA$_F$MP (Compound 8)

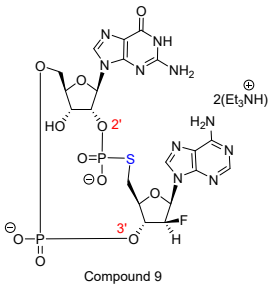


Compound 8

Ammonium hydroxide (5ml, 30 % NH_4OH in water) was added to 5'-Iodo 2'Fluoro adenosine guanosine phosphorothioate **IVc** (48 mg, 0.038 mmol) and stirred for 24 h at RT. The subsequent steps follow that described for compound 6. Off-white solid, 11% yield.

^{1}H NMR (800 MHz, D_2O) δ 8.24 (s, 1H), 8.14 (s, 1H), 8.04 (s, 1H), 6.35 (dt, *J* = 17.5, 2.3 Hz, 2H), 5.99 (d, *J* = 7.9 Hz, 1H), 5.70 (dd, *J* = 4.7, 2.4 Hz, 1H), 5.64 (dd, *J* = 4.7, 2.5 Hz, 1H), 5.50 – 5.43 (m, 2H), 5.15 (dtd, *J* = 13.7, 6.8, 3.2 Hz, 2H), 4.54 (d, *J* = 7.4 Hz, 1H), 4.39 (q, *J* = 2.6 Hz, 1H), 4.21 (dd, *J* = 5.2, 2.5 Hz, 3H), 3.42 – 3.36 (m, 2H), 3.03 (ddd, *J* = 13.9, 11.2, 2.9 Hz, 2H). ^{31}P NMR (203 MHz, D_2O) δ 18.78, -1.24. ^{13}C NMR (201 MHz, D_2O) δ 158.85, 154.51, 153.53, 151.99, 151.33, 148.31, 140.03, 138.80, 118.82, 116.43, 91.87, 90.92, 86.79, 86.63, 85.18, 84.15, 80.34, 76.60, 72.42, 71.69, 65.83, 46.57, 30.80, 21.63, 10.42, 8.12. HRMS (ESI-) m/z calcd for $C_{20}H_{22}FN_{10}O_{11}P_2S$ $[M-H]^-$ 691.064956, found 691.06582. Note that M refers to fully protonated phosphate.

Endo-S-cGA$_{F2}$MP (Compound 9)

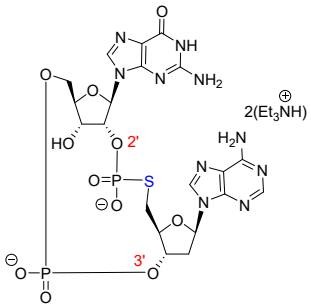


Compound 9

Ammonium hydroxide (5ml, 30 % NH_4OH in water) was added to 5'-Iodo 2'Fluoro adenosine guanosine phosphorothioate precursor (48 mg, 0.038 mmol) and stirred for 24 h at RT. The subsequent steps follow that described for compound 6. Off-white solid, 8% yield.

^{1}H NMR (800 MHz, D_2O) δ 8.29 – 8.20 (m, 2H), 8.12 (s, 1H), 6.53 (dd, *J* = 21.5, 3.1 Hz, 1H), 6.02 (d, *J* = 8.2 Hz, 1H), 5.51 – 5.39 (m, 2H), 5.34 (d, *J* = 3.2 Hz, 1H), 5.16 (dd, *J* = 14.2, 7.9 Hz, 2H), 4.25 – 4.18 (m, 3H), 4.09 – 3.99 (m, 1H), 3.00 (dq, *J* = 23.8, 8.1, 7.4 Hz, 2H). ^{31}P NMR (203 MHz, D_2O) δ 19.09, -1.74. ^{13}C NMR (201 MHz, D_2O) δ 177.96, 174.63, 158.88, 153.98, 153.77, 152.28, 150.61, 148.56, 141.60, 117.97, 116.06, 93.77, 92.82, 84.54, 84.27, 84.23, 83.56, 83.47, 83.03, 78.68, 78.55, 76.66, 71.95, 66.27, 61.62, 46.57, 42.15, 31.35, 29.49, 26.40, 21.10, 20.41, 13.14, 10.41, 8.12. HRMS (ESI-) m/z calcd for $C_{20}H_{22}FN_{10}O_{11}P_2S$ $[M-H]^-$ 691.064956, found 691.06633. Note that M refers to fully protonated phosphate.

Endo-S-cGA$_{H}$MP (Compound 10)

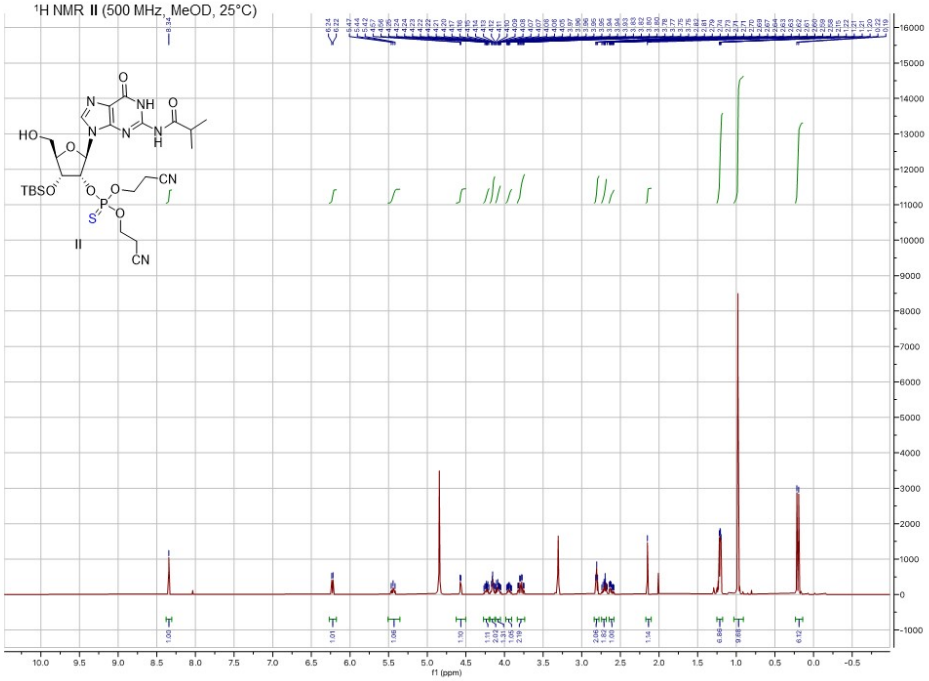


Compound 10

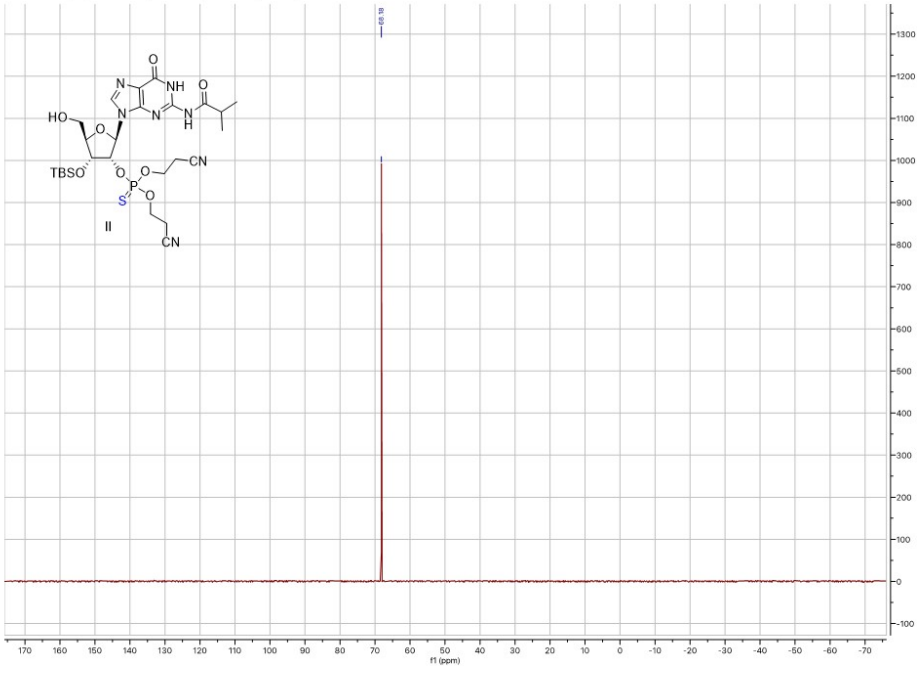
Ammonium hydroxide (5ml, 30 % NH_4OH in water) was added to 5'-Iodo 2'deoxyadenosine guanosine phosphorothioate **IVe** (47 mg, 0.038 mmol) and stirred for 24 h at RT. The subsequent steps follow that described for compound 6. Off-white solid, 26% yield.

^{1}H NMR (800 MHz, D_2O) δ 8.23 (d, *J* = 30.6 Hz, 3H), 6.45 (t, *J* = 6.9 Hz, 1H), 6.02 (d, *J* = 8.1 Hz, 1H), 5.45 (ddd, *J* = 11.9, 8.1, 4.1 Hz, 1H), 5.19 (tt, *J* = 6.6, 3.1 Hz, 1H), 4.37 (dt, *J* = 16.5, 4.8 Hz, 2H), 4.21 (dtt, *J* = 8.9, 6.6, 2.3 Hz, 2H), 3.02 – 2.99 (m, 1H), 2.99 – 2.94 (m, 4H), 2.75 (ddd, *J* = 14.6, 6.4, 3.3 Hz, 2H), 2.14 (d, *J* = 1.7 Hz, 2H). ^{31}P NMR (203 MHz, D_2O) δ 19.39, -1.13. ^{13}C NMR (201 MHz, D_2O) δ 215.33, 178.97, 174.63, 158.99, 154.43, 153.73, 151.01, 140.33, 84.80, 84.66, 84.33, 84.29, 84.08, 76.78, 76.40, 71.87, 65.90, 61.62, 46.58, 42.15, 37.18, 31.67, 30.13, 21.73, 20.41, 13.14, 10.41, 8.12. HRMS (ESI-) m/z calcd for $C_{20}H_{23}N_{10}O_{11}P_2S$ $[M-H]^-$ 673.074378, found 673.07426. Note that M refers to fully protonated phosphate.

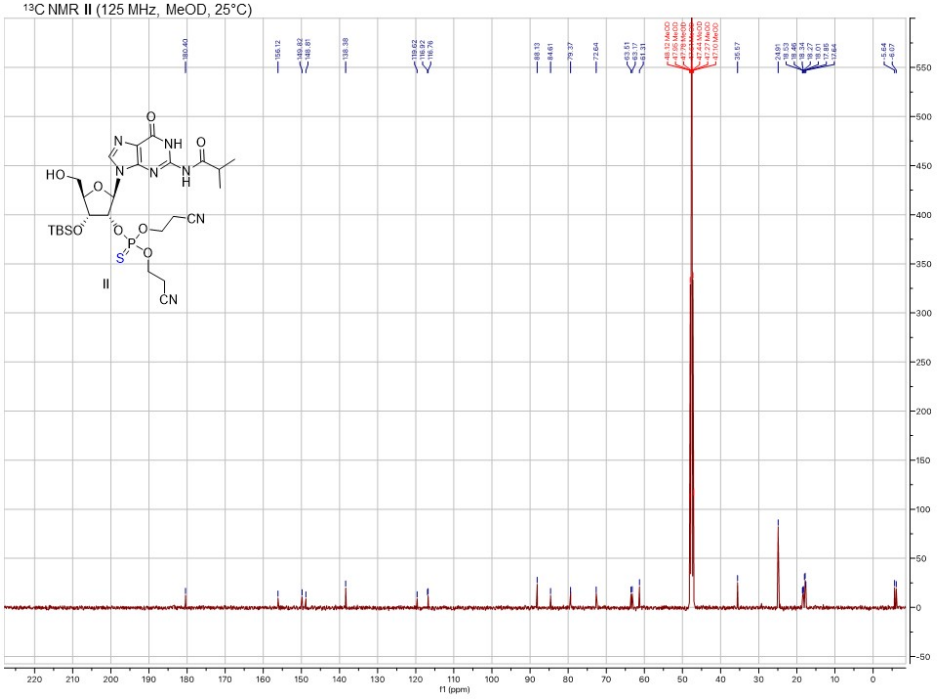
iii. NMR Spectra



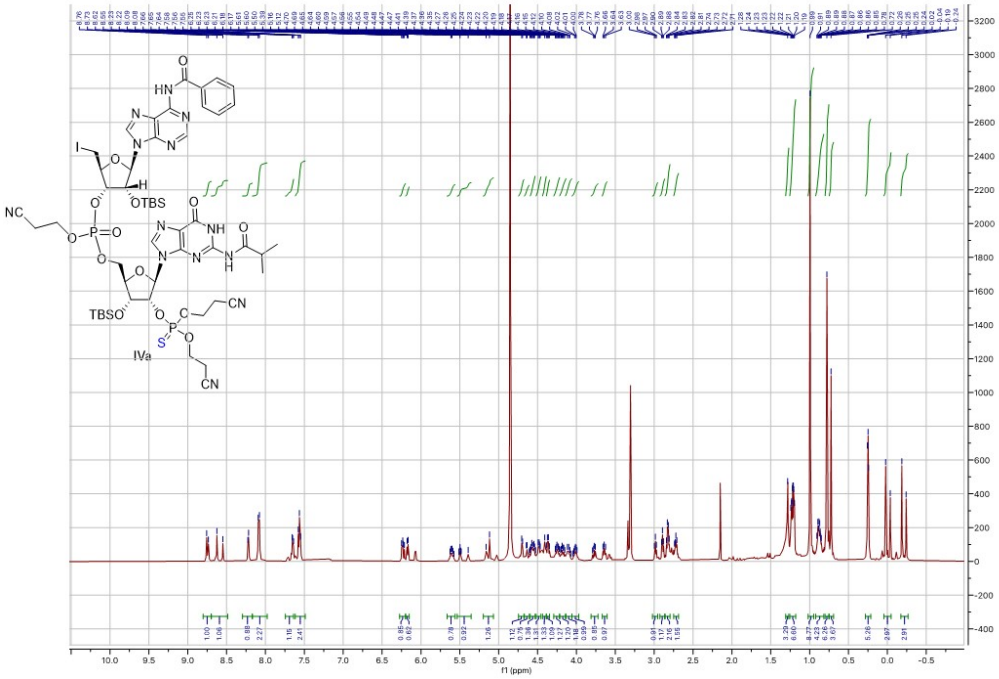
^{31}P {^{1}H} NMR **II** (202 MHz, MeOD, 25°C)



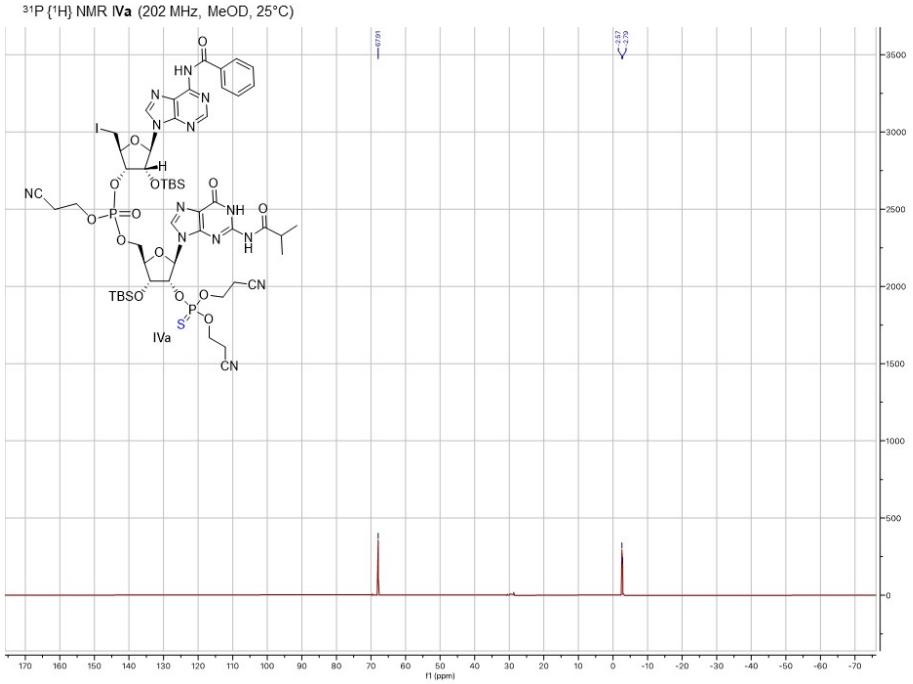
^{13}C NMR **II** (125 MHz, MeOD, 25°C)



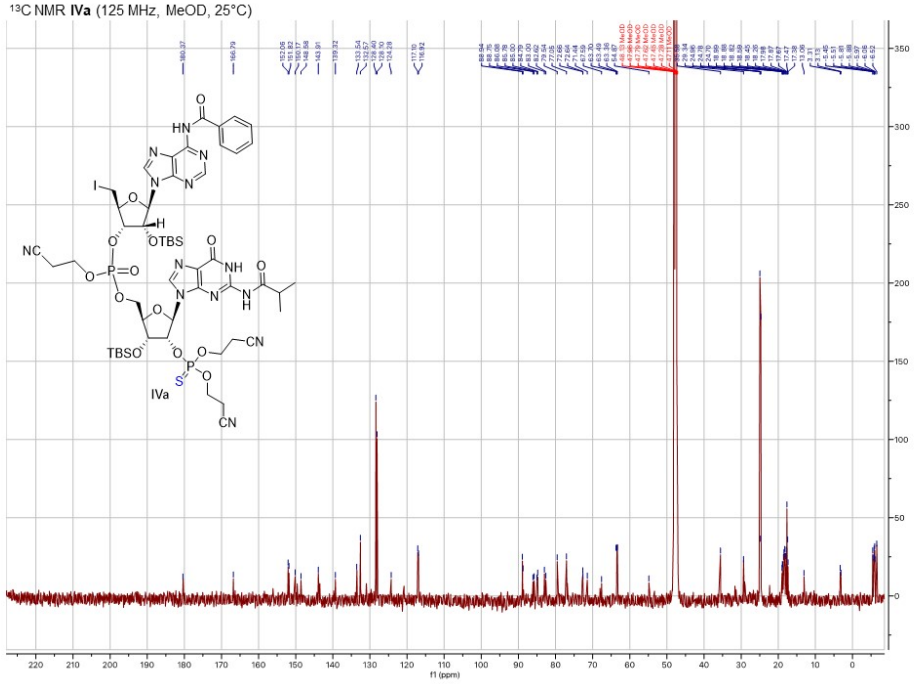
^{1}H NMR **IVa** (500 MHz, MeOD, 25°C)



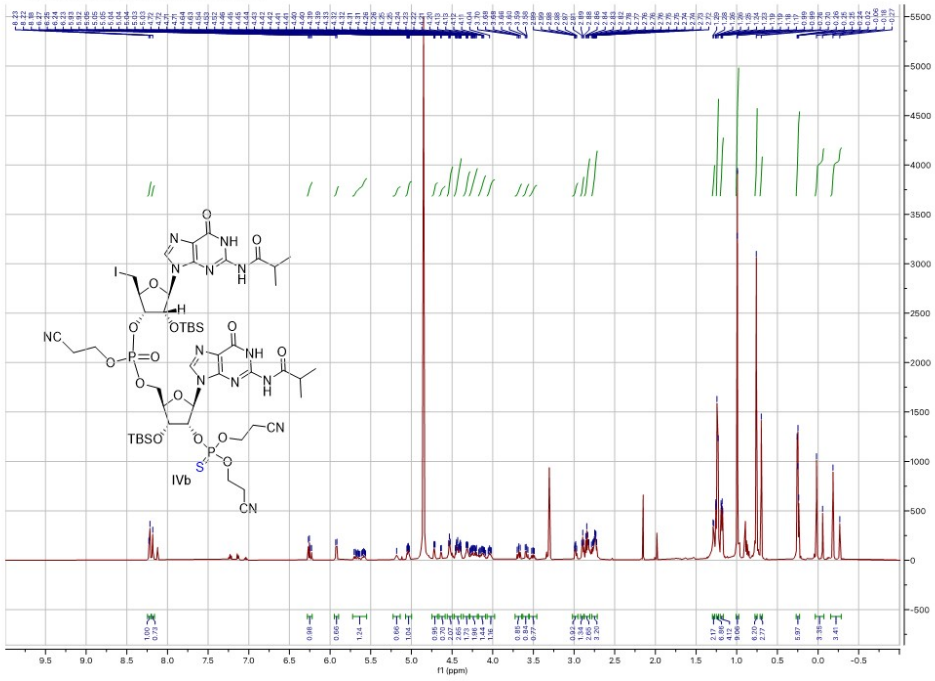
^{31}P {^{1}H} NMR **IVa** (202 MHz, MeOD, 25°C)



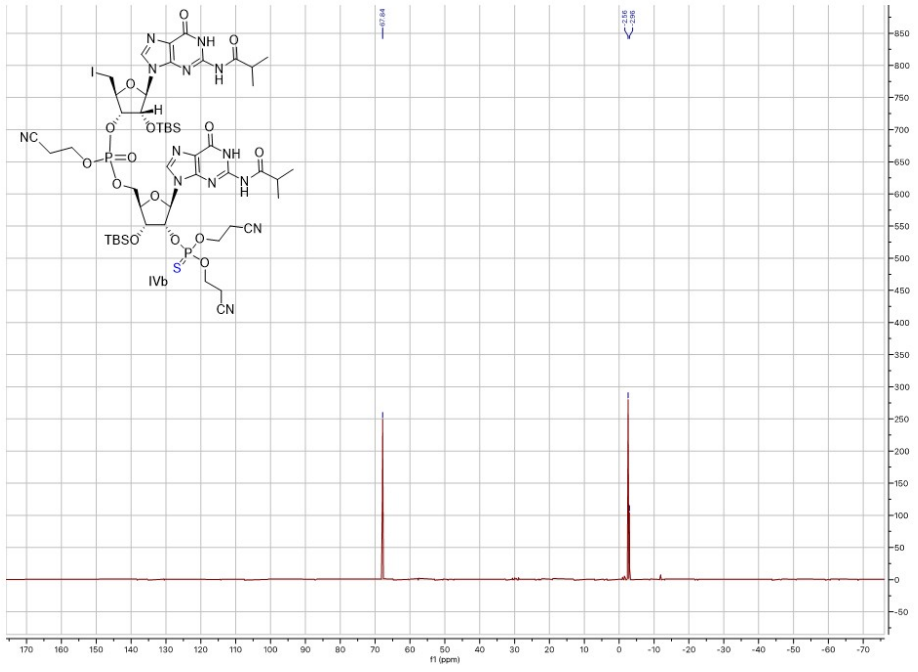
^{13}C NMR **IVa** (125 MHz, MeOD, 25°C)



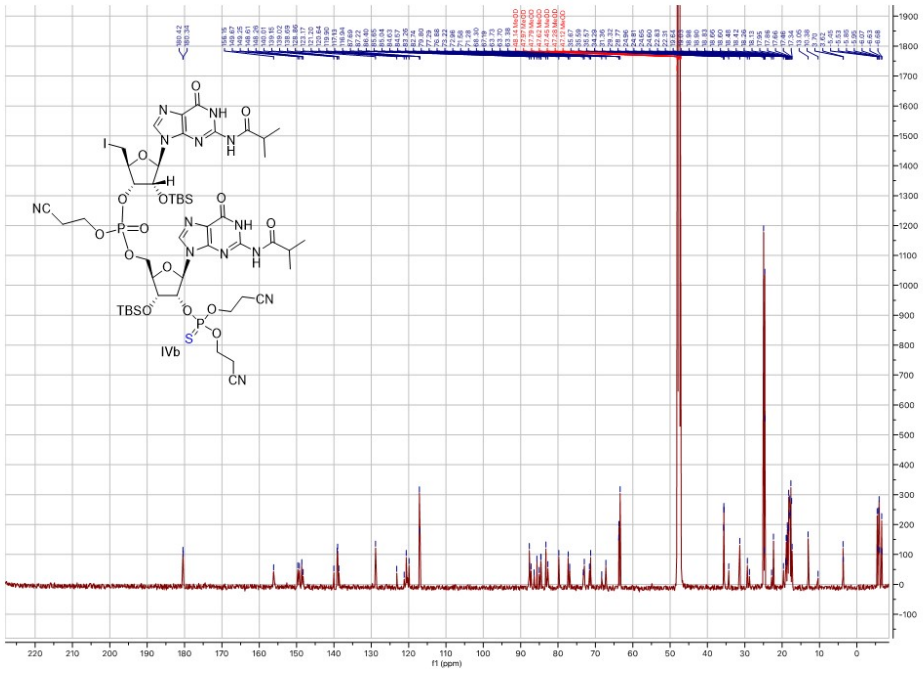
^{1}H NMR **IVb** (500 MHz, MeOD, 25°C)



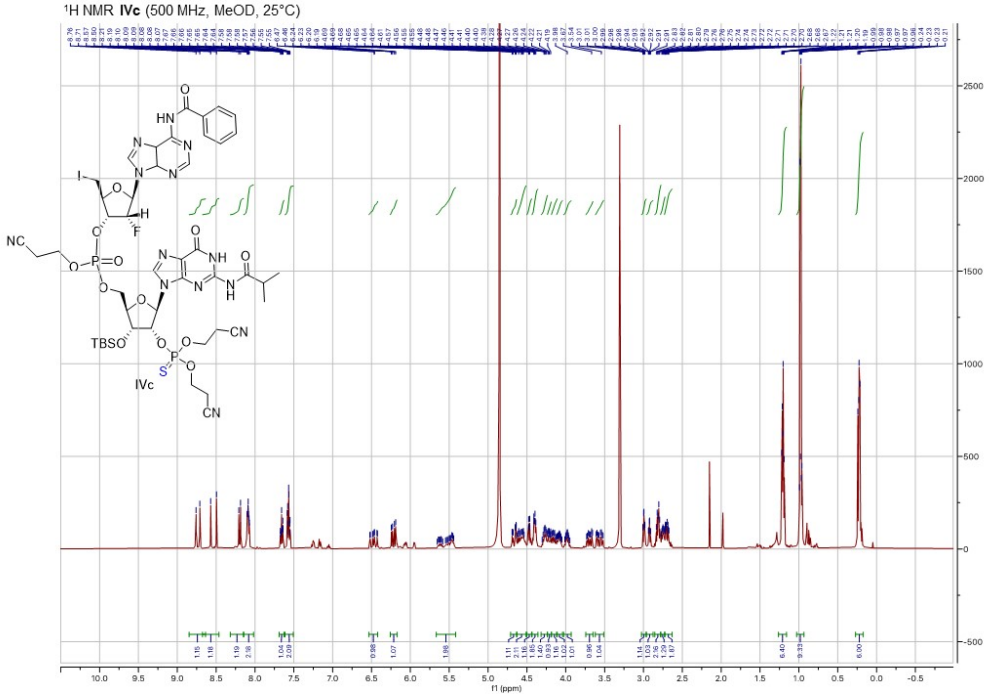
^{31}P {^{1}H} NMR **IVb** (202 MHz, MeOD, 25°C)



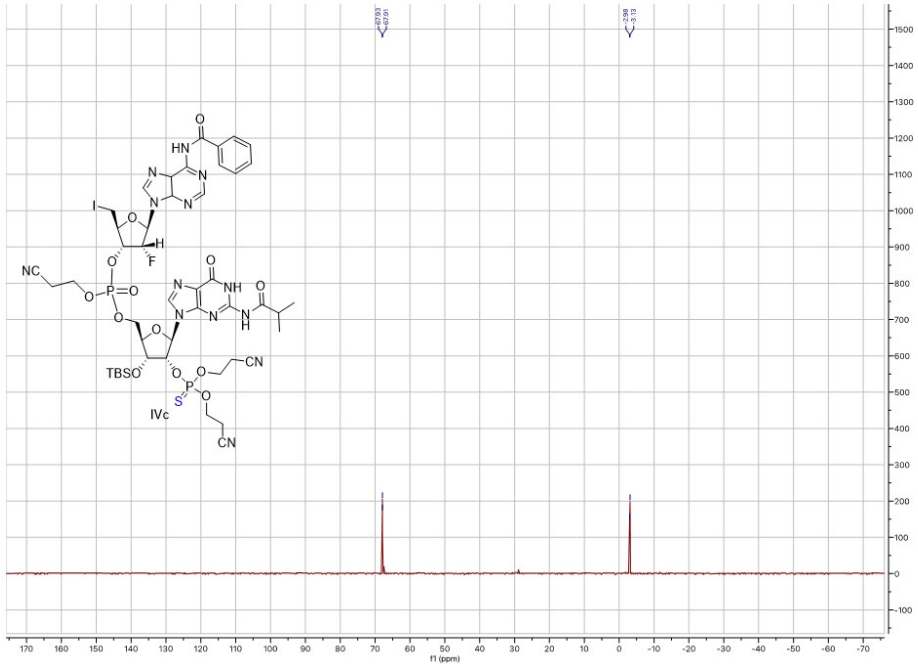
^{13}C NMR **IVb** (125 MHz, MeOD, 25°C)



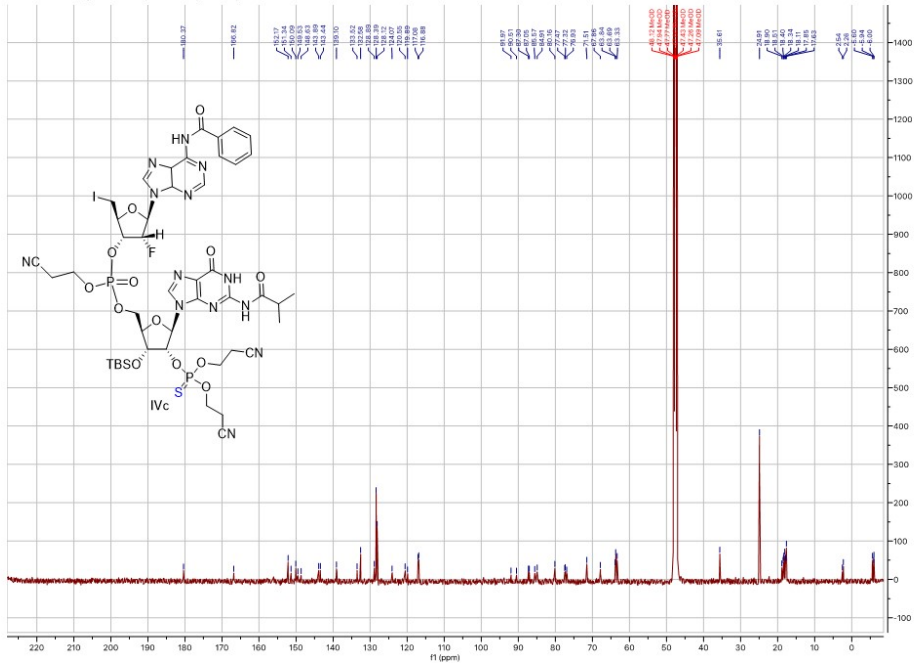
^{1}H NMR **IVc** (500 MHz, MeOD, 25°C)



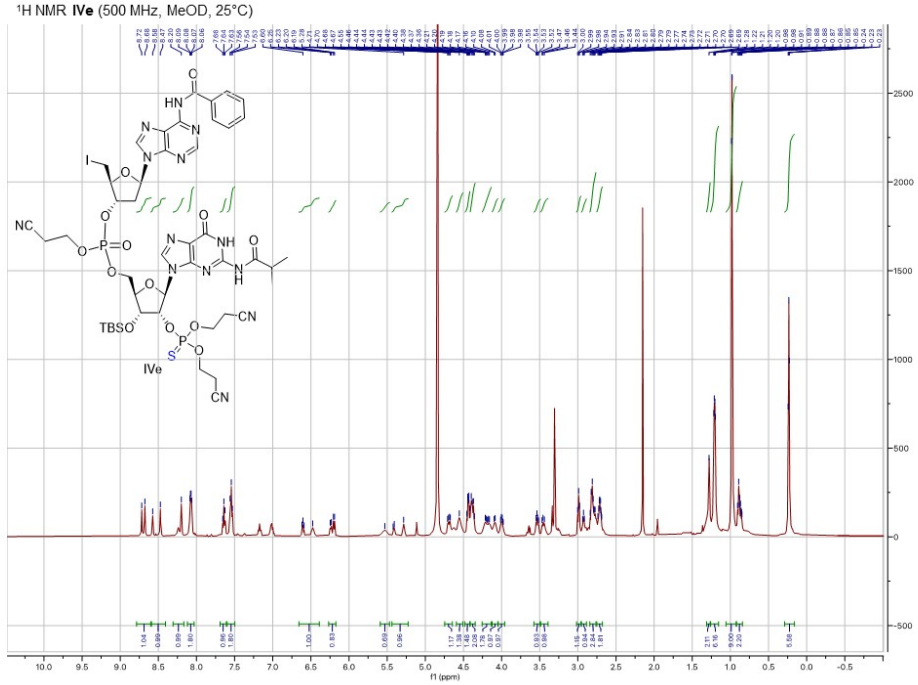
^{31}P {^{1}H} NMR **IVc** (202 MHz, MeOD, 25°C)



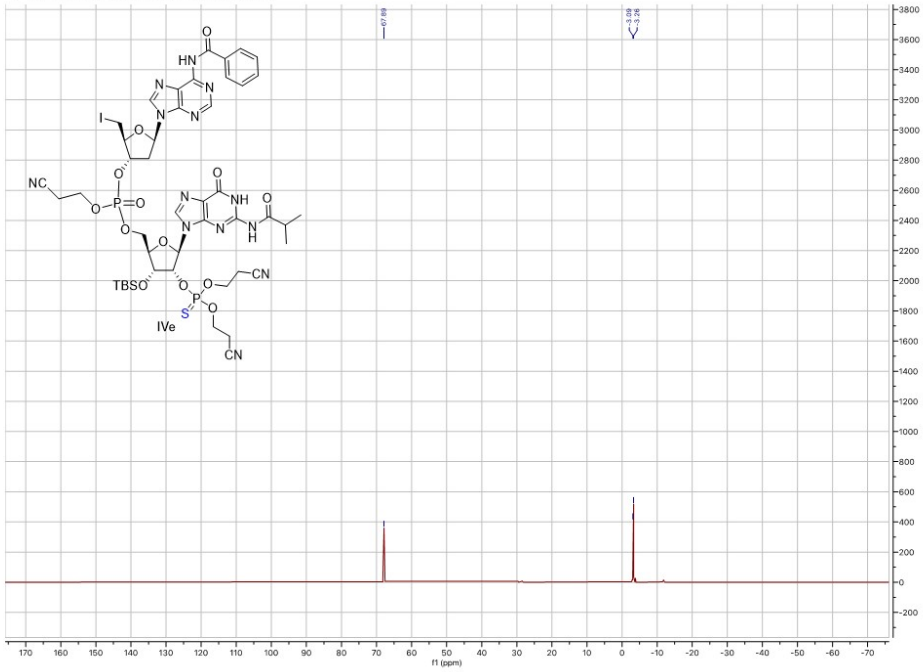
^{13}C NMR **IVc** (125 MHz, MeOD, 25°C)



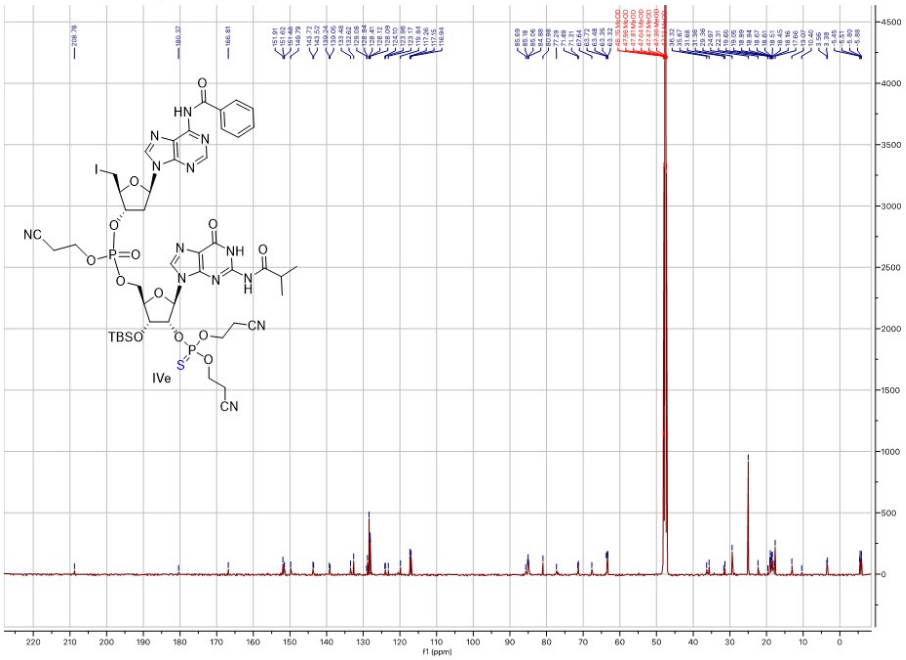
^{1}H NMR **IVe** (500 MHz, MeOD, 25°C)



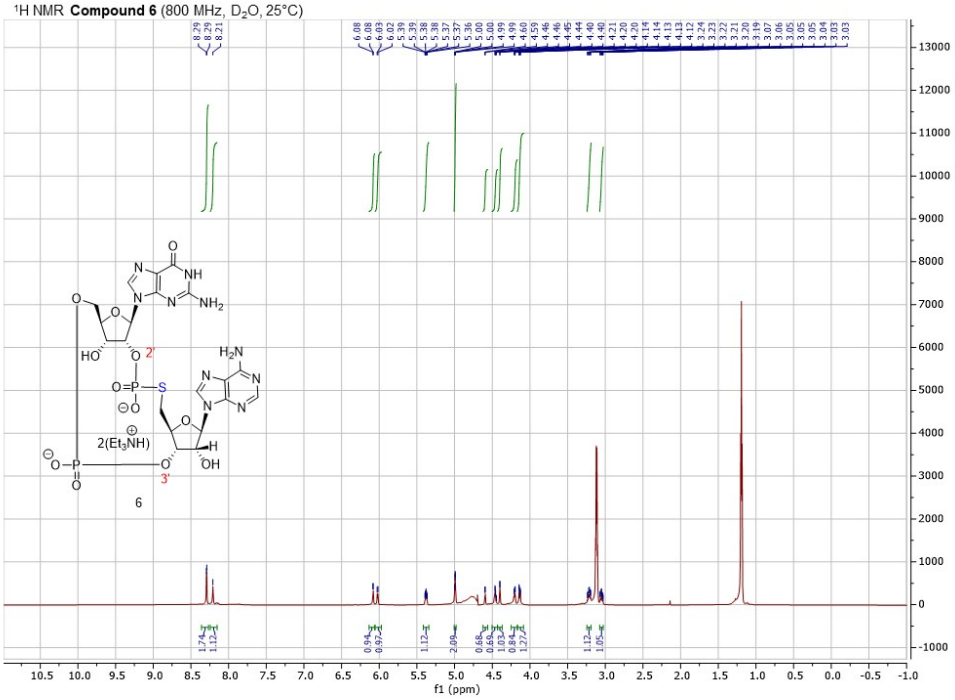
^{31}P {^{1}H} NMR **IVe** (202 MHz, MeOD, 25°C)



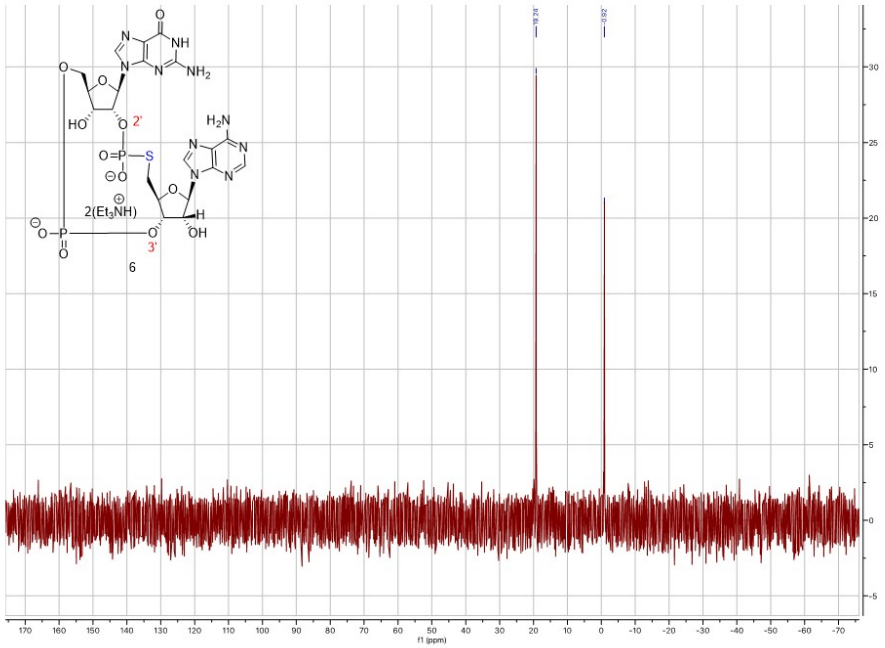
^{13}C NMR **IVe** (125 MHz, MeOD, 25°C)



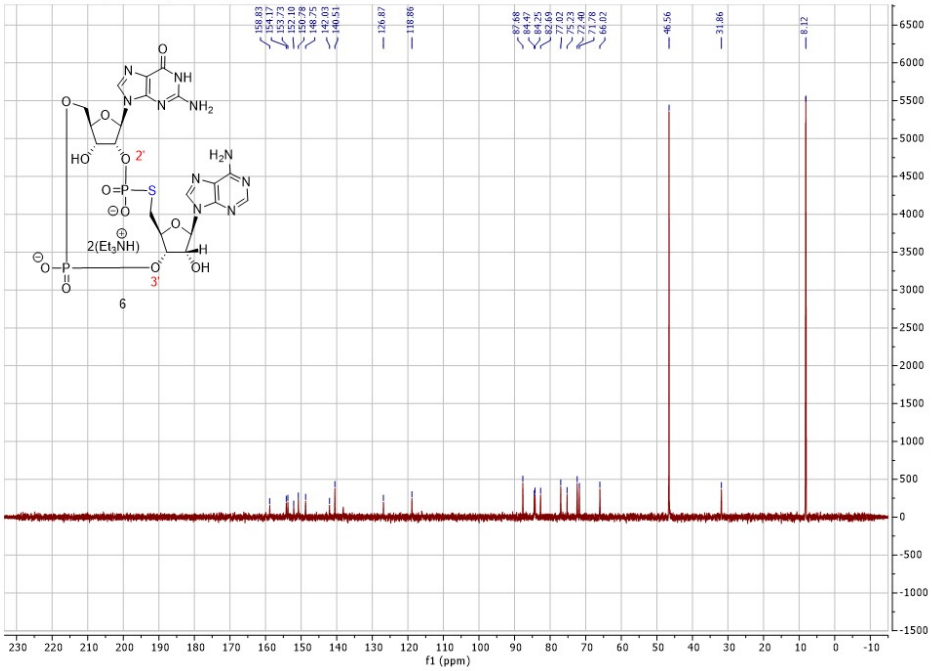
^{1}H NMR **Compound 6** (800 MHz, D_2O, 25°C)



^{31}P {^{1}H} NMR **Compound 6** (202 MHz, D_2O, 25°C)

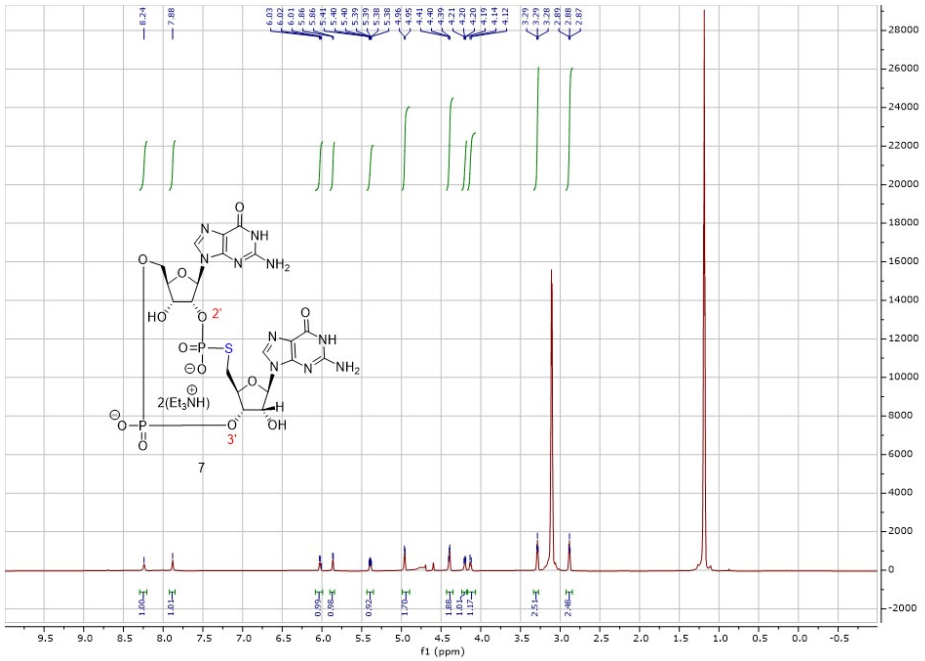


^{13}C NMR **Compound 6** (200 MHz, D_2O, 25°C)



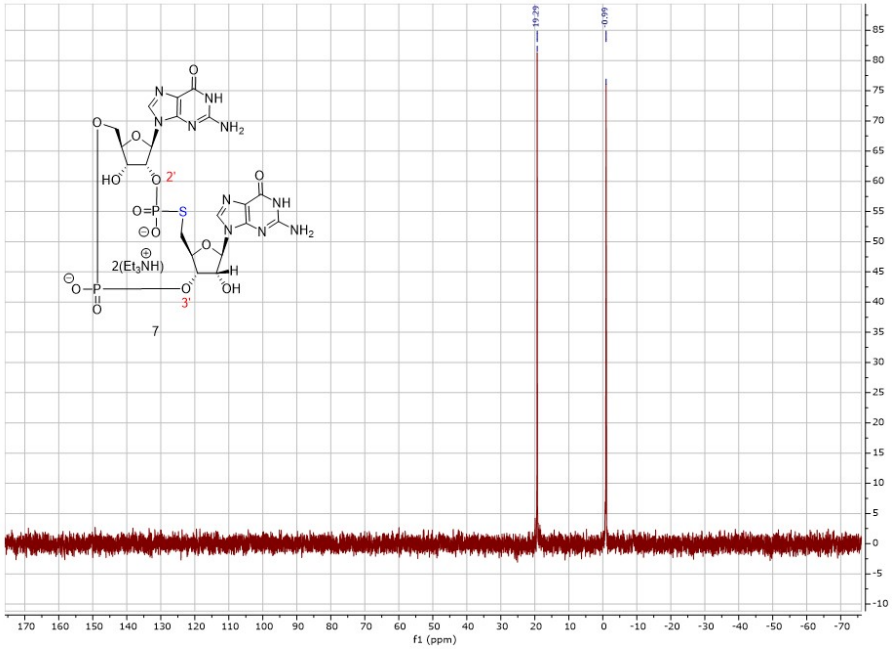
6

^{1}H NMR **Compound 7** (800 MHz, D_2O, 25°C)

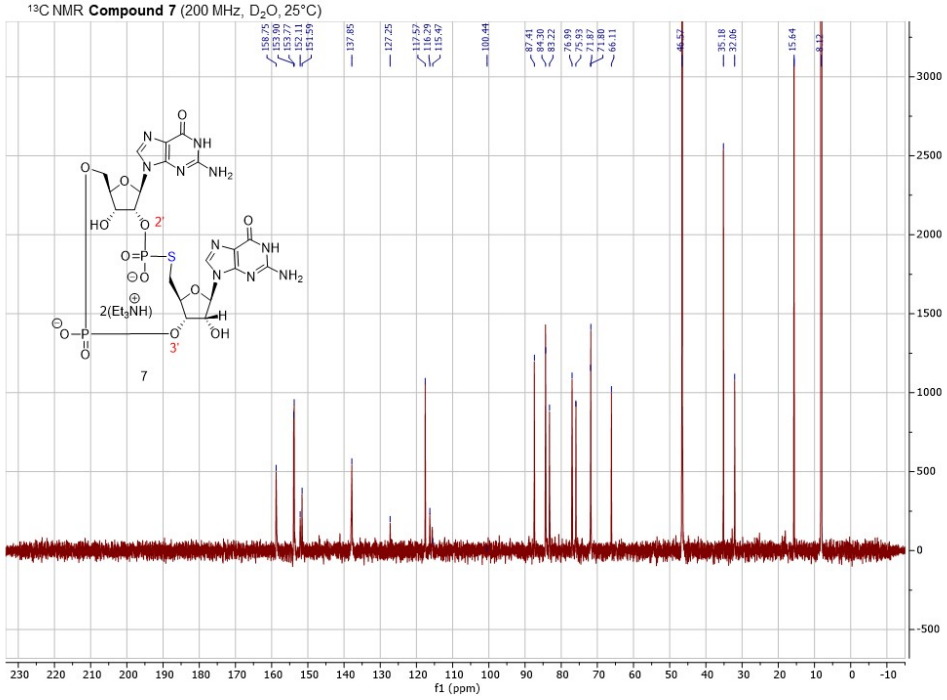


7

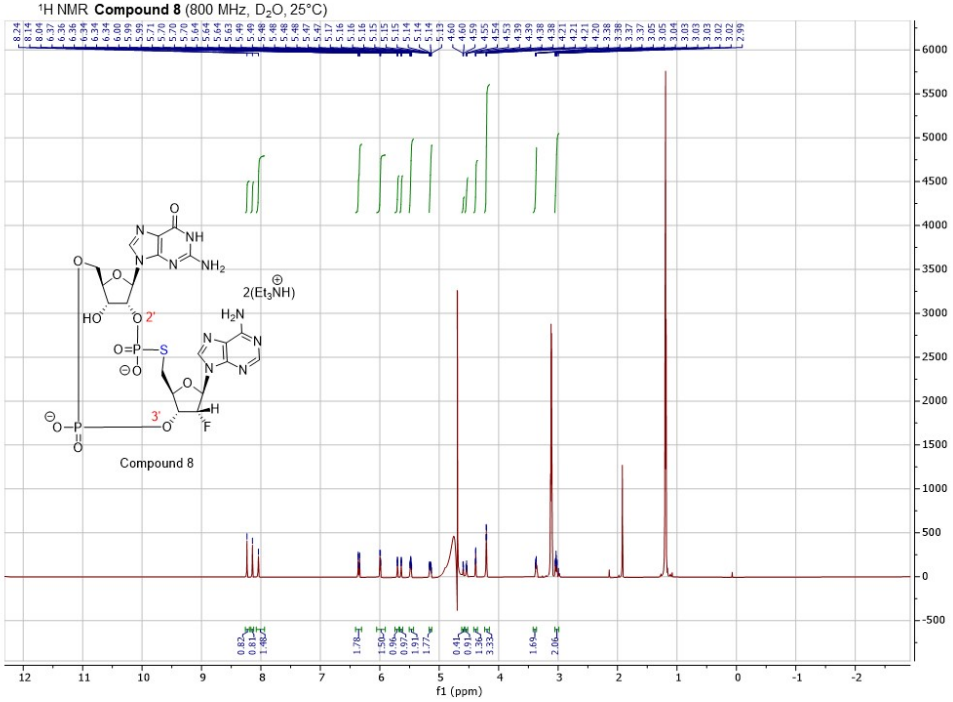
^{31}P {^{1}H} NMR **Compound 7** (202 MHz, D_2O, 25°C)



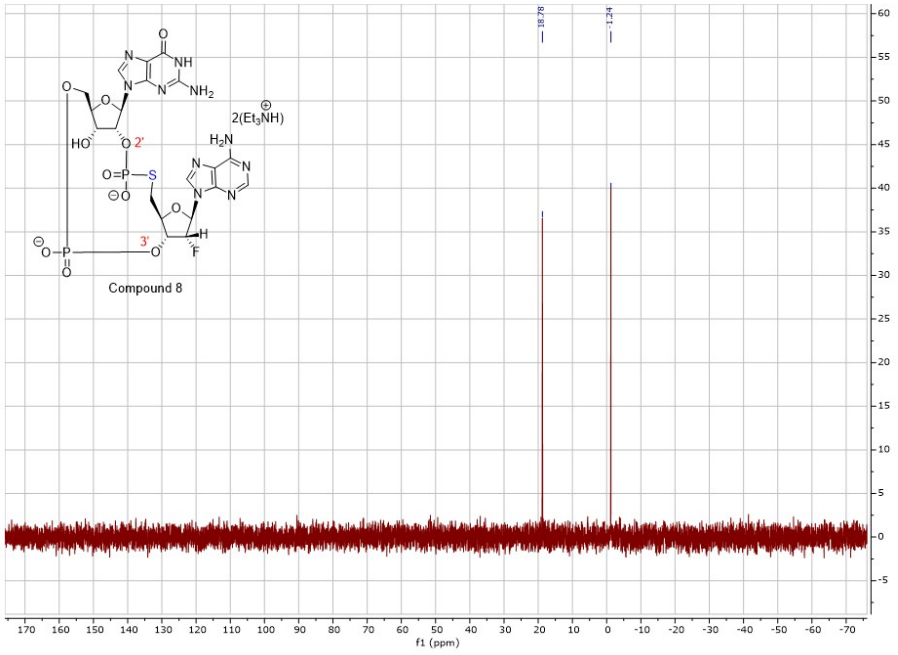
^{13}C NMR **Compound 7** (200 MHz, D_2O, 25°C)



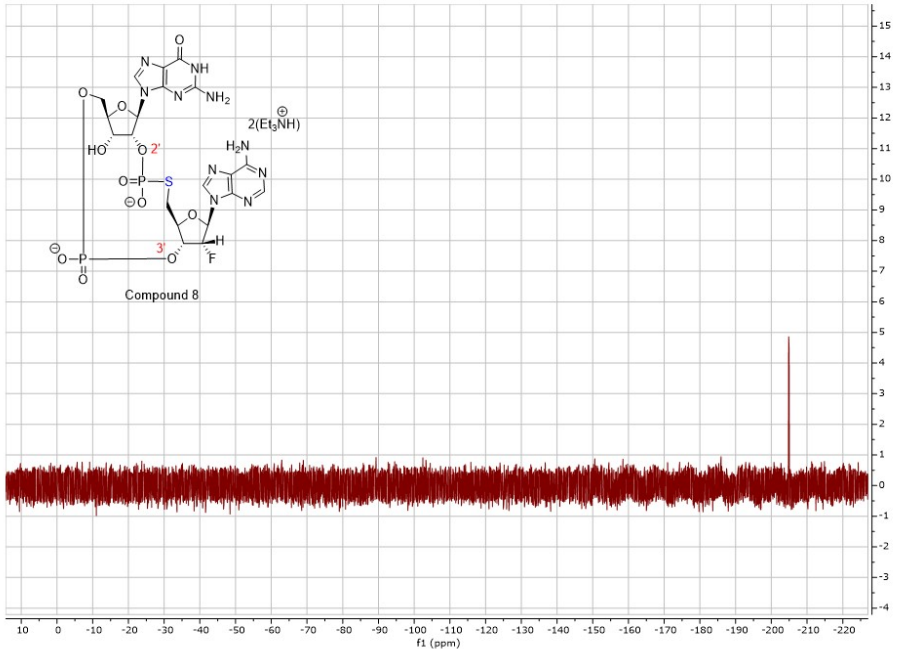
^{1}H NMR **Compound 8** (800 MHz, D_2O, 25°C)



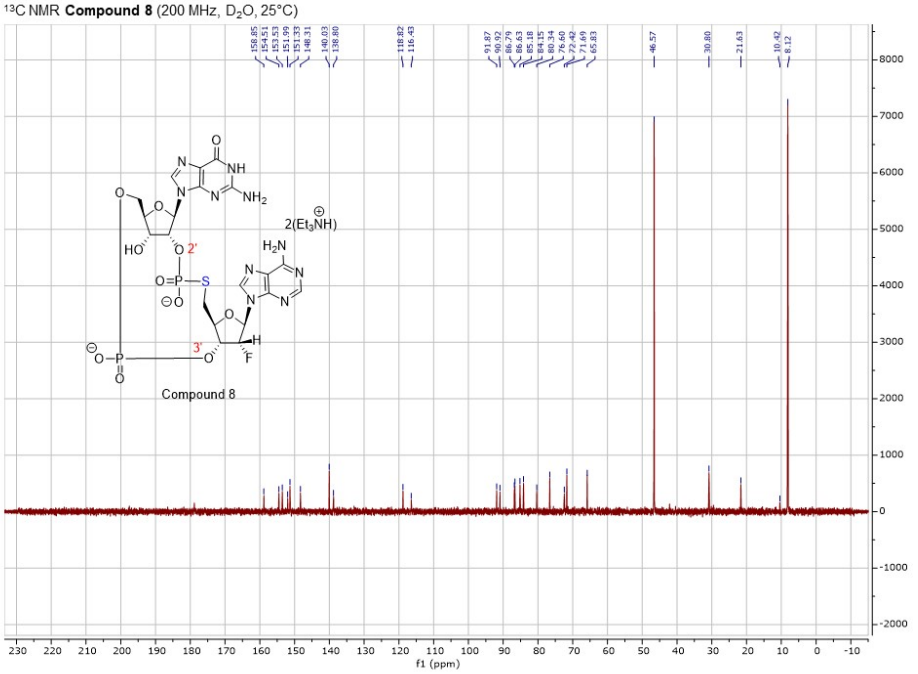
^{31}P {^{1}H} NMR **Compound 8** (202 MHz, D_2O, 25°C)



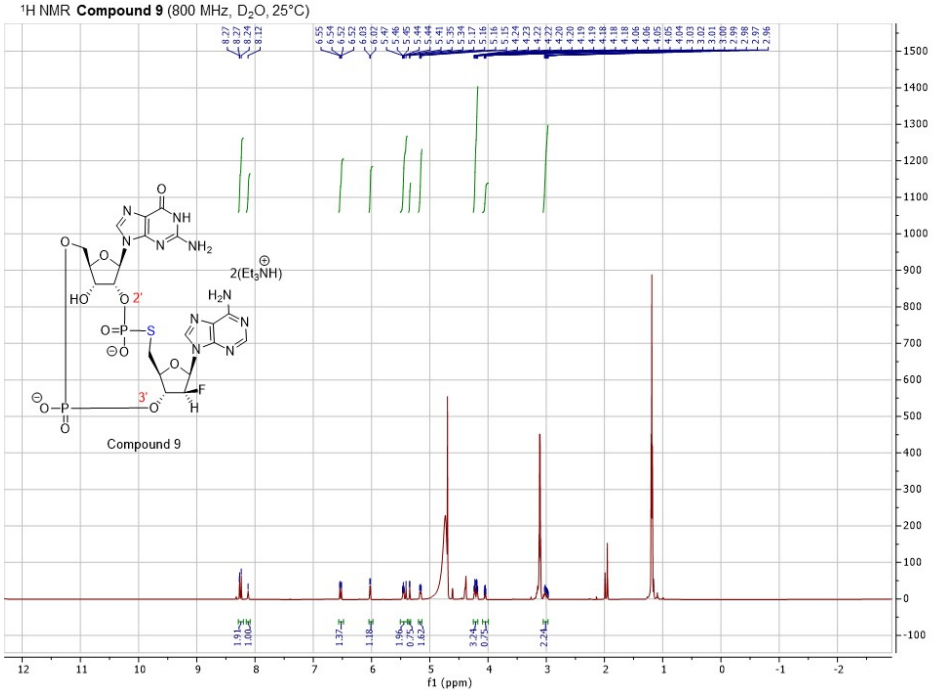
^{19}F NMR **Compound 8** (D_2O, 25°C)



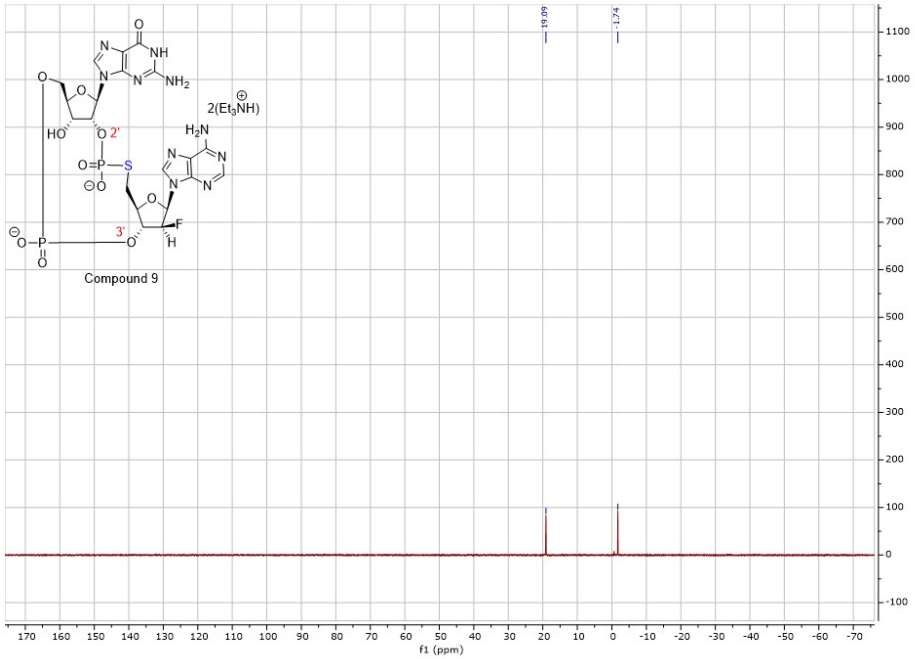
^{13}C NMR **Compound 8** (200 MHz, D_2O, 25°C)



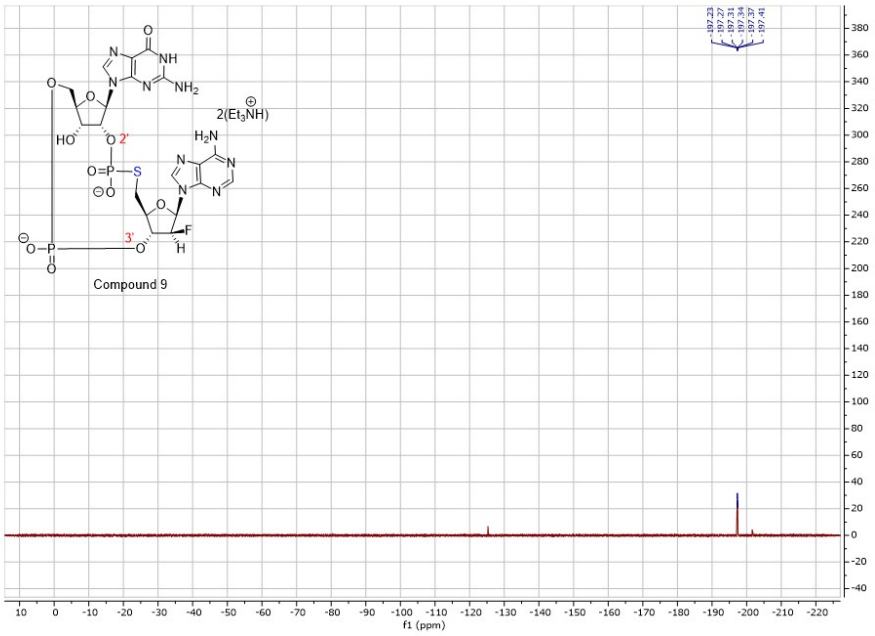
^{1}H NMR **Compound 9** (800 MHz, D_2O, 25°C)



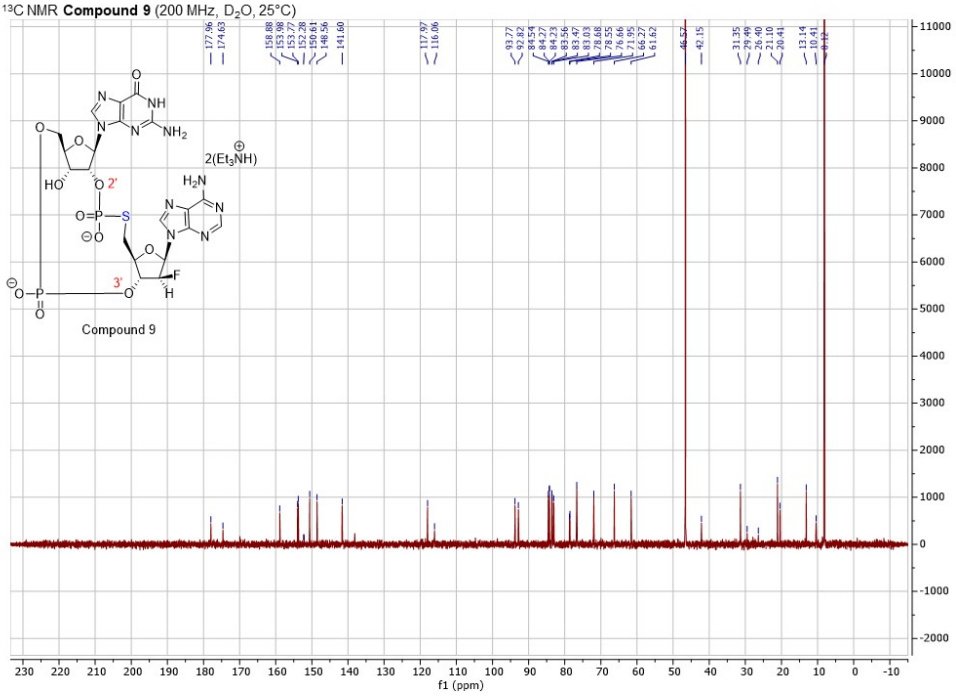
^{31}P {^{1}H} NMR **Compound 9** (202 MHz, D_2O, 25°C)



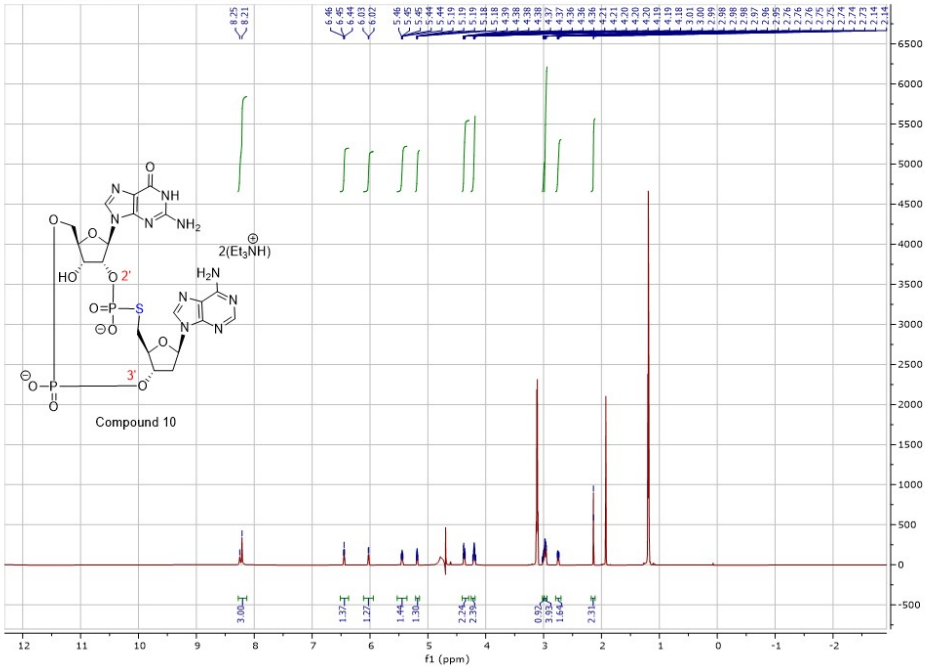
^{19}F NMR **Compound 9** (D_2O, 25°C)



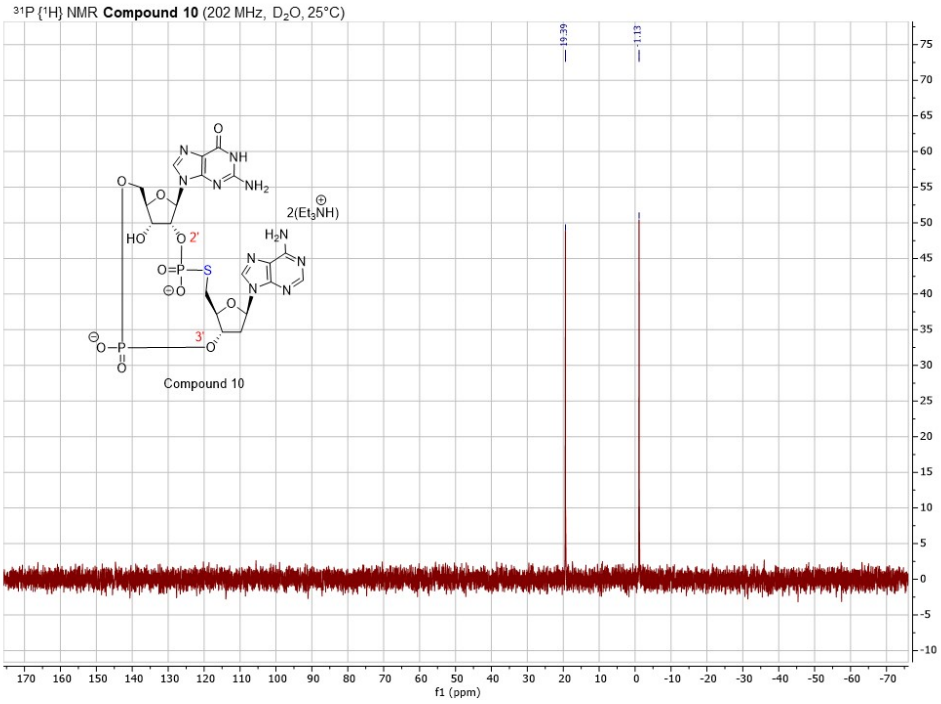
^{13}C NMR **Compound 9** (200 MHz, D_2O, 25°C)

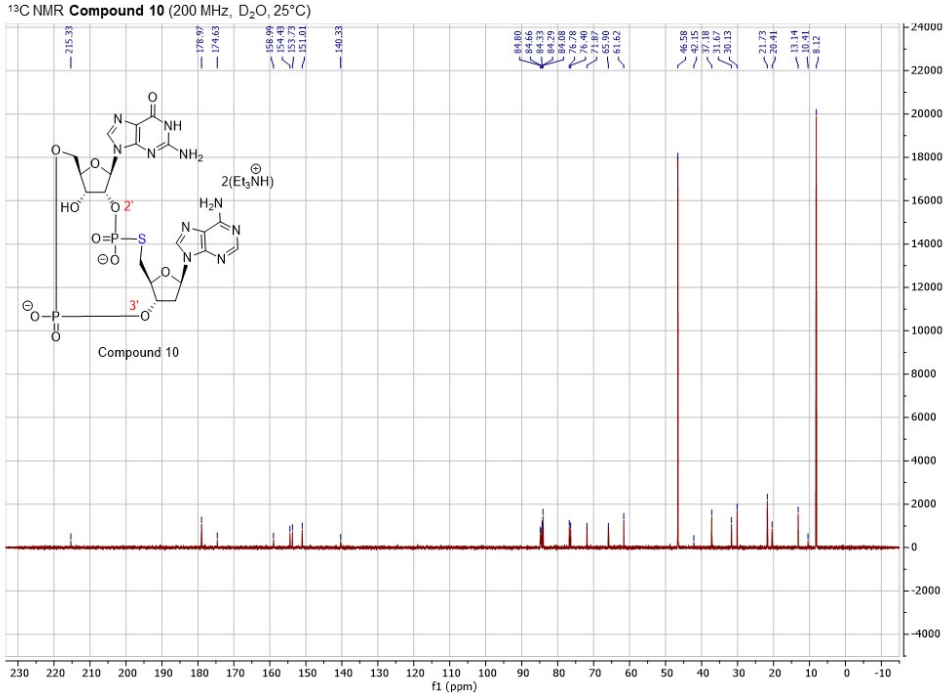


^{1}H NMR **Compound 10** (800 MHz, D_2O, 25°C)



^{31}P {^{1}H} NMR **Compound 10** (202 MHz, D_2O, 25°C)




13C NMR Compound 10 (200 MHz, D2O, 25°C)
215.33
178.97
174.63
158.99
154.43
153.23
151.01
140.33
84.80
84.56
84.33
84.29
84.08
76.78
76.40
71.87
65.90
61.62
46.58
42.15
37.18
31.67
30.13
21.73
20.41
13.14
10.41
8.12
2(Et3NH)
Compound 10
f1 (ppm)