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Supporting Information

Direct High-Throughput Screening Assay for mRNA Cap Guanine-N7 Methyltransferase Activity

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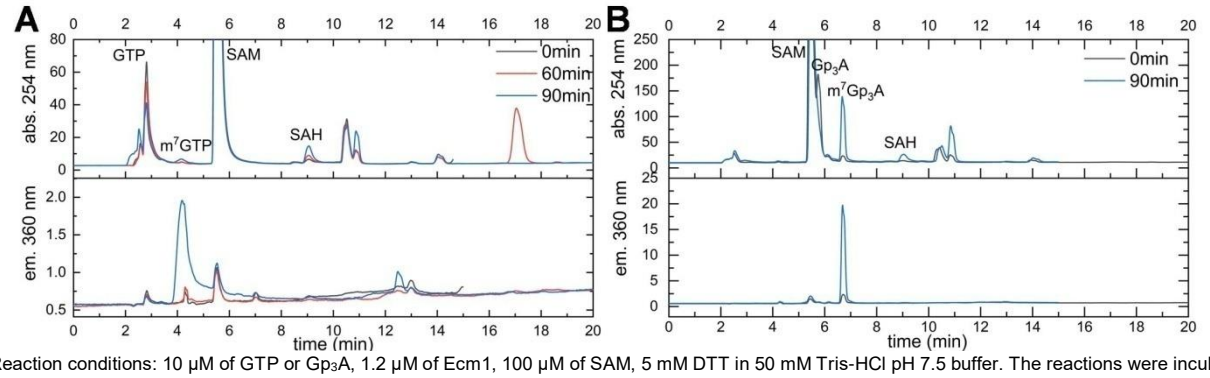
Figure S1. Comparison of **A)** GTP and **B)** GpppA Ecm1-catalyzed N7-methylation reaction progress monitored by RP HPLC



Reaction conditions: 10 µM of GTP or Gp_3A, 1.2 µM of Ecm1, 100 µM of SAM, 5 mM DTT in 50 mM Tris-HCl pH 7.5 buffer. The reactions were incubated at 37 °C and stopped by incubation at 98 °C for 2 min.
RP HPLC analysis conditions: Elution with linear gradient of methanol (0-25% within 15 min) in 0.05 M ammonium acetate buffer pH 5.9.

Figure S2. Synthesis of alkyne-modified non-fluorescent precursors

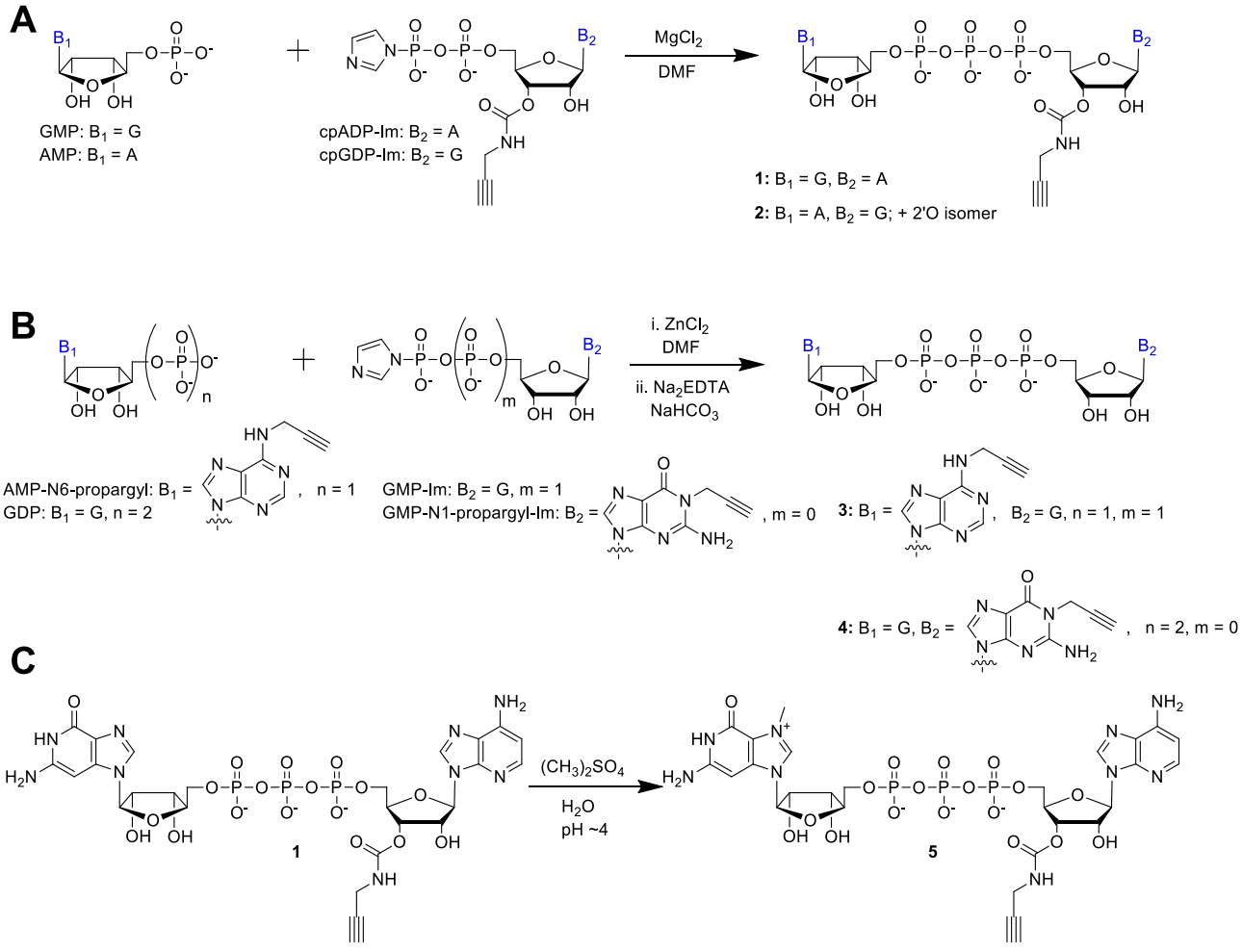
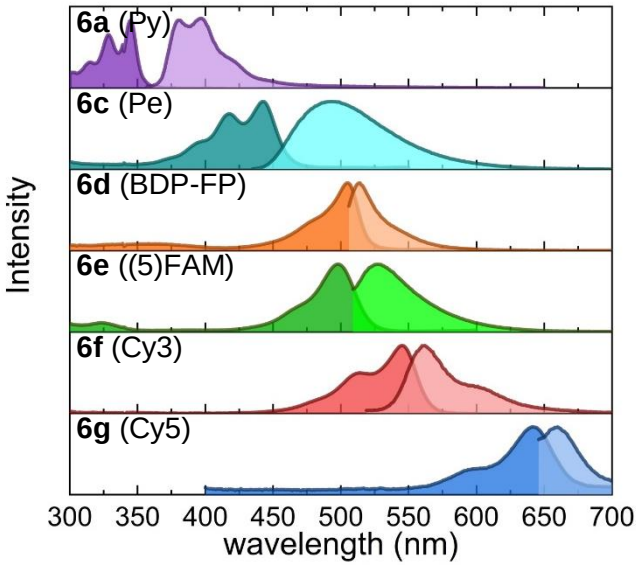
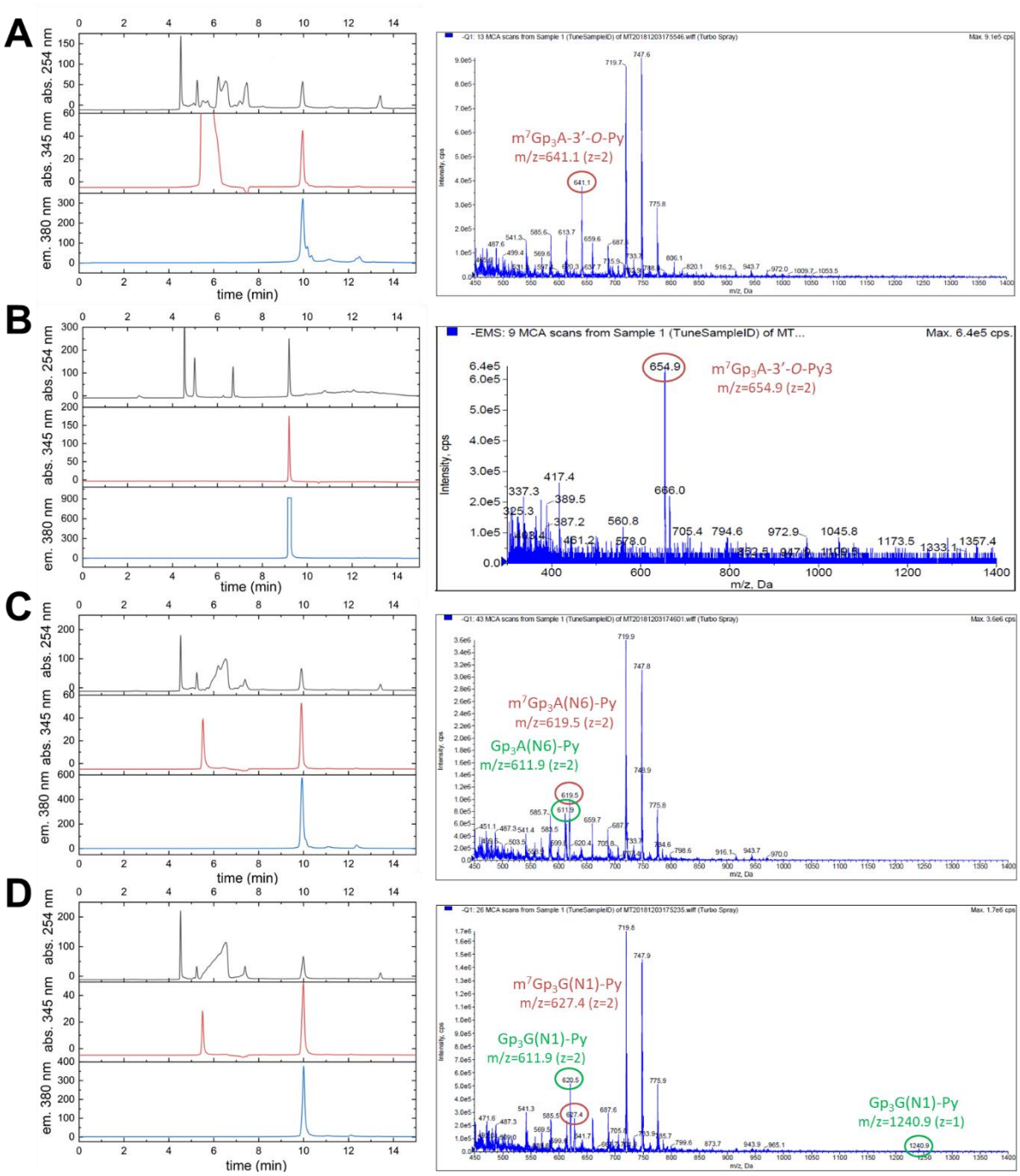


Figure S3. Absorption and emission spectra of GpppA analogs labelled with different fluorescent tags



Conditions for emission spectra: 1 µM probe in 50 mM Tris-HCl (pH 7.5) at 30 °C; excitation at the absorption maximum.

Figure S4. Comparison of Ecm1-catalyzed N7-methylation of probes **6a-b**, **8** and **9** using RP HPLC and MS

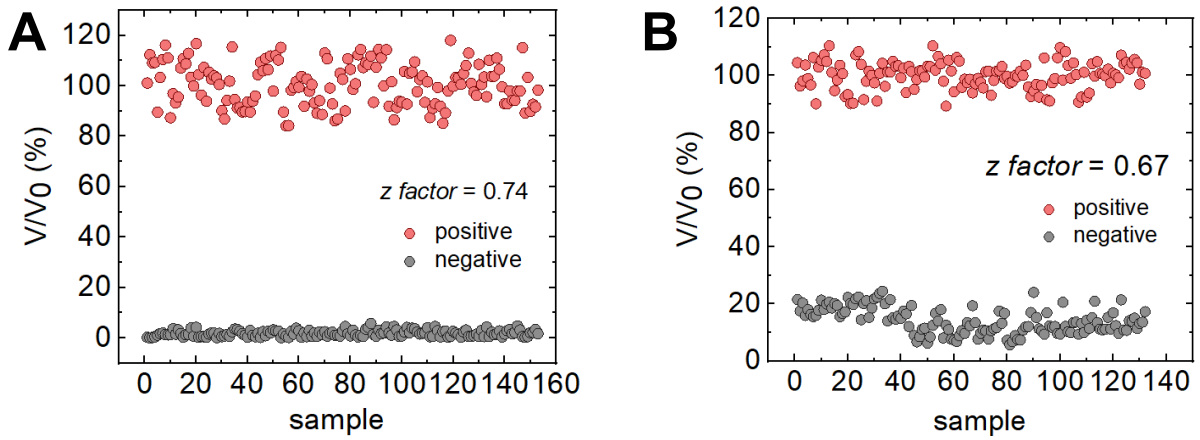


Reaction conditions: 100 µM of A) **6a**, B) **6b**, C) **8** or D) **9**, 1 µM of Ecm1, 500 µM of SAM, 5 mM DTT in 50 mM Tris-HCl pH 7.5 buffer. The reactions were incubated at 37 °C for 3 h and stopped by 2 min incubation at 98 °C.

RP HPLC analysis conditions: Elution with linear gradient of acetonitrile (100% at 15 min) in 0.05 M ammonium acetate buffer pH 5.9.

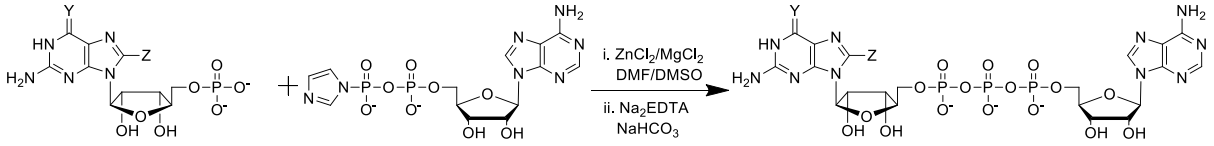
MS spectra show analysis of RP HPLC signal with t_R A), C) and D) 10 min or B) 9.2 min.

Figure S5. Determination of the *z* factor for the A) Ecm1 and B) RNMT-RAM kinetic assays



Reaction conditions for Ecm1: 2 µM of probe **6b**, 10 nM of Ecm1, 50 µM of SAM without (positive control) or with 25 µM of sinefungin (negative control). The experiment was carried out on plate reader in 50 mM Tris-HCl pH 7.5 buffer at 30 °C.
Reaction conditions for RNMT-RAM: 2 µM of probe **6b**, 20 nM of RNMT-RAM, 20 µM of SAM without (positive control) or with 30 µM of sinefungin (negative control). The experiment was carried out on plate reader in 50 mM Tris-HCl pH 7.5 buffer at 30 °C.

Figure S6. Synthesis of potential nucleotide-derived inhibitors



Ia: Y = O, Z = $-S-CH_3$

Ib: Y = O, Z = $-S-C_6H_5$

IIa: Y = S, Z = H

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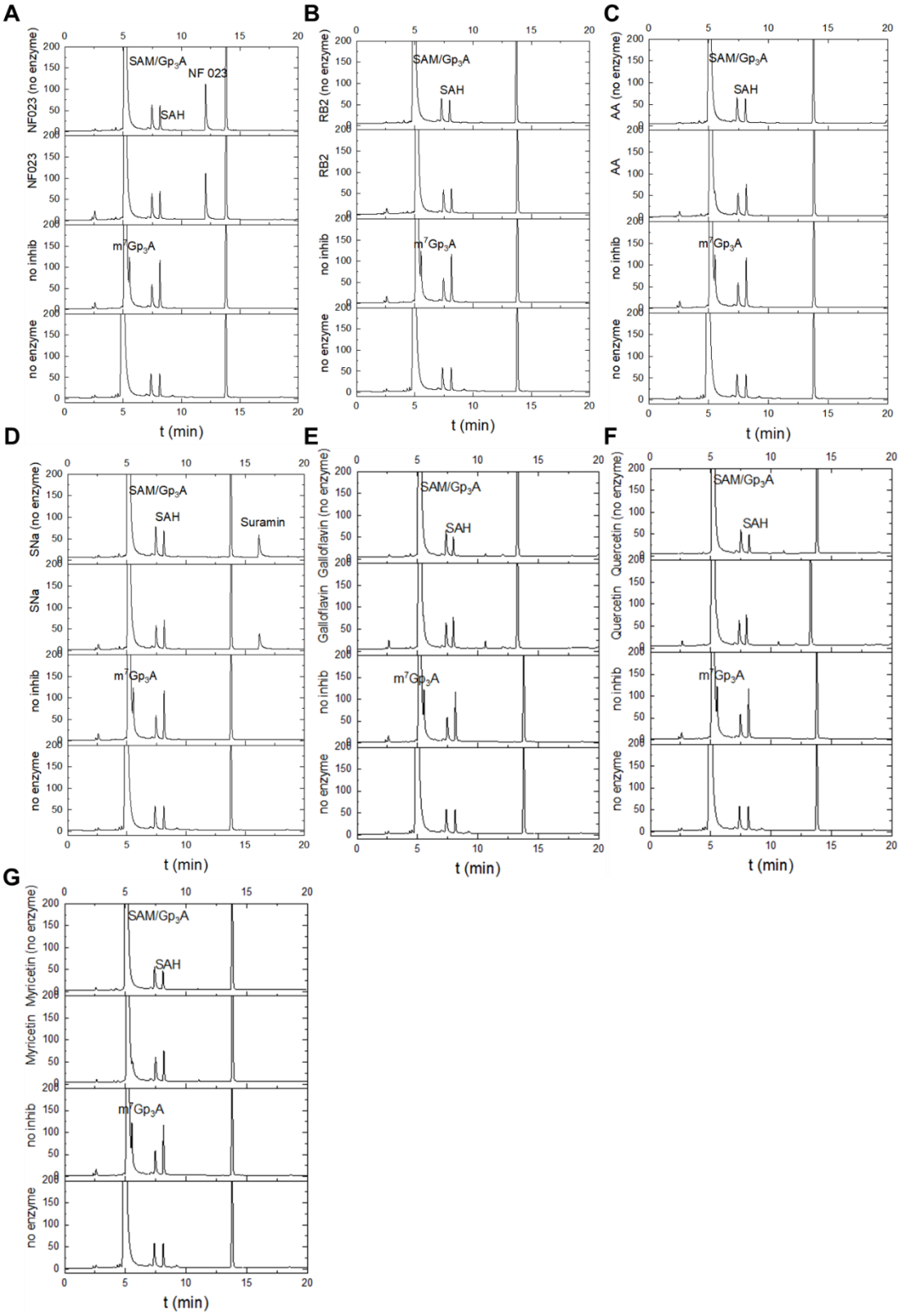
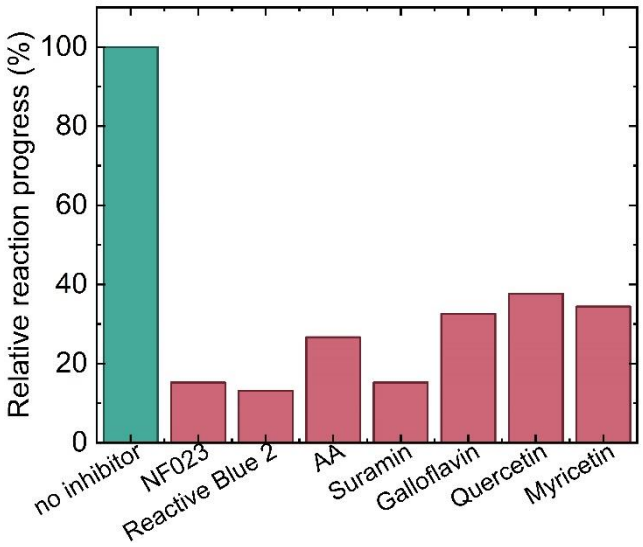
Figure S7. Inhibition of Ecm1 by four most potent LOPAC®1280 inhibitors verified by RP HPLC



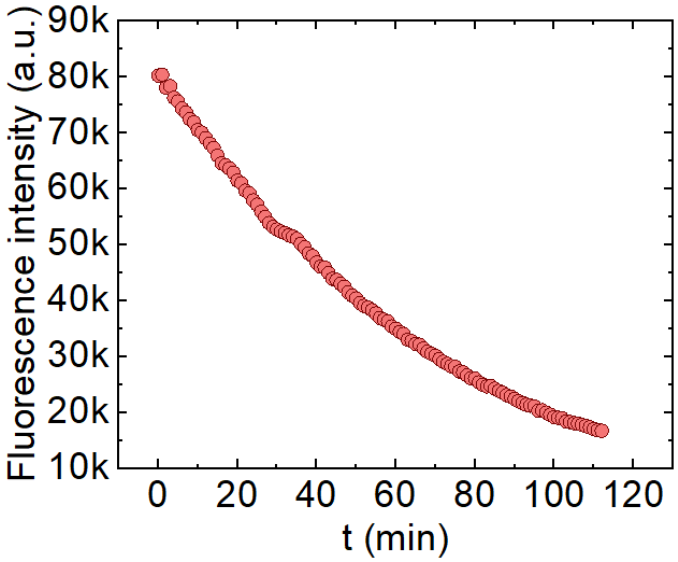
Reaction conditions: 10 µM of GpppA, 250 µM of SAM, 100 nM of Ecm1 and 2 µM of inhibitor in total volume 200 µl of 50 mM Tris-HCl pH 7.5 buffer. The reactions were incubated for 1.5 h at 30 °C with 300 rpm mixing and stopped by 2 min incubation at 95 °C.
RP HPLC analysis conditions: Elution with linear gradient of methanol (50% at 15 min) in 0.05 M ammonium acetate buffer pH 5.9.

Figure S8. Relative progress of Ecm1 catalyzed methylation of GpppA in the presence of selected inhibitors from LOPAC®1280 library



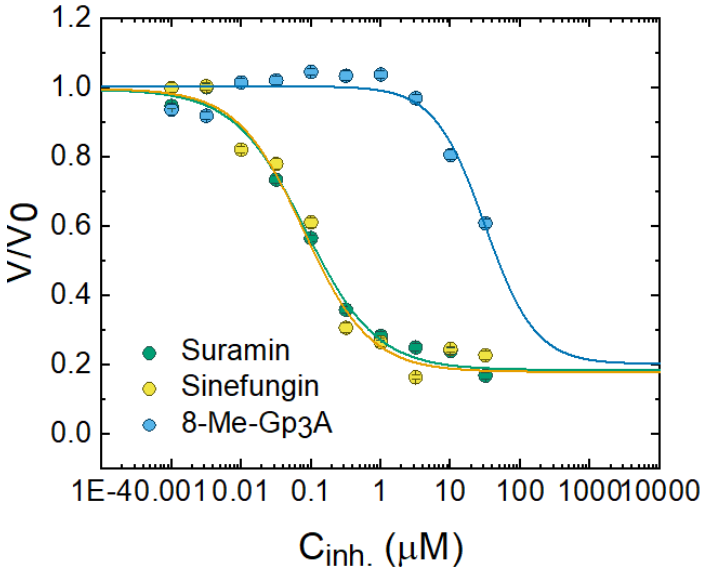
Data taken from chromatograms presented in Figure S7.

Figure S9. N7-methylation of probe **6b** by VCE



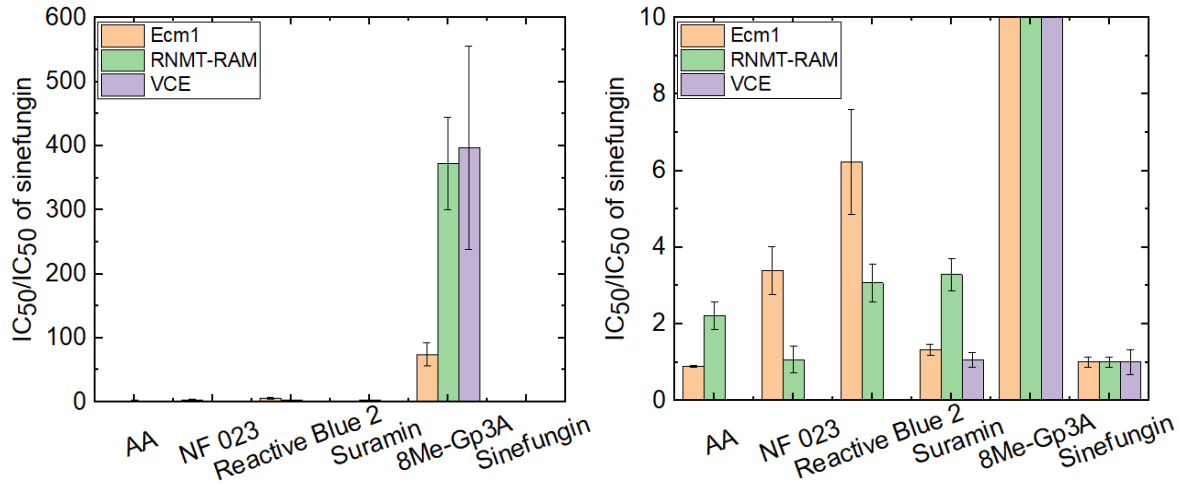
Reaction conditions: 2 μM of probe **6b**, 5U of VCE, 50 μM of SAM. The experiment was carried out on plate reader in 50 mM Tris-HCl pH 7.5 buffer at 30 °C.

Figure S10. Inhibition of VCE N7-methyltransferase activity towards probe **6b** by selected compounds and sinefungin as a reference



All reaction mixtures contained probe **6b** (2 µM), SAM (50 µM) and VCE (5U) in 50 mM Tris-HCl buffer pH 7.5. The mixtures were incubated at 30 ºC in a 96-well plate. The fluorescence measurements were carried out for excitation at 345 nm and emission at 378 nm using plate reader. Data shown are mean values of 3 independent experiments ± SD. The determined IC_{50} values are shown in Table 4.

Figure S11. Comparison of IC_{50} values for selected inhibitors in reference to IC_{50} for sinefungin



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Supplementary Tables

Table S1. Yields of the synthesis of fluorescently labelled nucleotides

Compound number	HPLC yield	Yield after purification
6a	89%	34% [p]
6b	94%	61% [p]
6c	85%	10% [a]
6d	93%	53% [p]
6e	83%	52% [p]
6f	79%	9% [p]
6g	72%	10% [a]
7	92%	28% [p]
8	96%	37% [p]
9	92%	42% [p]
10a	65%	43% [p]
10b	88%	41% [p]

[a] purification with analytical RP HPLC. [b] purification with semipreparative RP HPLC.

Table S2. Conditions recommended to use in Ecm1 activity assay

Sample composition	Optimal probe concentration	2 µM
	Optimal SAM concentration	50 µM
	Optimal enzyme concentration	10 nM
	Buffer	50 mM Tris-HCl pH 7.5
Measurement conditions	Excitation/emission wavelength of probe	345 nm/378 nm
	Temperature	30 °C
	Pre-incubation conditions	15 min/300 rpm plus 20 min measurement without enzyme

Table S3. Conditions recommended to use in RNMT–RAM activity assay

Sample composition	Optimal probe concentration	2 µM
	Optimal SAM concentration	20 µM
	Optimal enzyme concentration	20 nM
	Buffer	50 mM Tris-HCl pH 7.5
Measurement conditions	Excitation/emission wavelength of probe	345 nm/378 nm
	Temperature	30 °C
	Pre-incubation conditions	15 min/300 rpm plus 20 min measurement without enzyme

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Table S4. IC_{50} values calculated for compounds selected from LOPAC®1280 library

Lp	Compound	Full name	IC_{50} (μM)	p
1.	P1_F5	Aurintricarboxylic acid	0.036 ± 0.005	-2.79 ± 0.76
2.	P1_F8	Sandoz 58-035	> 100	-
3.	P2_B3	2-(methylthio)adenosine 5'diphosphate trisodium salt hydrate	1.73 ± 0.24	-3.43 ± 0.79
4.	P2_D11	p-Benzoquinone	3.3 ± 1.6	-0.60 ± 0.17
5.	P2_G10	ARL 67156 trisodium salt	> 100	-
6.		Sinefungin	0.020 ± 0.004	-1.30 ± 0.17
7.	P4_A2	Icaritin	38.1 ± 11.8	-3.33 ± 5.46
8.	P4_B10	Cefsulodin sodium salthydrate	0.34 ± 0.05	-2.11 ± 0.40
9.	P4_C10	Caffeic acid phenethynyl ester	5.26 ± 0.78	-1.60 ± 0.27
10.	P4_C11	Lumefantrine	> 100	-
11.	P4_D6	(-)-Cotinine	> 100	-
12.	P4_D9	Cefotaxime sodium salt	13.0 ± 1.1	-2.80 ± 0.51
13.	P4_H8	Calcimycin	13.4 ± 1.4	-2.80 ± 0.69
14.	P6_A4	Bisdemethoxycurcumin	3.28 ± 0.82	-0.71 ± 0.11
15.	P7_C11	GW5074	2.26 ± 0.23	-1.87 ± 0.30
16.	P7_E11	GW7647	10.8	-18.5
17.	P8_H4	6-Hydroxy-DL-DOPA	0.31 ± 0.01	-3.49 ± 0.92
18.	P8_H5	Hispidin	1,30 ± 0.13	-1.49 ± 0.24
19.	P9_G2	Mifamurtide	0.23 ± 0.03	-2.09 ± 0.49
20.	P10_B11	Myricetin	0.14 ± 0.01	-2.71 ± 0.49
21.	P10_F7	Morin	0.43 ± 0.05	-1.75 ± 0.27
22.	P10_F11	MRS2159	0.75 ± 0.06	-1.97 ± 0.30
23.	P11_A11	NF 023	0.015	-0.84 ± 0.14
24.	P11_D2	JNJ-40418677	14.3 ± 2.3	-1.06 ± 0.19
25.	P11_E8	Gossypol from cotton seeds	0.39 ± 0.05	-1.79 ± 0.25
26.	P11_H8	Nordihydroguaiaretic acid from Larreadivaricata (creosote bush)	0.89 ± 0.24	-1.25 ± 0.37
27.	P12_B4	Oleic Acid	2.00 ± 0.49	-1.14 ± 0.30
28.	P12_D6	Piceatannol	1.00 ± 0.05	-3.00 ± 0.54
29.	P13_B4	Protoporphyrin IX	0.30 ± 0.03	-1.88 ± 0.52
30.	P13_B10	Pyridostatintrifluoroacetate salt	2.45 ± 0.58	-1.92 ± 0.40
31.	P13_D10	Quercetin dihydrate	0.12 ± 0.02	-1.34 ± 0.25
32.	P13_G11	Candesartan cilexetil	4.66 ± 0.23	-2.71 ± 0.20
33.	P14_A5	Ro 8-4304	0.43 ± 0.03	-1.58 ± 0.13
34.	P14_A7	Auranofin	3.38 ± 0.98	-1.43 ± 0.42
35.	P14_B3	Rottlerin	2.78 ± 0.16	-3.47 ± 1.12

36.	P14_B5	Reactive Blue 2	0.043 ± 0.007	-1.33 ± 0.21
37.	P14_B9	N-Oleoyldopamine	47.4	-6.71
38.	P14_D6	7,8-Dihydroxyflavone hydrobromide	0.92 ± 0.07	-1.56 ± 0.14
39.	P14_D9	SCH-202676 hydrobromide	1.96 ± 0.12	-3.19 ± 0.39
40.	P15_D6	Tetradecylthioacetic acid	5.90 ± 0.52	-2.61 ± 0.39
41.	P15_D11	Tyrphostin 23	3.19 ± 0.24	-1.86 ± 0.20
42.	P15_F7	TTNPB	32.2 ± 1.4	-5.00
43.	P15_G3	Suraminhexasodium	0.046 ± 0.006	-1.38 ± 0.19
44.	P16_C2	Tyrphostin 47	0.35 ± 0.04	-2.58 ± 0.46
45.	P16_D2	Tyrphostin 51	0.15 ± 0.02	-1.28 ± 0.18
46.	P16_E8	Galloflavin	0.12 ± 0.02	-2.13 ± 0.63
47.	P16_G4	XCT790	> 100	--
48.	P16_H3	IPA-3	13.2 ± 4.4	-0.38 ± 0.06
49.	P3_A7	Bupropion hydrochloride	> 100	-
50.	P3_A8	Arvanil	17.4 ± 3.6	-0.98 ± 0.18
51.	P3_A10	CGP-7930	> 100	-
52.	P3_B9	PF 3845 hydrate	> 100	-
53.	P3_G7	BTCP hydrochloride	> 100	-
54.	P3_G8	Rhodblock 6	> 100	-
55.	P3_G9	Caffeine	> 100	-
56.	P3_G10	Chlorpropamide	34.3	-13.44
57.	P3_G5	Alfuzosin hydrochloride	> 100	-

Dark green – compounds with IC_{50} < 0.05 µM
Light green – compounds with IC_{50} between 0.05 µM and 0.5 µM
Yellow – sinefungin control

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Experimental Procedures

General Information

Synthesized non-fluorescent nucleotides were purified by ion-exchange chromatography on DEAE-Sephadex A-25 (HCO_3^- form) column. A column was loaded with reaction mixture and washed through with excess of water to remove metal (II) salt/EDTA complex. The nucleotides were eluted using a linear gradient of triethylammonium bicarbonate buffer (TEAB) in deionized water. The collected fractions were analyzed spectrophotometrically at 260 nm. After evaporation under reduced pressure with repeated additions of ethanol to decompose TEAB, compounds were isolated as triethylammonium (TEA) salts. Yields were calculated based on either sample weight or (preferably) optical milliunits (opt.mu) of the product. Optical unit measurements were performed in 0.1 M phosphate buffer pH 7 at 260 nm.

Analytical HPLC was performed on Agilent Tech. Series 1200 using (RP)Supelcosil LC-18-T HPLC column (4.6 x 250 mm, flow rate 1.3 mL/min) with a different linear gradient of methanol or acetonitrile in 0.05 M ammonium acetate buffer (pH 5.9). UV-detection was performed at 260 nm, as well as fluorescent label absorption maximum and fluorescence detection (at 360 nm for non-labelled compounds and at fluorescence tag emission maximum for labelled nucleotides).
Semipreparative HPLC was performed on the same apparatus equipped with Discovery RP Amide C-16 HPLC column (25 cm x 21.2 mm, 5 µm, flow rate 5.0 mL/min) or Grace Vision HT C18 HL column (250 cm x 22 mm, 10 µm, flow rate 5.0 mL/min) with different linear gradients of acetonitrile in 0.05 M ammonium acetate buffer (pH 5.9). UV-detection was performed at 260 nm, as well as fluorescent label absorption maximum and fluorescence detection (at 360 nm for non-labelled compounds and at fluorescence tag emission maximum for labelled nucleotides).
HPLC methods used in the studies are listed below:

Method name	Eluent A	Eluent B	Eluent gradient	Column
A	0.05 M ammonium acetate buffer (pH 5.9)	ACN	0 – 100% (0 – 15 min)	(RP)Supelcosil LC-18-T
P1	0.05 M ammonium acetate buffer (pH 5.9)	70% of eluent A/30% of ACN	0 – 50% 0 – 240 min	Grace Vision HT C18 HL
P2	0.05 M ammonium acetate buffer (pH 5.9)	70% of eluent A/30% of ACN	0 – 50% 0 – 120 min	Discovery RP Amide C-16 HPLC column
P3	0.05 M ammonium acetate buffer (pH 5.9)	70% of eluent A/30% of ACN	0 – 50% 0 – 90 min	Discovery RP Amide C-16 HPLC column
P4	0.05 M ammonium acetate buffer (pH 5.9)	70% of eluent A/30% of ACN	0 – 50% 0 – 60 min	Grace Vision HT C18 HL
P5	0.05 M ammonium acetate buffer (pH 5.9)	ACN	0 – 100% 0 – 120 min	Grace Vision HT C18 HL
P6	0.05 M ammonium acetate buffer (pH 5.9)	ACN	0 – 100% 0 – 120 min	Discovery RP Amide C-16 HPLC column
P7	0.05 M ammonium acetate buffer (pH 5.9)	ACN	0 – 100% 0 – 240 min	Grace Vision HT C18 HL

The structure and homogeneity of each final product was confirmed by RP HPLC and high resolution mass spectrometry HRMS (ESI-). Intermediate products were characterized by low resolution MS (ESI-) or ^{1}H NMR, ^{1}H-^{1}H COSY and ^{31}P NMR spectroscopy. Mass spectra were recorded on Thermo Scientific LTQ Orbitrap Velos (high resolution) and Sciex QTRAP 3200 (low resolution) spectrometers. Routine ^{1}H NMR, COSY and ^{31}P NMR spectra were run in pure D_2O at 25 °C and nucleotide concentration of 1–5 mM on a Bruker AVANCE III spectrometer at 500.24 MHz (^{1}H, ^{1}H-^{1}H COSY) and 202.5 MHz (^{31}P) or Varian INOVA 400 MHz at 399.94 (^{1}H, ^{1}H-^{1}H COSY) and 161.90 (^{31}P). The ^{1}H chemical shifts were determined relative to internal standard sodium 3-trimethylsilyl-[2,2,3,3-$^2H_4$]-propionate (TSP), and the ^{31}P chemical shifts relative to external standard 20% $^2H_3PO_4$.

The Ecm1 protein was expressed and purified at the Laboratory of Bioorganic Chemistry, Centre of New Technologies, University of Warsaw, Poland. The RNMT-RAM protein was expressed and purified at the Centre of Gene Regulation and Expression, School of Life Sciences, University of Dundee, Dundee, UK. The vaccinia capping enzyme (VCE) and *S*-adenosyl-L-methionine (SAM) were purchased from New England BioLabs.

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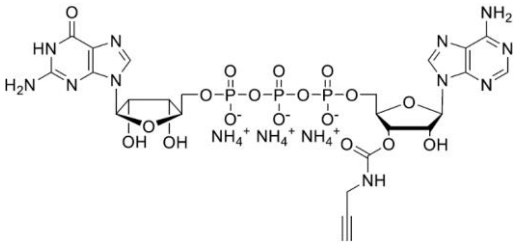
Chemical Synthesis

All solvents and chemical reagents for non-fluorescent nucleotides synthesis were purchased from Sigma-Aldrich and used without any pre-treatement. Dye azides used for CuAAC reactions: pyrene, perylene, BDP-FL, cyanine 3 and cyanine 5 with PEG linker and azide moiety were purchased from Lumiprobe. 5-N-(2-azidoethyl)-fluorescein amide ((5)FAM) was synthesized as described previously[1]. Guanosine, guanosine 5'-monophosphate disodium salt and adenosine 5'-monophosphae free acid form were purchased from Sigma Aldrich. LOPAC®1280 library of pharmacologically active compounds was purchased from Sigma Aldrich.
Propargyl-modified mononucleotide analogues were all synthesized in our laboratory as described previously.[2]
Synthesis of the following C8- and C6- modified nucleotides has also been described: 8-Me-GMP,[3] 8-CF_3-Gp_3A[4] and 6S-GMP[5]. Synthesis of 8-Ph-GMP will be published elsewhere.

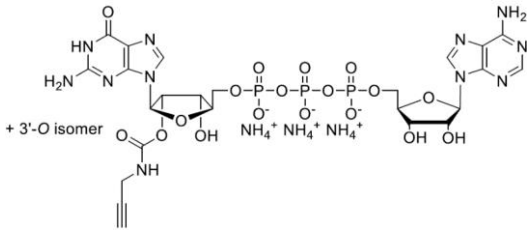
Synthesis of alkyne-modified non-fluorescent nucleotides

*P1-5'-guanosine-P3-(N-propargyl)-3'-carbamoyloadenosine triphosphate ammonium salt (Gp_3A-3'-O-cp, **1**)*



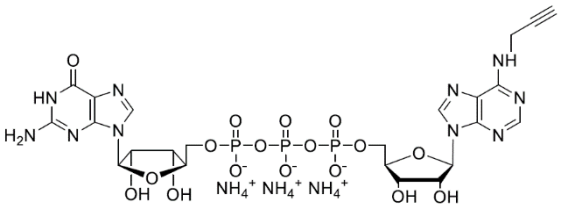
Triethylammonium salt of GMP (70 mg, 0.15 mmol) was mixed with lithium salt of cpADP-Im (41 mg, 0.072 mmol) and $MgCl_2$ (95 mg, 1 mmol) in DMF (1 mL). The reaction was carried out at a room temperature for 17.5 h and stopped by addition of 10 mL of H_2O. The reaction product was purified using semipreparative RP HPLC (Method P1). The collected eluate was lyophilized repeatedly to afford 16.5 mg (0.018 mmol) of **1** as a white solid and .11 mg (0.012 mmol) of 2'-*O* isomer. HPLC yield: 100%. Yield after purification: 41% (25% for isomer 3'-*O* only). HRMS (-)ESI *m/z* found: 852.0919, calc. for $C_{24}H_{29}N_{11}O_{18}P_3^-$: 852.0910; ^{1}H NMR (500 MHz, D_2O): 8.49 (1H, s, H_{C2}-Ado), 8.24 (1H, s, H_{C8}-Ado), 8.03 (1H, s, H_{C8}-Guo), 6.04 (1H, d, *J* = 6.9 Hz, $H_{1'}$-Ado), 5.78 (1H, d, *J* = 5.5 Hz, $H_{1'}$-Guo), 5.34 (1H, m, $H_{3'}$-Ado), 4.89 (1H, t, *J* = 5.9, $H_{2'}$-Ado), 4.67 (1H, t, *J* = 5.5, $H_{2'}$-Guo), 4.50 (2H, m), 4.28 (5H, m), 3.95 (2H, s, $H_{propargyl\ 3'\text{-}O}$), 2.63 (1H, s, $H_{propargyl\ 3'\text{-}O}$), ^{31}P NMR (202 MHz, D_2O): -10.42 (2P, m, P_α, P_γ), -22.09 (1P, t, *J* = 19.2 Hz, P_β).

*P1-5'-((N-propargyl)-2'(3)-carbamoylo)guanosine-P3-3'-adenosine triphosphate ammonium salt (2'-O/3'-O-cp-Gp_3A, **2**)*



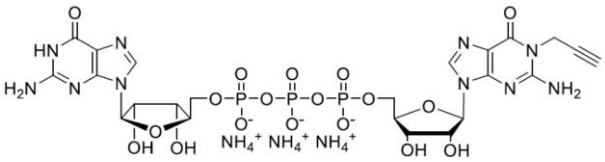
Triethylammonium salt of AMP (38 mg, 0.085 mmol) was mixed with lithium salt of 2'/3'-*O*-cpGDP-Im (18.8 mg, 0.031 mmol) and $MgCl_2$ (46 mg, 0.484 mmol) in DMSO (0.5 mL) and DMF (0.1 mL). The reaction was carried out at a room temperature for 6 h and stopped by addition of 4 mL of H_2O. The reaction product was purified using semipreparative RP HPLC (Method P2). The collected eluate was lyophilized repeatedly to afford 10 mg (0.011 mmol) of **2** as a white solid. HPLC yield: 100%. Yield after purification: 35%. HRMS (-)ESI *m/z* found: 852.0924, calc. for $C_{24}H_{29}N_{11}O_{18}P_3^-$: 852.0910; ^{1}H NMR (500 MHz, D_2O): 8.41 (1H, s, H_{C2}-Ado (2'-*O* isomer)), 8.40 (1H, s, H_{C2}-Ado (3'-*O* isomer)), 8.18 (1H, s, H_{C8}-Ado (3'-*O* isomer)), 8.18 (1H, s, H_{C8}-Ado (2'-*O* isomer)), 8.02 (1H, s, H_{C8}-Guo (3'-*O* isomer)), 7.92 (1H, s, H_{C8}-Guo (2'-*O* isomer)), 6.03 (1H, d, *J* = 4.7 Hz, $H_{1'}$-Ado (2'-*O* isomer)), 6.02 (1H, d, *J* = 5.1 Hz, $H_{1'}$-Ado (3'-*O* isomer)), 5.88 (1H, d, *J* = 3.0 Hz, $H_{1'}$-Guo (2'-*O* isomer), 5.78 (1H, d, *J* = 7.2 Hz, $H_{1'}$-Guo (3'-*O* isomer), 5.34 (1H, m, $H_{2'}$-Guo (2'-*O* isomer)), 5.31 (1H, m, $H_{3'}$-Guo (3'-*O* isomer)), 4.91 (1H, m, $H_{2'}$-Guo (3'-*O* isomer)), 4.65 (3H, m, $H_{2'}$-Ado (3'O isomer), $H_{3'}$-Guo (2'O isomer), one more H from 2'O isomer), 4.52 (2H, m), 4.42 (1J, m, $H_{4'}$-Guo (3'-*O* isomer), 4.35-4.26 (11H, m), 3.93 (1H, s, $H_{propargyl}$ (3'-*O* isomer)), 3.88 (1H, m, $H_{propargyl}$ (2'-*O* isomer), 2.63 (1H, s, $H_{propargyl}$ (3'-*O* isomer)), 2.59 (1H, t, *J* = 2.4 Hz, $H_{propargyl}$ (2'-*O* isomer). ^{31}P NMR (202 MHz, D_2O): -10.42 (2P, m, P_α, P_γ), -22.04 (1P, t, *J* = 18.6 Hz, P_β).

*P1-5'-guanosine-P3-3'-N6-propargyloadenosine triphosphate ammonium salt (Gp_3A(N6)prop, **3**)*



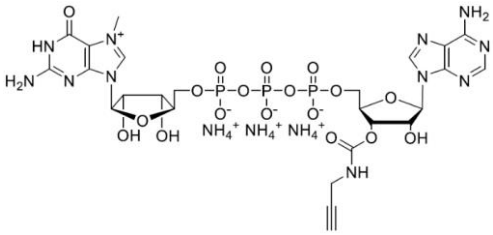
Triethylammonium salt of AMP-N6-propargyl (45 mg, 0.093 mmol) was mixed with sodium salt of GDP-Im (23 mg, 0.043 mmol) and $ZnCl_2$ (40 mg, 0.295 mmol) in DMF (0.5 mL). The reaction was carried out at a room temperature for 22 h and stopped by addition of Na_2EDTA (110 mg, 0.295 mmol) in 10 mL of H_2O. The pH was adjusted to 7.0 by addition of $NaHCO_3$. The reaction product was purified using semipreparative RP HPLC (Method P4). The collected eluate was lyophilized repeatedly to afford 5.4 mg (6.3 µmol) of **3** as a white solid. HPLC yield: 77%. Yield after purification: 15%. HRMS (-)ESI *m/z* found: 809.0862, calc. for $C_{23}H_{28}N_{10}O_{17}P_3^-$: 809.0852; ^{1}H NMR (500 MHz, D_2O): 8.38 (1H, s, H_{C2}-Ado), 8.23 (1H, s, H_{C8}-Ado), 8.02 (1H, s, H_{C8}-Guo), 6.06 (1H, d, *J* = 4.7 Hz, $H_{1'}$-Ado), 5.78 (1H, d, *J* = 5.0 Hz, $H_{1'}$-Guo), 4.65 (1H, t, *J* = 4.7 Hz, $H_{2'}$-Ado), 4.61 (1H, t, *J* = 5.0, $H_{2'}$-Guo), 4.51 (1H, t, *J* = 5.0, $H_{3'}$-Guo), 4.47 (1H, t, *J* = 4.7 Hz, $H_{3'}$-Ado), 4.36-4.24 (8H, m, $H_{4',5',5''}$-Ado, $H_{4',5',5''}$-Guo, $H_{propargyl\ N6}$), 2.00 (1H, s, $H_{propargyl\ N6}$), ^{31}P NMR (202 MHz, D_2O): -10.49 (2P, m, P_α, P_γ), -22.09 (1P, t, *J* = 18.4 Hz, P_β).

*P1-5'-guanosine-P3-(N-propargyl)-3'-carbamoyloadenosine triphosphate ammonium salt ($Gp_3G(N1)prop$, **4**)*



Sodium salt of GMP-N1-propargyl *P*-imidazolide (8.7 mg, 208 ODU, 17.2 µmol) was mixed with triethylammonium salt of GDP (17.6 mg, 27.4 µmol, 1.6 eq) and $ZnCl_2$ (19.1 mg, 140.5 µmol, 8.5 eq) in an anhydrous DMF (172 µL). Reaction was mixed overnight at a room temperature and progress was monitored with HPLC on the other day. The reaction was quenched with aqueous solution of Na_2EDTA (51.4 mg, 141 µmol) and pH was adjusted to pH = 6 with a solid $NaHCO_3$. Product was purified using RP-HPLC (Method 3). The collected eluate was lyophilized repeatedly to afford 7.9 mg (9.0 µmol) of **4** as a white solid. HPLC yield: 87%. Yield after purification: 52%. HRMS (-)ESI *m/z* found: 825.0814, calc. for $C_{23}H_{28}N_{10}O_{18}P_3^-$: 825.0801; ^{1}H NMR: (400 MHz, D_2O) δ 7.99 (2H, d, *J* = 4.8 Hz, H_{C8}-Guo), 5.83 (1H, d, *J* = 5.6 Hz, $H_{1'}$-Guo), 5.81 (1H, d, *J* = 5.6 Hz, $H_{1'}$-Guo), 4.82 (2H, overlapped with HDO peak, $H_{propargyl\ N1}$), 4.79 (2H, overlapped with HDO peak, $H_{2'}$-Guo), 4.52-4.48 (2H, m, $H_{3'}$-Guo), 4.31-4.29 (2H, m, $H_{4'}$-Guo), 4.27-4.23 (4H, m, $H_{5',5''}$-Guo), 2.73 (1H, t, *J* = 2.5 Hz, $H_{propargyl\ N1}$); ^{31}P NMR: (162 MHz, D_2O) δ -10.57 (dtd, J_1 = 19.3, J_2 = 6.0, J_3 = 1.2 Hz, P_α, P_γ), -22.31 (t, *J* = 19.3 Hz, P_β).

*P1-5'-(7-methyl)guanosine-P3-(N-propargyl)-3'-carbamoyloadenosine triphosphate ammonium salt (m^7Gp_3A-3'-O-cp, **5**)*

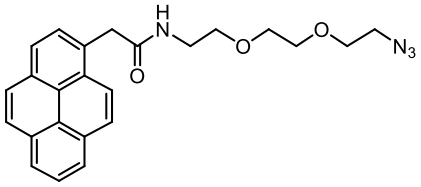


Ammonium salt 3'-*O*-Gp_3Acp (**1**) (1.0 mg, 1.1 µmol) was dissolved to 0.18 M concentration in 60 µL of H_2O and the pH was adjusted to 4 by 30% acetic acid. Then 10 equivalents of $(CH_3)_2SO_4$ were added every 5 min to the reaction mixture under stirring. 10% KOH was added if necessary to keep the pH at 4 value. The reaction was stopped after 4.5 h by addition of 0.8 mL of H_2O. Organic-soluble compounds were removed by extraction with diethyl ether repeated three times. The reaction product was purified using semipreparative RP HPLC (Method P1). The collected eluate was lyophilized repeatedly to afford 0.5 mg (0.54 µmol) of **5** as a white solid. HPLC yield: 93%. Yield after purification: 49%. HRMS (-)ESI *m/z* found: 866.1070, calc. for $C_{25}H_{31}N_{11}O_{18}P_3^-$: 866.1067; ^{1}H NMR (500 MHz, D_2O): 8.45 (1H, s, H_{C2}-Ado), 8.16 (1H, s, H_{C8}-Guo), 6.02 (1H, d, *J* = 6.0 Hz, $H_{1'}$-Ado), 5.84 (1H, d, *J* = 3.6 Hz, $H_{1'}$-Guo), 5.34 (1H, m, $H_{3'}$-Ado), 4.88 (1H, t, *J* = 6.0 Hz, $H_{2'}$-Ado), 4.46 (1H, dd, J_1 = 4.5 Hz, J_2 = 3.6 Hz, $H_{2'}$-Guo), 4.39 (2H, m), 4.37-4.33 (2H, m), 4.29-4.26 (3H, m), 4.00 (3H, s, $-CH_3$(N7)), 3.95 (2H, m, $H_{propargyl\ 3'\text{-}O}$), 2.63 (1H, m, $H_{propargyl\ 3'\text{-}O}$); ^{31}P NMR (202 MHz, D_2O): -10.67 (2P, m, P_α, P_γ), -22.19 (1P, t, *J* = 19.3 Hz, P_β).

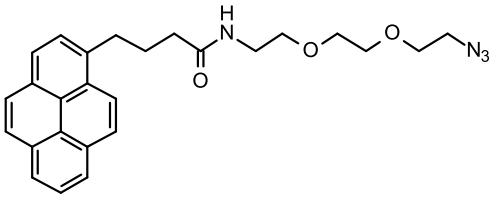
Synthesis of fluorescently labelled nucleotides

For the synthesis of fluorescently labelled nucleotide analogues dye azides were used. Pyrene azide (Py), perylene azide (Pe), BODIPY-FL azide (BODIPY-FL), cyanine 3 azide (Cy3) and cyanine 5 azide (Cy5) were purchased from Lumiprobe. Pyrene azide 3 (Py3) was purchased from APExBIO. 5-N-(2-azidoethyl)-fluorescein amide ((5)FAM) was synthesized as described before.[1] The structures of all used fluorescent tags are presented below.

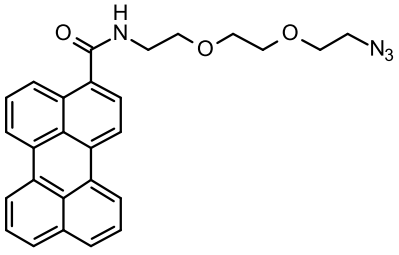
Pyrene azide (Py)



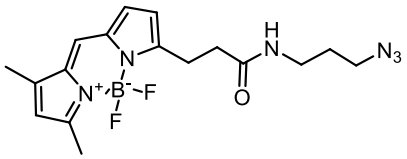
Pyrene azide 3(Py3)



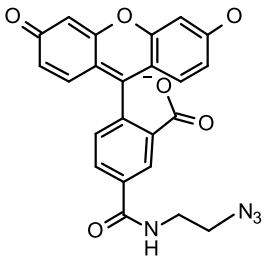
Perylene azide (Pe)



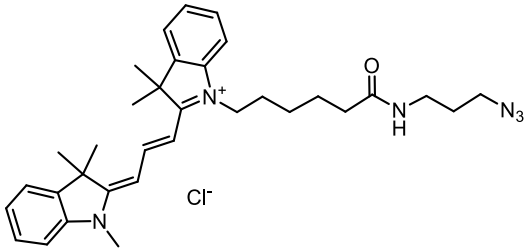
BDPY-FL azide (BDPY-FL)



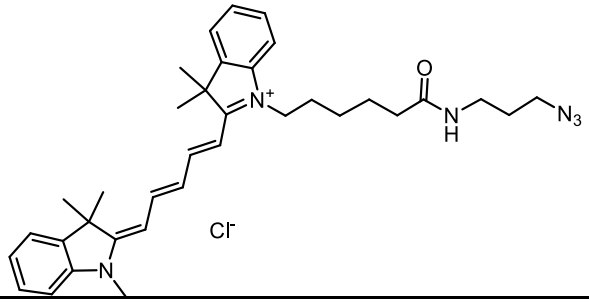
(5)-carboxyfluorescein azide ((5)FAM)



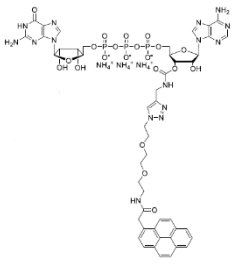
Cyanine 3 azide (Cy3)



Cyanine 5 azide (Cy5)

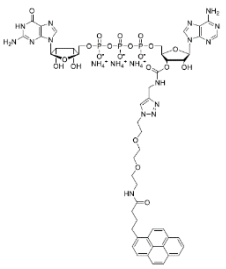


*Gp_3A-3'-O-Py (**6a**)*



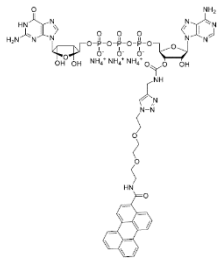
Aqueous solution of Gp_3A-3'-*O*-cp (**1**) ammonium salt (1.0 mg, 1.1 µmol, 40 µL, 0.027 M) was mixed with pyrene azide (**Py**) (1.8 mg, 4.3 µmol, 0.087 M) in 50 µL of DMSO following by addition of aqueous solutions of $CuSO_4 \cdot 5H_2O$ (0.75 mg, 3.0 µmol, 3.0 µL, 1.0 M) and sodium ascorbate (1.2 mg, 6.0 µmol, 6.0 µL, 1.0 M). The reaction was carried out at a room temperature for 4.5 h and stopped by addition of aqueous Na_2EDTA (3.7 mg, 10 µmol) in 0.9 mL of H_2O. The precipitated pyrene dye remains were centrifuged. The reaction product was purified using semipreparative RP HPLC (Method P6). The collected eluate was lyophilized repeatedly to afford 0.5 mg (0.38 µmol) of **6a** as a white solid. HPLC yield: 89%. Yield after purification: 34%.
HRMS (-)ESI *m/z* found: 1268.2778, calc. for $C_{48}H_{53}N_{15}O_{21}P_3^-$: 1268.2759.

*Gp_3A-3'-O-Py3 (**6b**)*



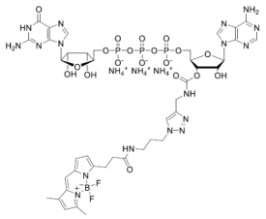
Aqueous solution of Gp_3A-3'-*O*-cp (**1**) ammonium salt (2.2 mg, 2.4 µmol, 6 µL, 0.40 M) was mixed with pyrene 3 azide (**Py3**) (2.0 mg, 4.5 µmol, 0.09 M) in 50 µL of DMSO following by addition of aqueous solutions of $CuSO_4 \cdot 5H_2O$ (0.75 mg, 3.0 µmol, 3.0 µL, 1.0 M) and sodium ascorbate (1.2 mg, 6.0 µmol, 6.0 µL, 1.0 M). The reaction was carried out at a room temperature for 5.5 h and stopped by addition of aqueous Na_2EDTA (7.4 mg, 20 µmol) in 0.7 mL of H_2O. The precipitated pyrene dye remains were centrifuged. The reaction product was purified using semipreparative RP HPLC (Method P5). The collected eluate was lyophilized repeatedly to afford 2.0 mg (1.4 µmol) of **6b** as a white solid. HPLC yield: 94%. Yield after purification: 61%.
HRMS (-)ESI *m/z* found: 1296.3072, calc. for $C_{50}H_{57}N_{15}O_{21}P_3^-$: 1296.3072.

*Gp_3A-3'-O-Pe (**6c**)*



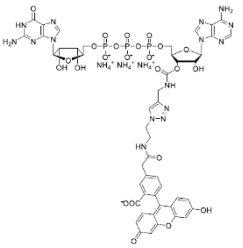
Gp_3A-3'-*O*-cp (**1**) ammonium salt (0.7 mg, 0.77 µmol) was mixed with perylene azide (**Pe**) (1.3 mg, 2.9 µmol, 0.057 M) in 50 µL of DMSO following by addition of aqueous solutions of $CuSO_4 \cdot 5H_2O$ (0.75 mg, 3.0 µmol, 3.0 µL, 1.0 M) and sodium ascorbate (1.2 mg, 6.0 µmol, 6.0 µL, 1.0 M). The reaction was carried out at a room temperature for 22 h and stopped by addition of aqueous Na_2EDTA (7.4 mg, 20 µmol) in 0.4 mL of H_2O. The precipitated pyrene dye remains were centrifuged. The reaction product was purified using analytical RP HPLC (Method A). The collected eluate was lyophilized repeatedly to afford 0.11 mg (0.08 µmol) of **6c** as a yellow solid. HPLC yield: 85%. Yield after purification: 10.5%.
HRMS (-)ESI *m/z* found: 11304.2762, calc. for $C_{51}H_{53}N_{15}O_{21}P_3^-$: 1304.2759.

*Gp_3A-3'-O-BDP-FL (**6d**)*



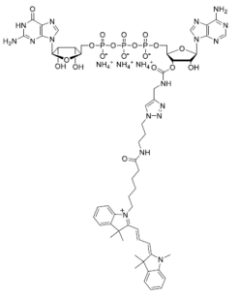
Aqueous solution of Gp_3A-3'-*O*-cp (**1**) ammonium salt (1.0 mg, 1.1 µmol, 40 µL, 0.027 M) was mixed with BDP-FL azide (**BDP-FL**) (2.0 mg, 5.3 µmol, 0.11 M) in 50 µL of DMSO following by addition of aqueous solutions of $CuSO_4 \cdot 5H_2O$ (0.75 mg, 3.0 µmol, 3.0 µL, 1.0 M) and sodium ascorbate (1.2 mg, 6.0 µmol, 6.0 µL, 1.0 M). The reaction was carried out at a room temperature for 7.5 h and stopped by addition of aqueous Na_2EDTA (5.6 mg, 15 µmol) in 0.95 mL of H_2O. The precipitated BDP-FL dye remains were centrifuged. The reaction product was purified using semipreparative RP HPLC (Method P6). The collected eluate was lyophilized repeatedly to afford 0.75 mg (0.59 µmol) of **6d** as an orange solid. HPLC yield: 93%. Yield after purification: 53%.
HRMS (-)ESI *m/z* found: 1226.2768, calc. for $C_{41}H_{50}BF_2N_{17}O_{19}P_3^-$: 1226.2748.

*Gp_3A-3'-O-(5)FAM (**6e**)*



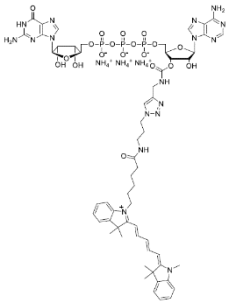
Aqueous solution of Gp_3A-3'-*O*-cp (**1**) ammonium salt (0.7 mg, 0.77 µmol) was mixed with N-(2-azidoethyl)-5-fluorescein-carboxoamide (0.7 mg, 1.58 µmol, 0.05 M) in 30 µL of DMSO following by addition of aqueous solutions of $CuSO_4 \cdot 5H_2O$ (0.75 mg, 3.0 µmol, 3.0 µL, 1.0 M) and sodium ascorbate (1.2 mg, 6.0 µmol, 6.0 µL, 1.0 M). The reaction was carried out at a room temperature for 3 h and stopped by addition of aqueous Na_2EDTA (7.4 mg, 20 µmol). The precipitated fluorescein dye remains were centrifuged. The reaction product was purified using semipreparative RP HPLC (Method P5). The collected eluate was lyophilized repeatedly to afford 0.54 mg (0.4 µmol) of **6e** as an orange solid. HPLC yield: 83%. Yield after purification: 52%.
HRMS (-)ESI *m/z*found: 1296.1993, calc. for $C_{47}H_{45}N_{15}O_{24}P_3^-$: 1296.1980.

*Gp_3A-3'-O-Cy3 (**6f**)*



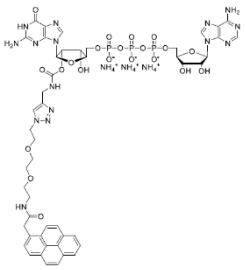
Aqueous solution of Gp_3A-3'-*O*-cp (**1**) ammonium salt (0.85 mg, 0.94 µmol, 10 µL, 0.09 M) was mixed with cyanine 3 azide (**Cy3**) (1.2 mg, 2.09 µmol, 0.05 M) in 40 µL of DMSO following by addition of aqueous solutions of $CuSO_4 \cdot 5H_2O$ (0.75 mg, 3.0 µmol, 3.0 µL, 1.0 M) and sodium ascorbate (1.2 mg, 6.0 µmol, 6.0 µL, 1.0 M). The reaction was carried out at a room temperature for 21 h and stopped by addition of aqueous Na_2EDTA (7.4 mg, 20 µmol) in 0.7 mL of H_2O. The precipitated cyanine dye remains were centrifuged. The reaction product was purified using semipreparative RP HPLC (Method P6). The collected eluate was lyophilized repeatedly to afford 0.12 mg (0.08 µmol) of **6f** as a red solid. HPLC yield: 79%. Yield after purification: 9%.
HRMS (-)ESI *m/z* found: 1390.4354, calc. for $C_{57}H_{71}N_{17}O_{19}P_3^-$: 1390.4330;

*Gp_3A-3'-O-Cy5 (**6g**)*



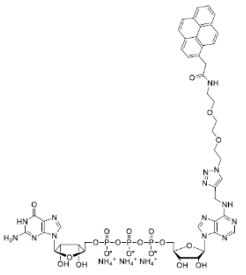
Aqueous solution of Gp_3A-3'-*O*-cp (**1**) ammonium salt (1.1 mg, 1.21 µmol) was mixed with cyanine 5 azide (**Cy5**) (2.5 mg, 4.16 µmol, 0.08 M) in 50 µL of DMSO following by addition of aqueous solutions of $CuSO_4 \cdot 5H_2O$ (0.75 mg, 3.0 µmol, 3.0 µL, 1.0 M) and sodium ascorbate (1.2 mg, 6.0 µmol, 6.0 µL, 1.0 M). The reaction was carried out at a room temperature for 4 h and stopped by addition of aqueous Na_2EDTA (7.4 mg, 20 µmol) in 0.4 mL of H_2O. The precipitated cyanine dye remains were centrifuged. The reaction product was purified using analytical RP HPLC (Method A). The collected eluate was lyophilized repeatedly to afford 0.18 mg (0.12 µmol) of **6g** as a blue solid. HPLC yield: 72%. Yield after purification: 10%.
HRMS (-)ESI *m/z* found: 1416.4506, calc. for $C_{59}H_{73}N_{17}O_{19}P_3^-$: 1416.4487;

*2'-O/3'-O-Py-Gp_3A (**7**)*



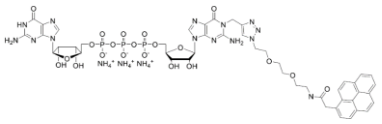
2'-*O*/3'-*O*-cp-Gp_3A (**2**) ammonium salt (0.5 mg, 0.55 µmol) was mixed with pyrene azide (**Py**) (1.5 mg, 3.6 µmol, 0.07 M) in 50 µL of DMSO following by addition of aqueous solutions of $CuSO_4 \cdot 5H_2O$ (0.75 mg, 3.0 µmol, 3.0 µl, 1.0 M) and sodium ascorbate (1.2 mg, 6.0 µmol, 6.0 µL, 1.0 M). The reaction was carried out at a room temperature for 20 h and stopped by addition of aqueous Na_2EDTA (7.4 mg, 20 µmol) in 0.7 mL of H_2O. The precipitated pyrene dye remains were centrifuged. The reaction product was purified using semipreparative RP HPLC (Method P5). The collected eluate was lyophilized repeatedly to afford 0.82 mg (0.16 µmol) of **7** as a white solid. HPLC yield: 92%. Yield after purification: 28%.
HRMS (-)ESI *m/z* found: 1268.2759, calc. for $C_{48}H_{53}N_{15}O_{21}P_3^-$: 1268.2759.

*$Gp_3A(N6)$-Py (**8**)*



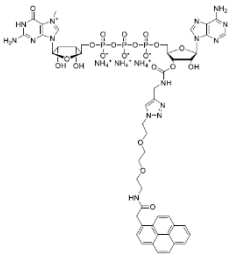
$Gp_3A(N6)$prop (**3**) ammonium salt (1.5 mg, 1.8 µmol, 0.058 M) was mixed with pyrene azide (**Py**) (1.2 mg, 2.9 µmol, 0.096 M) in 30 µL of DMSO following by addition of aqueous solutions of $CuSO_4 \cdot 5H_2O$ (0.38 mg, 1.5 µmol, 1.5 µL, 1.0 M) and sodium ascorbate (0.6 mg, 3.0 µmol, 3.0 µL, 1.0 M). The reaction was carried out at a room temperature for 48 h and stopped by addition of aqueous Na_2EDTA (7.4 mg, 20 µmol) in 0.7 mL of H_2O. The precipitated pyrene dye remains were centrifuged. The reaction product was purified using semipreparative RP HPLC (Method P5). The collected eluate was lyophilized repeatedly to afford 0.82 mg (0.64 µmol) of **8** as a white solid. HPLC yield: 96%. Yield after purification: 37%.
HRMS (-)ESI *m/z* found: 1225.2700, calc. for $C_{47}H_{52}N_{14}O_{20}P_3^-$: 1225.2701.

*$Gp_3G(N1)$-Py (**9**)*



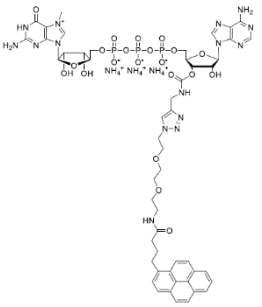
$Gp_3G(N1)$prop (**4**) triethylammonium salt (1.7mg, 1.9 µmol, 0.043 M) was mixed with pyrene azide (**Py**) (1.8 mg, 4.3 µmol, 0.096 M) in 45 µL of DMSO following by addition of aqueous solutions of $CuSO_4 \cdot 5H_2O$ (0.38 mg, 1.5 µmol, 1.5 µL, 1.0 M) and sodium ascorbate (0.6 mg, 3.0 µmol, 3.0 µL, 1.0 M). The reaction was carried out at a room temperature for 48 h and stopped by addition of aqueous Na_2EDTA (7.4 mg, 20 µmol) in 0.7 mL of H_2O. The precipitated pyrene dye remains were centrifuged. The reaction product was purified using semipreparative RP HPLC (Method P5). The collected eluate was lyophilized repeatedly to afford 1.05 mg (0.82 µmol) of **9** as a white solid. HPLC yield: 92%. Yield after purification: 42%.
HRMS (-)ESI *m/z* found: 1241.2653, calc. for $C_{47}H_{52}N_{14}O_{21}P_3^-$: 1241.2650.

*m^7Gp_3A-3'-O-Py (**10a**)*



Aqueous solution of m^7Gp_3A-3'-*O*-cp (**5**) ammonium salt (0.5 mg, 0.54 µmol, 10 µL, 0.54 M) was mixed with pyrene azide (**Py**) (2.0 mg, 4.8 µmol, 0.069 M) in 70 µL of DMSO following by addition of aqueous solutions of $CuSO_4 \cdot 5H_2O$ (0.75 mg, 3.0 µmol, 3.0 µL, 1.0 M) and sodium ascorbate (1.2 mg, 6.0 µmol, 6.0 µL, 1.0 M). The reaction was carried out at a room temperature for 4.5 h and stopped by addition of aqueous Na_2EDTA (5.6 mg, 15 µmol) in 0.95 mL of H_2O. The precipitated pyrene dye remains were centrifuged. The reaction product was purified using semipreparative RP HPLC (Method P6). The collected eluate was lyophilized repeatedly to afford 0.48 mg (0.31 µmol) of **10a** as a white solid. HPLC yield: 65%. Yield after purification: 43%.
HRMS (-)ESI *m/z* found: 1282.2929, calc. for $C_{49}H_{55}N_{15}O_{21}P_3^-$: 1282.2915.

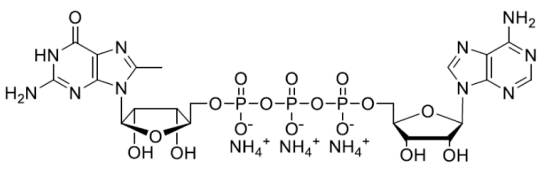
*m^7Gp_3A-3'-O-Py3 (**10b**)*



m^7Gp_3A-3'-*O*-cp (**5**) ammonium salt (0.3 mg, 0.36 µmol, 0.007 M) was mixed with pyrene 3 azide (**Py3**) (0.63 mg, 1.4 µmol, 0.028 M) in 50 µL of DMSO following by addition of aqueous solutions of $CuSO_4 \cdot 5H_2O$ (0.75 mg, 3.0 µmol, 3.0 µL, 1.0 M) and sodium ascorbate (1.2 mg, 6.0 µmol, 6.0 µL, 1.0 M). The reaction was carried out at a room temperature for 3 h and stopped by addition of aqueous Na_2EDTA (7.4 mg, 20 µmol) in 0.7 mL of H_2O. The precipitated pyrene dye remains were centrifuged. The reaction product was purified using semipreparative RP HPLC (Method P5). The collected eluate was lyophilized repeatedly to afford 0.2 mg (0.15 µmol) of **10b** as a white solid. HPLC yield: 88%. Yield after purification: 41%.
HRMS (-)ESI *m/z* found: 1311.3257, calc. for $C_{52}H_{61}N_{15}O_{20}P_3^-$: 1311.3301

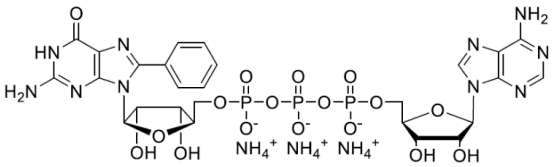
Synthesis of potential inhibitors

P1-5'-(C8-methyl)guanosine-P3-3'-adenosine triphosphate ammonium salt (8-Me-Gp_3A)



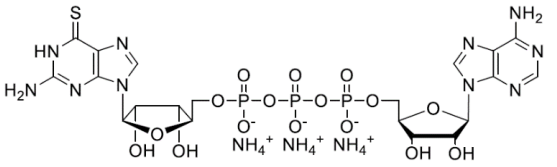
Triethylammonium salt of 8-Me-GMP (5.7 mg, 11.9 µmol) was mixed with sodium salt of ADP-Im (20.9 mg, 40.0 µmol) and $ZnCl_2$ (15.7 mg, 115 µmol) in DMF (0.1 mL). The reaction was carried out at 35 °C for 24 h and stopped by addition of Na_2EDTA (44 mg, 118 umol) dissolved in 1 mL of H_2O and $NaHCO_3$ to pH 7. The reaction product was purified using semipreparative RP HPLC (Method P1). The collected eluate was lyophilized repeatedly to afford 1.9 mg (2.3 µmol) of 8-Me-Gp_3A as a white solid. HPLC yield: 89%. Yield after purification: 19%. HRMS (-)ESI *m/z* found: 785.0860, calc. for $C_{21}H_{28}N_{10}O_{17}P_3^-$: 785.0852. ^{1}H NMR (500 MHz, D_2O): 8.47 (1H, s, H_{C2}-Ado), 8.24 (1H, s, H_{C8}-Ado), 6.03 (1H, d, J = 5.4 Hz, $H_{1'}$-Ado), 5.74 (1H, d, J = 6.8 Hz, $H_{1'}$-Guo), 5.08 (1H, dd, J_1 = 5.5 Hz, J_2 = 6.8 Hz, $H_{2'}$-Guo), 4.66 (1H, t, J = 5.4 Hz, $H_{2'}$-Ado), 4.55 (1H, dd, J_1 = 3.9 Hz, J_2 = 4.9 Hz, $H_{3'}$-Guos), 4.52 (1H, dd, J_1 = 3.9 Hz, J_2 = 4.9 Hz), 4.37 (1H, m), 4.34-4.22 (5H, m), 2.49 (3H, s, $H_{methyl\ (C8)}$), ^{31}P NMR (202 MHz, D_2O): -10.48 (2P, d, J = 18.8 Hz, P_α, P_γ), -22.25 (1P, t, J = 18.8 Hz, P_β).

P1-5'-(C8-phenyl)guanosine-P3-3'-adenosine triphosphate ammonium salt (8-Ph-Gp$_3$A)



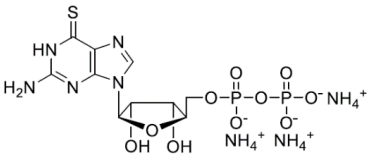
Triethylammonium salt of 8-Ph-GMP (5 mg, 9.3 µmol) was mixed with sodium salt of ADP-Im (10.5 mg, 20.1 µmol) and mixture of $ZnCl_2$ (18 mg, 132 µmol) and $MgCl_2$(10 mg, 105 µmol) in DMSO (0.1 mL) and DMF (0.1 mL). The reaction was carried out at a room temperature for 22 h and stopped by addition of Na_2EDTA (56 mg, 150µmol) in 0.8 mL of H_2O. The pH was adjusted to 7.0 by addition of $NaHCO_3$. The reaction product was purified using semipreparative RP HPLC (Method P7). The collected eluate was lyophilized repeatedly to afford 2.6 mg (2.8 µmol) of 8-Ph-Gp$_3$A as a white solid. HPLC yield: 93%. Yield after purification: 31%. HRMS (-)ESI *m/z* found: 847.1025, calc. for $C_{26}H_{30}N_{10}O_{17}P_3^-$: 847.1009; ^{1}H NMR (500 MHz, D_2O): 8.47 (1H, s, H_{C2}-Ado), 8.14 (1H, s, H_{C8}-Ado), 7.59 (5H, s, $H_{phenyl\ (C8)}$), 5.98 (1H, d, *J* = 5.6 Hz, $H_{1'}$-Ado), 5.60 (1H, d, *J* = 6.2 Hz, $H_{1'}$-Guo), 5.31 (1H, t, *J* = 6.2 Hz, $H_{2'}$-Guo), 4.63 (1H, t, *J* = 5.6 Hz, $H_{2'}$-Ado), 4.51 (2H, m), 4.36 (1H, m), 4.30 (3H, m), 4.24 (1H, m), 4.21 (1H, m), ^{31}P NMR (202 MHz, D_2O): -10.40 (2P, m, P_α, P_γ), -22.17 (1P, t, *J* = 19.2 Hz, P_β).

P1-5'-(6-thio)guanosine-P3-3'-adenosine triphosphate ammonium salt ($^{6\text{-}S}$Gp$_3$A)



Triethylammonium salt of $^{6\text{-}S}$GMP (7.0mg, 14.6 µmol) was mixed with sodium salt of ADP-Im (15.4 mg, 29.5 µmol) and $ZnCl_2$ (24.2 mg, 178 µmol) in DMF (0.25 mL). The reaction was carried out at 35 °C for 4 h and stopped by addition of Na_2EDTA (70 mg, 188 umol) dissolved in 2 mL of H_2O and $NaHCO_3$ to pH 7. The reaction product was purified using semipreparative RP HPLC (Method P1). The collected eluate was lyophilized repeatedly to afford 8.0 mg (8.9 µmol) of $^{6\text{-}S}$Gp$_3$A as a white solid. HPLC yield: 70%. Yield after purification: 61%. HRMS (-)ESI *m/z* found: 787.0475, calc. for $C_{20}H_{26}N_{10}O_{16}P_3S^-$: 787.0467. ^{1}H NMR (500 MHz, D_2O): 8.32 (1H, s, H_{C2}-Ado), 8.16 (1H, s, H_{C8}-Ado or H_{C8}-Guo), 8.15 (1H, s, H_{C8}-Ado or H_{C8}-Guo), 6.04 (1H, d, *J* = 4.5Hz, $H_{1'}$-Ado), 5.80 (1H, d, *J* = 5.2 Hz, $H_{1'}$-Guo), 4.67 (1H, t, *J* = 5.2 Hz, $H_{2'}$-Guo), 4.65 (1H, t, *J* = 4.5, $H_{2'}$-Ado), 4.49 (2H, m), 4.35 (1H, m), 4.31 (3H, m), 4.27 (2H, m), ^{31}P NMR (202 MHz, D_2O): -10.54 (2P, d, *J* = 18.9 Hz, P_α, P_γ), -22.04 (1P, t, *J* = 18.9 Hz, P_β).

(6-thio)guanosine-diphosphate ammonium salt ($^{6\text{-}S}$GDP)



Triethylammonium salt of $^{6\text{-}S}$GMP (7.0 mg, 14.6 µmol) was mixed with triethylammonium salt of PO_4^{3-} (14.0 mg, 35.2 µmol) and $ZnCl_2$ (30.0 mg, 220 µmol) in DMF (0.32 mL). The reaction was carried out at 35 °C for 24 h and stopped by addition of Na_2EDTA (85 mg, 228 µmol) dissolved in 5 mL of H_2O and $NaHCO_3$ to pH 7. The reaction product was purified using semipreparative RP HPLC (Method P1). The collected eluate was lyophilized repeatedly to afford 1.5 mg (1.2 µmol) of $^{6\text{-}S}$GDP as a white solid. HPLC yield: 56%. Yield after purification: 21%. HRMS (-)ESI *m/z* found: 457.9946, calc. for $C_{10}H_{14}N_5O_{10}P_2S^-$: 457.9942. ^{1}H NMR (500 MHz, D_2O): 8.29 (1H, s, H_{C8}), 5.94 (1H, d, *J* = 5.7 Hz, $H_{1'}$), 4.81 (1H, m, $H_{2'}$), 4.58 (1H, dd, J_1 = 5.0 Hz, J_2 = 3.9Hz, $H_{3'}$), 4.35 (1H, m, $H_{4'}$), 4.22 (2H, dd, J_1 = 3.4 Hz, J_2 = 5.3 Hz, $H_{5',5''}$), ^{31}P NMR (202 MHz, D_2O): -8.22 (1P, m, P_β), -10.01 (1P, d, *J* = 19.8 Hz, P_α).

Ecm1 and RNMT-RAM Expression and Purification

Methyltransferase gene form *E. Cuniculi* called Ecm1 cloned in pEX-K4 cloning vector (purchased from Eurofins) was amplified by PCR using primers that introduced BamHI and HindIII restriction sites compatible with the cloning sites of pQE-30 expression vector. Inserted ECM1 gene was flanked at the 5′ end by a sequence that encoded His-tag and Sumo-tag. Ecm1 methyltransferase (MTase) was overexpressed as a fusion protein with His-tagged Sumo in the pLysS (DE3) *E. coli* strain (Stratagene). 6xHis-Sumo-Ecm1 expression was induced with 0.3 mM IPTG at an optical density of 0.8, and the cells were further cultured for 16 h at 18 °C. The cells were lysed in buffer containing 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 5% glycerol, 5 mM β-mercaptoethanol (2-ME), 10 mM imidazole, 1 mg/mL lysozyme with additon of a mixture of protease inhibitors. After 30 min, the NaCl concentration was increased to 500 mM. The lysate was sonicated, centrifuged and clarified by filtration. The cleared lysate was then loaded on a 5 mL HisTrap Crude column (GE Healthcare) previously equilibrated with a buffer containing 50 mM Tris-HCl (pH 8.0), 500 mM NaCl, 10 mM imidazole, 5% glycerol and 5 mM 2-ME. 6xHis-Sumo-Ecm1 was eluted with buffer containing 300 mM imidazole. For removal of *N*-terminal tag, His6_Ulp1 Sumo protease (MCLAB) was added to pooled fractions containing 6x-His-SUMO-Ecm1. The mixture was dialyzed at 4 °C overnight against a buffer containing 50 mM Tris-HCl (pH 8.0), 500 mM NaCl, 5% glycerol, 0.5 mM EDTA, 0.5 mM DTT and 10 mM imidazole. The dialyzed sample was loaded on the HisTrap Crude column previously equilibrated with dialysis buffer. Flow-through was collected as a fraction containing Ecm1 MTase separated from *N*-terminal tag (6xHis-Sumo) and His-tagged Sumo protease. The flow-through fraction was further polished on a Superdex 75 Prep Grade HiLoad 26/600 gel filtration column (GE Healthcare). Fractions containing Ecm1 MTase were concentrated to 10.5 µM, flash-frozen and stored at -80 °C in a buffer containing 30 mM Tris-HCl (pH 8.0), 200 mM NaCl, 5% glycerol, 0.5 mM EDTA and 0.5 mM DTT.

Human N7-methyltransferase RNMT in complex with RAM subunit was expressed and purified as described previously.[6]

UV-Vis and Fluorescence Measurements

Absorption and Fluorescence Measurements. Absorption spectra were measured in 50 mM Tris-HCl, pH 7.5, on a Shimadzu UV-1800 spectrophotometer, with a CPS-100 holder for temperature control, using a quartz 10 × 2 mm cuvette. Emission and excitation spectra were measured in 50 mM Tris-HCl, pH 7.5, on a Cary Eclipse spectrofluorometer (Agilent), equipped with a xenon lamp, under temperature-controlled conditions at 30 °C using a quartz 10 × 4 mm cuvette. Before each measurement, the buffer was degassed. Emission spectra were recorded for all excitation maxima, and excitation spectra were recorded for all emission maxima.

Quantum Yield (QY) Determination. Relative QYs of all compounds were determined according to a previously reported procedure.[7] For all labeled nucleotides, the experiments were carried out in a 10 × 10 mm quartz cuvette in 50 mM Tris-HCl, pH 7.5, at 25 °C. QY values for pyrene-labeled nucleotides were determined at an excitation of 345 nm relative to quinine sulfate in 0.1 M H_2SO_4 (QY = 54%).[8] QY of perylene-labeled GpppA was determined at an excitation of 435 nm relative to perylene-azide in ethanol (QY = 100%), according to the manufacturer (https://www.lumiprobe.com/). QYs of BDP-FL-labeled nucleotides were determined at an excitation of 475 nm relative to BDP-FL-azide in ethanol (QY = 97%), according to the manufacturer (https://www.lumiprobe.com/). QYs of 5-FAM-labeled nucleotides were determined at an excitation of 475 nm relative to fluorescein in 0.1 M NaOH (QY = 79%).[9] QY of cyanine 3 (Cy3)-labeled GpppA was determined at an excitation of 520 nm relative to Cy3-azide in ethanol (QY = 31%), according to the manufacturer (https://www.lumiprobe.com/). QY of cyanine 5 (Cy5)-labeled GpppA was determined at an excitation of 610 nm relative to Cy5-azide in ethanol (QY = 20%), according to the manufacturer (https://www.lumiprobe.com/). The same excitation wavelengths and slit widths were used for samples and references. To minimize the reabsorption effect, absorption of a sample at the excitation wavelength was kept under 0.1. For QY calculations the following formula was applied:

$$QY_{sample} = QY_{ref}\frac{S_{sample}}{S_{ref}}\frac{f_{ref}}{f_{sample}}\frac{\eta^2_{sample}}{\eta^2_{ref}}, \qquad (1)$$

where *S* is the integral area under the fluorescence emission spectrum; η is the refractive index of the solvent; and *f* is the absorption factor defined as

$$f = 1 - 10^{-A(\lambda_{exc})}, \qquad (2)$$

Point Fluorescence Measurements. All experiments involving monitoring of the enzymatic N7-MTase process were performed on a Synergy™ H1 plate reader in Greiner 96-well black, flat-bottom, non-binding plates. For all measurements with probe **6b** fluorescence detection at 345 nm excitation wavelength and 378 nm emission wavelength was registered. For other probes excitation and emission corresponded to its absorption and emission maxima.

Monitoring of N7-MTase Activity

Steady-State Parameter Determination. Point fluorescence measurements were performed to determine kinetic parameters of enzymatic N7-methylation of probe **6b** by Ecm1 and RNMT-RAM. The experiments were performed in 96-well black, non-binding assay plates at 30 °C. Each well contained a substrate (0–15 μM probe **6b**), the SAM cosubstrate (50 μM for Ecm1 or 20 μM for RNMT-RAM), and a protein (10 nM Ecm1 or 20 nM RNMT-RAM) in 50 mM Tris-HCl, pH 7.5. Studies on N7-methylation of the SAM cosubstrate were carried out in 50 mM Tris-HCl, pH 7.5, with a substrate (2 μM probe **6b**), the SAM cosubstrate (0–100 μM), and a protein (10 nM Ecm1 and 20 nM RNMT-RAM) at 30 °C. The reaction components for both experiments were preincubated for 15 min at 30 °C with mixing at 300 rpm, and the enzyme solutions were prepared separately in the same buffer at a concentration up to 150 nM (Ecm1) and 300 nM (RNMT-RAM). Before adding an enzyme, the plate was additionally incubated in a plate reader for 20 min at 30 °C with mixing at 300 rpm and point fluorescence registered every minute. Immediately before fluorescence reading, 10 μL of an enzyme solution was added into each well to a total reaction volume of 150 μL. The initial rates were calculated by fitting a linear curve to the first 10 points (10 minutes) for Ecm1 or 20 points (20 minutes) for RNMT-RAM. We measured fluorescence intensity changes until complete signal saturation, thus being able to convert the initial rates from au/min to μM/min. To calculate kinetic parameters, the Michaelis–Menten model was applied. To obtain the relationship between the initial rate of the reaction and the substrate concentration, the following equation was fitted:

$$V = \frac{E_t k_{cat}[S]}{K_M + [S]}, \qquad (3)$$

where K_M is the Michaelis-Menten constant; [*S*] is the concentration of probe **6b**; E_t is the enzyme concentration; k_{cat} is the catalytic constant; and V_{max} is the maximum rate, calculated as $E_t k_{cat}$. To determine K_M, k_{cat}, and V_{max} we used the GraphPad Prism 7 software.

Screening of the LOPAC®1280 Library. Screening of the LOPAC®1280 library was performed using point fluorescence measurements in 96-well black, non-binding assay plates at 30 °C. Each well contained 50 mM Tris-HCl, pH 7.5, a substrate (2 μM probe **6b**), the SAM cosubstrate (50 μM), the Ecm1 enzyme (10 nM), an inhibitor (10 μM), and 3% of DMSO (compounds from LOPAC®1280 library are dissolved in pure DMSO). The reaction components were preincubated for 15 min at 30 °C with mixing at 300 rpm. Before adding the enzyme, the plate was additionally incubated in a plate reader for 20 min at 30 °C with mixing at 300 rpm and point fluorescence registered every minute. Immediately before fluorescence reading, 10 μL of an enzyme solution was added into each well to a total reaction volume of 150 μL. Initial rates were calculated by fitting a linear curve to the first 10 points (10 minutes).

IC_{50} Determination. Inhibition parameters of enzymatic N7-methylation of probe **6b** by Ecm1, RNMT-RAM, and VCE were determined using point fluorescence measurements in 96-well black, non-binding assay plates at 30 °C. Each well contained 50 mM Tris-HCl, pH 7.5, a substrate (2 μM probe **6b**), the SAM cosubstrate (50 μM for Ecm1 and VCE or 20 μM for RNMT-RAM), an enzyme (10 nM Ecm1, 20 nM RNMT-RAM, or 5U of VCE), and an inhibitor (half-log dilutions $\log C_{inh} \in <-2.5;2>$ for nucleotide-like inhibitors or $\log C_{inh} \in <-3;1.5>$ for compounds from the LOPAC®1280 library). During the experiment, point fluorescence (exc. 345 nm, em. 378 nm) was registered. If the inhibitor fluorescence interfered with the probe **6b** emission, a control measurement of the inhibitor solution was performed under the same conditions and then the result was subtracted from the inhibition data.
To determine IC_{50} parameters, a four-parameter dose-response equation was fitted as follows:

$$\frac{V}{V_0} = A1 + \frac{A2-A1}{1+\frac{(C_{inh.})^p}{(IC_{50})^p}}, \qquad (4)$$

where A1 and A2 are the bottom and top asymptotes, respectively; $C_{inh.}$ the inhibitor concentration; p is the Hill coefficient, and V/V_0 is the ratio of the initial reaction rate with the inhibitor to that without the inhibitor.

***z*-Factor Determination**

To determine *z*-factor values of the Ecm1 and RNMT-RAM assays point fluorescence measurements were performed in 96-well black, non-binding assay plates at 30 °C. The plates included positive and negative controls in a 1:1 ratio. The positive controls consisted of a mixture of 50 mM Tris-HCl (pH 7.5), a substrate (2 μM probe **6b**), the SAM cosubstrate (50 μM for Ecm1 and 20 μM for RNMT-RAM), and an enzyme (10 nM Ecm1 or 20 nM RNMT-RAM). To the negative controls, sinefungin was added at a concentration causing complete reacion inhibition (25 μM for Ecm1 and 20 μM for RNMT-RAM). The reaction components were preincubated for 15 min at 30 °C with mixing at 300 rpm. Before adding an enzyme, the plate was additionally incubated in a plate reader for 20 min at 30 °C with mixing at 300 rpm and point fluorescence was registered every minute. Immediately before fluorescence reading, 10 μL of an enzyme solution was added into each well to a total reaction volume of 150 μL. The initial rates were calculated by fitting a linear curve to the first 10 points (10 minutes) for Ecm1 or 20 points (20 minutes) for RNMT-RAM. Finally, *z*-factor values were calculated as follows:

$$z = 1 - \frac{3*(\sigma_{pos}+\sigma_{neg})}{|\mu_{pos}-\mu_{neg}|}, \qquad (5)$$

where σ is the standard deviation, and μ is the average value of the initial rate for the positive (pos) and negative (neg) controls, respectively.

SUPPORTING INFORMATION

Author Contributions

R.K., J.K., and J.J. designed the study; R.K. synthesized fluorescent probes and performed the experiments; M.F. synthesized C8- and 6S-modified nucleotides and participated in fluorescence measurements; A.M. synthesized C8-modified monophosphate precursors; P.W. synthesized *P*-imidazolide of (N6-propargil)-2′/3′-carbamoyladenosine di- and monophosphate; M.S. provided Ecm1; M.K. synthesized compound **4**; V.H.C. provided RNMT-RAM. R.K., J.K., and J.J. wrote the first draft of the manuscript. All authors contributed to the writing of the manuscript and approved the final version of the manuscript.

Supporting References

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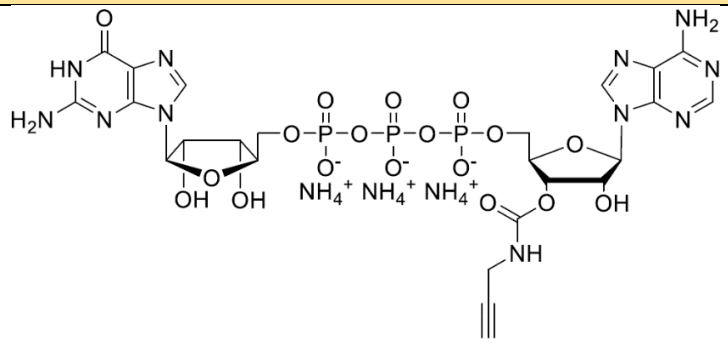
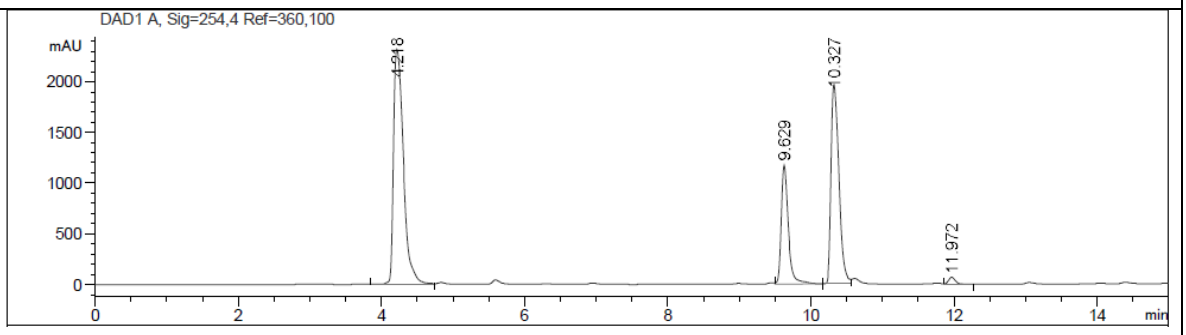
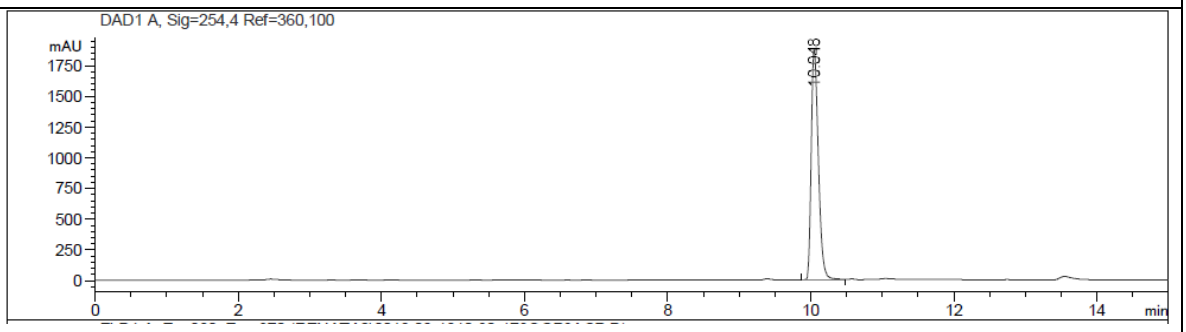
HPLC profiles, HR MS and NMR spectra

P^1-5'-guanosine-P^3-3'-carbamoyloadenosine triphosphate ammonium salt (Gp_3A-3'-*O*-cp, **1**)	
Formula	
HPLC reaction profile	
HPLC profile	

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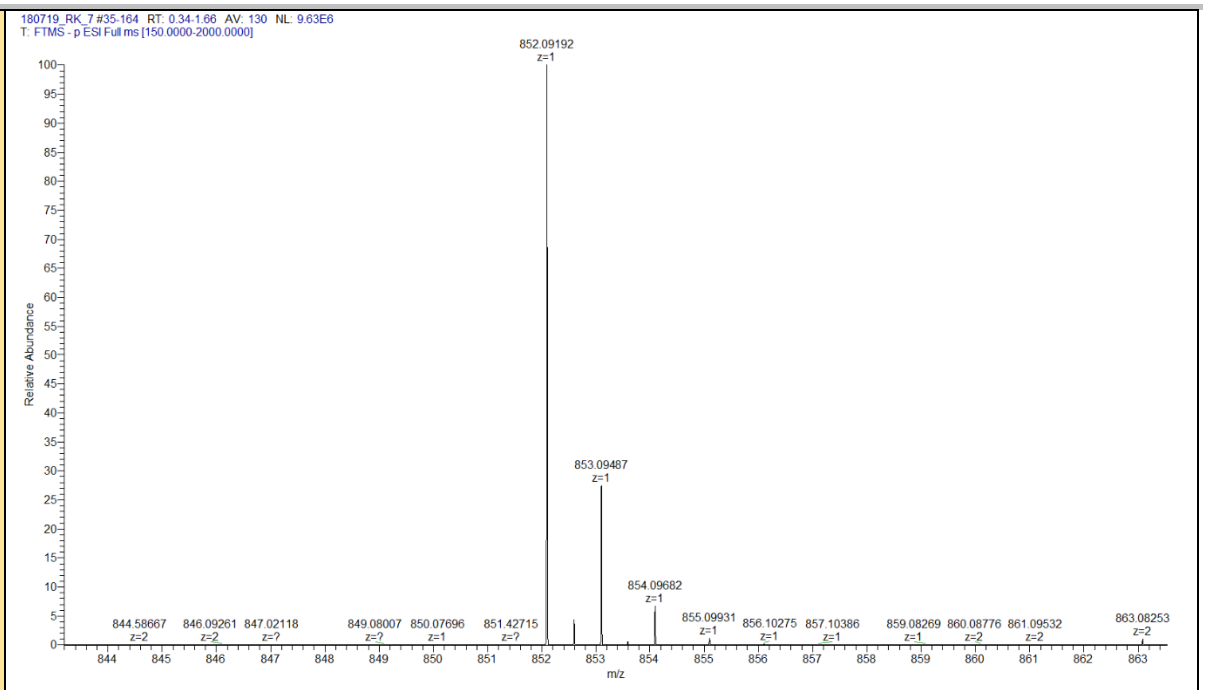
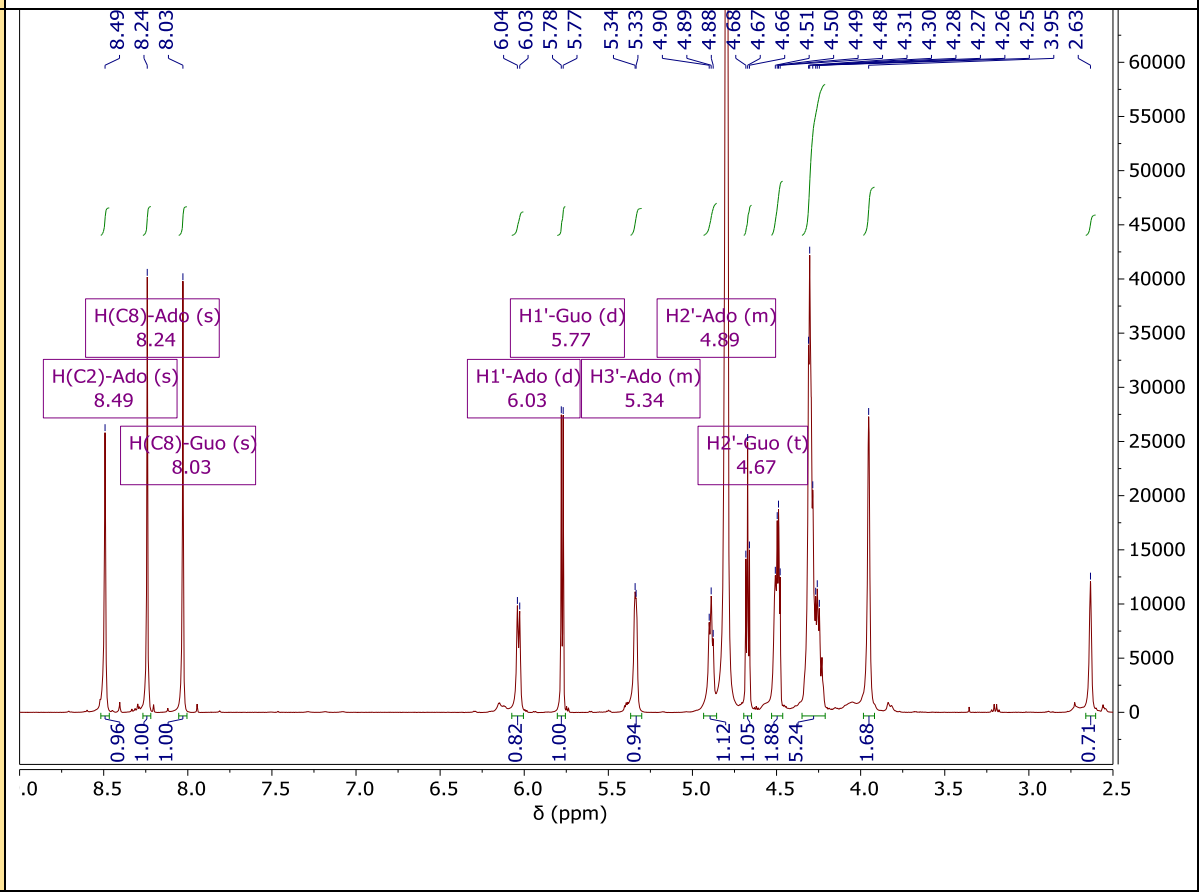
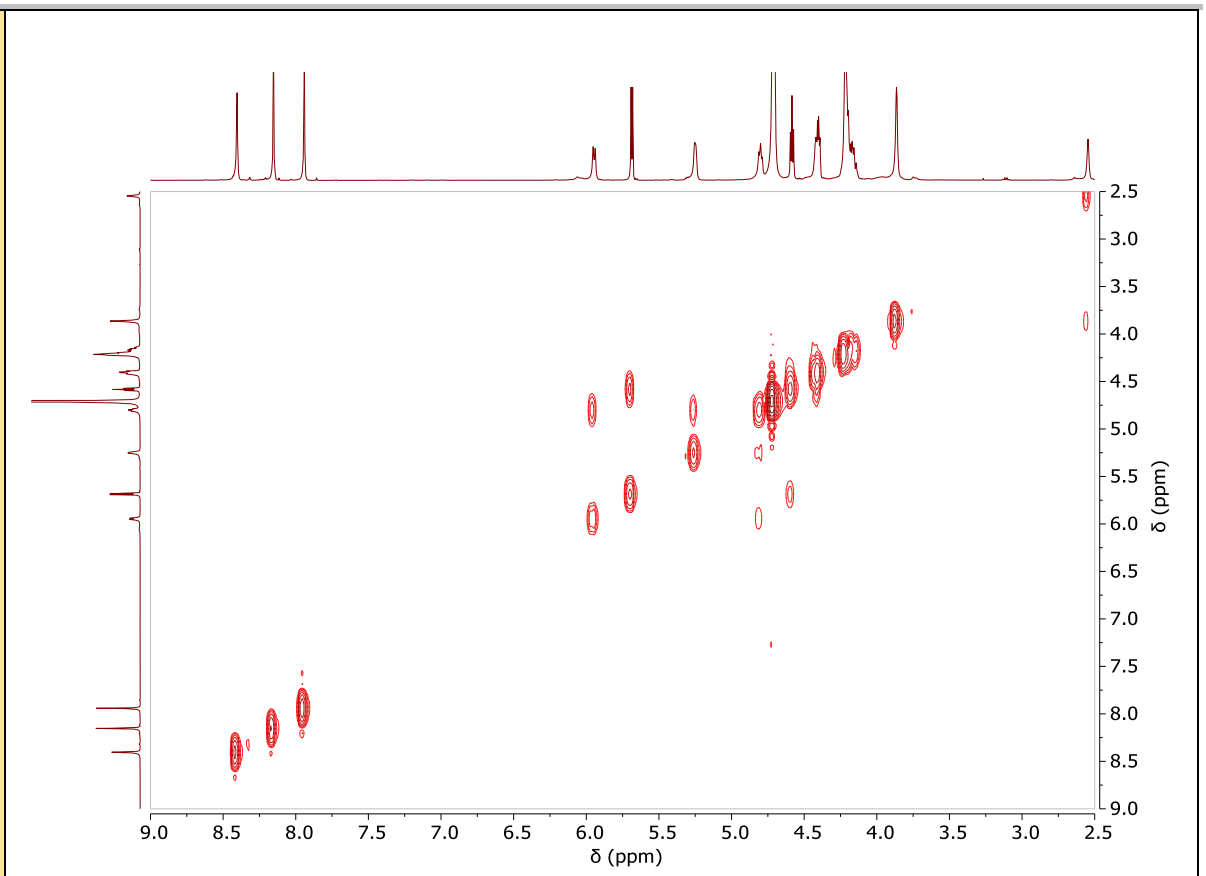


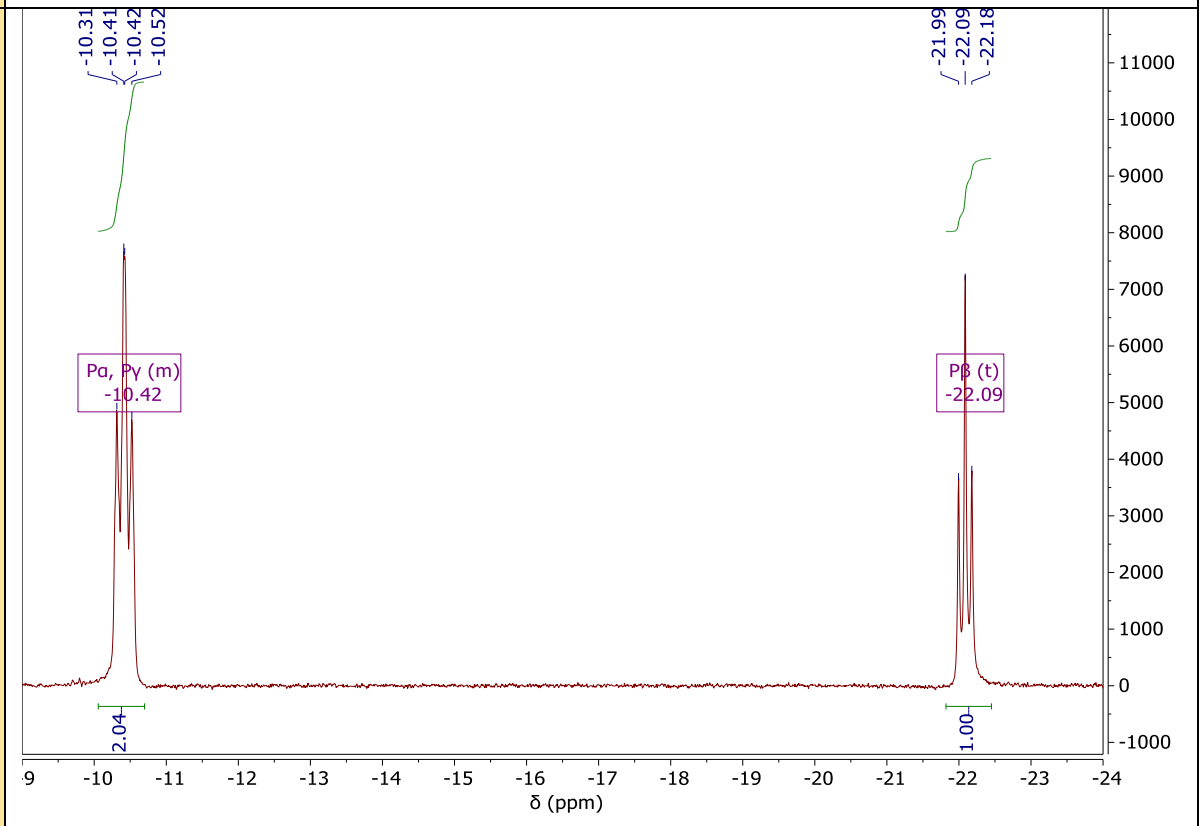
^{1}H NMR (500 MHz, D_2O, 25°C)

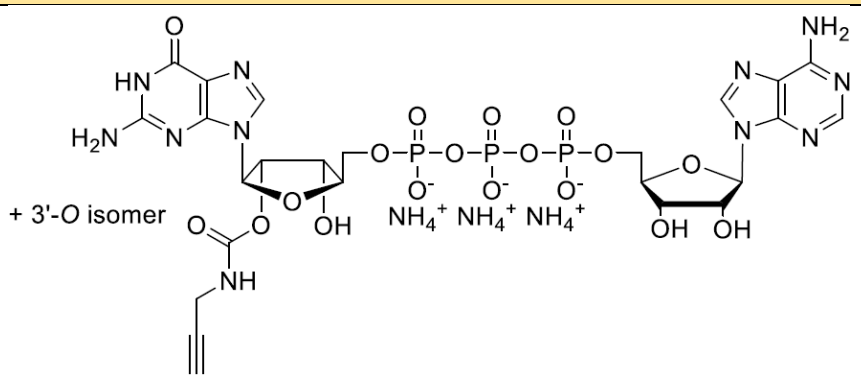
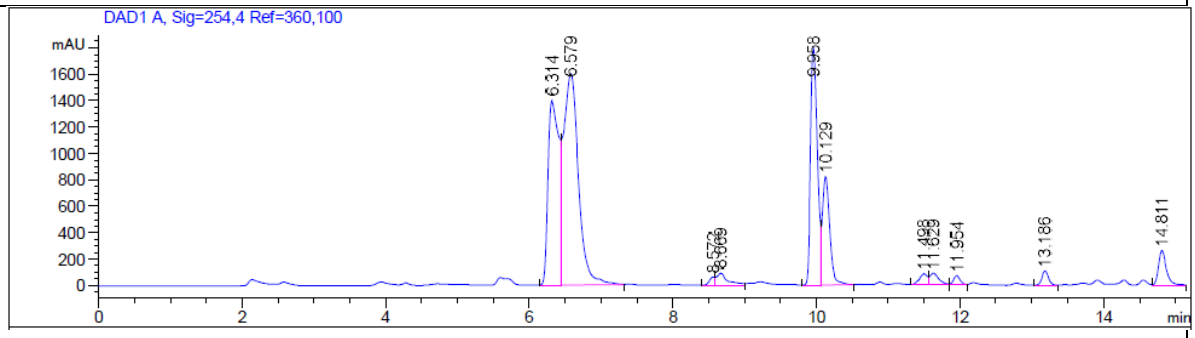
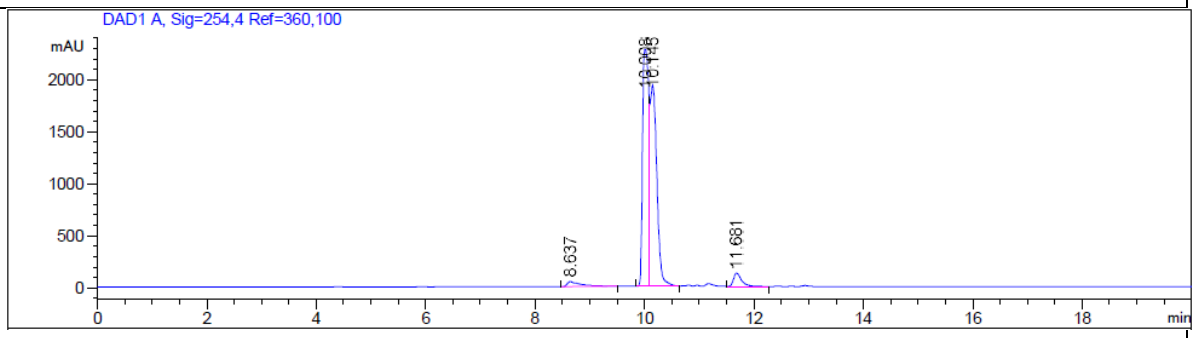


^{1}H-^{1}H COSY (500 MHz, D_2O, 25°C)



^{31}P NMR (202 MHz, D_2O, 25°C)



P^1-5'-(2'(3)-carbamoylo)guanosine-P^3-3'-adenosine triphosphate ammonium salt (2'-*O*/3'-*O*-cp-Gp_3A, **2**)	
Formula	
HPLC reaction profile	
HPLC profile	

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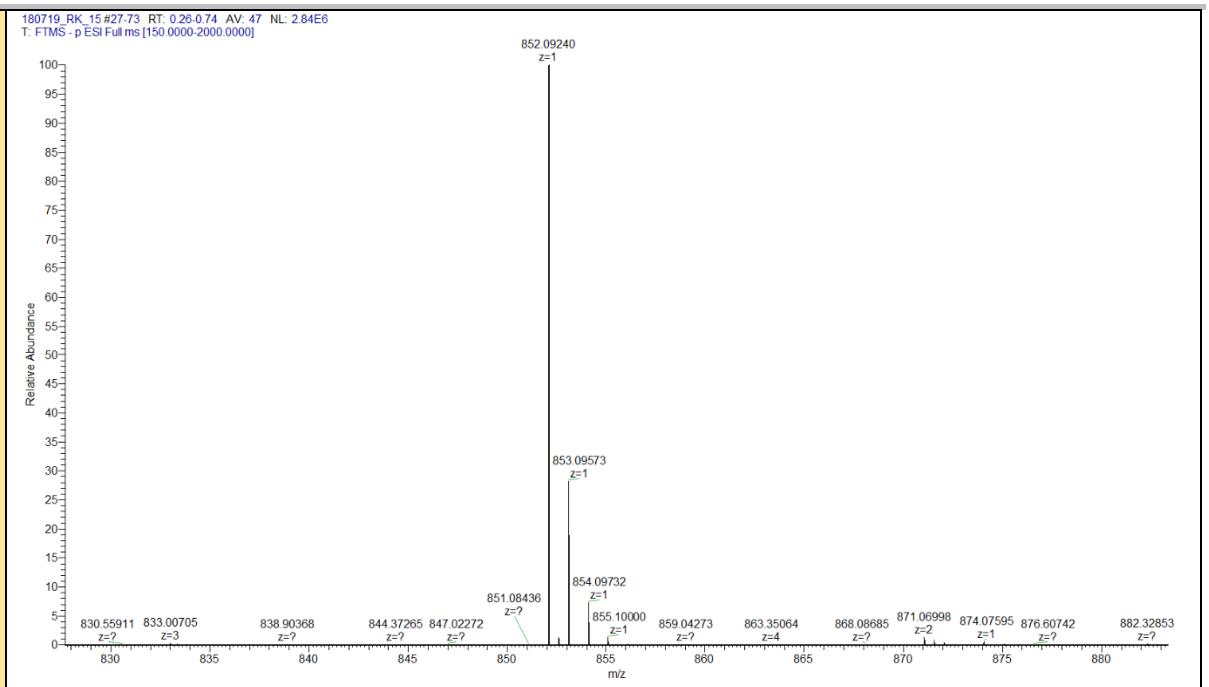
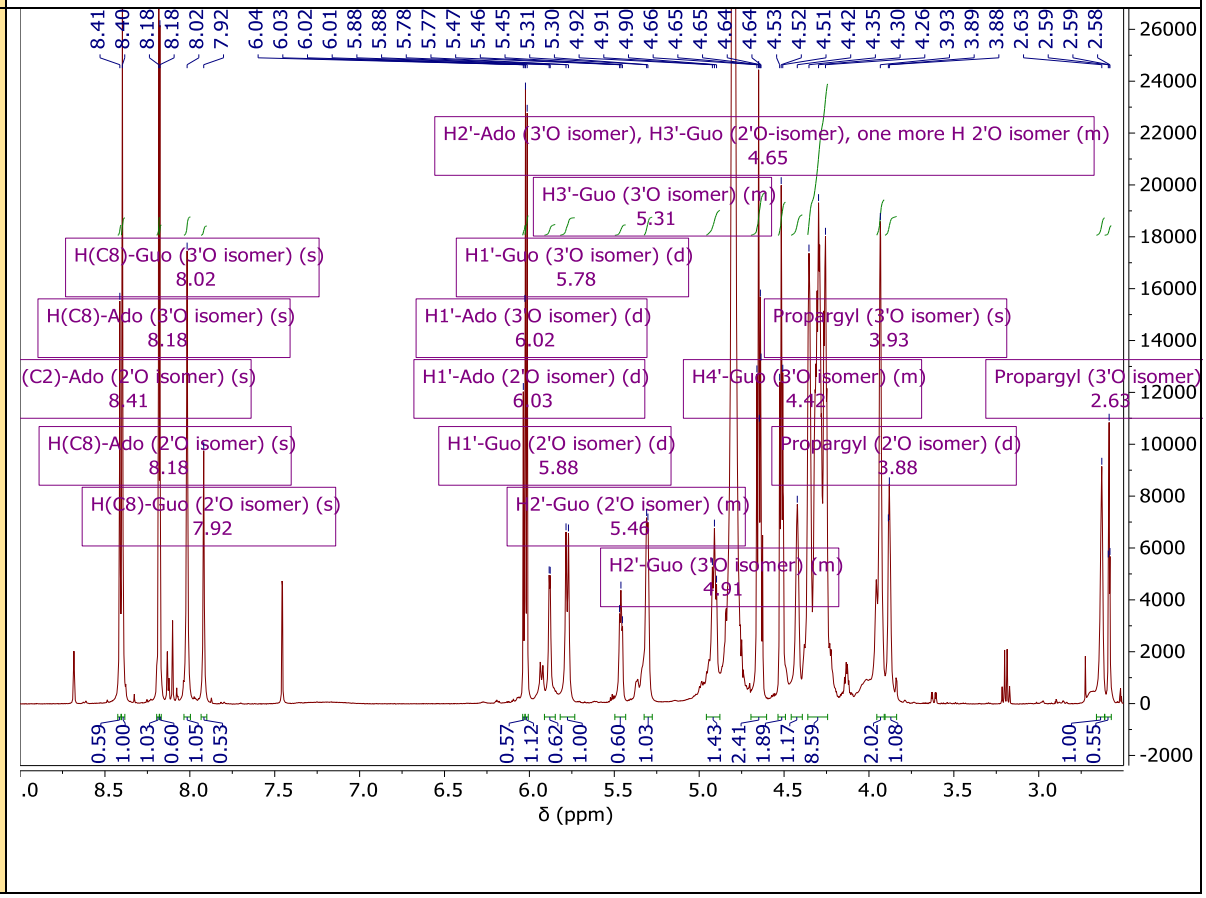
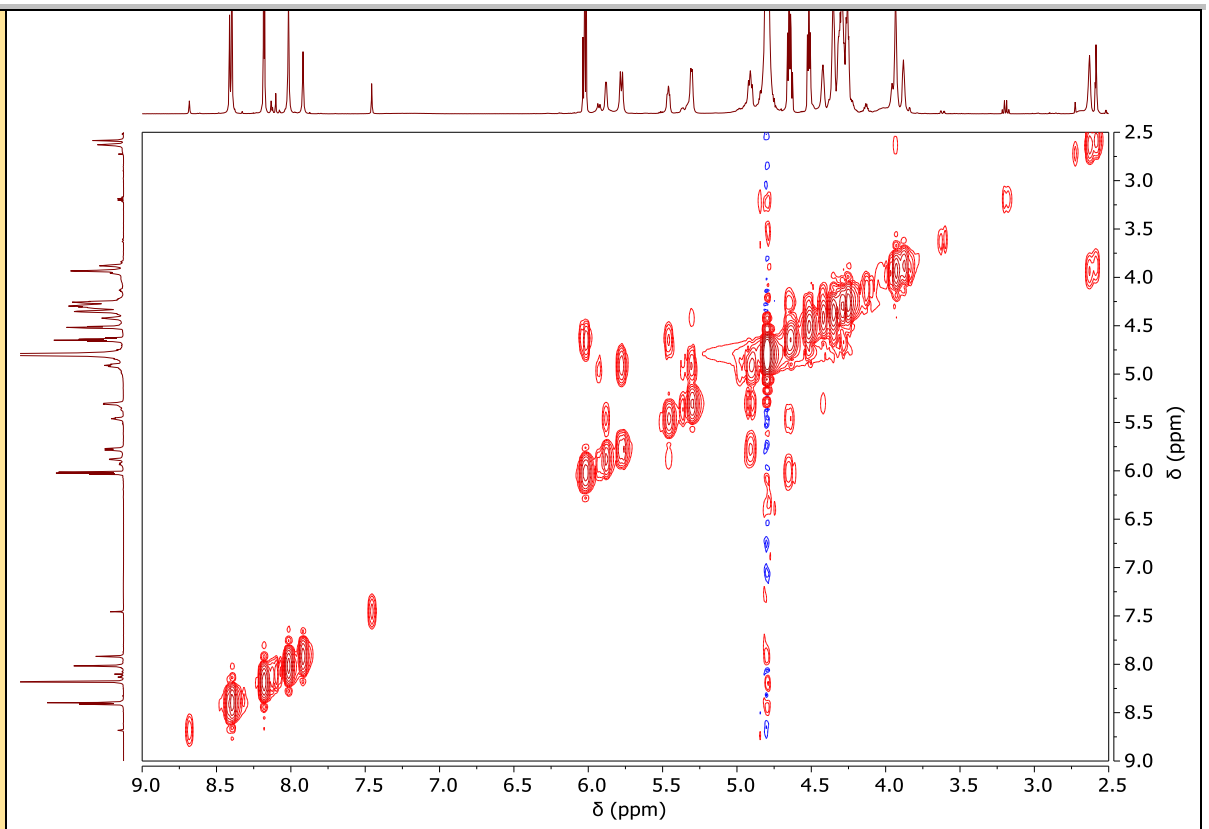


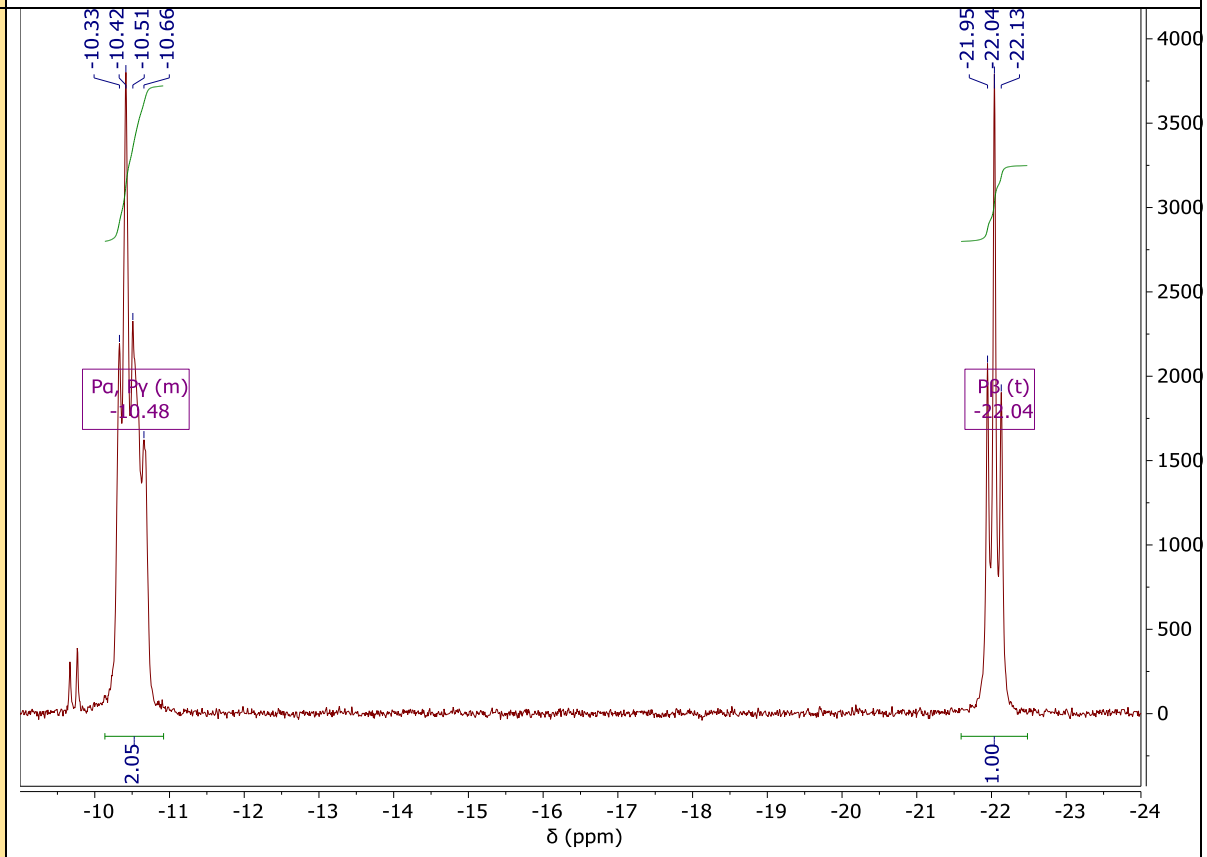
^{1}H NMR (500 MHz, D_2O, 25°C)



^{1}H-^{1}H COSY (500 MHz, D_2O, 25°C)



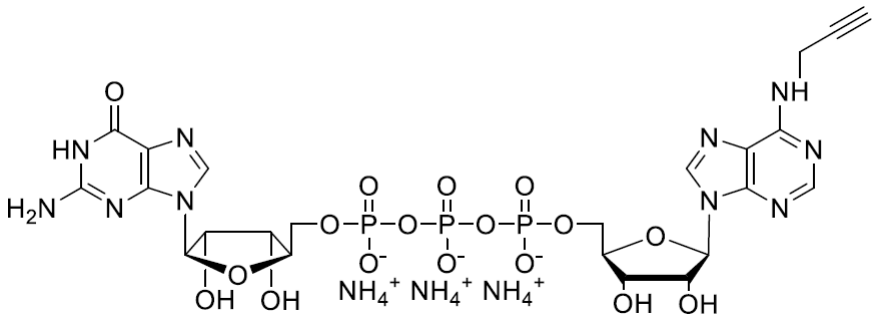
^{31}P NMR (202 MHz, D_2O, 25°C)



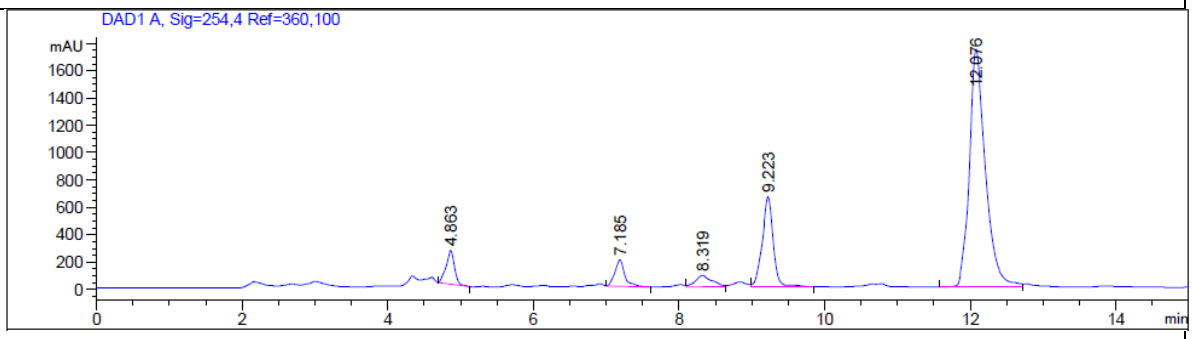
SUPPORTING INFORMATION

P[1]-5'-guanosine-P[3]-3'-N6-propargyladenosine triphosphate ammonium salt ($Gp_3A(N6)prop$, 3)

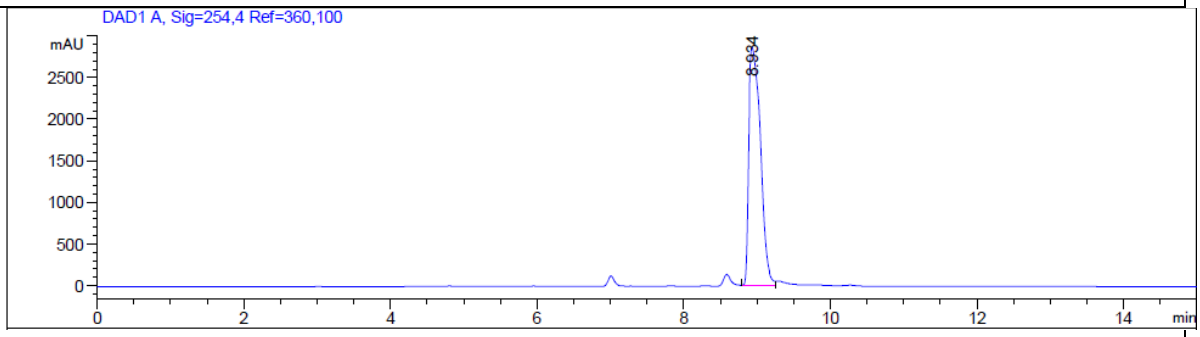
Formula



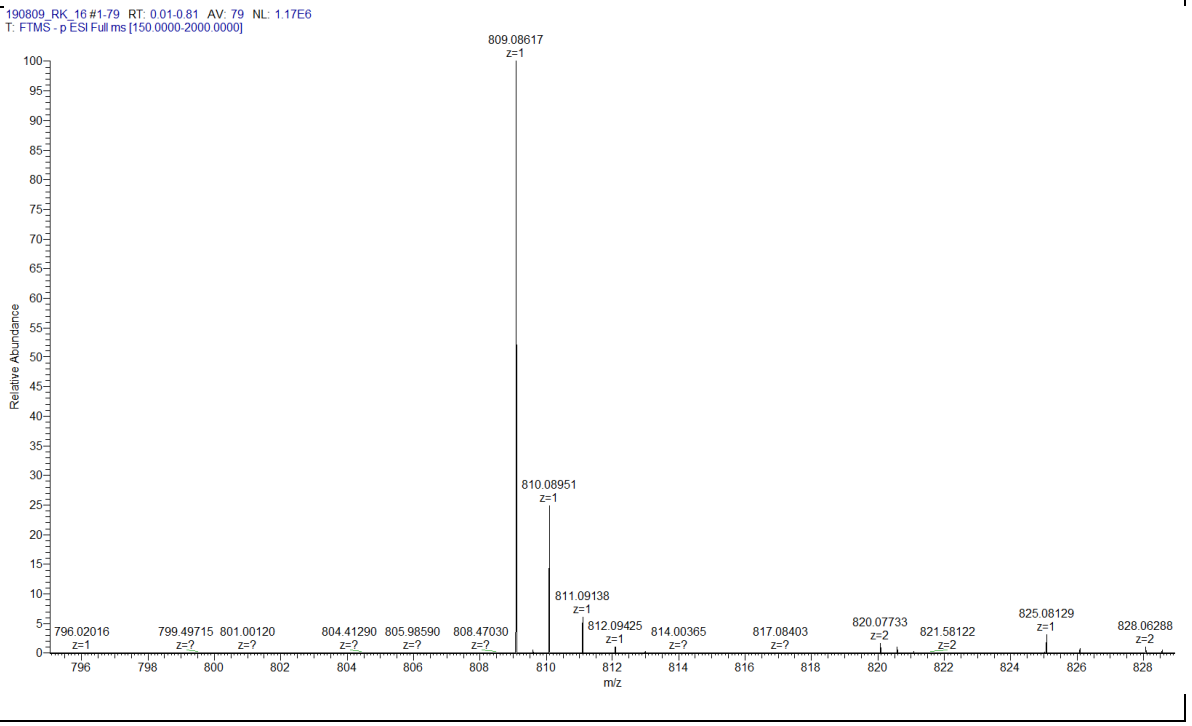
HPLC reaction profile



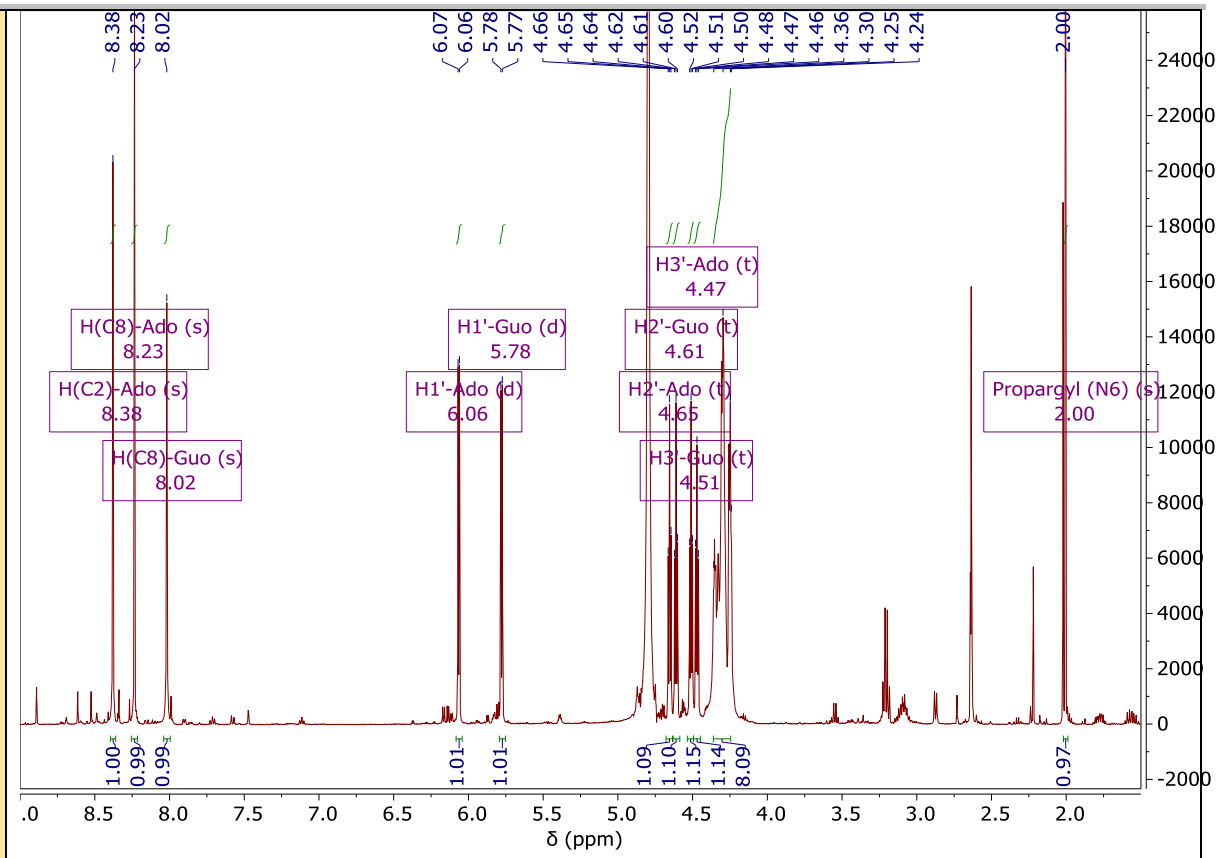
HPLC profile



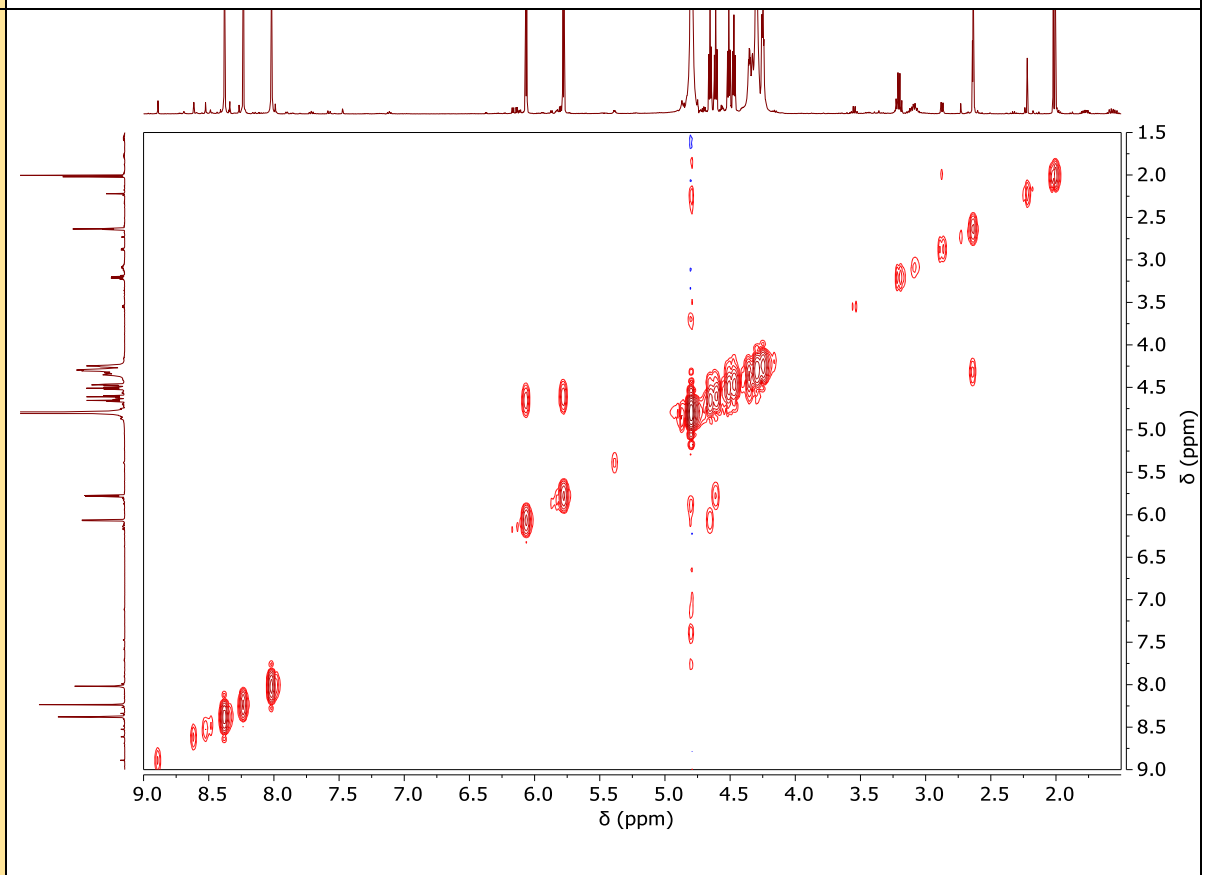
HRMS (-)ES



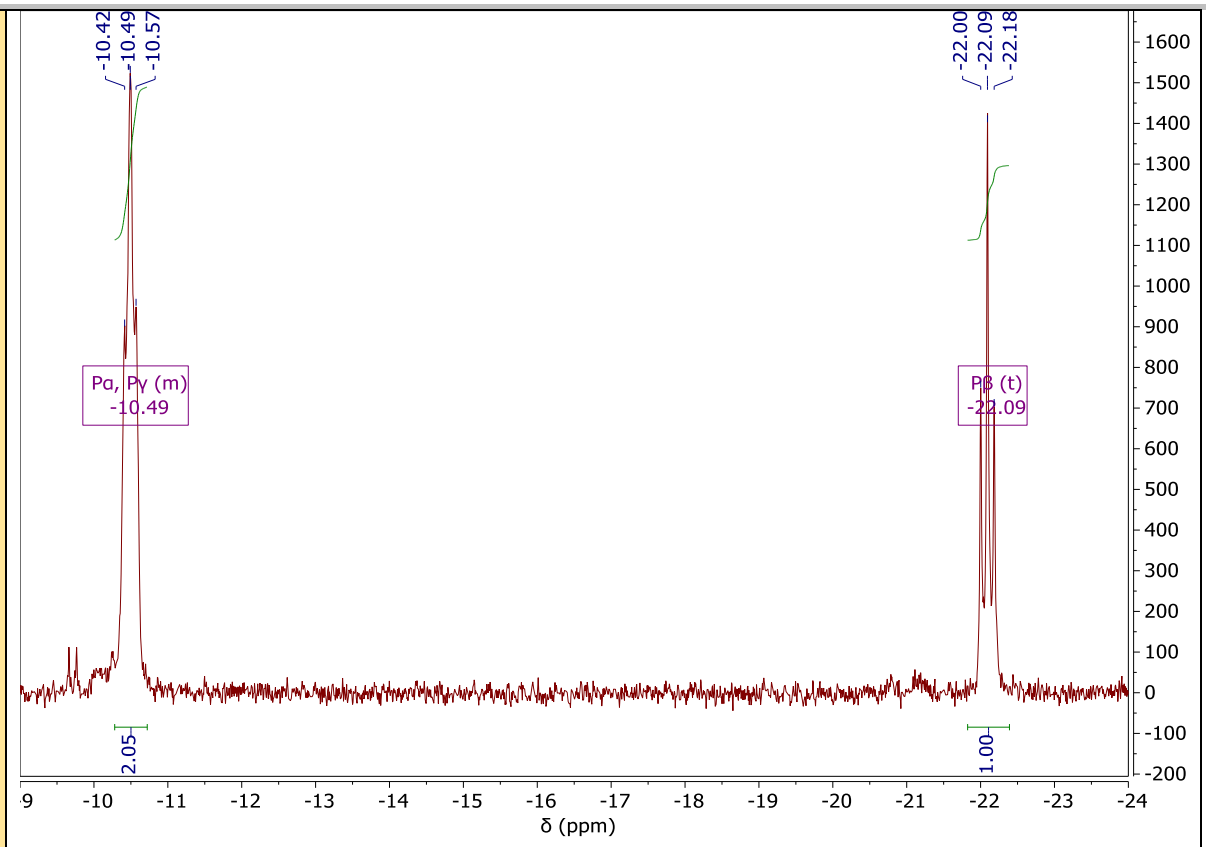
^{1}H NMR (500 MHz, D_2O, 25°C)



^{1}H-^{1}H COSY (500 MHz, D_2O, 25°C)

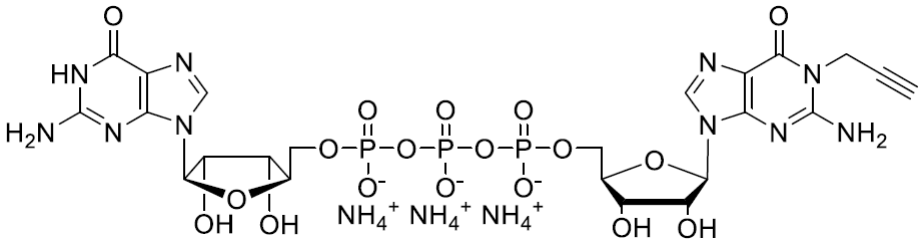


^{31}P NMR (202 MHz, D_2O, 25°C)

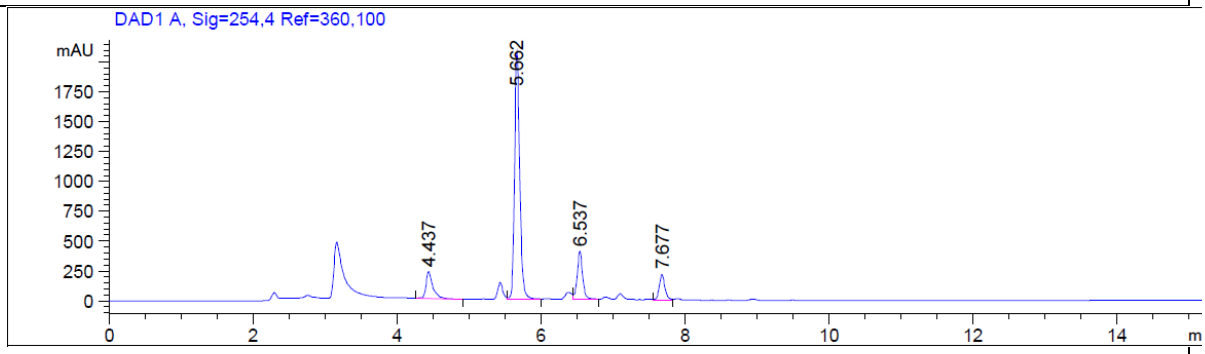


P[1]-5'-guanosine-P[3]-3'-N1-propargylguanosine triphosphate ammonium salt ($Gp_3G(N1)prop$, **4**)

Formula



HPLC reaction profile



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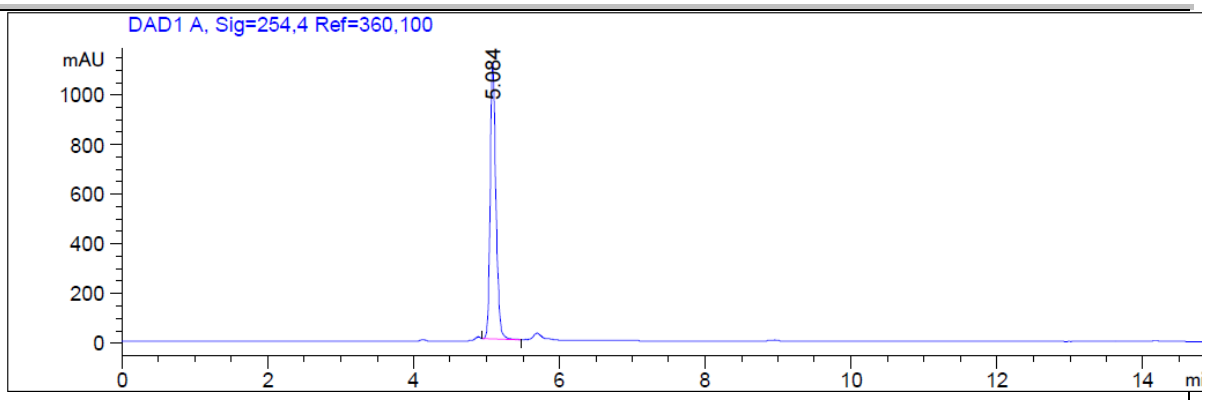

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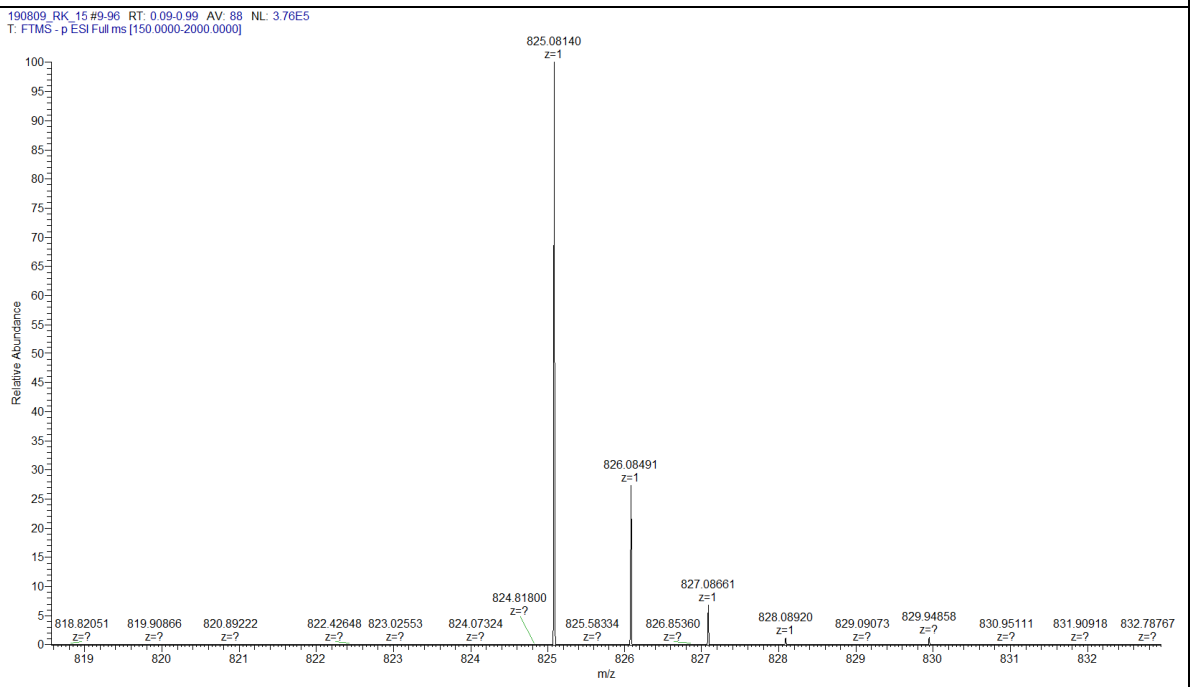
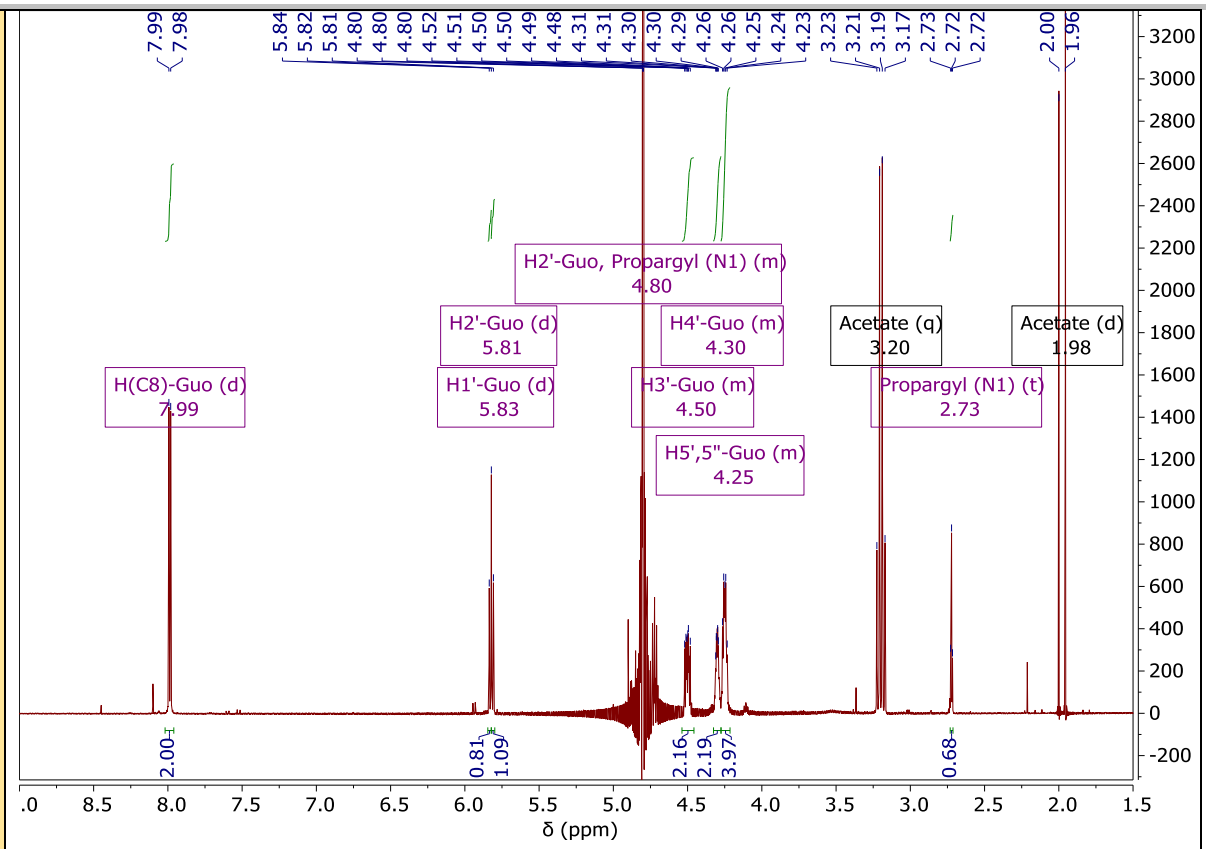
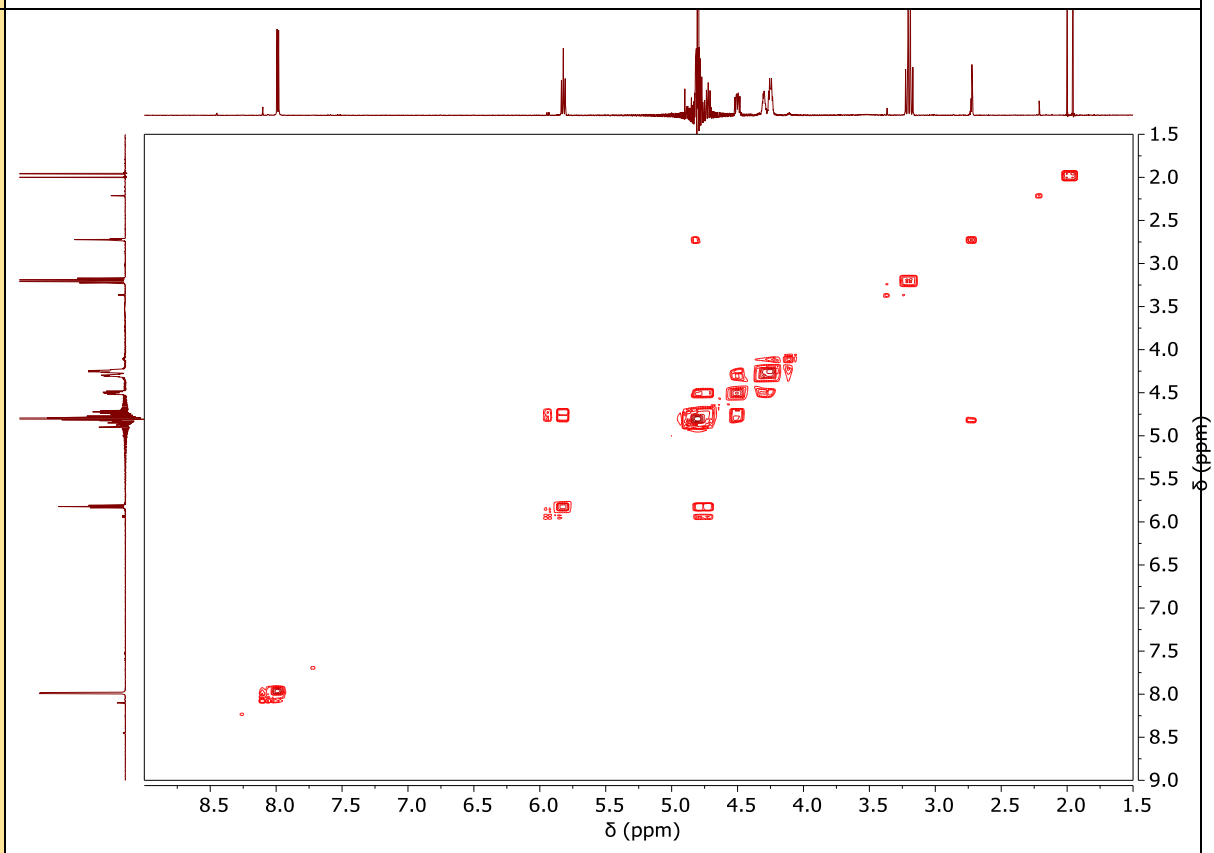


SUPPORTING INFORMATION

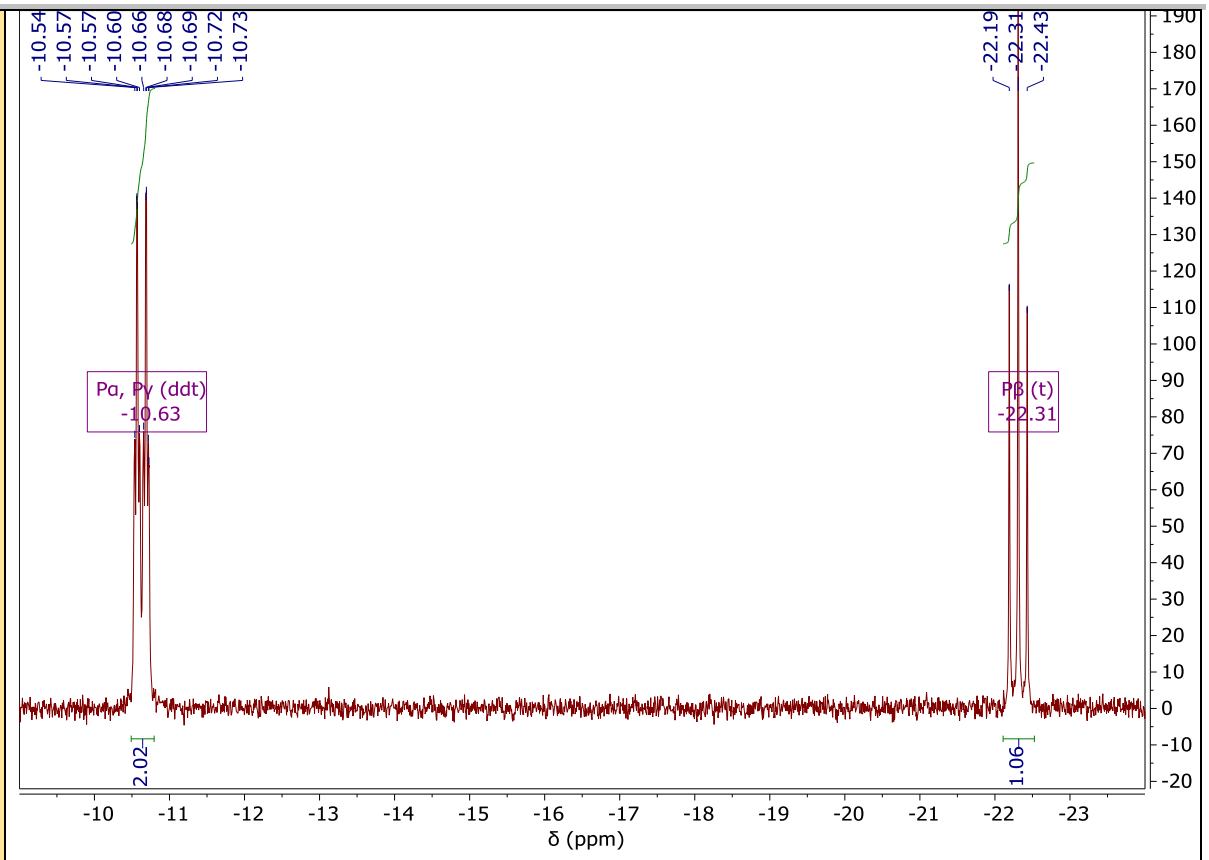
^{1}H NMR (500 MHz, D_2O, 25°C)



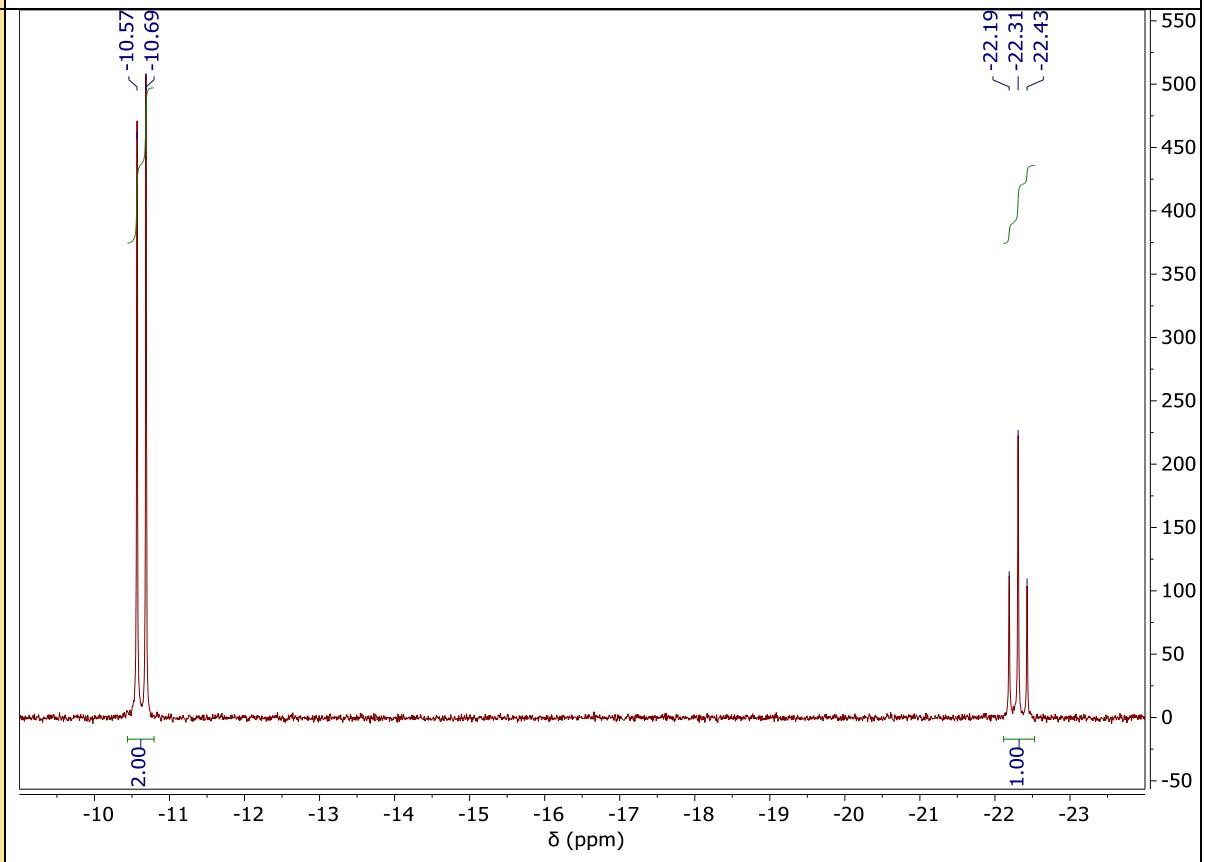
^{1}H-^{1}H COSY (500 MHz, D_2O, 25°C)

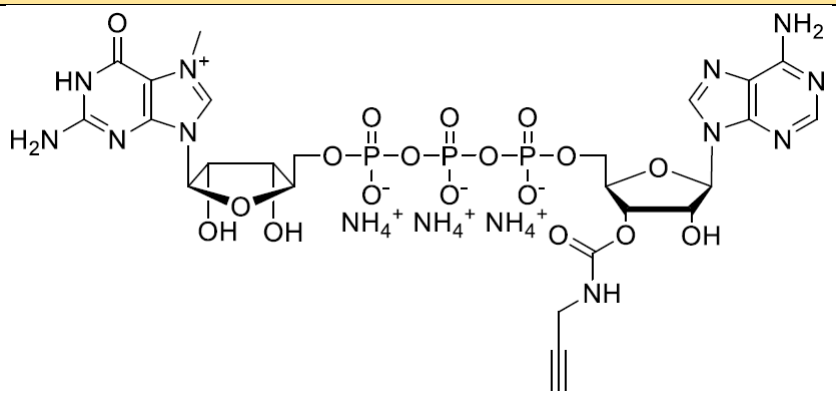
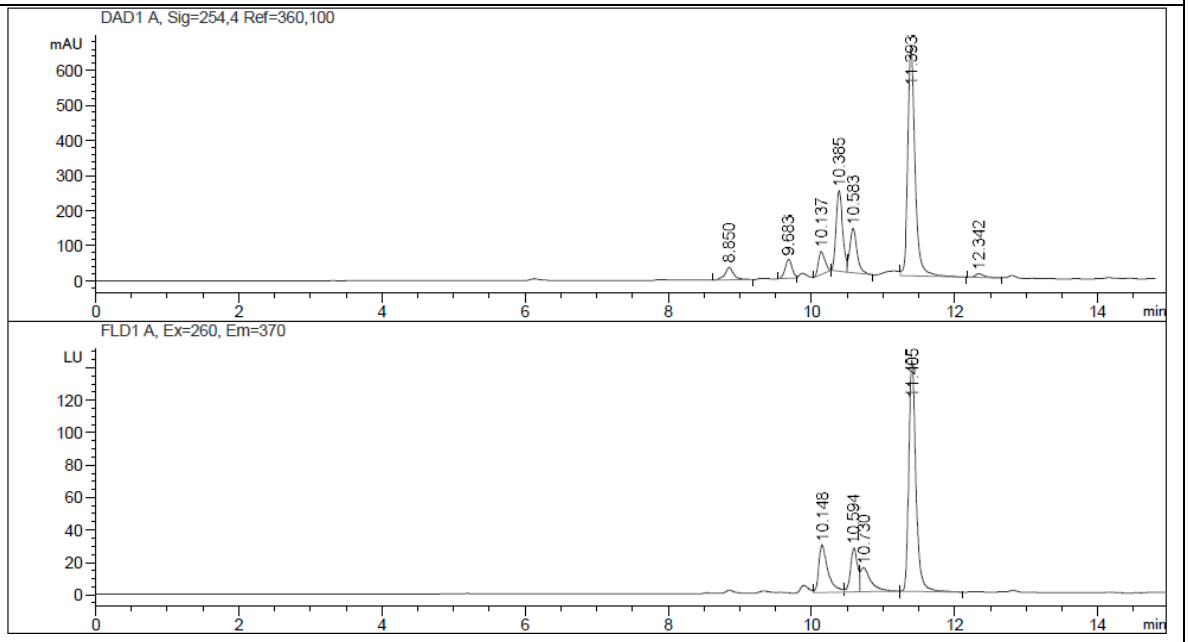


^{31}P NMR (202 MHz, D_2O, 25°C)



^{31}P NMR (202 MHz, D_2O, 25°C)
proton decoupled



P^1-5'-(7-methyl)guanosine-P^3-3'-carbamoyloadenosine triphosphate ammonium salt (m^7Gp_3A-3'-*O*-cp, **5**)	
Formula	
HPLC reaction profile	

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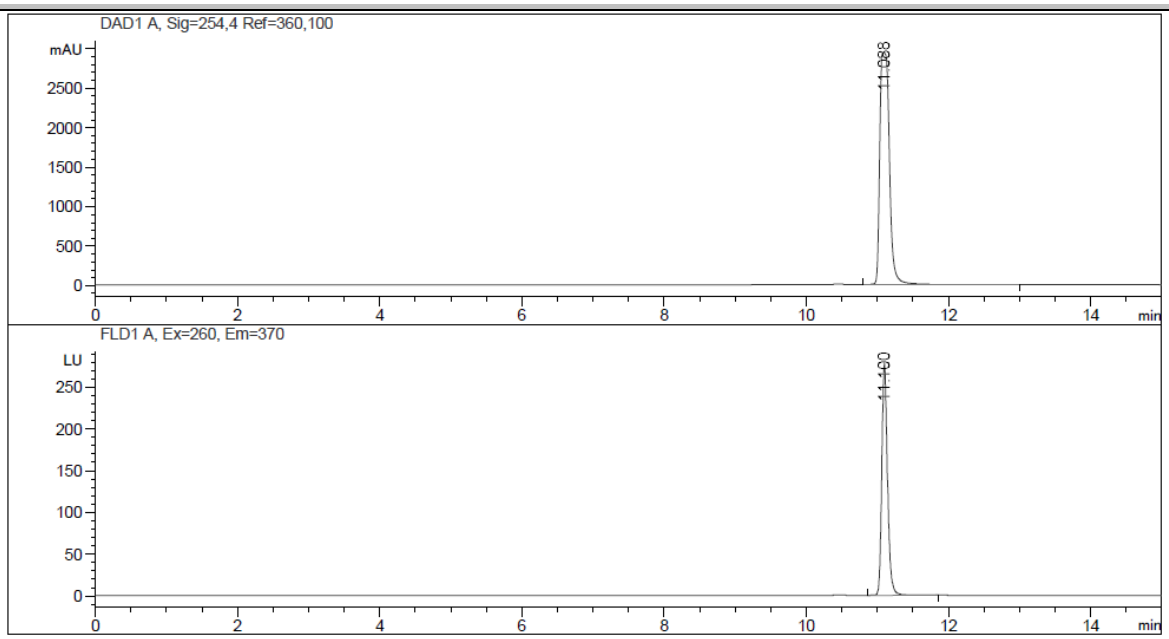

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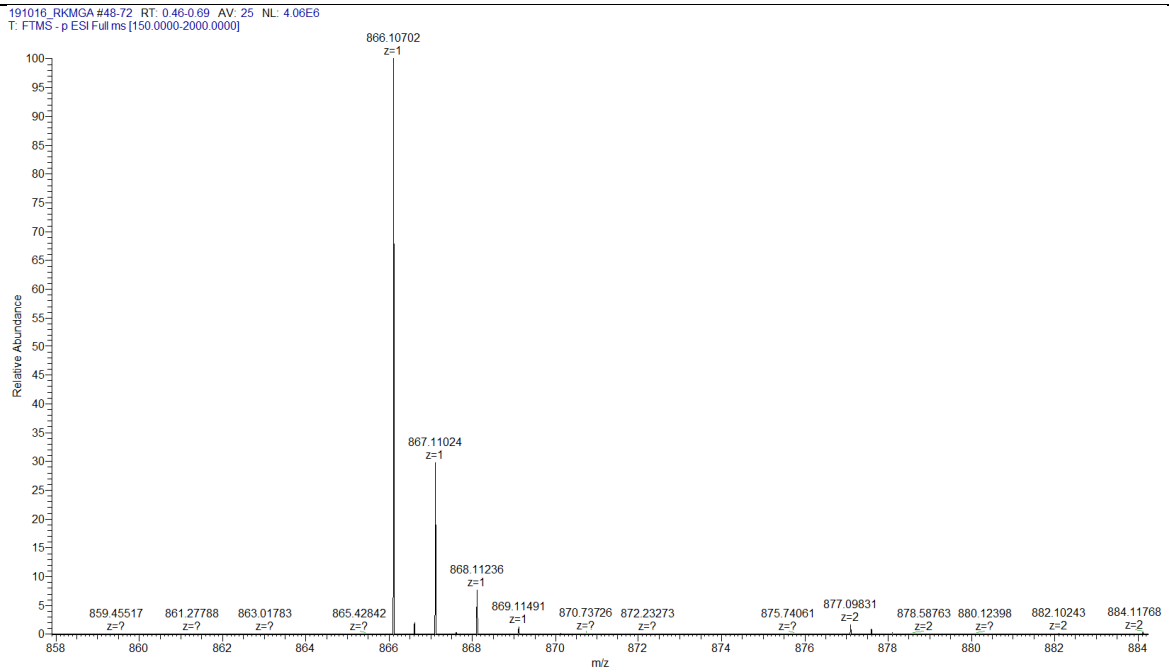
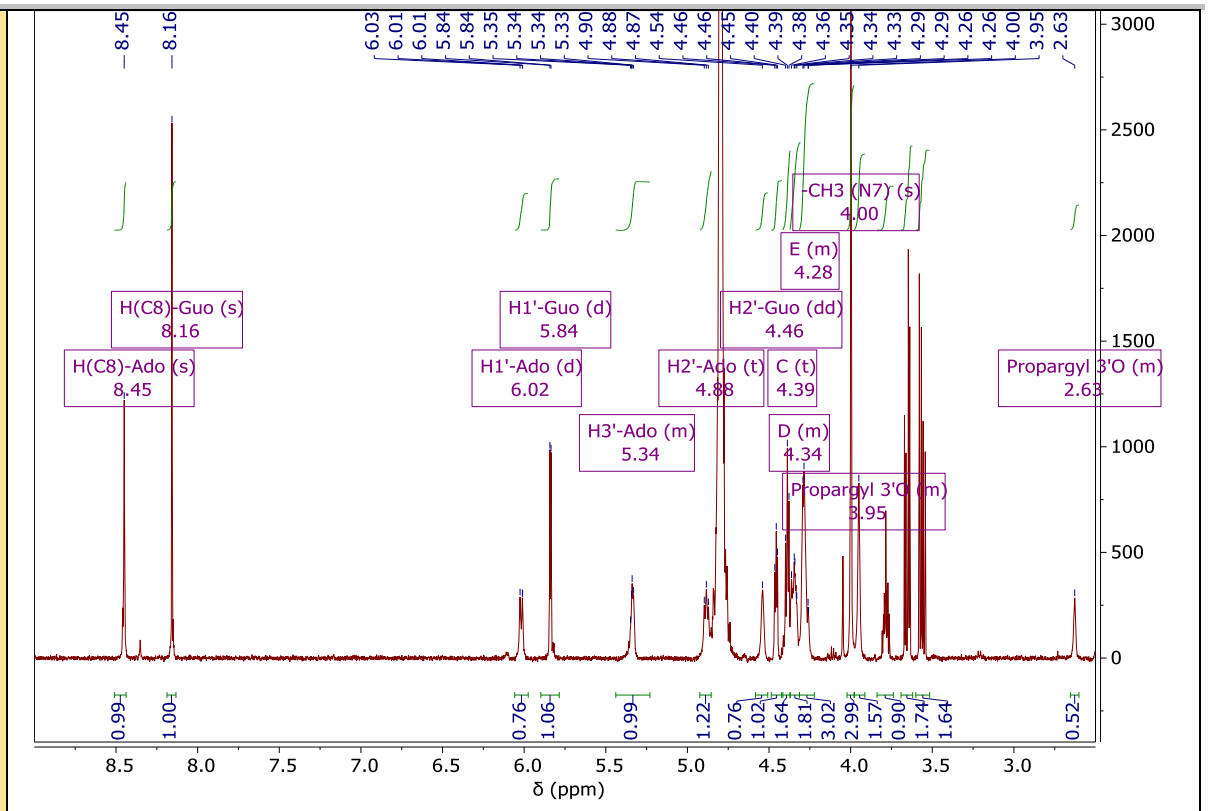
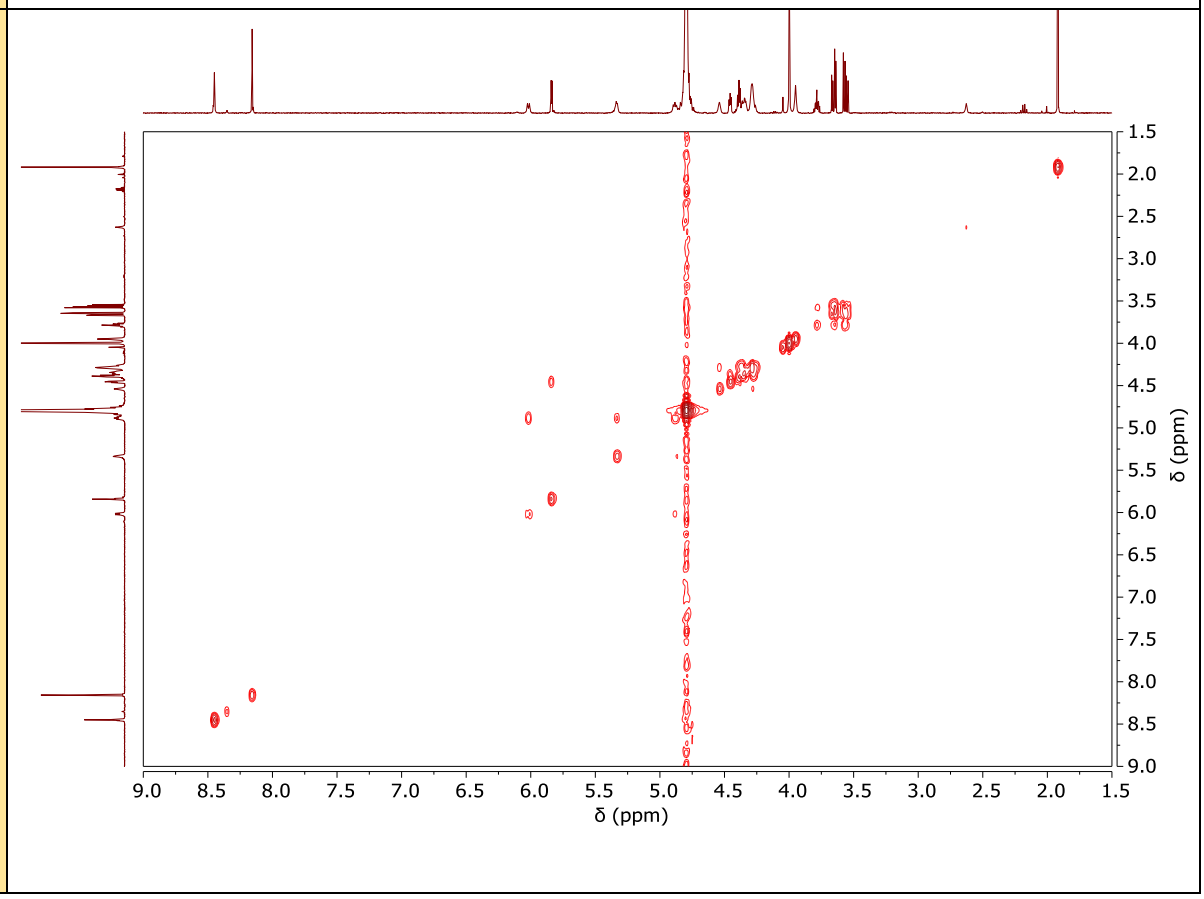


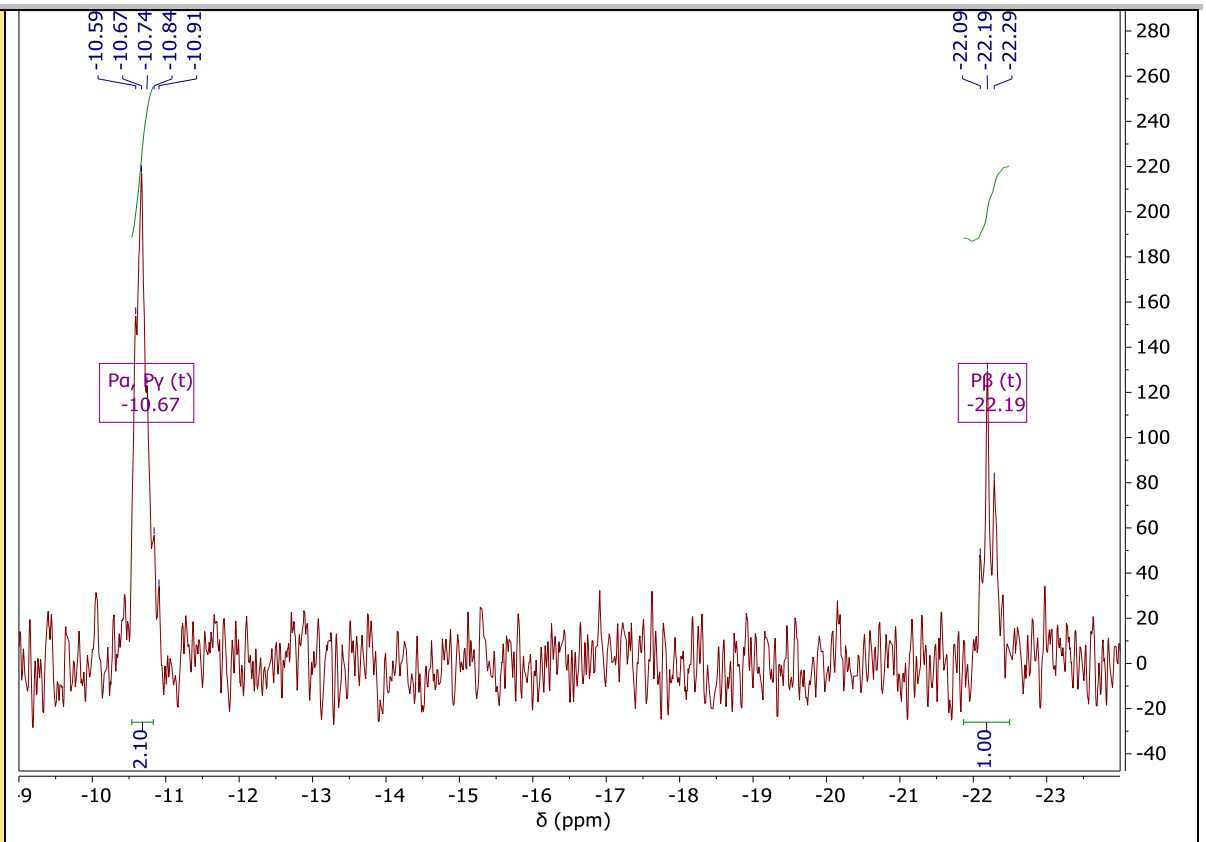
^{1}H NMR (500 MHz, D_2O, 25°C)



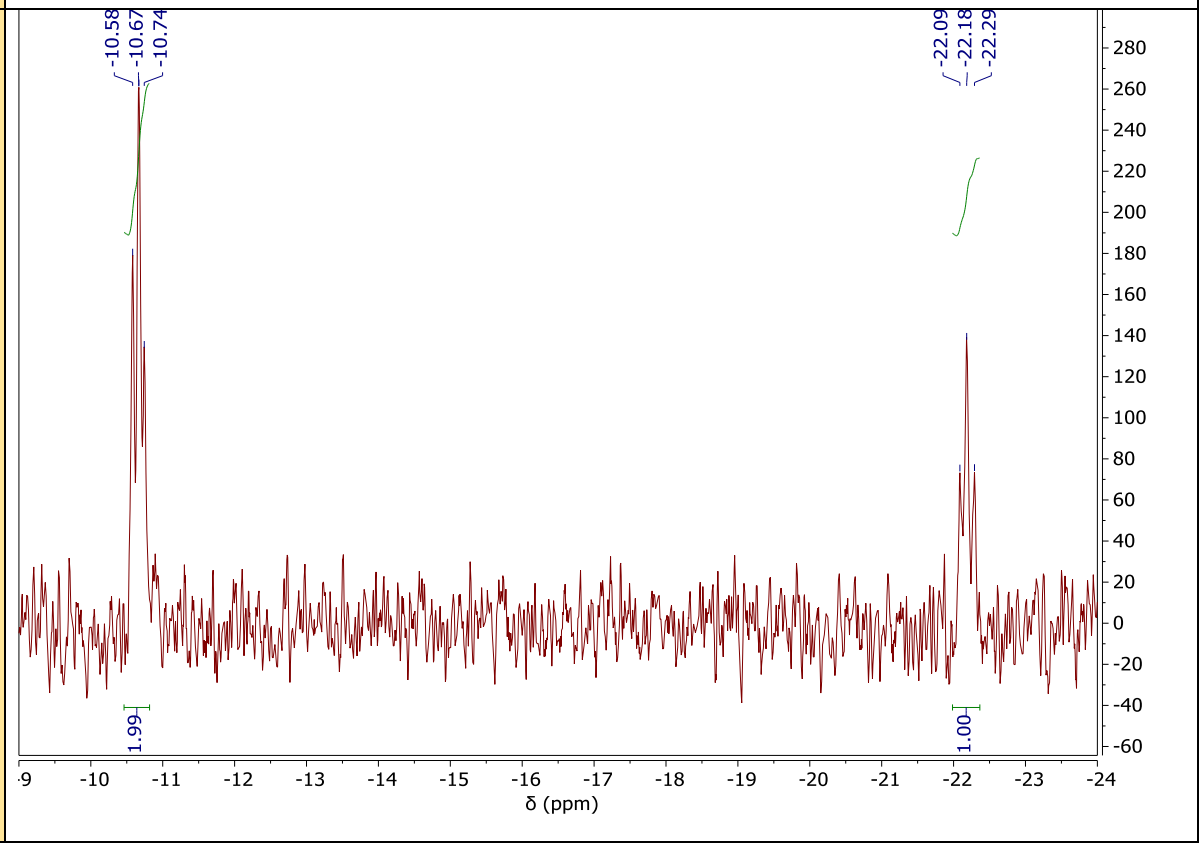
^{1}H-^{1}H COSY (500 MHz, D_2O, 25°C)

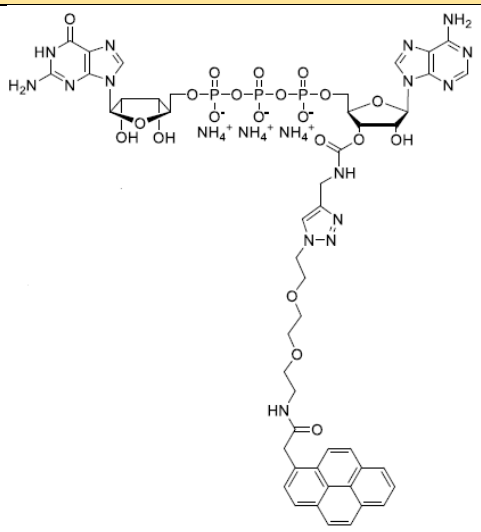
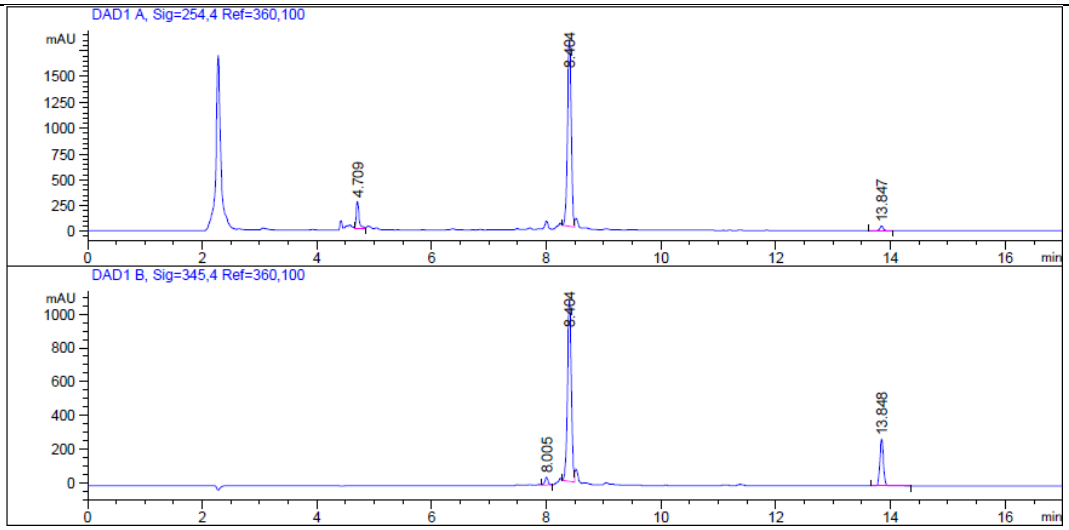


^{31}P NMR (202 MHz, D_2O, 25°C)



^{31}P NMR (202 MHz, D_2O, 25°C)
proton decoupled



Gp_3A-3'-*O*-Py (**6a**)	
Formula	
HPLC reaction profile	

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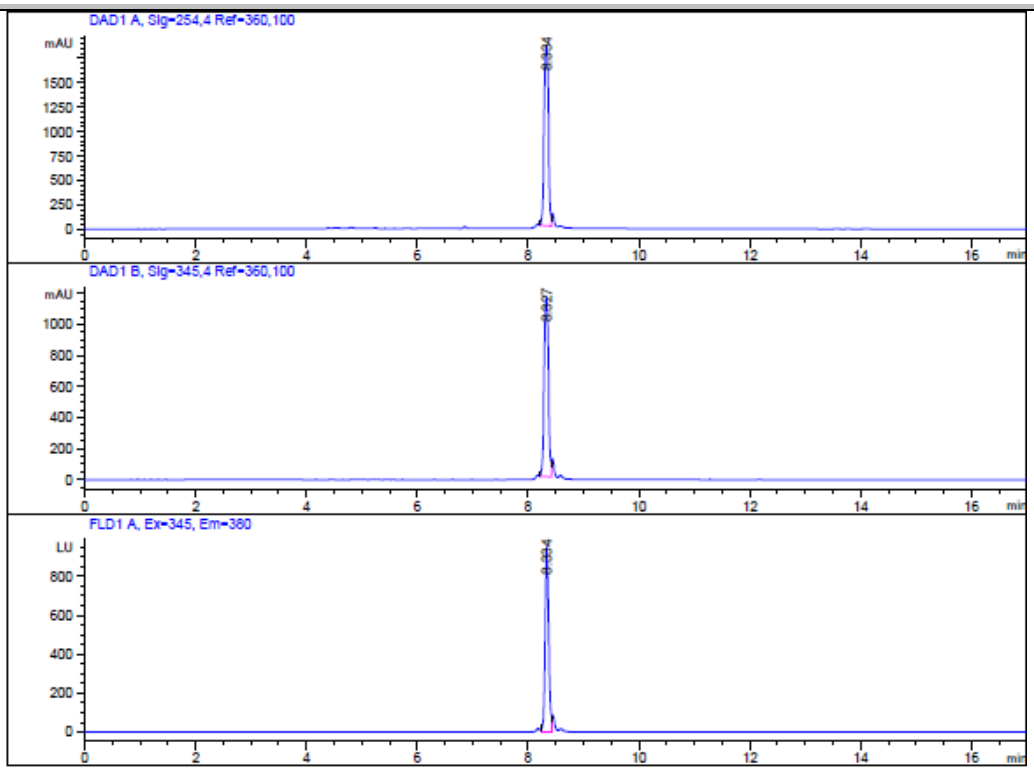

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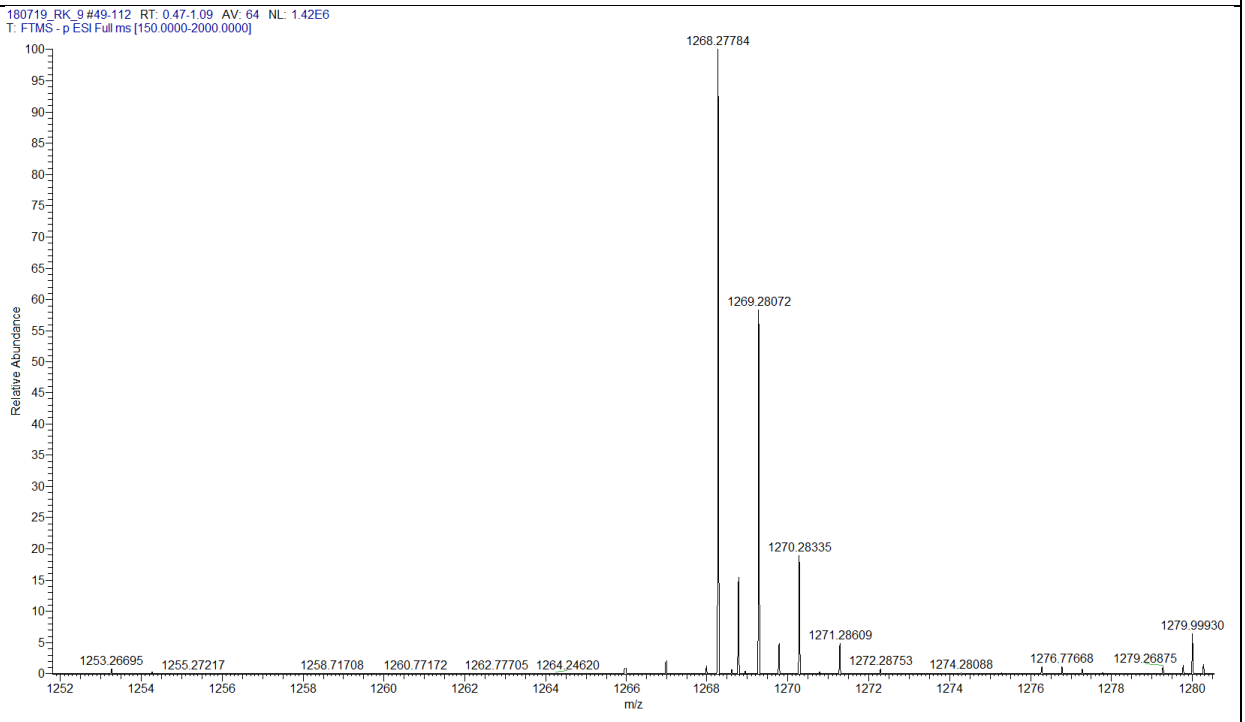
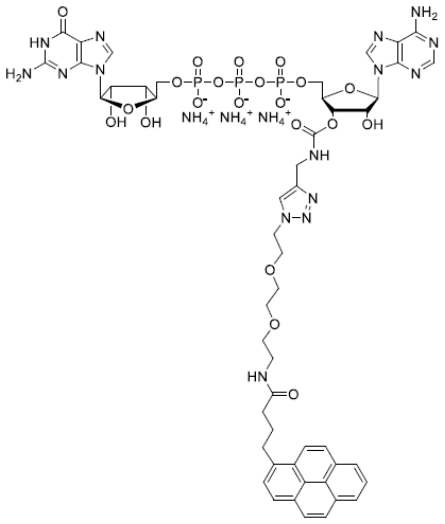


Gp$_3$A-3'-*O*-Py3 (**6b**)

SUPPORTING INFORMATION

Formula



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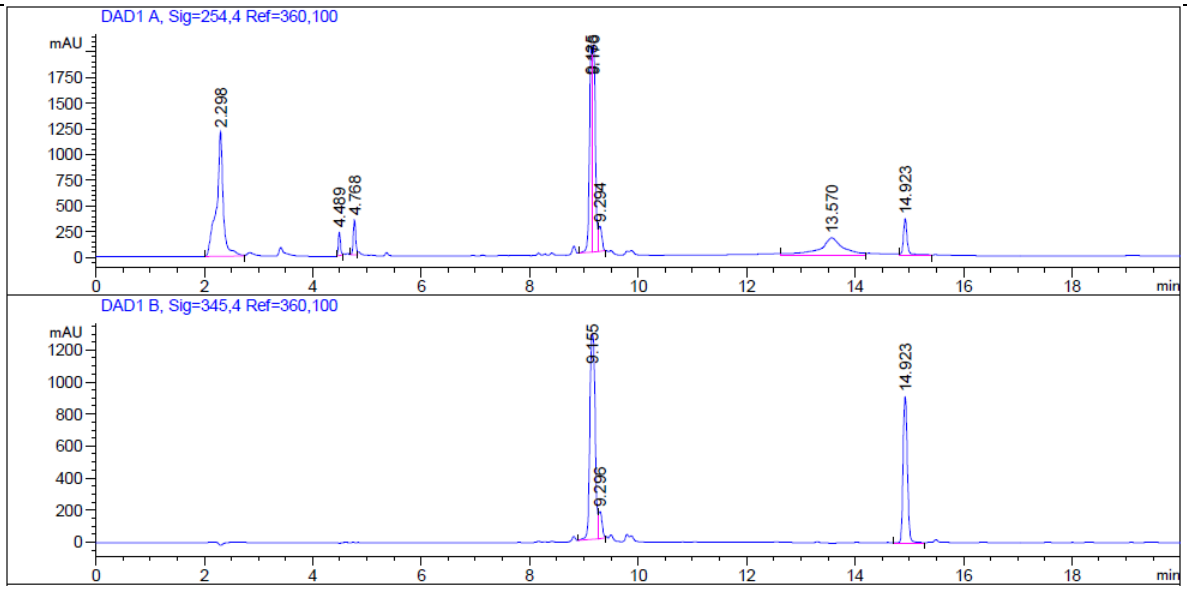

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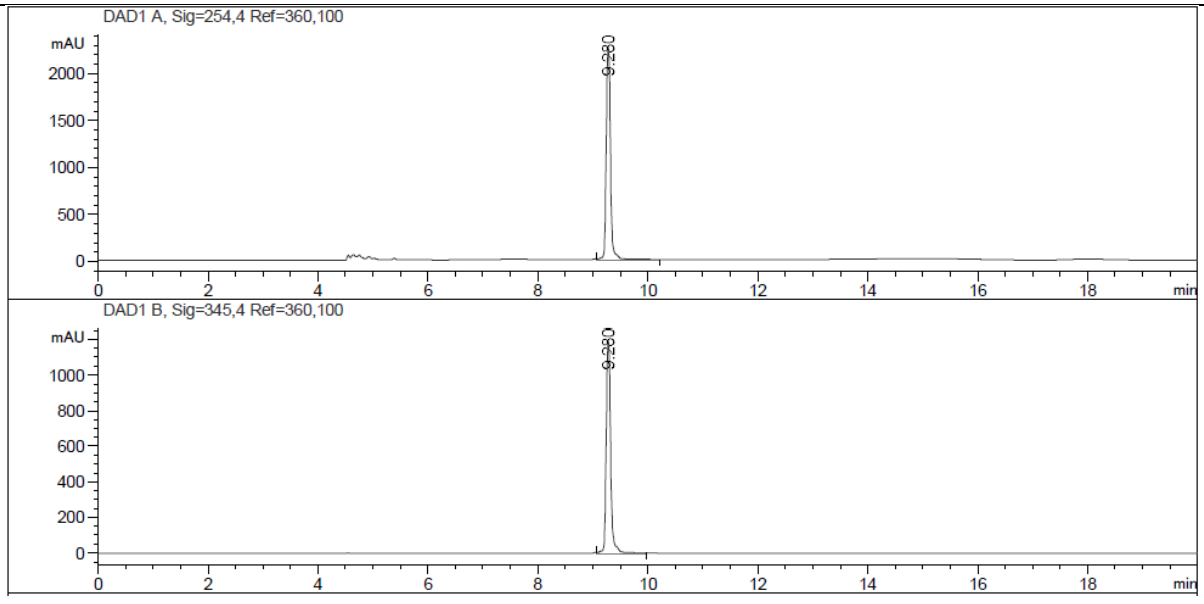
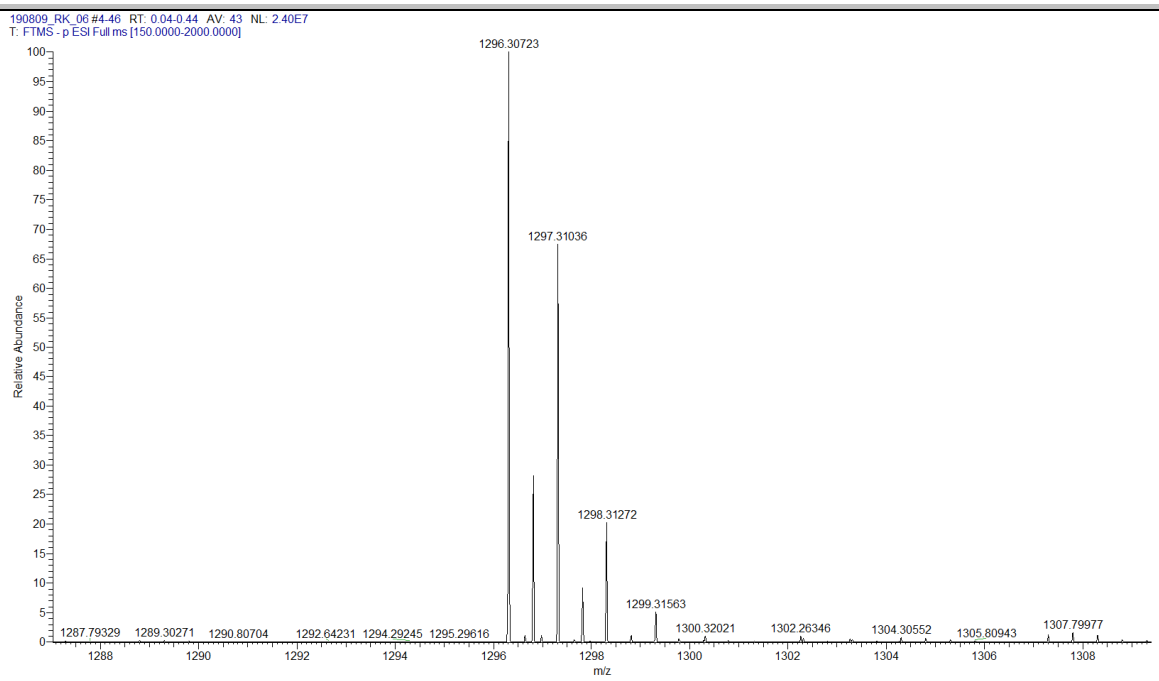


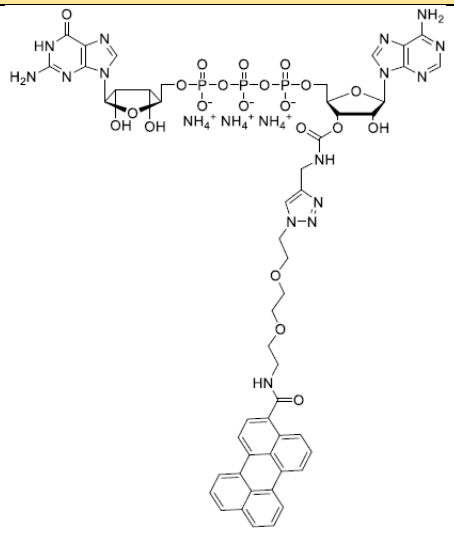
SUPPORTING INFORMATION

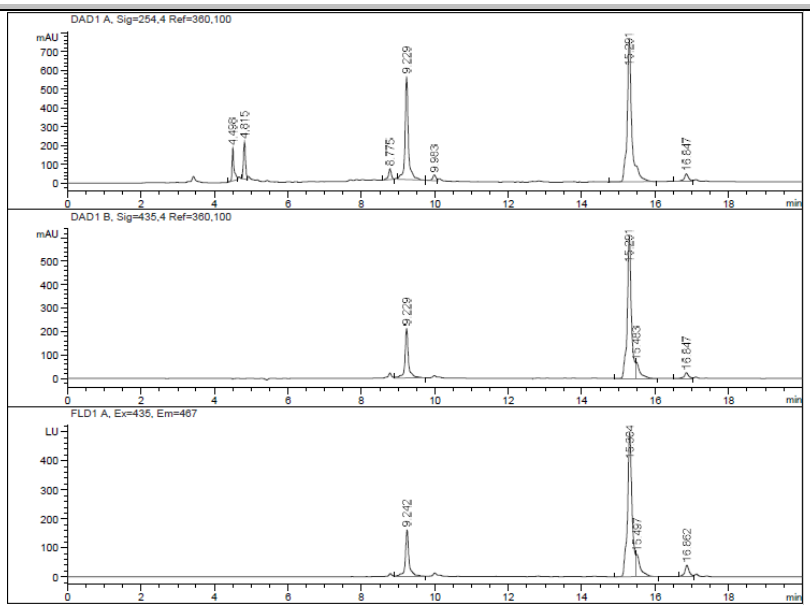
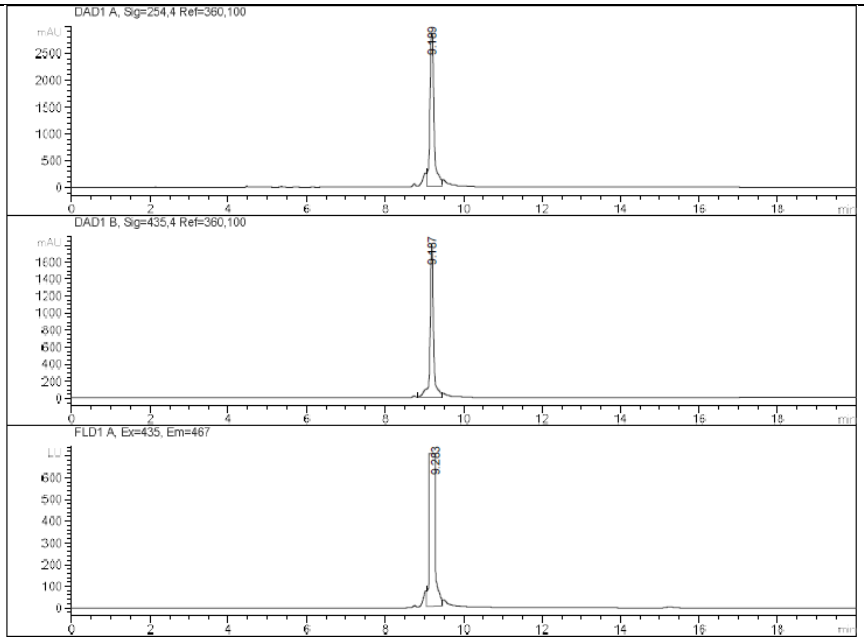
HRMS (-)ES



Formula

Gp$_3$A-3'-*O*-Pe (**6c**)



HPLC reaction profile	
HPLC profile	

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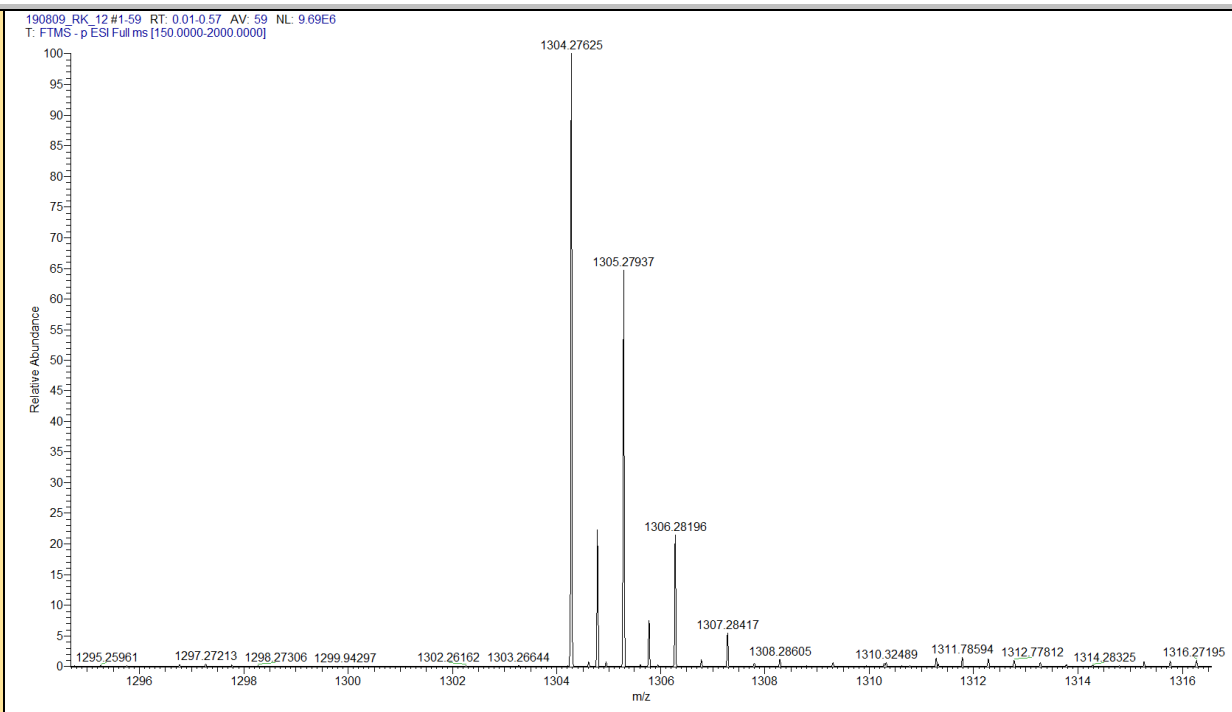
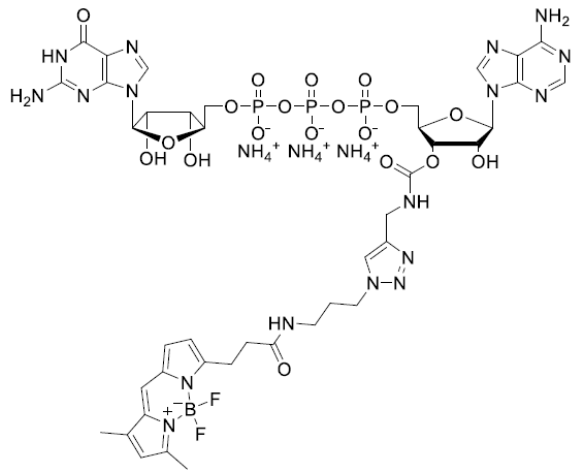
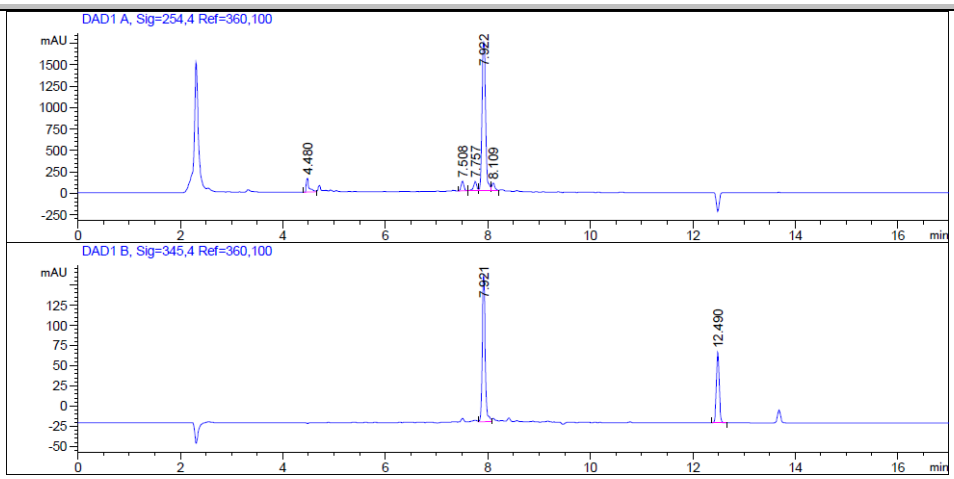


Formula

Gp$_3$A-3'-*O*-BDP-FL (**6d**)



HPLC reaction profile



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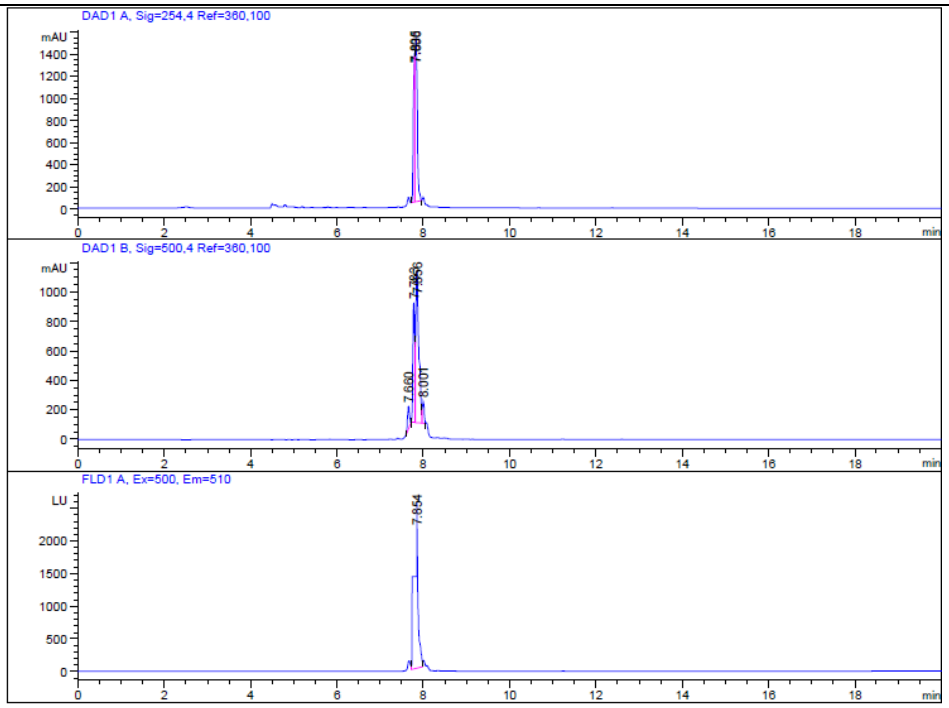
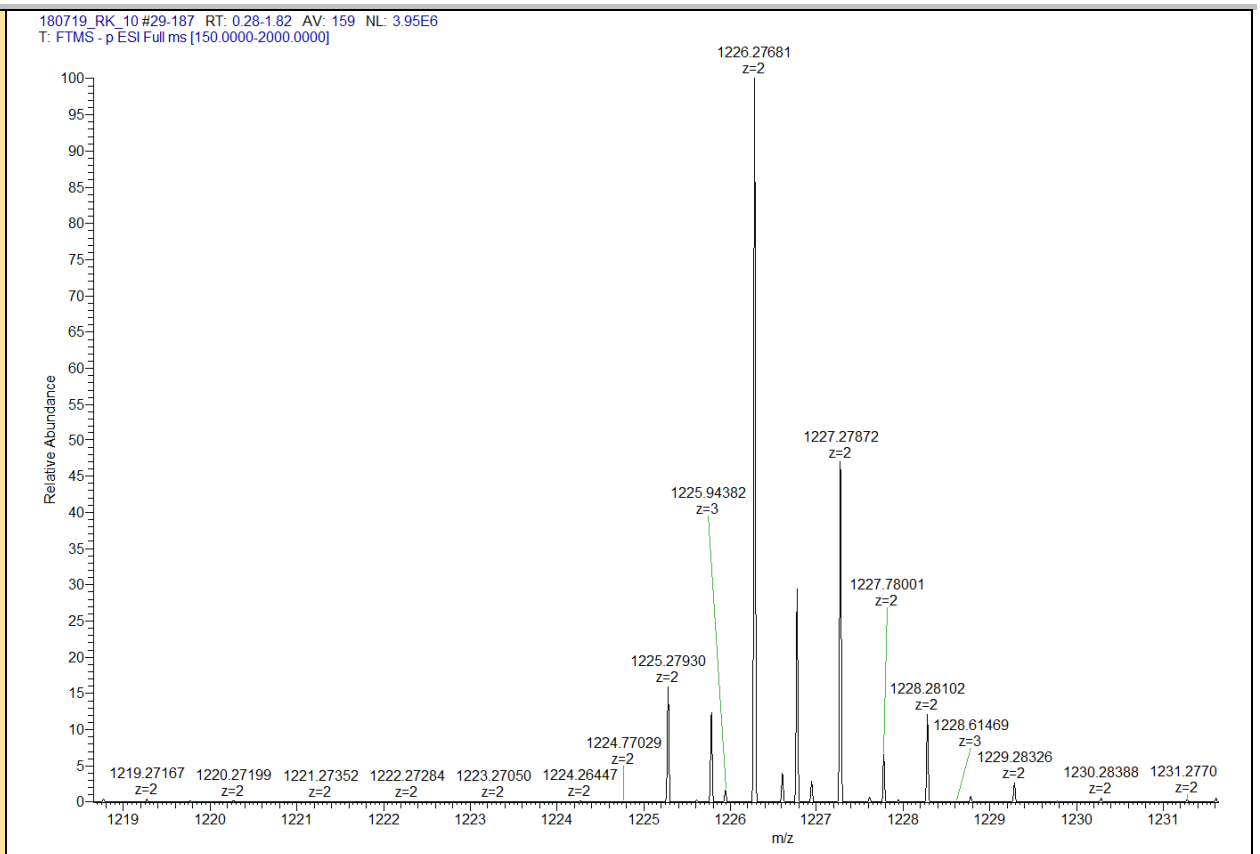
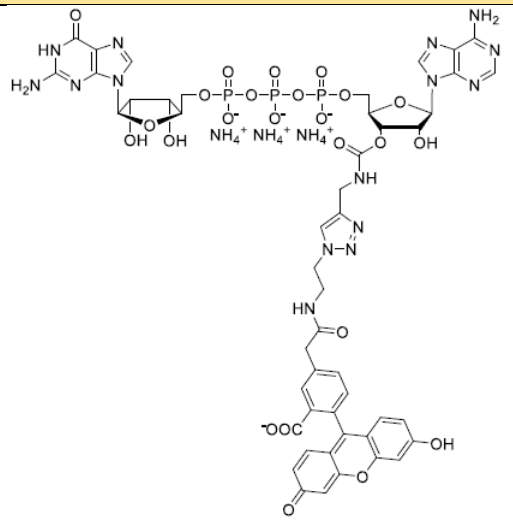


HRMS (-)ES

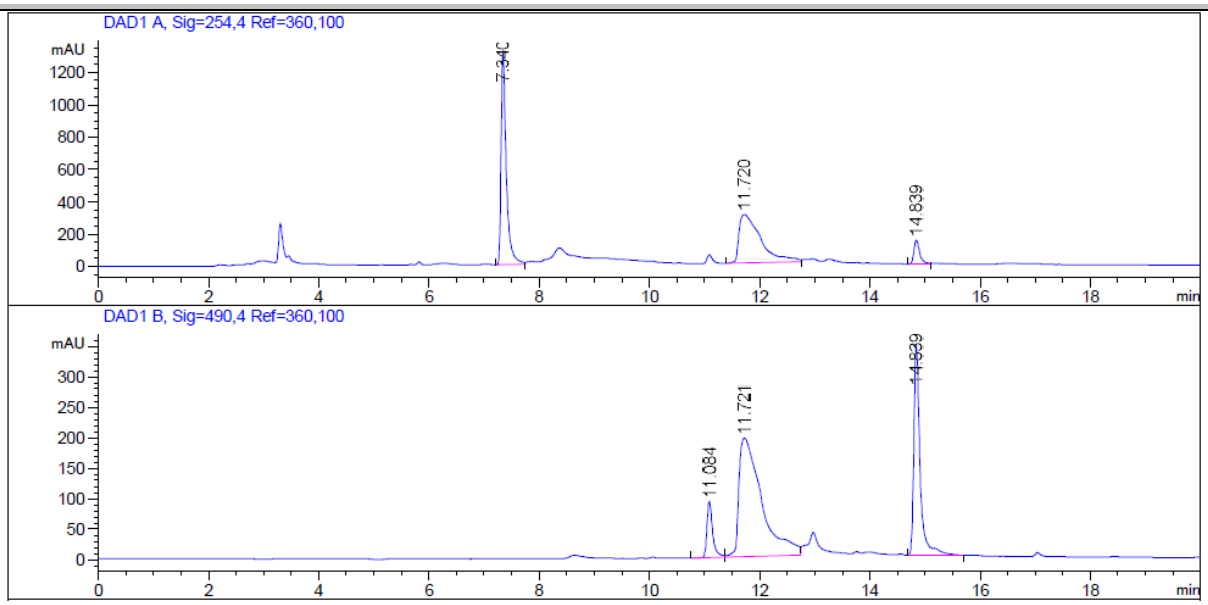


Formula

Gp$_3$A-3'-*O*-(5)FAM (**6e**)



HPLC reaction profile



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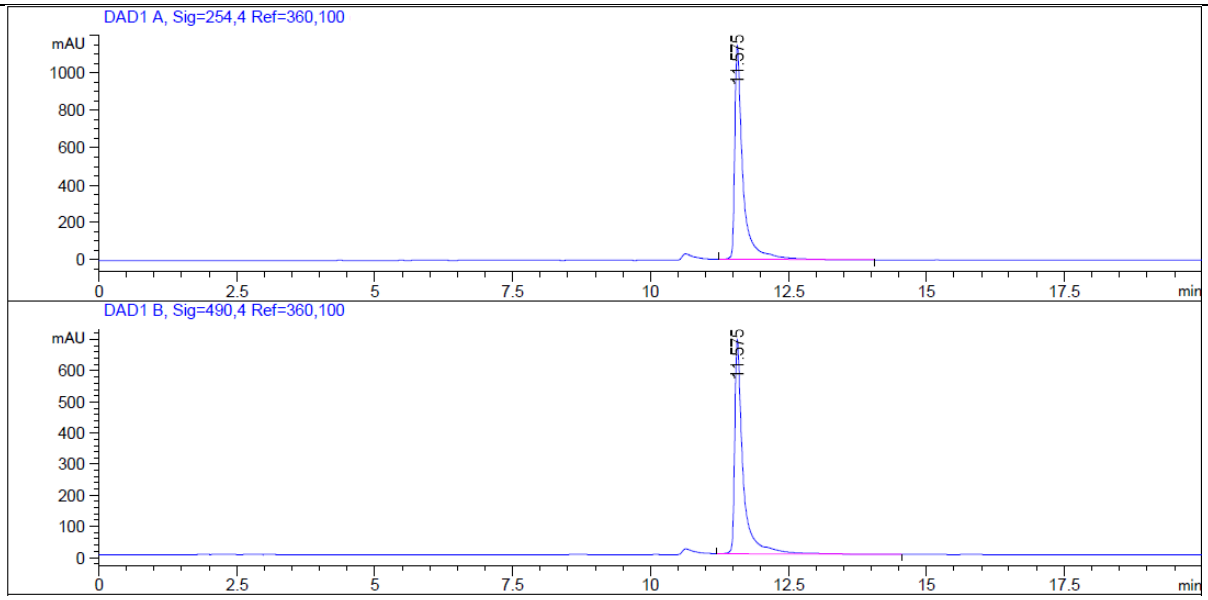
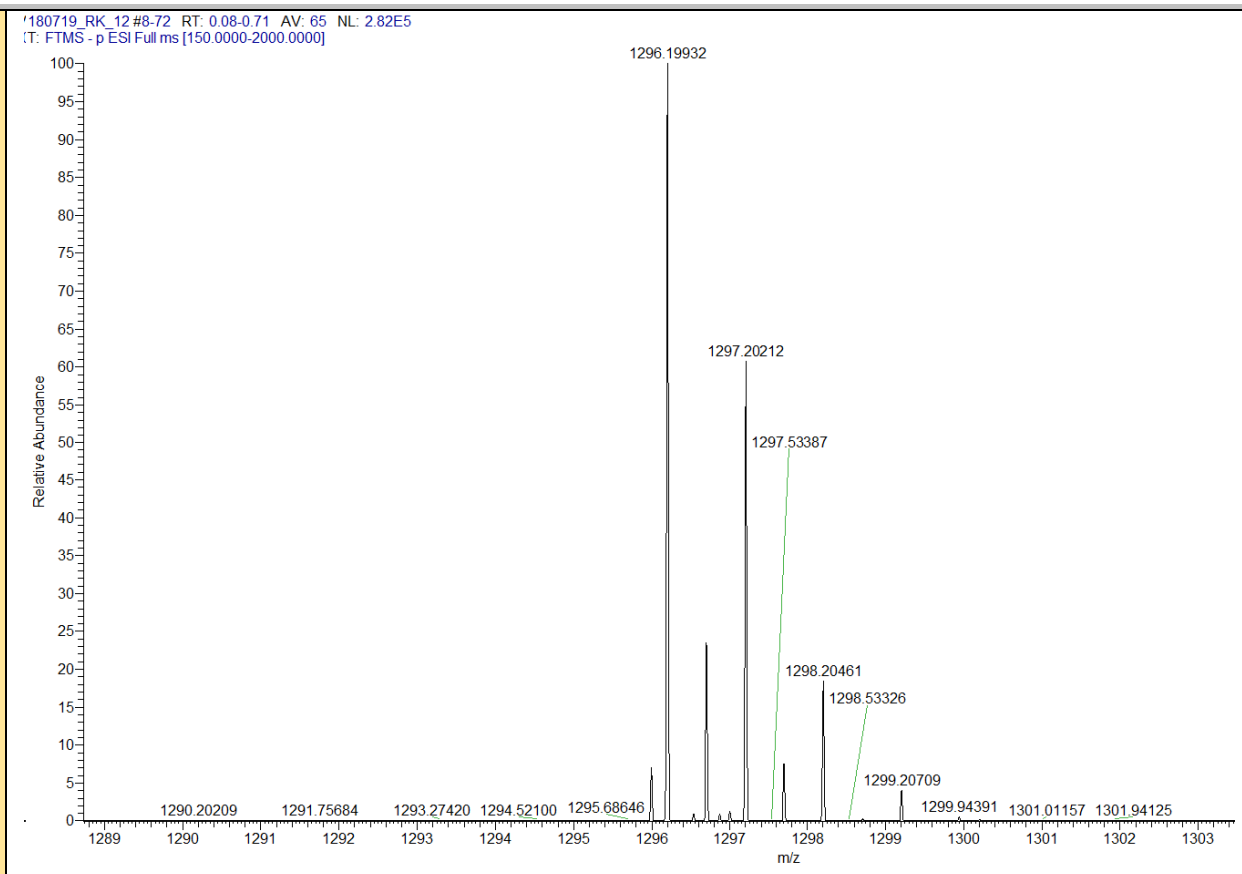
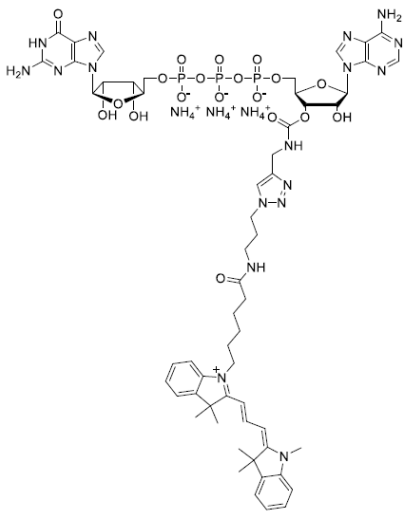


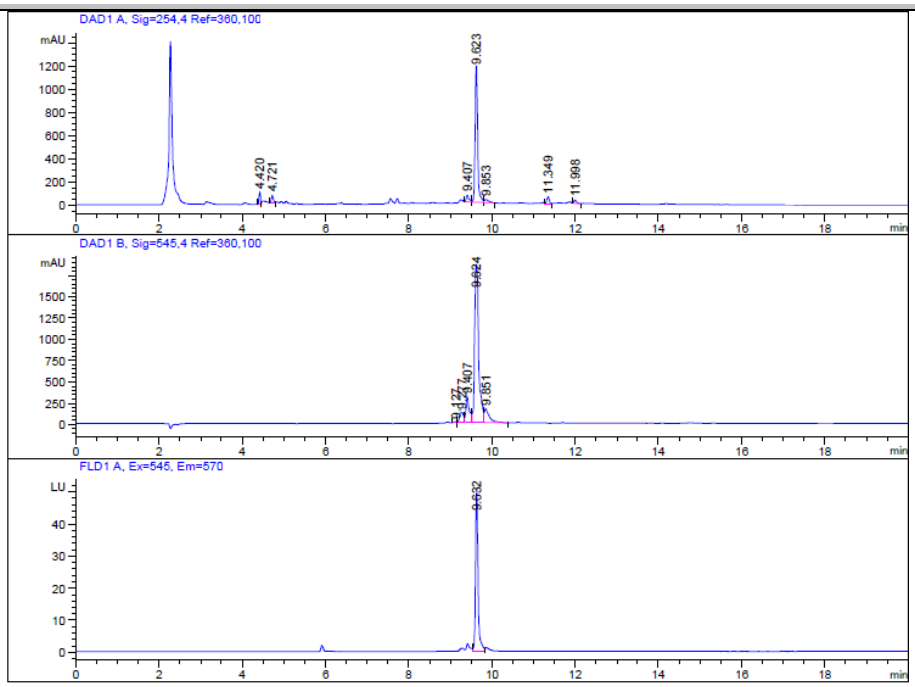
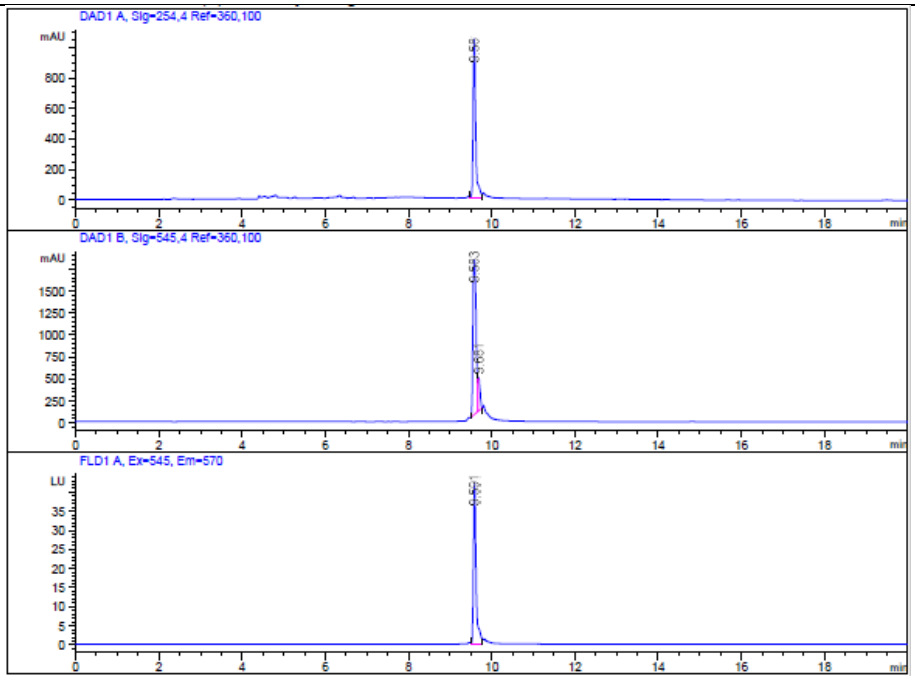
HRMS (-)ES



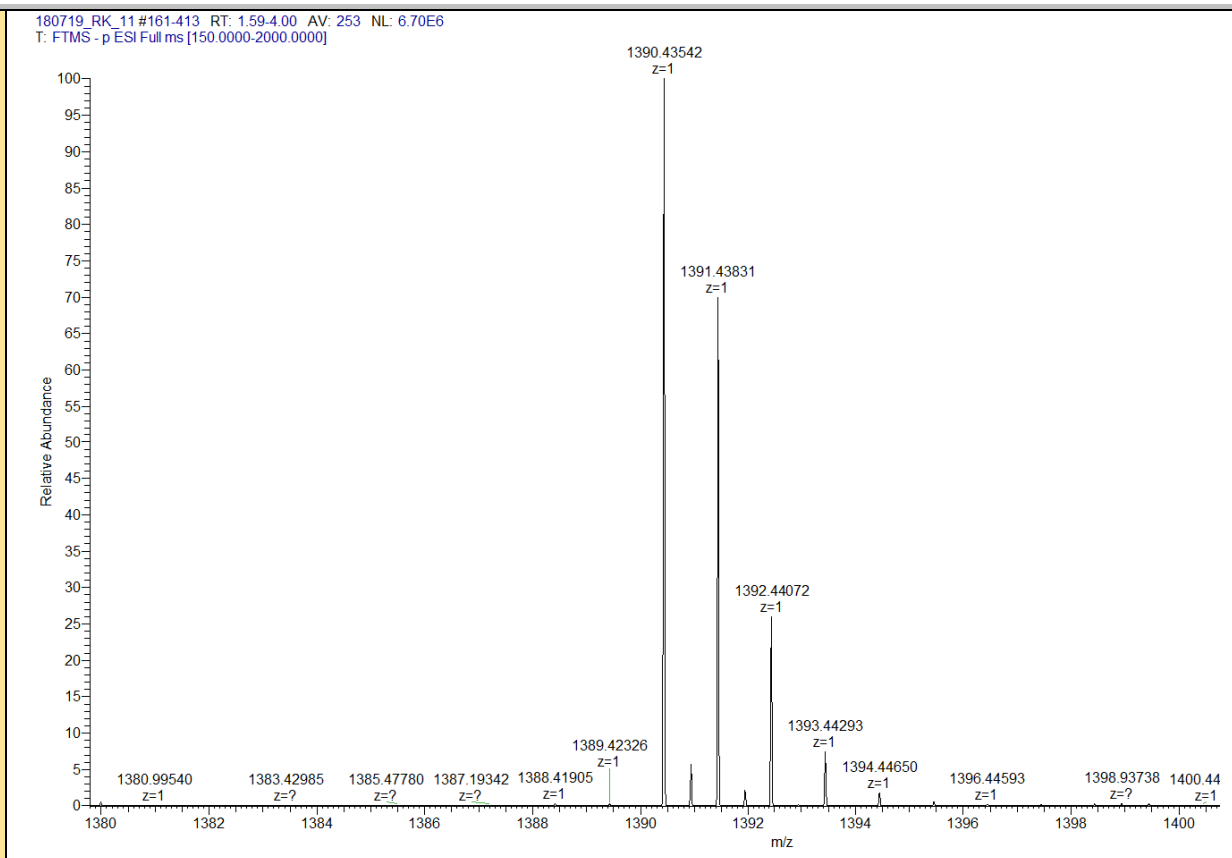
Formula

Gp_3A-3'-*O*-Cy3 (**6f**)



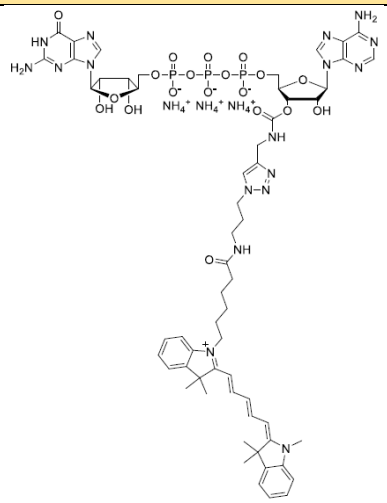
HPLC reaction profile	
HPLC profile	

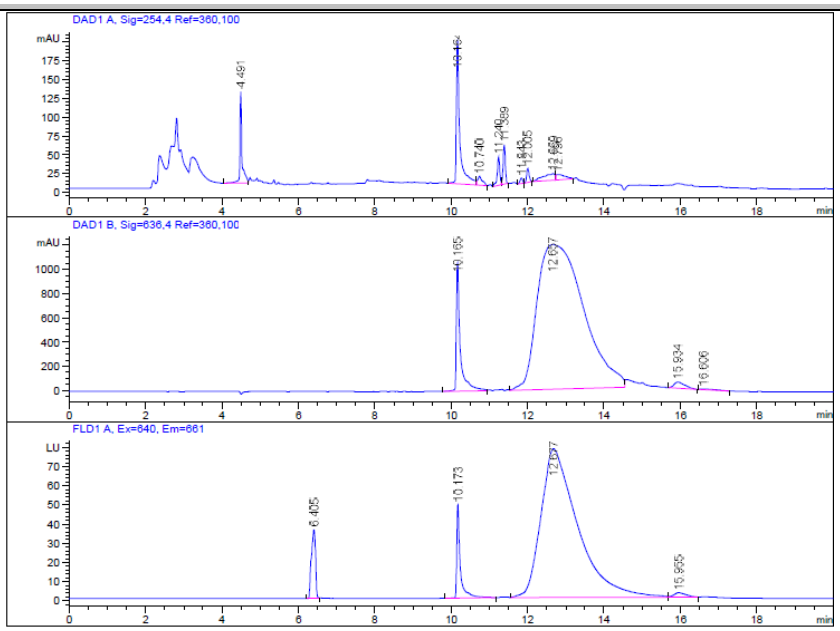
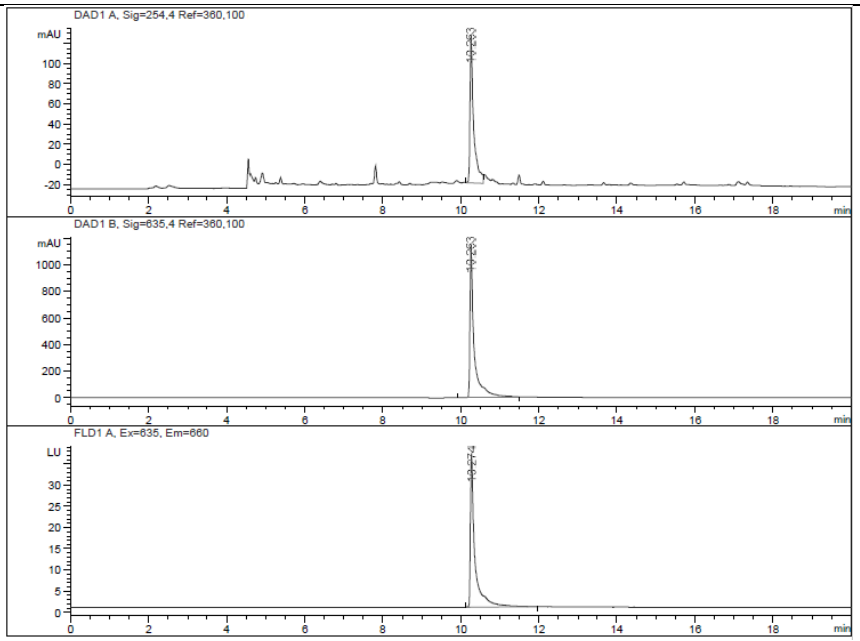
HRMS (-)ES



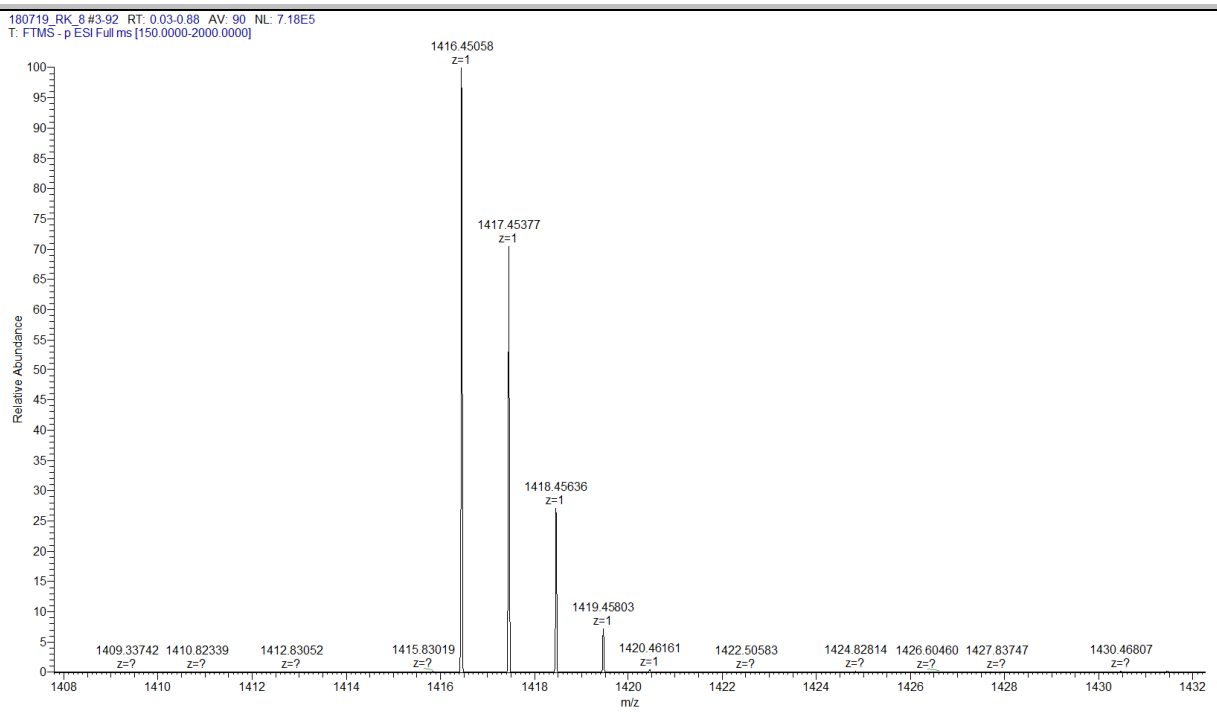
Formula

Gp_3A-3'-*O*-Cy5 (**6g**)



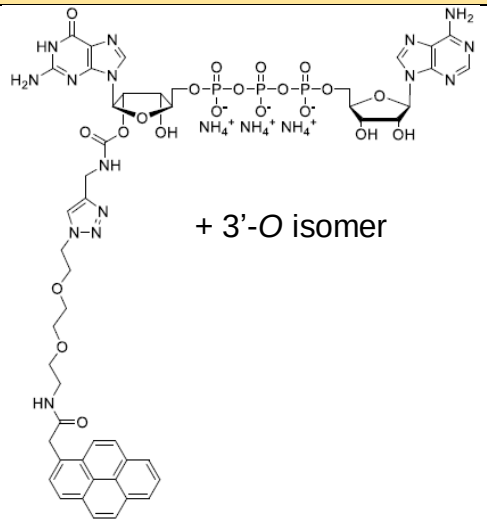
HPLC reaction profile	
HPLC profile	

HRMS (-)ES

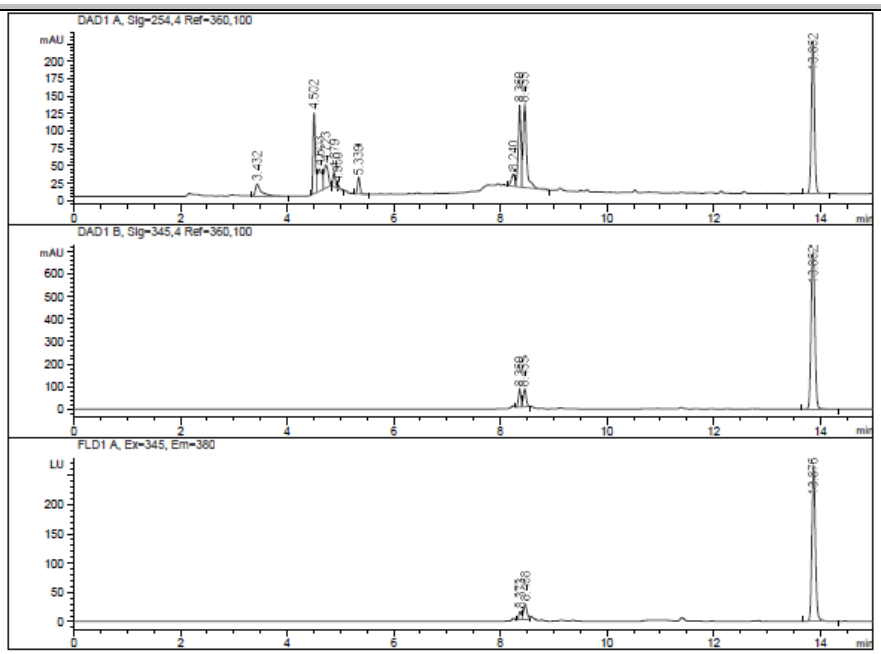
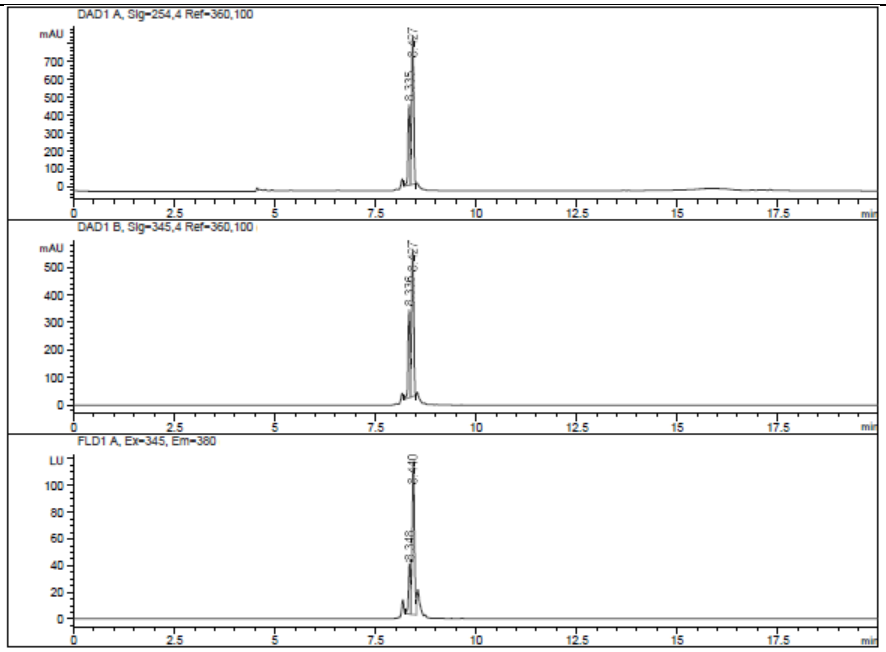


Formula

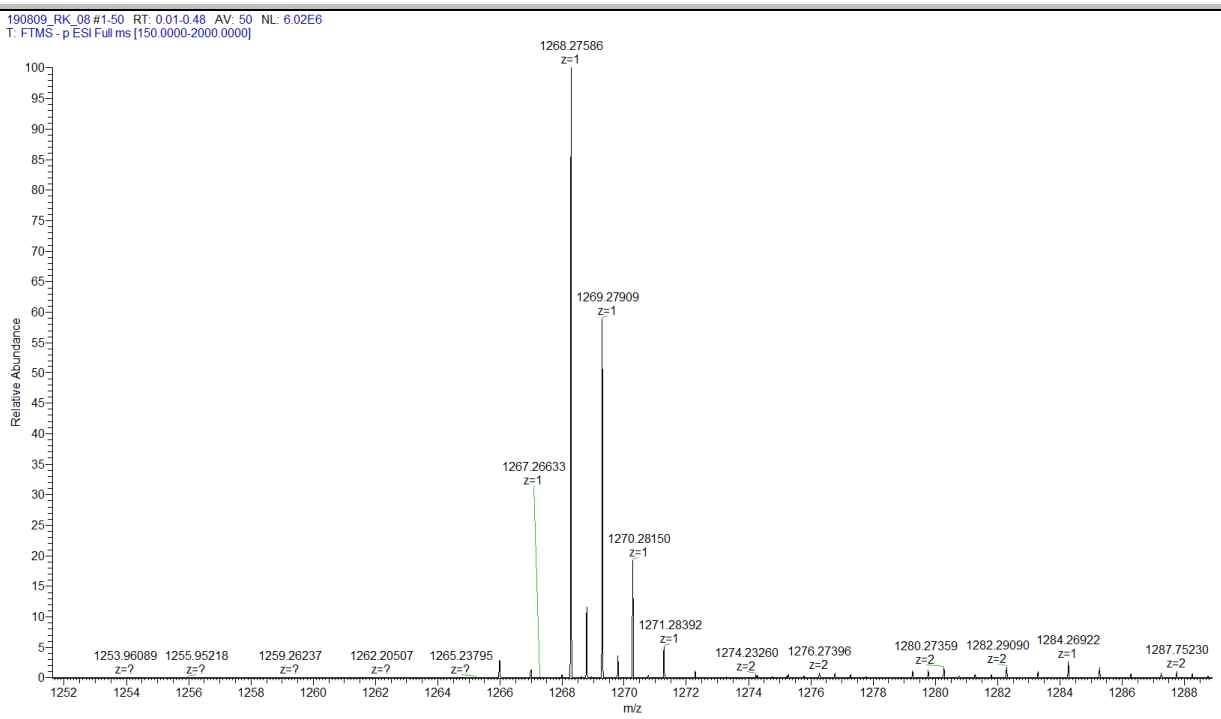
2'-O/3'-*O*-Py-Gp$_3$A (**7**)



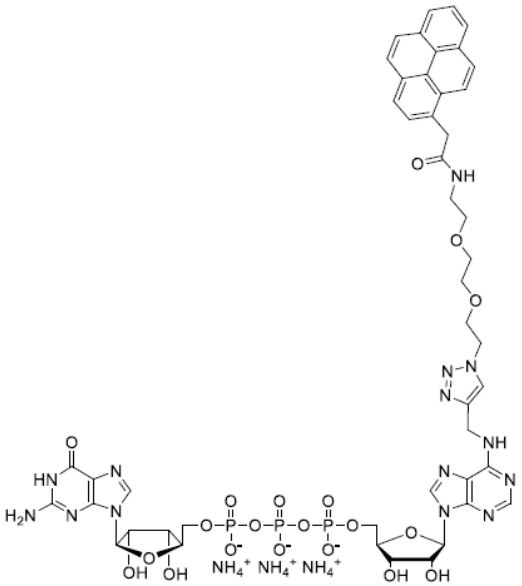
+ 3'-*O* isomer

HPLC reaction profile	
HPLC profile	

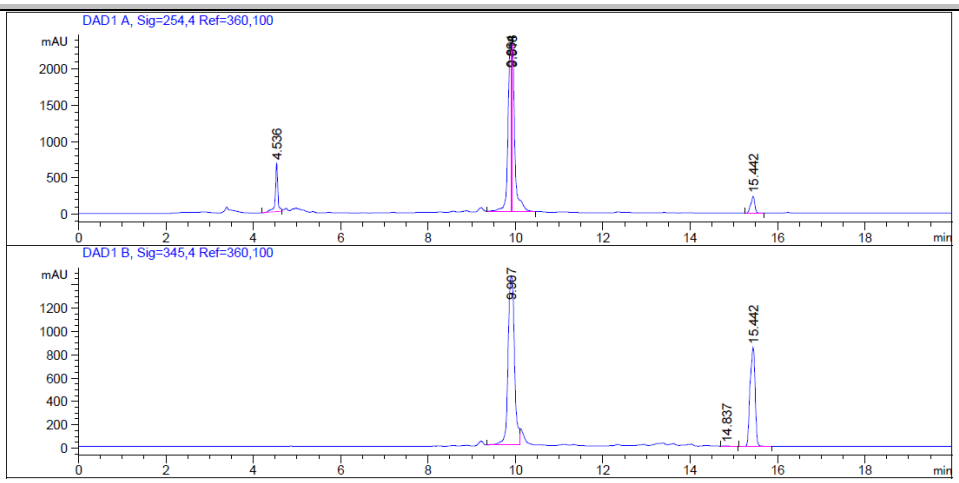
HRMS (-)ES



Gp$_3$A(N6)-Py(**8**)
Formula



HPLC reaction profile



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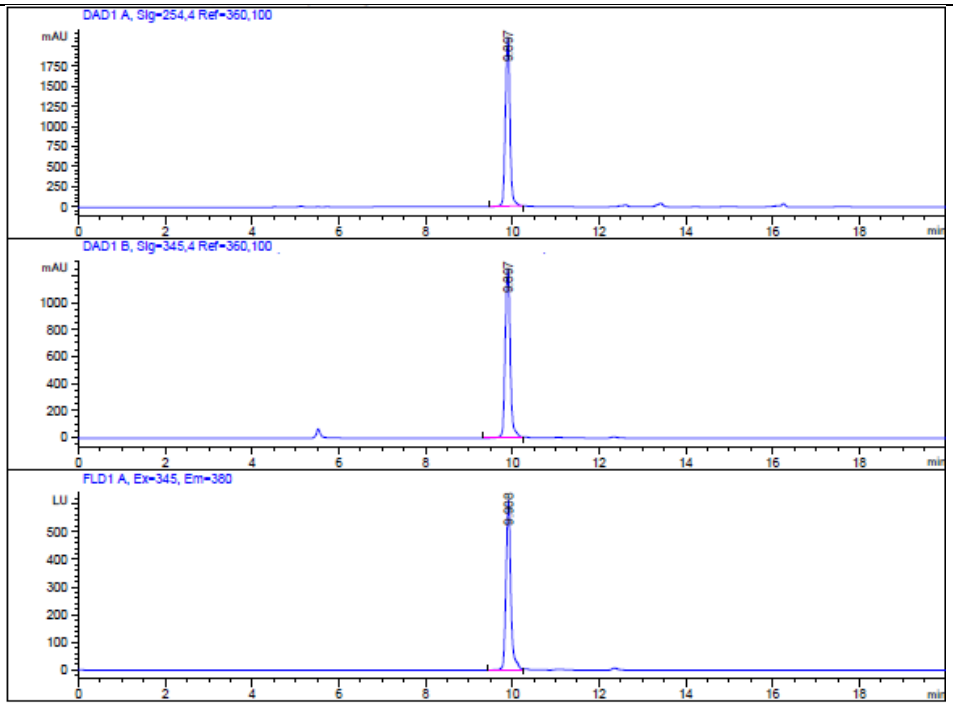
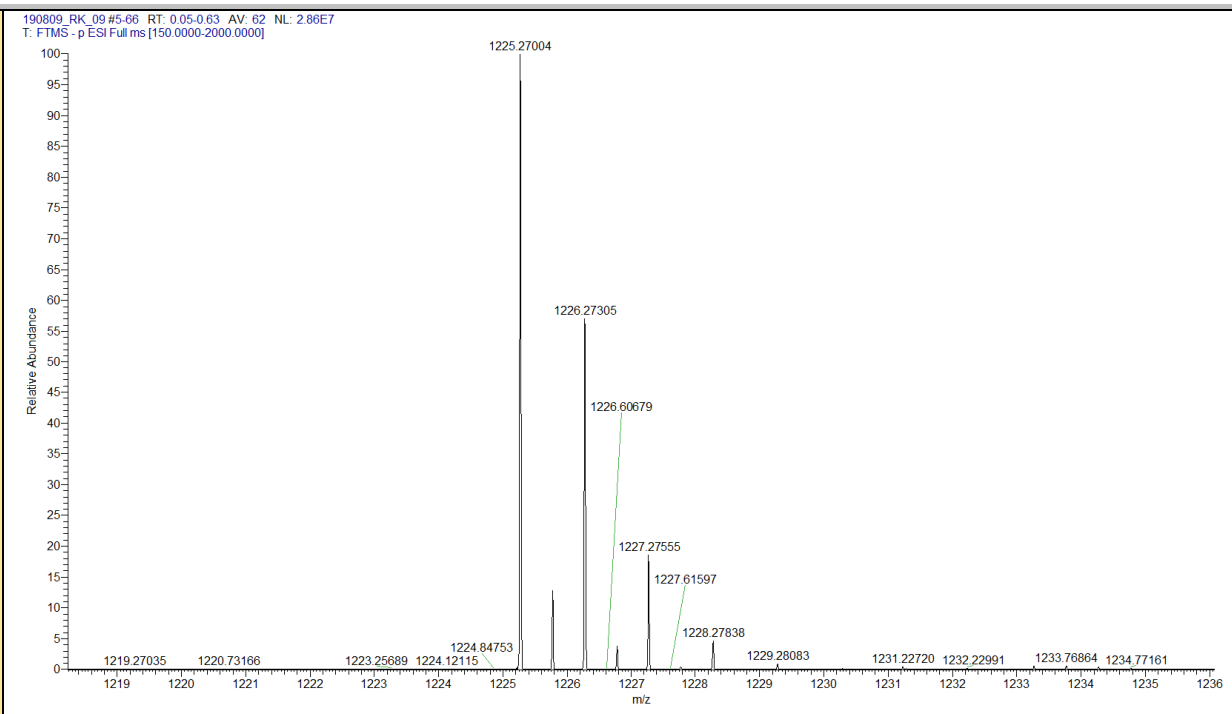


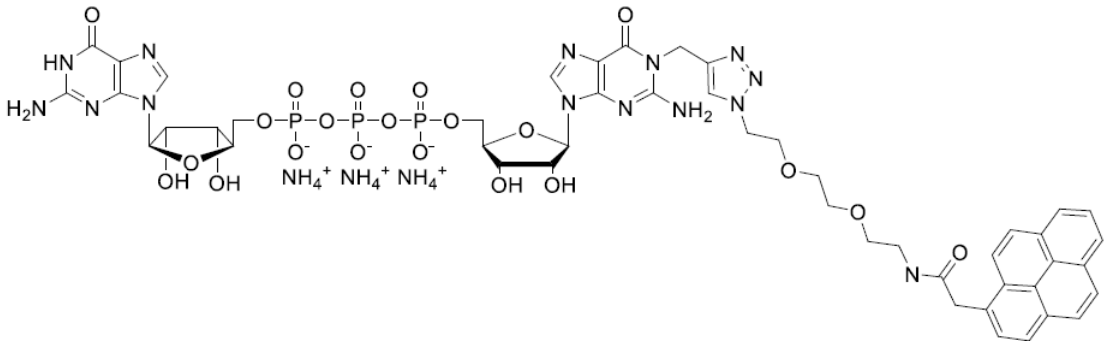
SUPPORTING INFORMATION

HRMS (-)ES



Gp$_3$G(N1)-Py(9)

Formula



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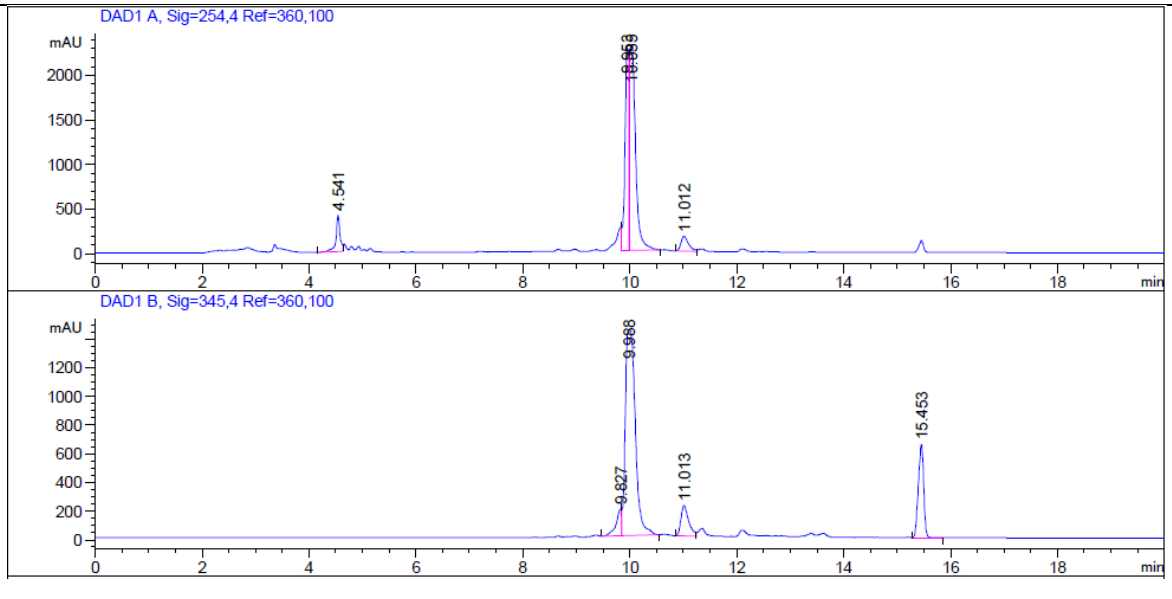

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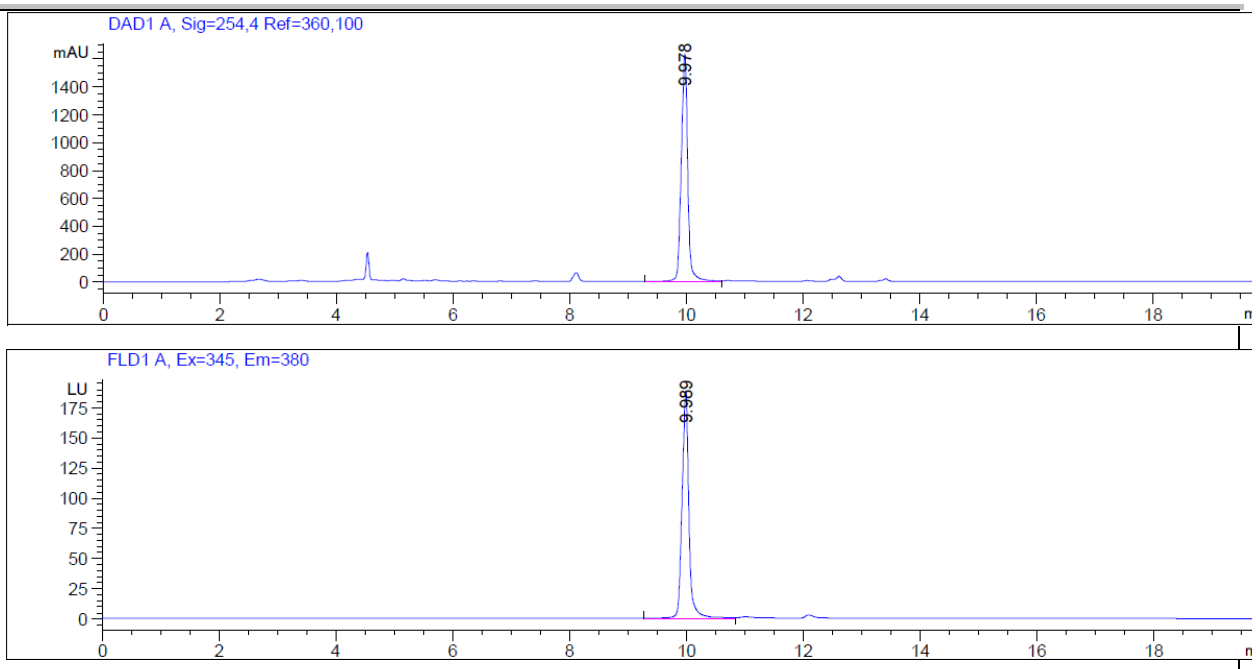
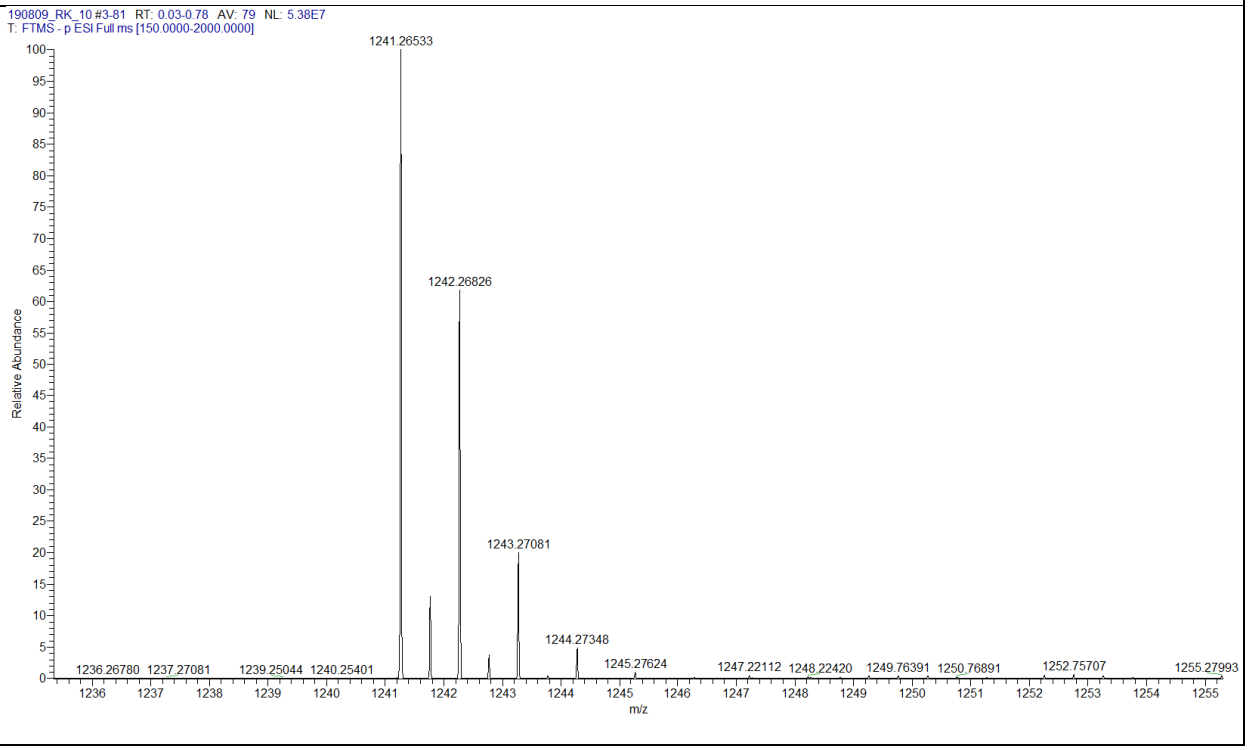
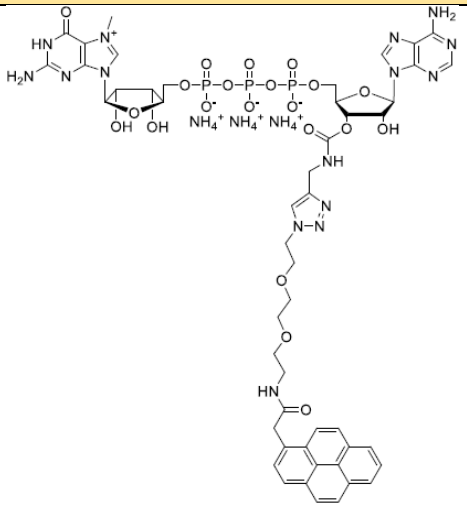
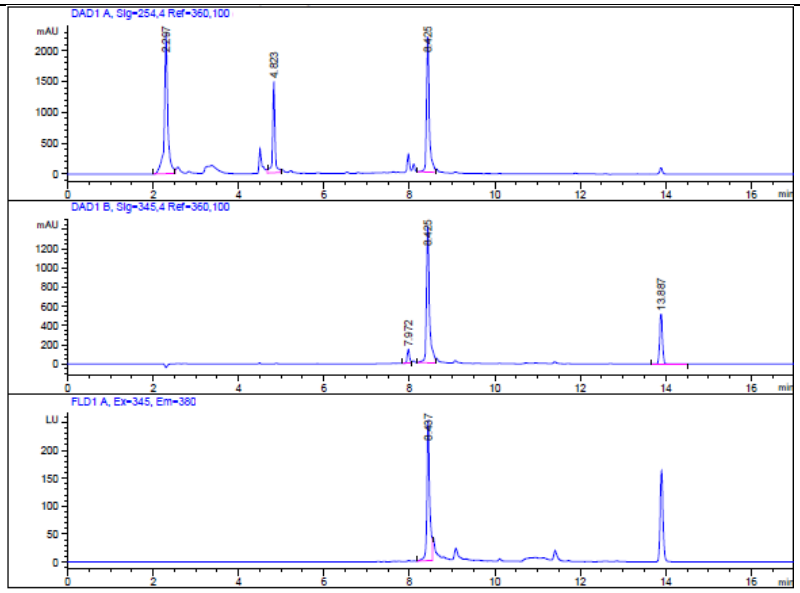
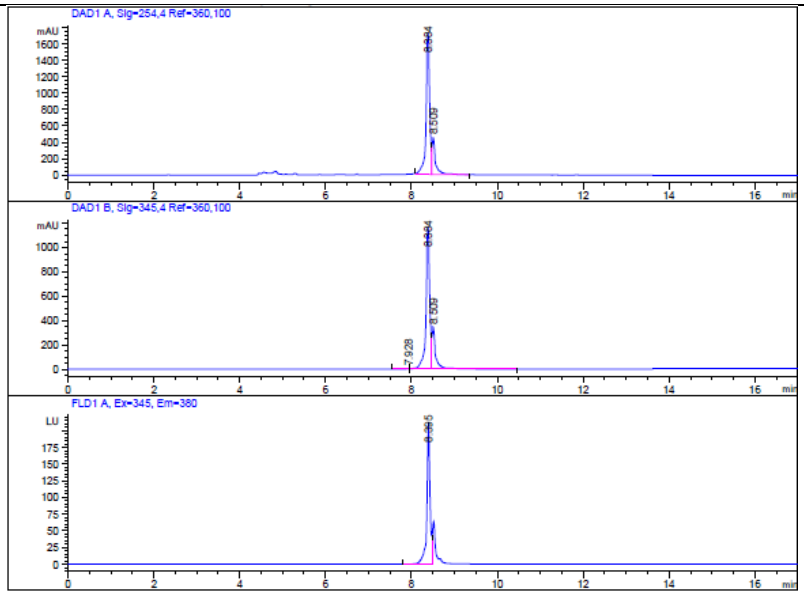
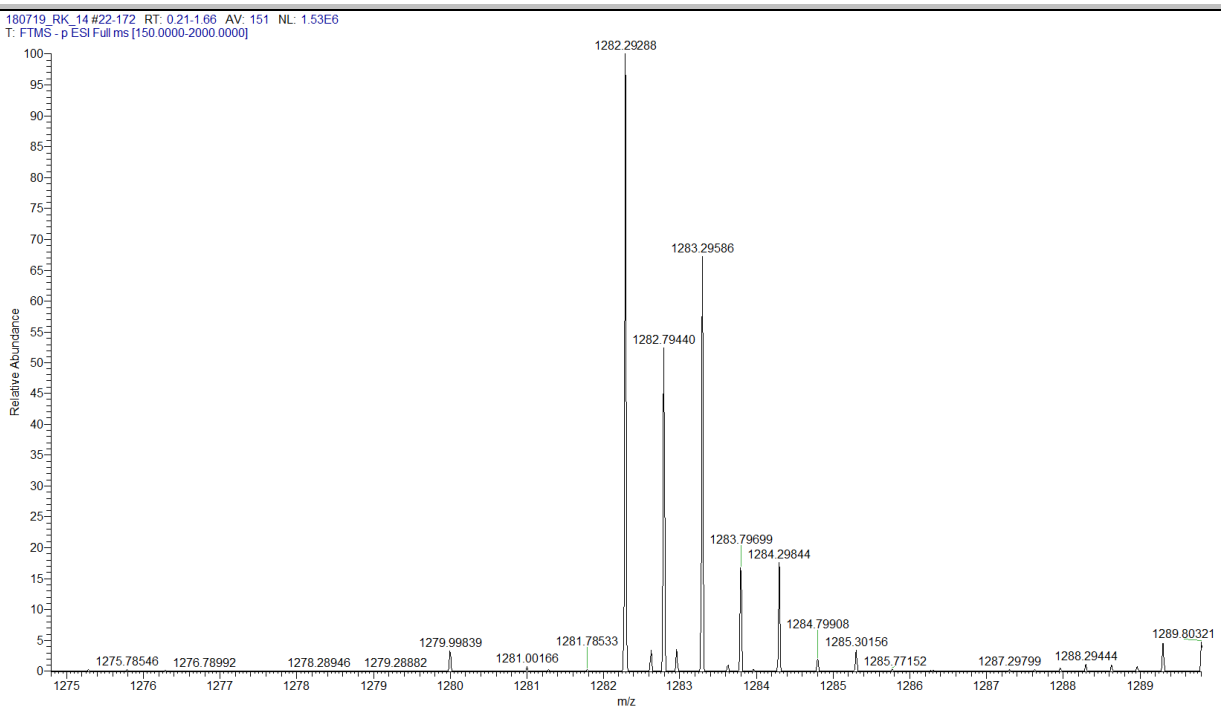


HRMS (-)ES



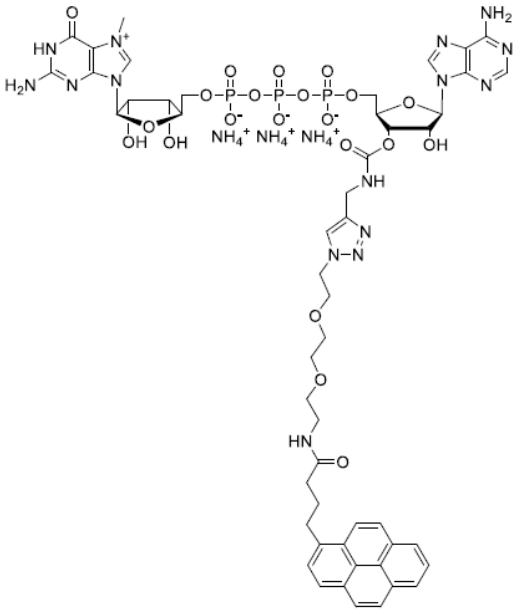
m^7Gp_3A-3'-*O*-Py (**10a**)	
Formula	
HPLC reaction profile	
HPLC profile	

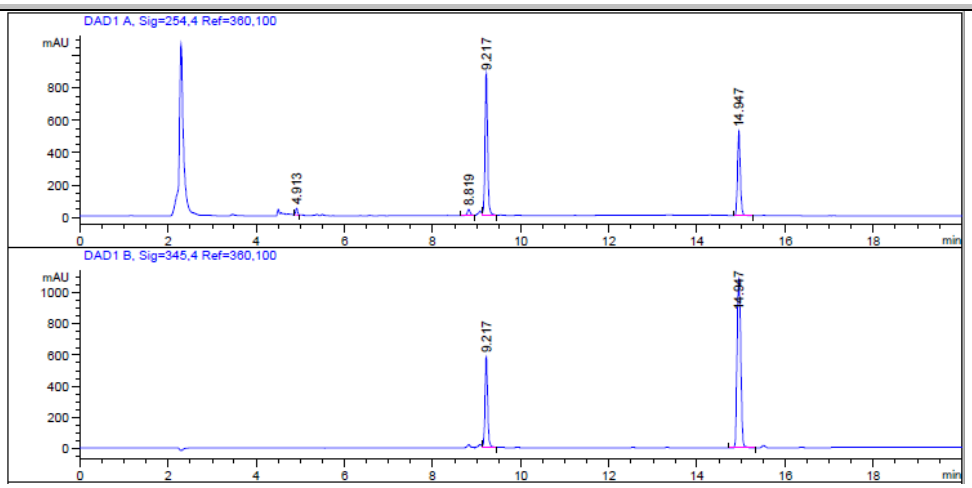
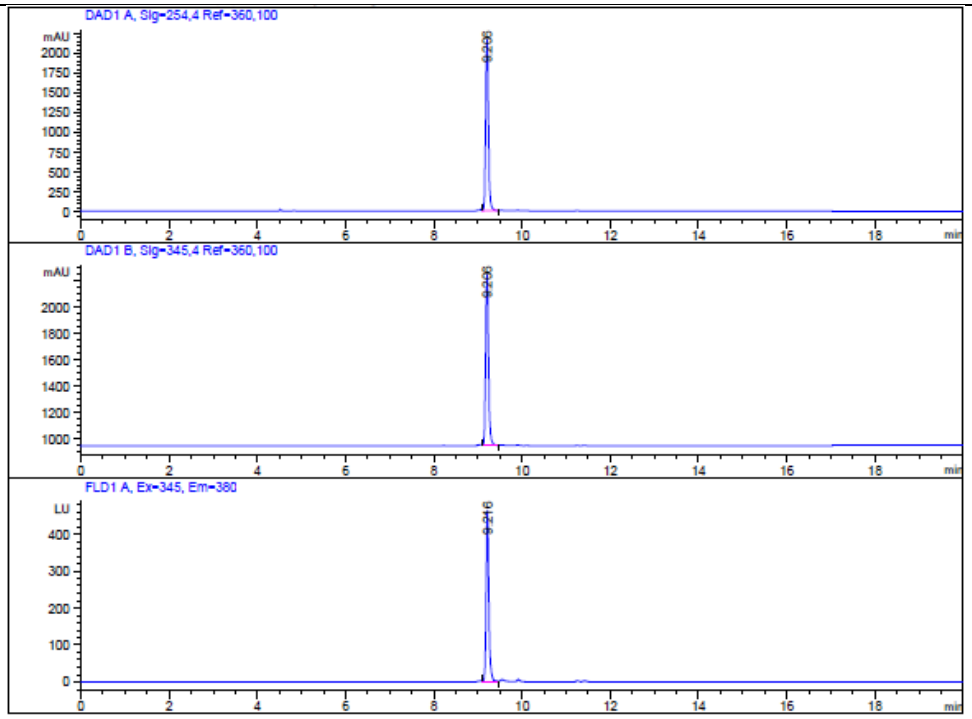
HRMS (-)ES



Formula

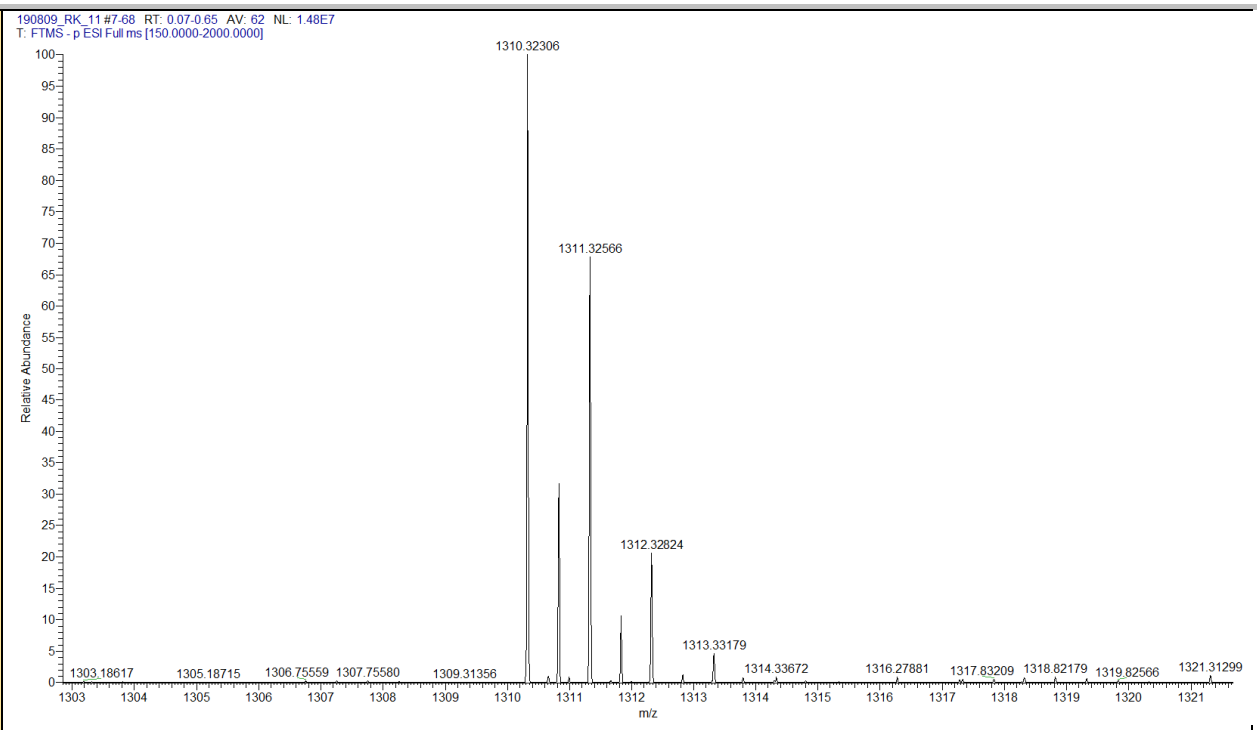
m^{7}Gp$_3$A-3'-*O*-Py3 (**10b**)



HPLC reaction profile	
HPLC profile	

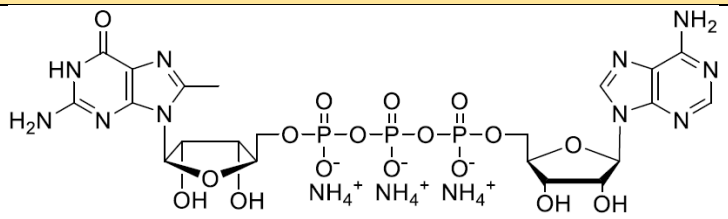
SUPPORTING INFORMATION

HRMS (-)ES

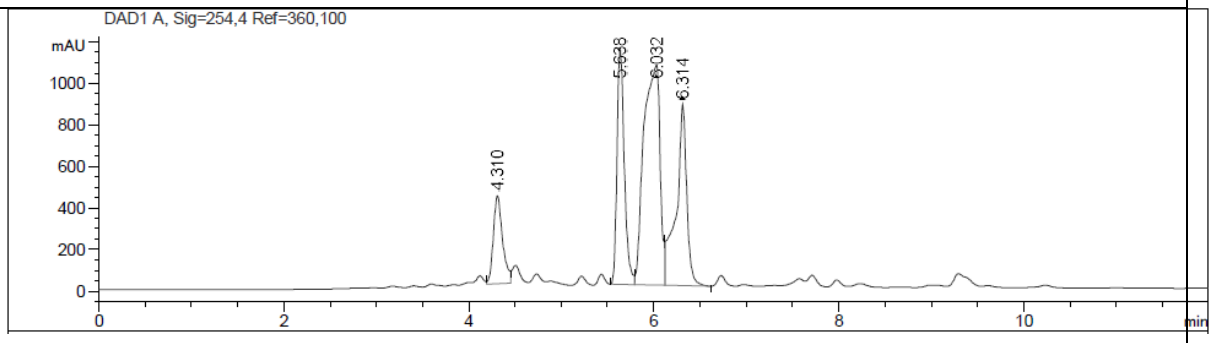


P[1]-5'-(*C*8-methyl)guanosine-P[3]-3'-adenosine triphosphate ammonium salt (8-Me-Gp$_3$A)

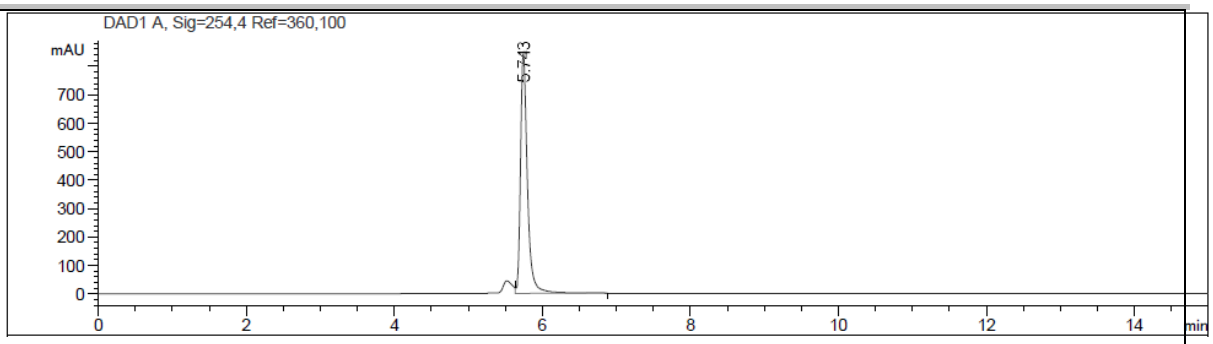
Formula



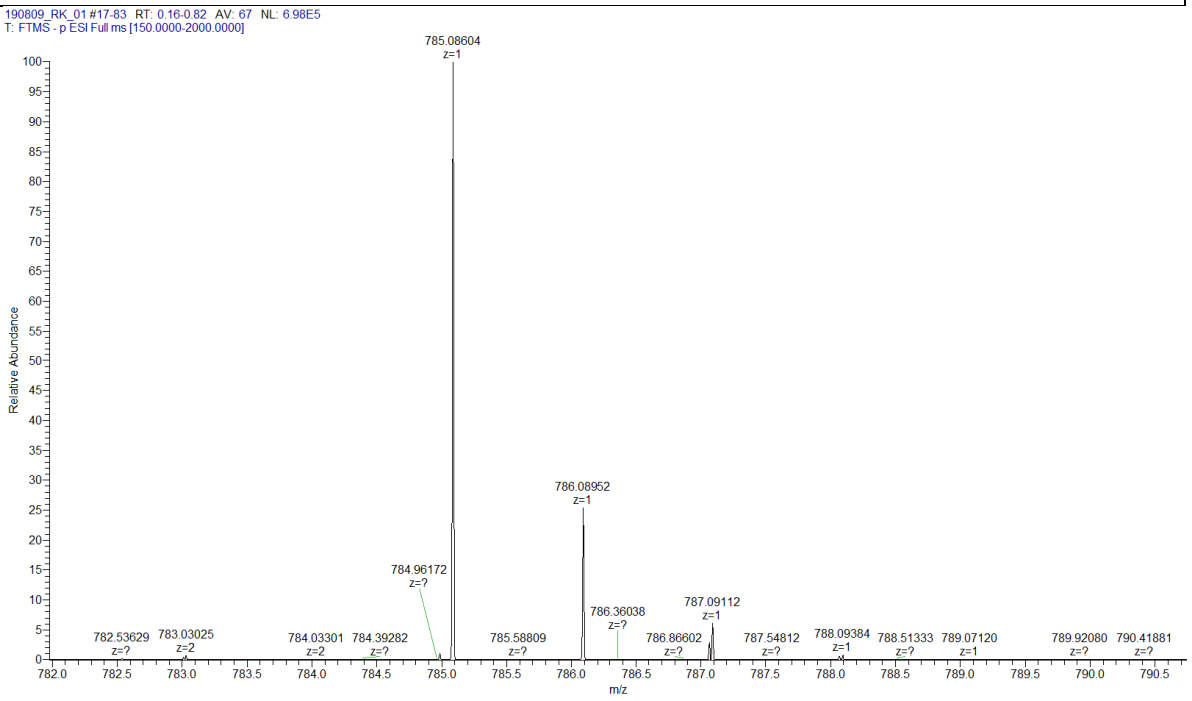
HPLC reaction profile



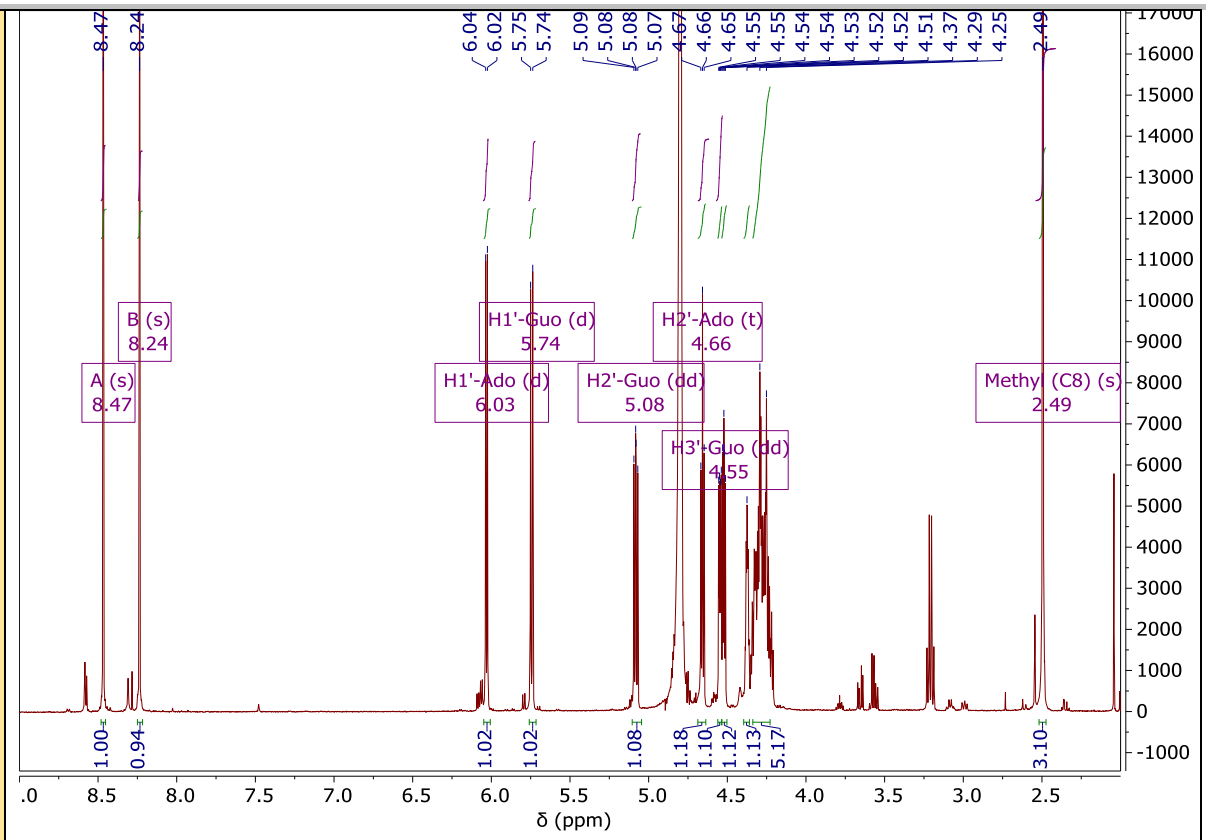
HPLC profile



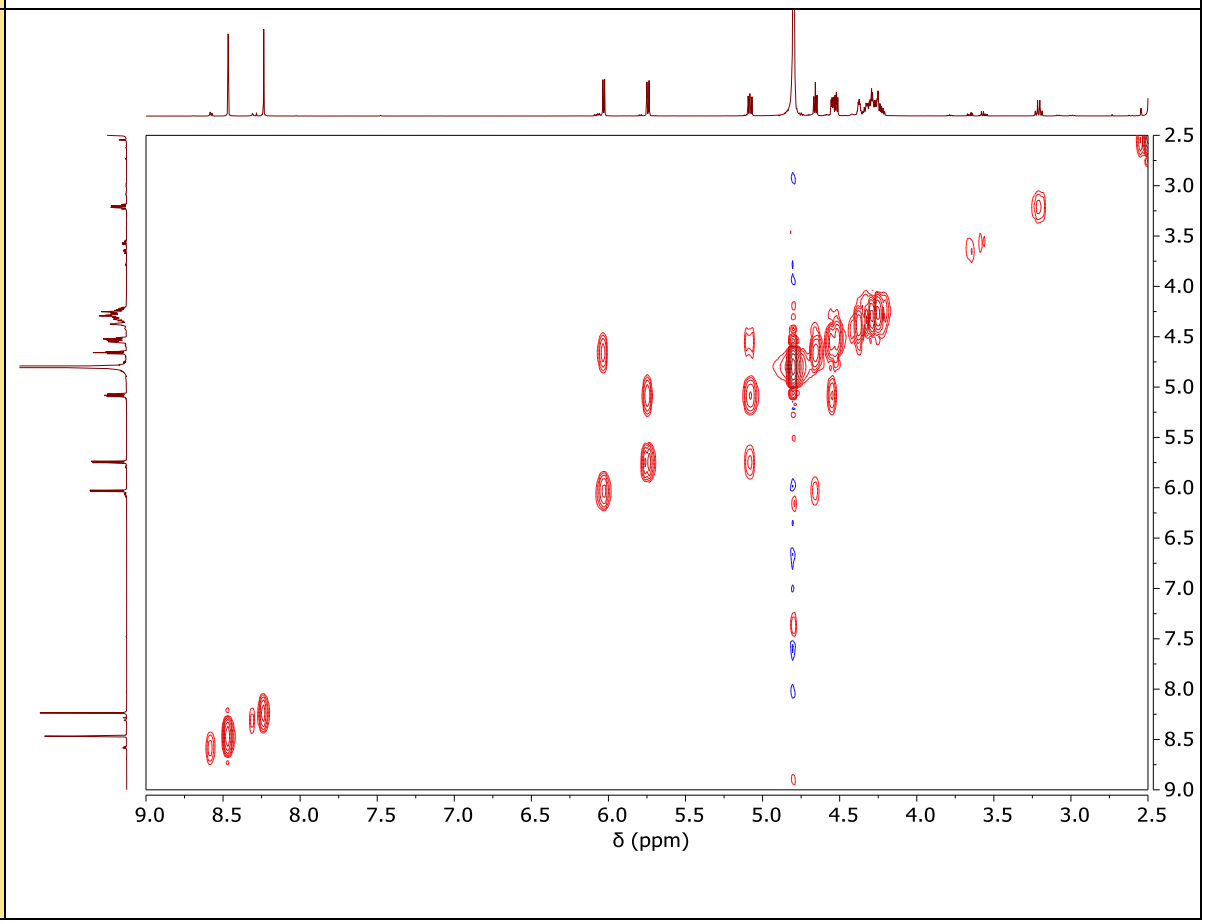
HRMS (-)ES



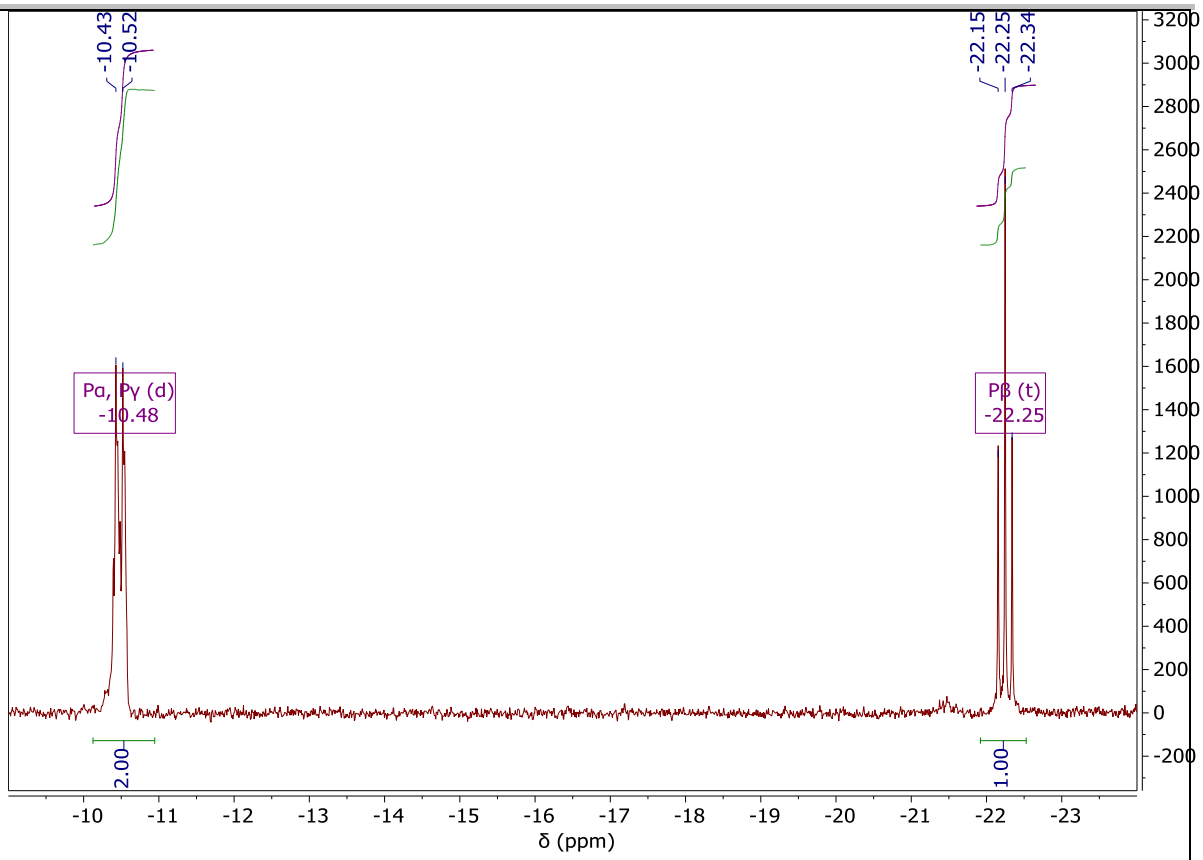
^{1}H NMR (500 MHz, D_2O, 25°C)



^{1}H-^{1}H COSY (500 MHz, D_2O, 25°C)

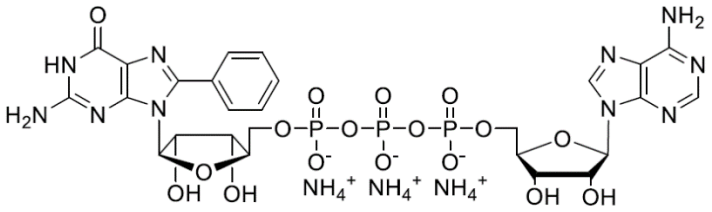


^{31}P NMR (202 MHz, D_2O, 25°C)

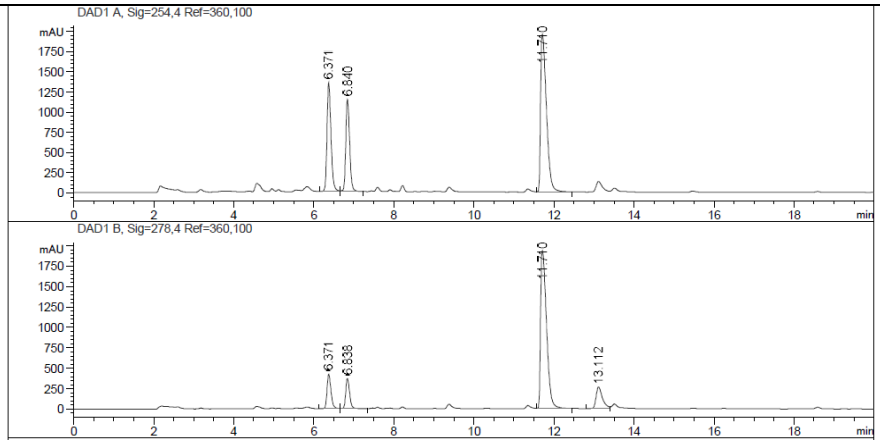


P^1-5'-(*C*8-phenyl)guanosine-P^3-3'-adenosine triphosphate ammonium salt (8-Ph-Gp$_3$A)

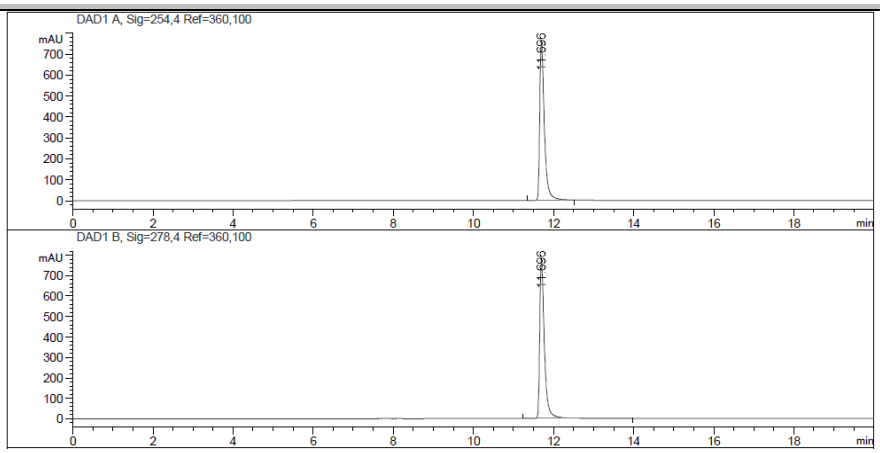
Formula



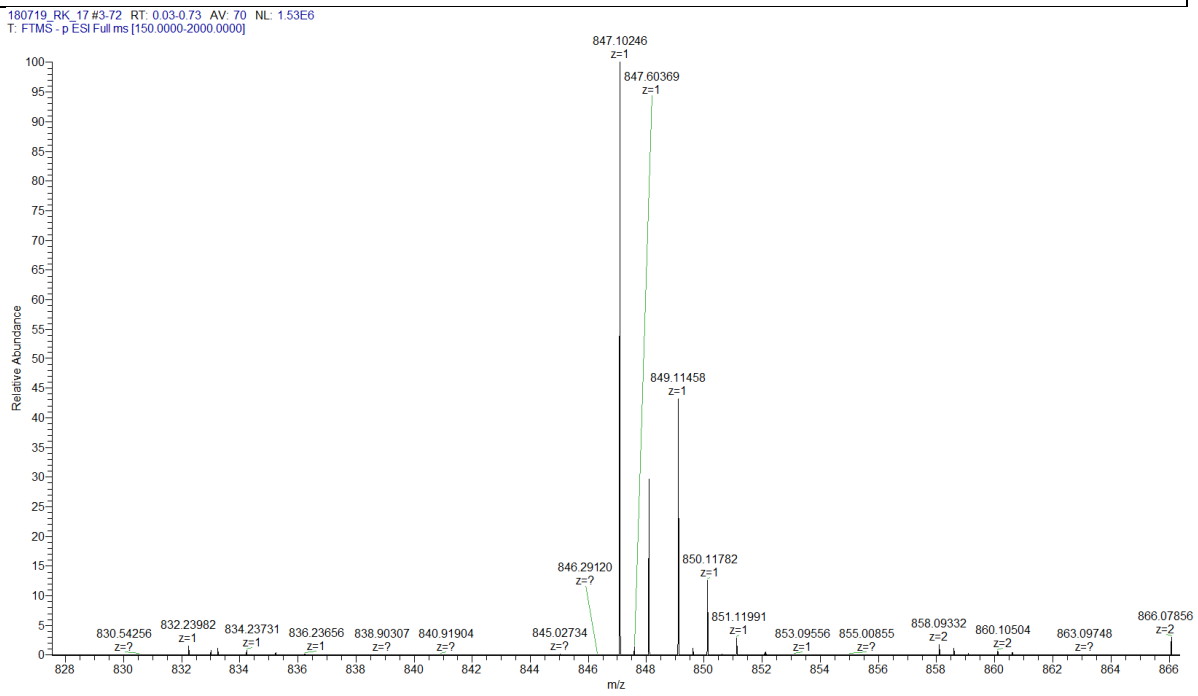
HPLC reaction profile



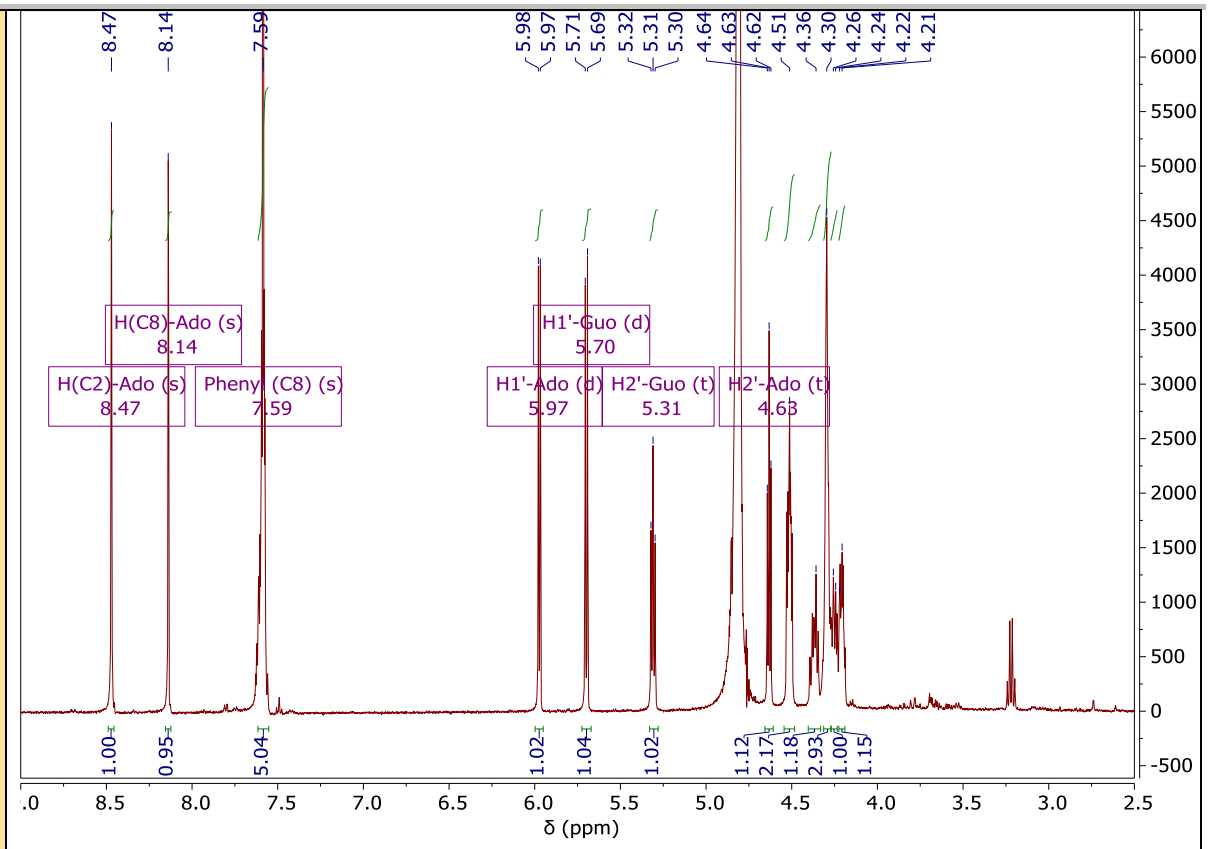
HPLC profile



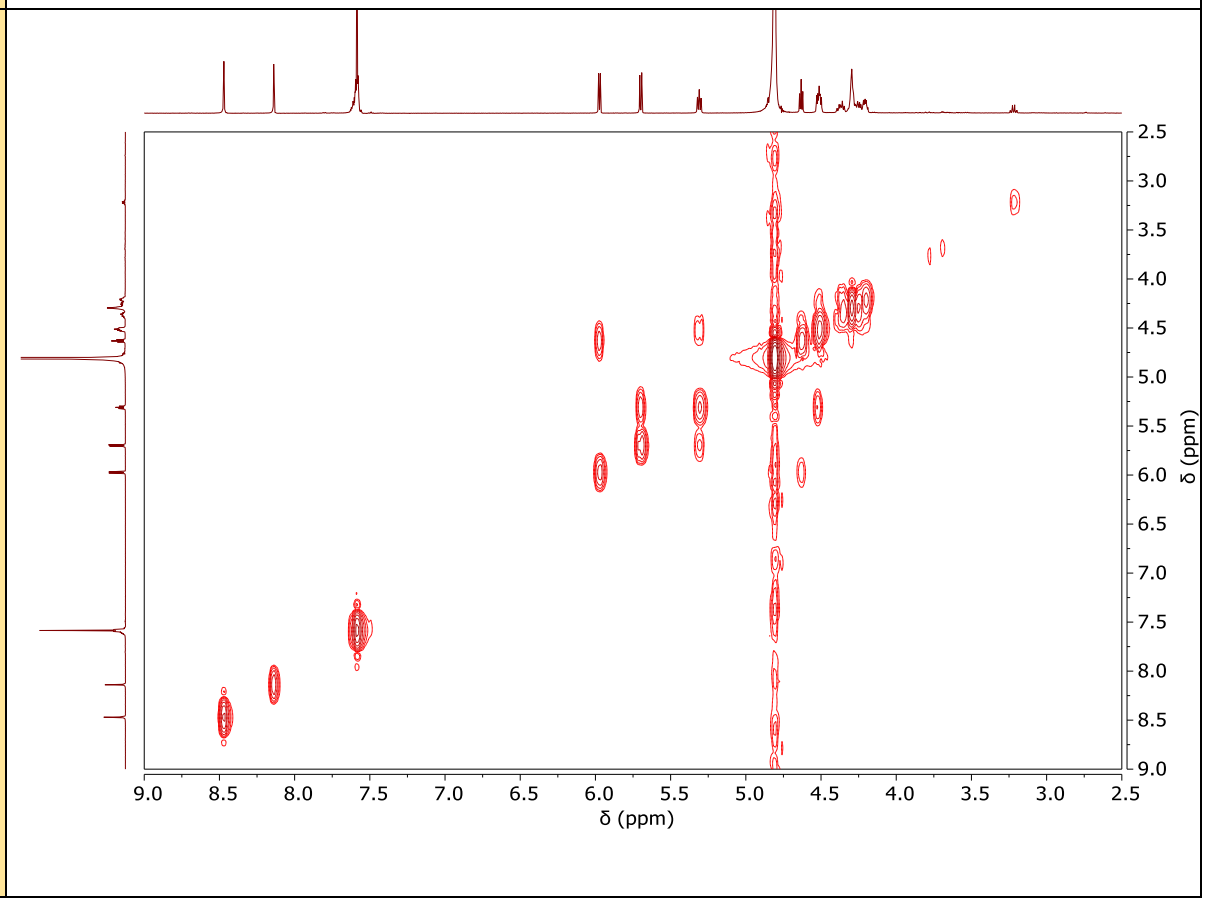
HRMS (-)ES



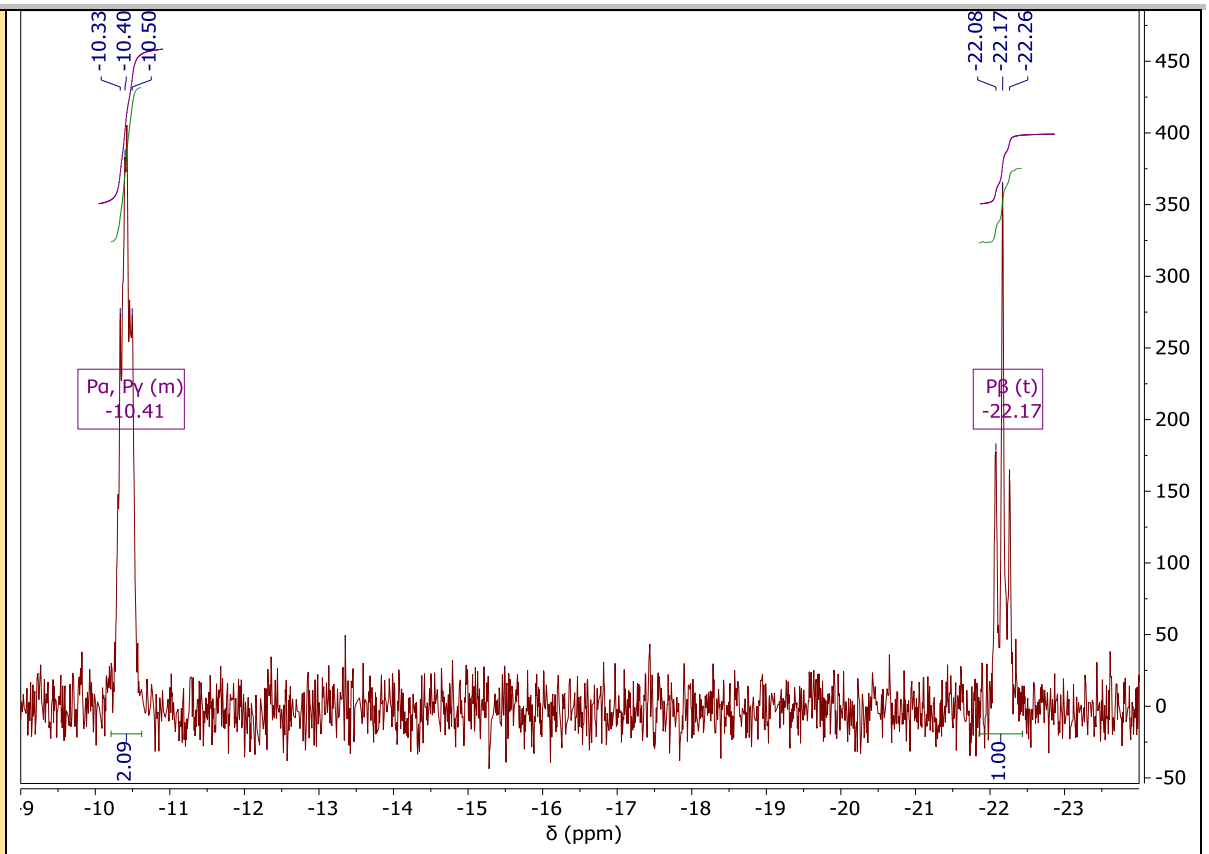
^{1}H NMR (500 MHz, D_2O, 25°C)



^{1}H-^{1}H COSY (500 MHz, D_2O, 25°C)

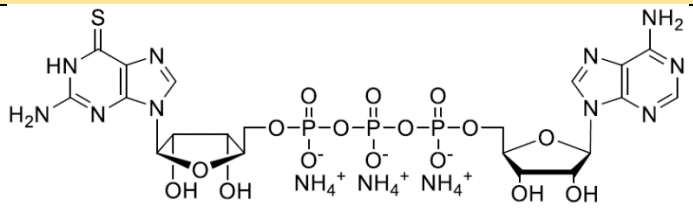


^{31}P NMR (202 MHz, D_2O, 25°C)

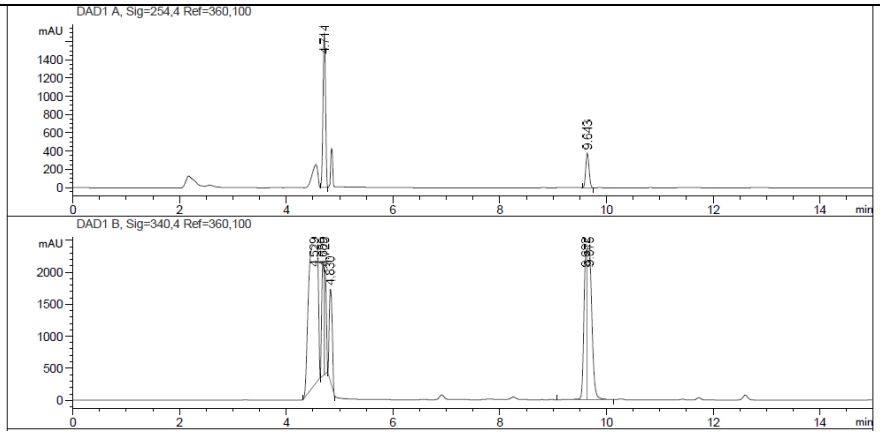


P^1-5'-(6-thio)guanosine-P^3-3'-adenosine triphosphate ammonium salt ($^{6\text{-S}}$Gp$_3$A)

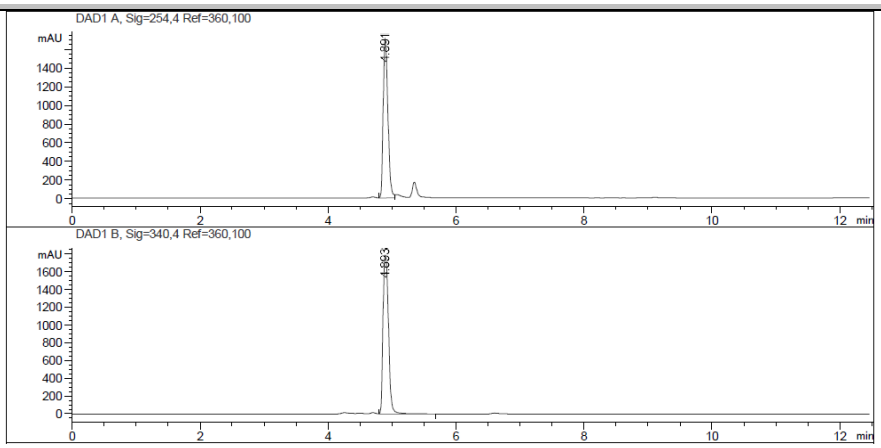
Formula



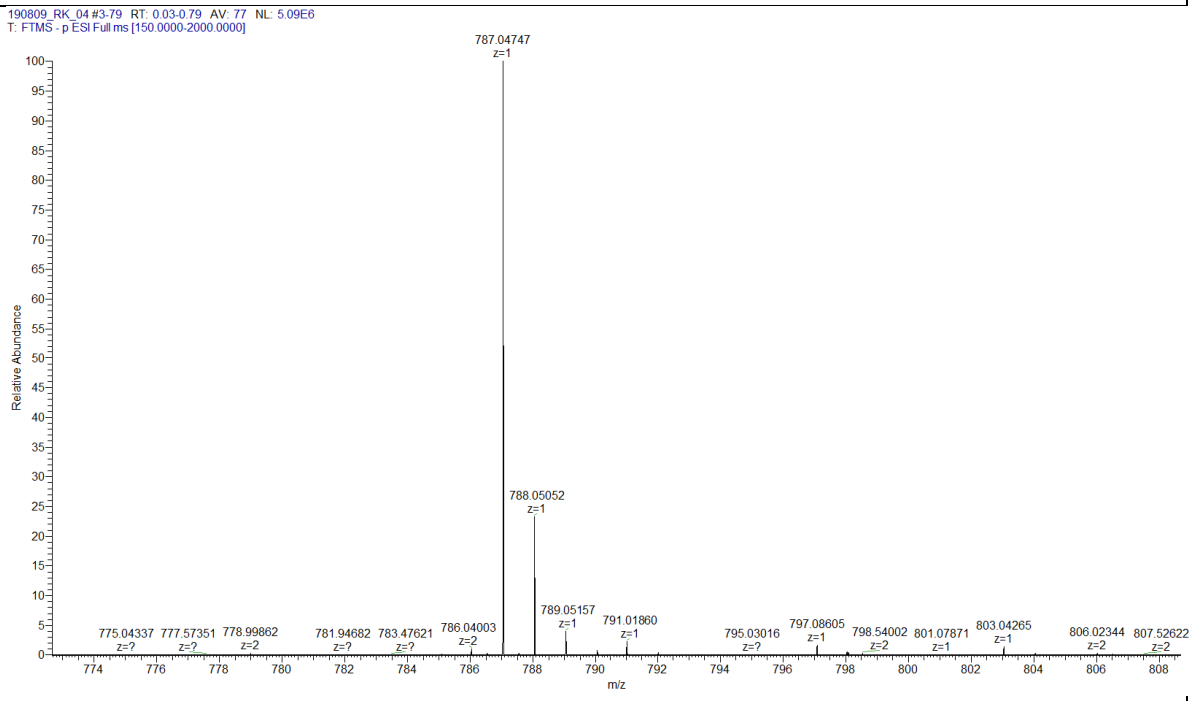
HPLC reaction profile



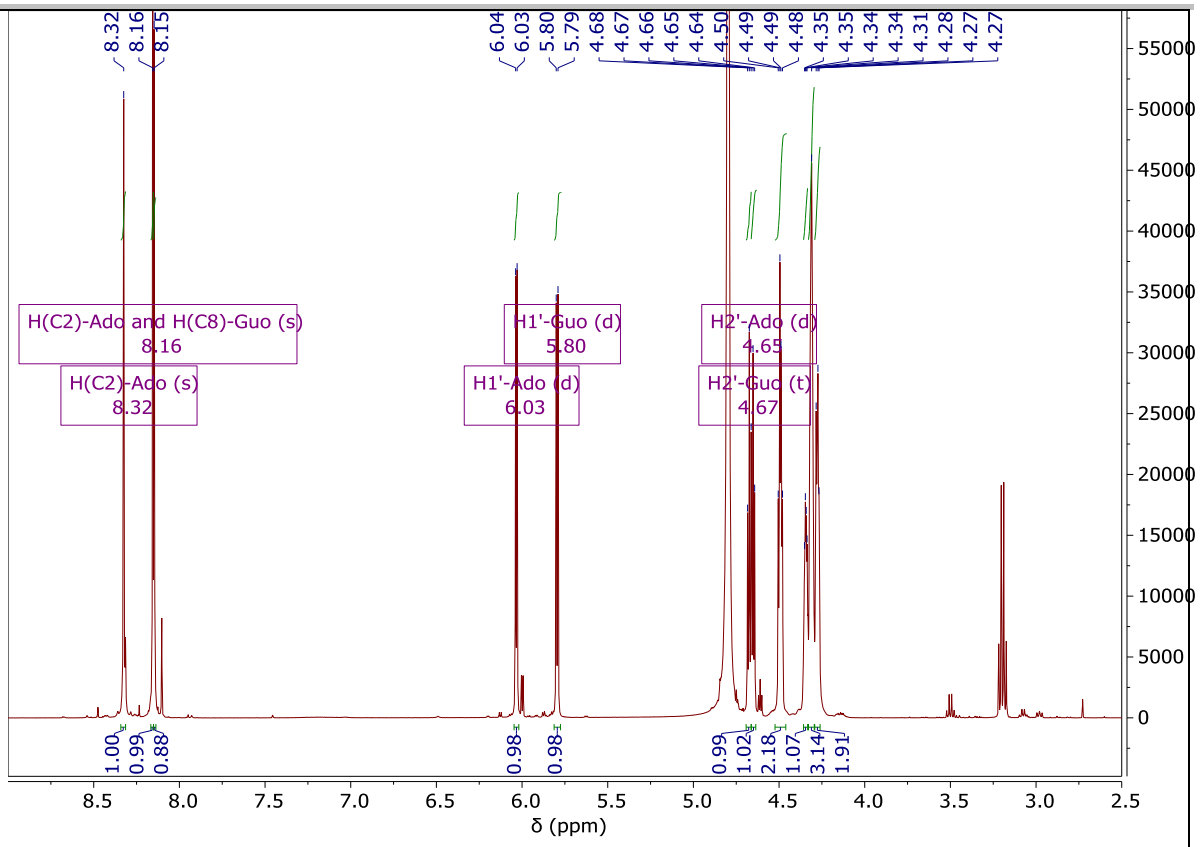
HPLC profile



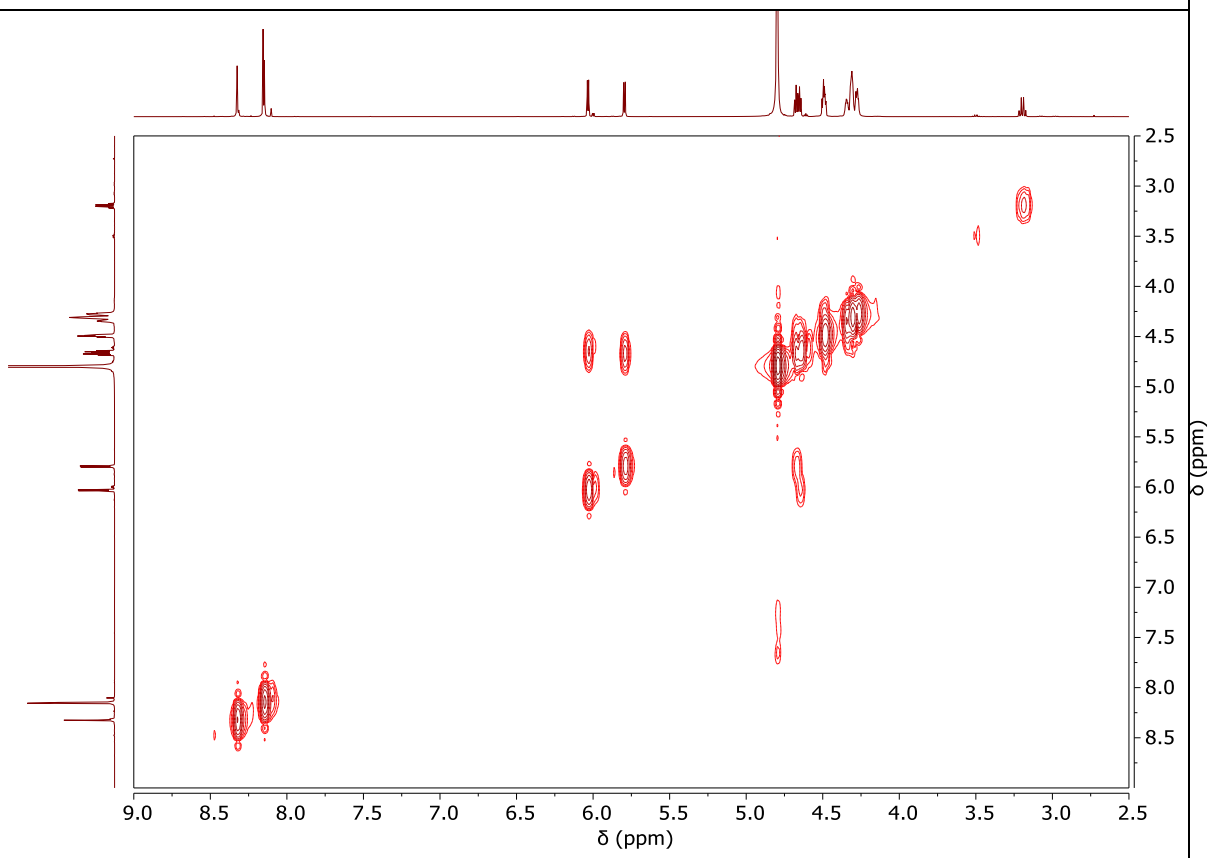
HRMS (-)ES



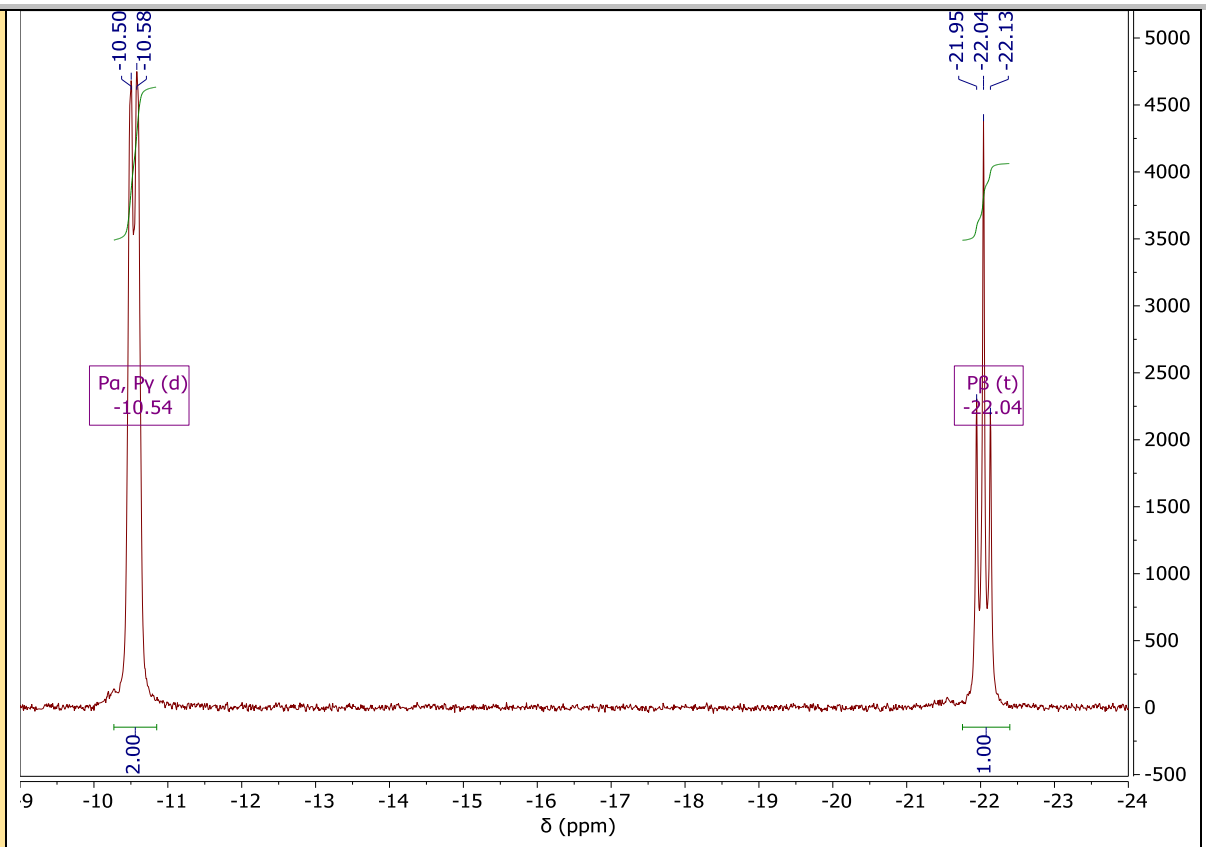
^{1}H NMR (500 MHz, D_2O, 25°C)

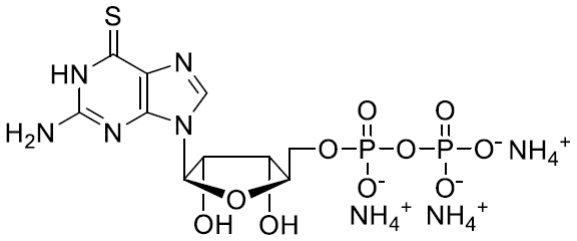
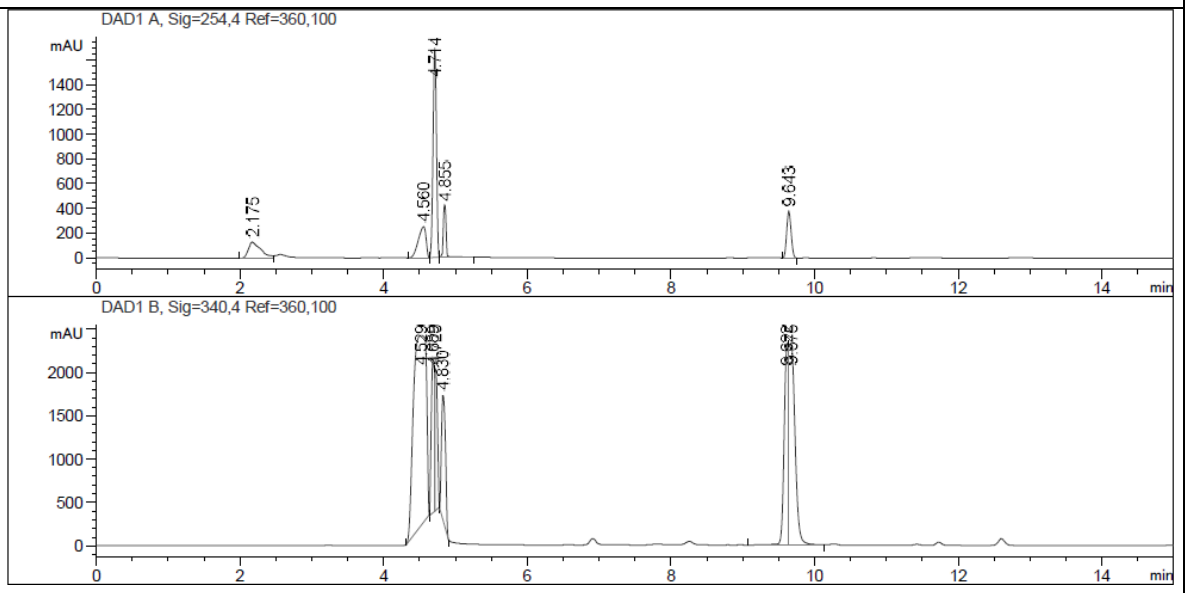
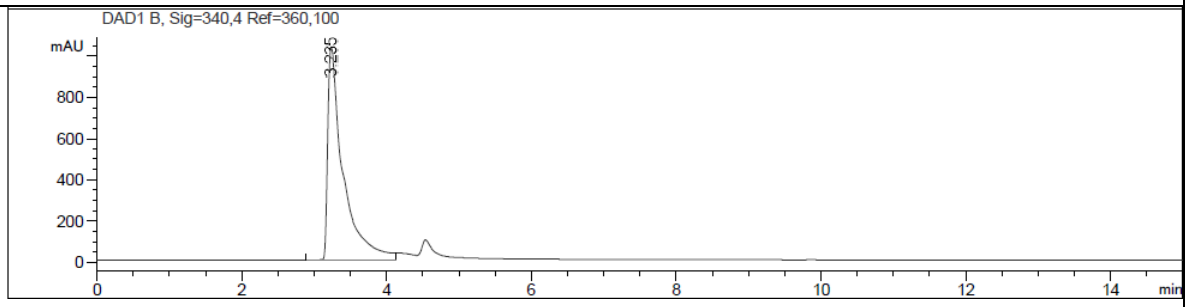


^{1}H-^{1}H COSY (500 MHz, D_2O, 25°C)

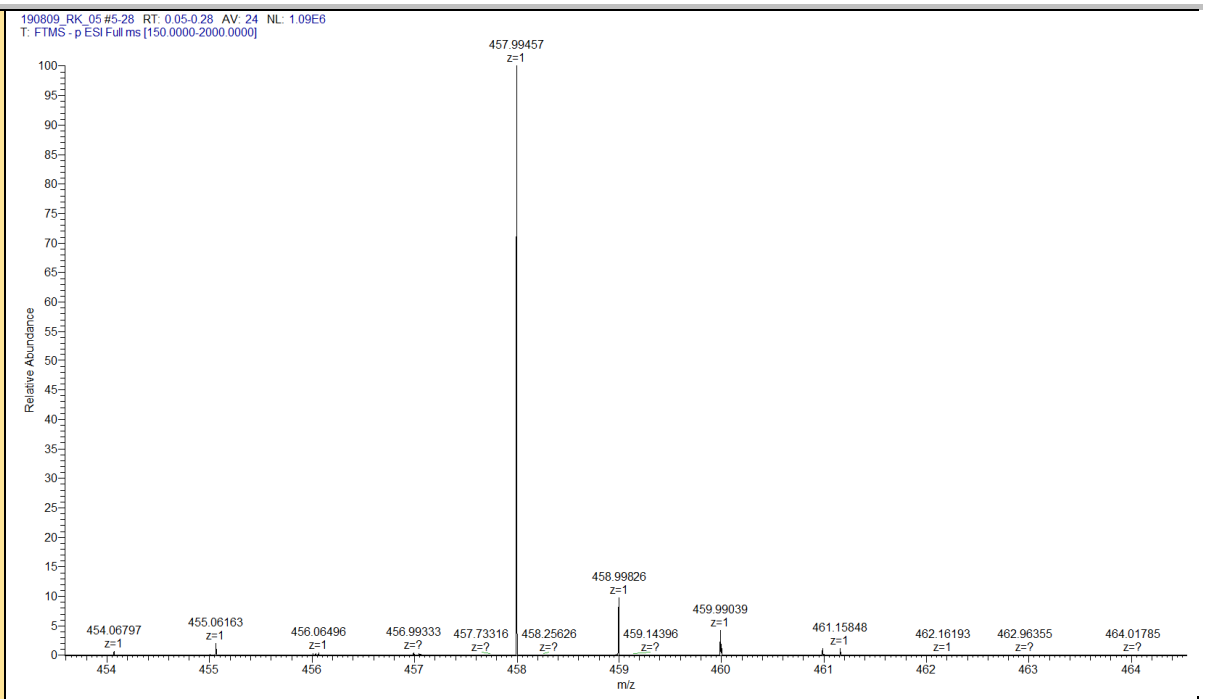


^{31}P NMR (202 MHz, D_2O, 25°C)

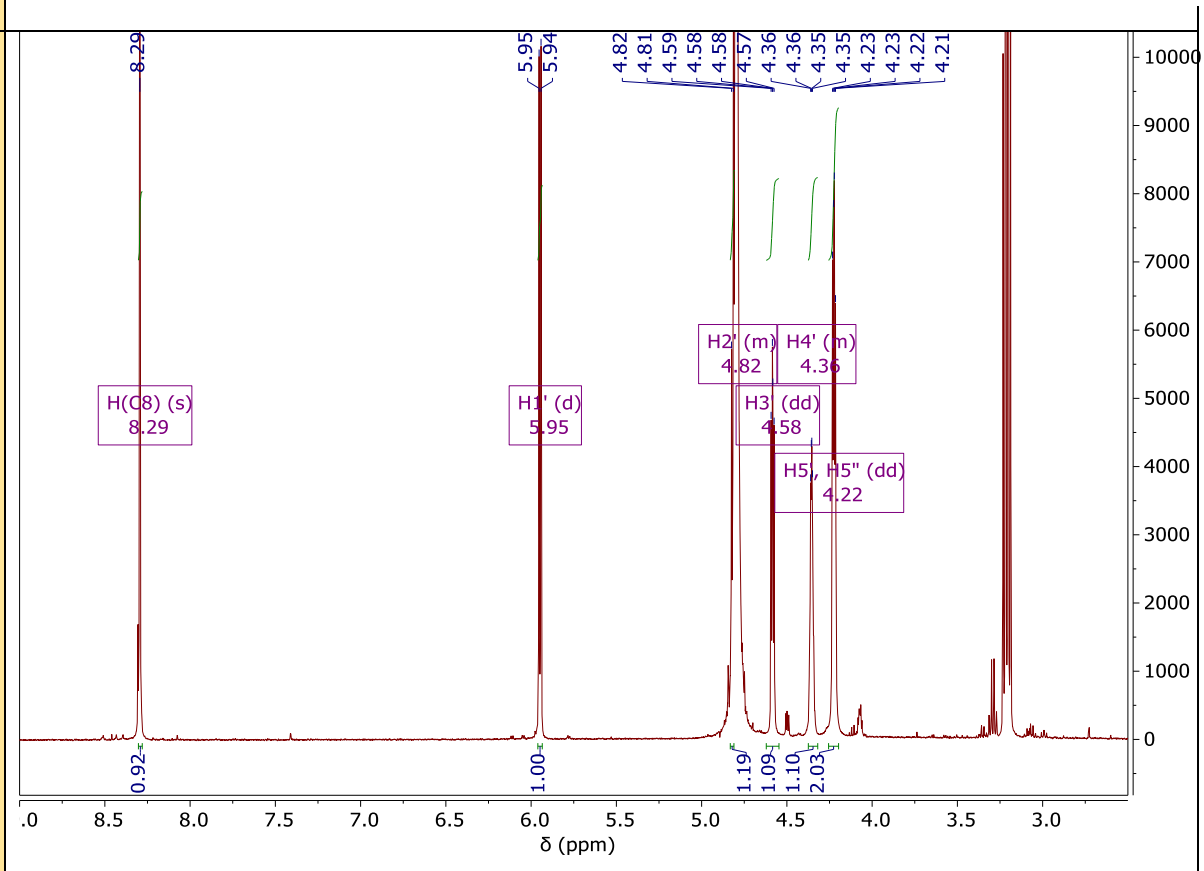


P^1-5'-(6-thio)guanosine diphosphate ammonium salt ($^{6\text{-}S}$GDP)	
Formula	
HPLC reaction profile	
HPLC profile	

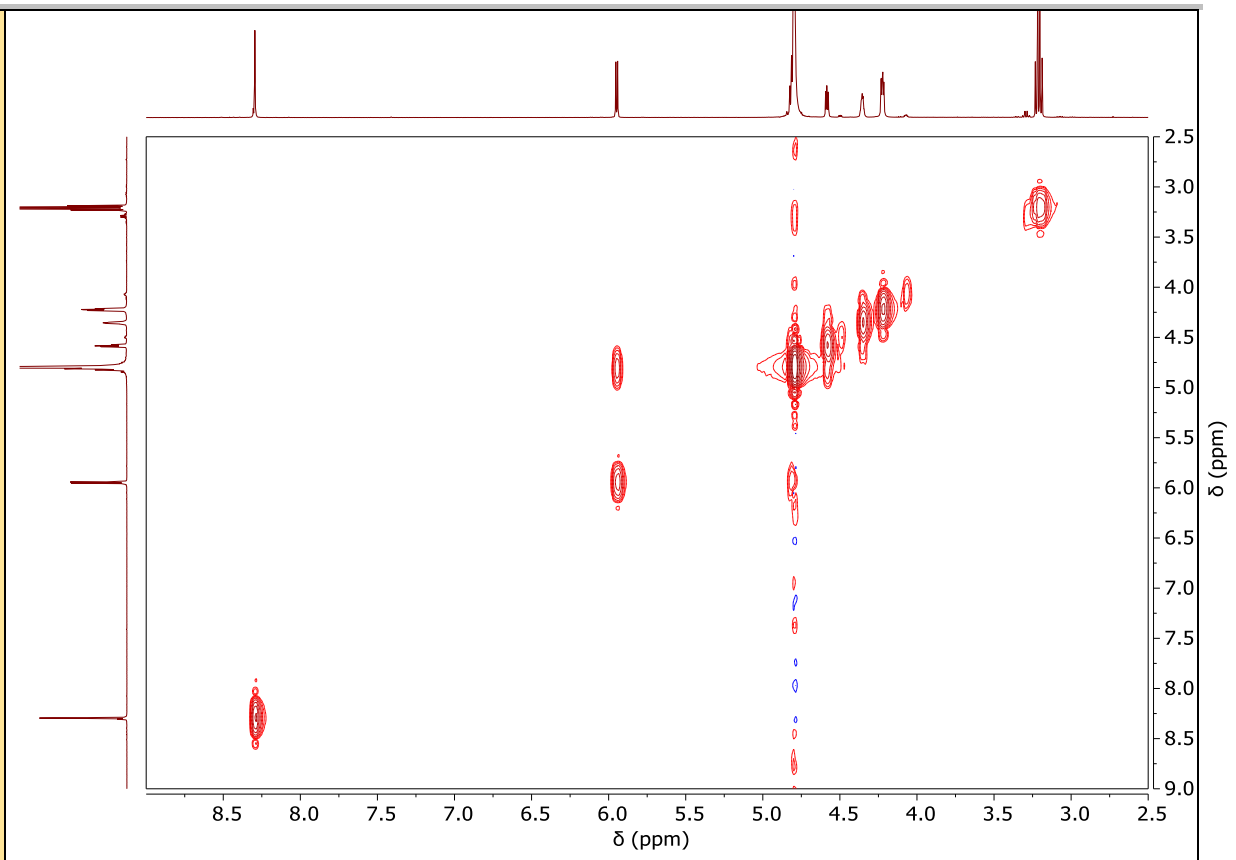
HRMS (-)ES



^{1}H NMR (500 MHz, D_2O, 25°C)



^{1}H-^{1}H COSY
(500 MHz, D_2O, 25°C)



^{31}P NMR (202 MHz, D_2O, 25°C)