

Towards superior mRNA caps accessible by click chemistry: synthesis and translational properties of triazole-bearing oligonucleotide cap analogs

Mateusz Kozarski ^{a, b}, Karolina Drazkowska^b, Marcelina Bednarczyk ^{a, b}, Marcin Warminski ^a, Jacek Jemielity ^{b*}, Joanna Kowalska ^{a*}

^aDivision of Biophysics, Institute of Experimental Physics, Faculty of Physics, University of Warsaw, Pasteura 5, 02-093 Warsaw, Poland

^bCentre of New Technologies, University of Warsaw, Banacha 2c, 02-097 Warsaw, Poland

List of contents

MATERIALS AND METHODS	2
TABLES	2
TABLE S1. HPLC METHODS USED WITH AGILENT TECHNOLOGIES SERIES 1200 OR SHIMADZU RF-20A.....	2
TABLE S2. THE AFFINITY OF CAP ANALOGS FOR EIF4E AT 20 °C DETERMINED USING FQT EXPERIMENTS.	3
TABLES S3. NMR SPECTRA, HR MS, HPLC PROFILES OF SYNTHETIZED MODIFIED NUCLEOTIDES AND CAP ANALOGUES.	3
5'-N ₃ APG 12A	3
5'-N ₃ A _M PG 12B.....	7
5'-N ₃ C ₂ H ₄ PA _M PG 12C.....	10
M ⁷ GPPP-TR-APG 1	12
M ⁷ GPPP-TR-A _M PG 2	15
M ⁷ GPPP-CH ₂ -TR-APG 3.....	17
M ⁷ GPPP-CH ₂ -TR-A _M PG 4	20
M ⁷ GPPP-C ₂ H ₄ -APG 5	22
M ⁷ GPPP-C ₂ H ₄ -A _M PG 6	25
M ⁷ GPPPPOCH ₂ -TR-APG 7	28
M ⁷ GPPPPOCH ₂ -TR-A _M PG 8.....	31
M ⁷ GPPP-TR-C ₂ H ₄ PA _M PG 9	34
M ⁷ GPPPG-CARB-CH ₂ -TR-APG (BOTH ISOMERS) 10A AND 10B.....	37
2'-OME-3'-OC ₃ H ₃ -ADENOSINE	42
5'-MONOPHOSPHATE 2'-OME-3'-OC ₃ H ₃ ADENOSINE (2'-OME-3'-OC ₃ H ₃ AMP)	44
M ⁷ GPPPA-2'-OME-3'-OC ₃ H ₃	47
M ⁷ GPPPA _M -3'-OCH ₂ -TR-G 11.....	50
FIGURES	53
FIGURE S1. MRNA PURITY	53
FIGURE S2. LUCIFERASE ACTIVITY IN RABBIT RETICULOCYTE LYSATE	55
FIGURE S3. SUSCEPTIBILITY TO DEGRADATION BY HDCP1/DCP2 COMPLEX.	55

Materials and methods

Solvents, chemical reagents, and starting materials were purchased from commercial sources. Phosphoramidites were purchased from ChemGenes (2'-O-PivOM-5'-DMT-A (N-PAC) 3'-CEP) and Link Technologies (2'-OMe-Bz-A-CE). Solid support for solid phase synthesis was purchased from GE Healthcare (Primer Support™ 5G ribo G 300 (309 μmol ribo G/g). C- phosphonate nucleotide analogues were obtained according to the previously described procedures (Walczak et al.). ¹²-Cyanoethyl 2-bromoethyl N, N-diisopropylamino Phosphite was synthesized as previously described (Hebert et al.). ² The analytical analysis and purifications were performed using Agilent Technologies Series 1200 with UV detection at 254 nm wavelength and fluorescent detection (ex, 260 nm; em. 370 nm) and Shimadzu RF-20A. For chemical reaction analysis the Gemini 3 μm NX-C18 110 Å 150 x 4.6 mm column was used with linear gradient of methanol in 0.05 M ammonium buffer, pH 5.9. For purification the Gemini 5 μm NX-C18 110 Å 150 x 10 mm column (column A) or HiChrom Vydac Denali 5 μm C18 120 Å 150 x 10 mm (column B) or Dr. Maisch VisionHT C18 Highload 25 cm x 2 cm, 10 μm (column C) were used with linear gradient of acetonitrile in 0.05 M ammonium buffer, pH 5.9. Additionally, for purification, ion-exchange chromatography on DEAD Sephadex resin A-25 (HCO₃⁻ form) was used. To confirm the purity and structure of intermediate and final products high resolution mass spectrometry (HRMS) and nuclear magnetic resonance (NMR) were used. Mass spectra was recorded using Thermo Scientific LTQ Orbitrap Velos spectrometer, with the negative electrospray ionization HRMS (-) ESI. NMR spectra were recorded using a Bruker AVANCE III HD 500 MHz spectrometer equipped with a high-stability temperature unit with 5 mm PAB- BOBB/19F-1H/D Z-GRD Z119470/0188 probe at 500.24MHz (¹H NMR) and 202.49 MHz (³¹P NMR) or Varian 400 MHz with OneNMR_W016 probe at 399.90 MHz (1H NMR) and 161.89 MHz (³¹P NMR). The ¹H NMR and ³¹P NMR chemical shifts (δH and δP) were reported in ppm and referenced to the respective standards: sodium 3-(trimethylsilyl)-2,2,3,3-tetradeuteropropionate (TSP, internal standard) and 20% phosphoric acid in D₂O (external standard).

Tables

Table S1. HPLC methods used with Agilent Technologies Series 1200 or Shimadzu RF-20A

Analytical methods			
Method	Buffer A	Buffer B	Gradient
A	0.05 M ammonium buffer, pH 5.9	0.05 M ammonium buffer, pH 5.9/MeOH (v/v, 1/1)	Linear gradient 0%- 50% of buffer B; 15 min; 1ml/min
B	0.05 M ammonium buffer, pH 5.9	0.05 M ammonium buffer, pH 5.9/MeOH (v/v, 1/1)	Linear gradient 0%- 100% of buffer B; 15 min; 1ml/min
C	0.05 M ammonium	0.05 M ammonium buffer,	Linear gradient 0%- 50% of buffer B; 7.5 min; 1ml/min

	buffer, pH 5.9	pH 5.9/MeOH (v/v, 1/1)	
Preparative methods			
D	0.05 M ammonium buffer, pH 5.9	0.05 M ammonium buffer, pH 5.9/Acetonitrile (30%)	Linear gradient 0%- 50% of buffer B; 60 min; 4.7 ml/min

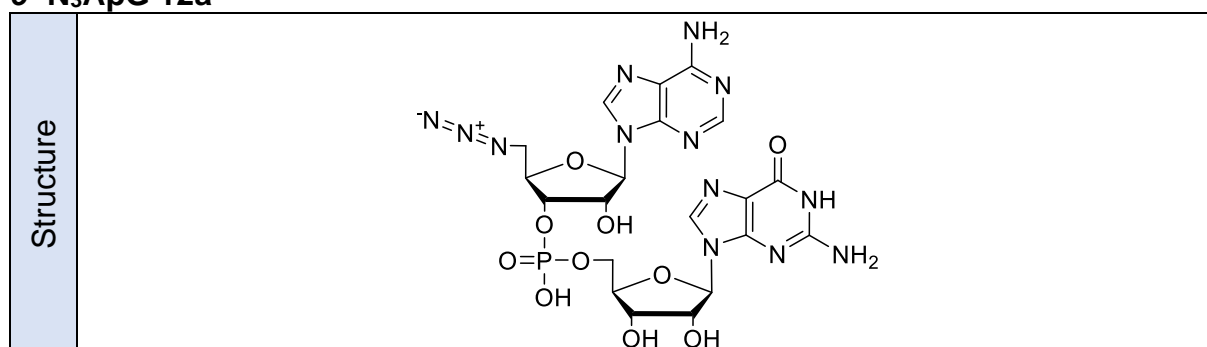
Table S2. The affinity of cap analogs for eIF4E at 20 °C determined using FQT experiments.

The experiments were performed in triplicate. The values in table are presented as weighted averages.

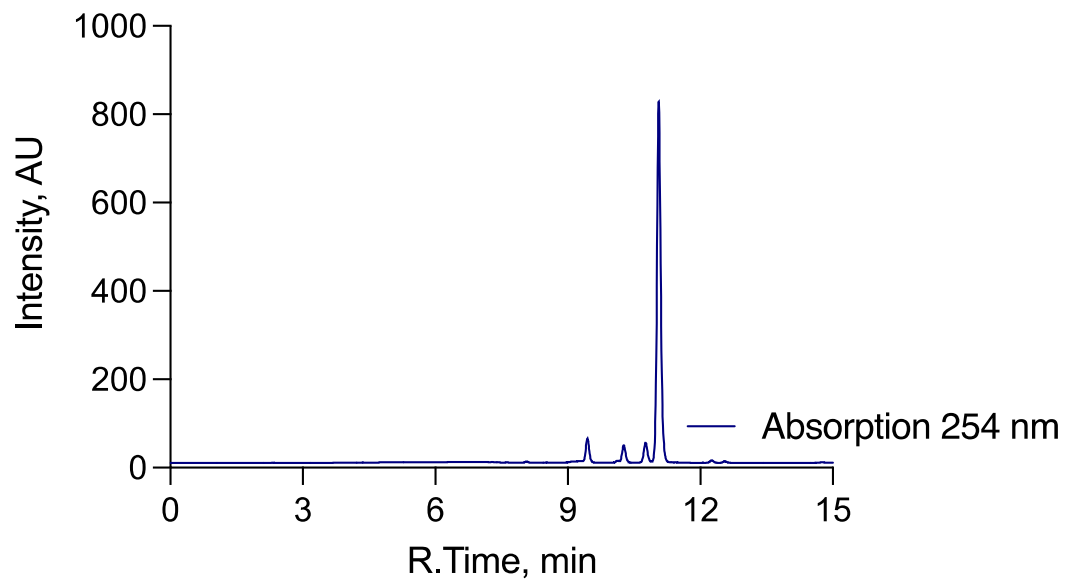
Cap No.	K_{As} (μM^{-1})
m ⁷ GpppA _m pG	29.6 ± 2.3
2	23.8 ± 3.6
4	29.7 ± 6.0
6	30.7 ± 6.9
8	20.3 ± 3.4
9	136.1 ± 13.2
10b	35.3 ± 8.0

Tables S3. NMR spectra, HR MS, HPLC profiles of synthesized modified nucleotides and cap analogues.

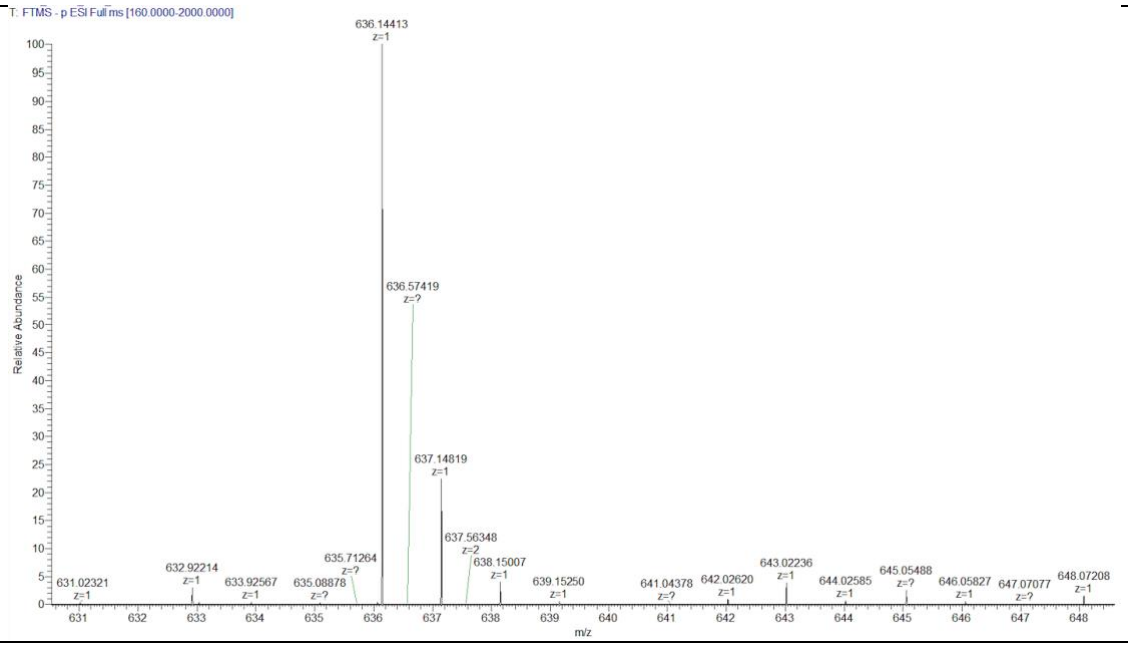
5'-N₃ApG 12a



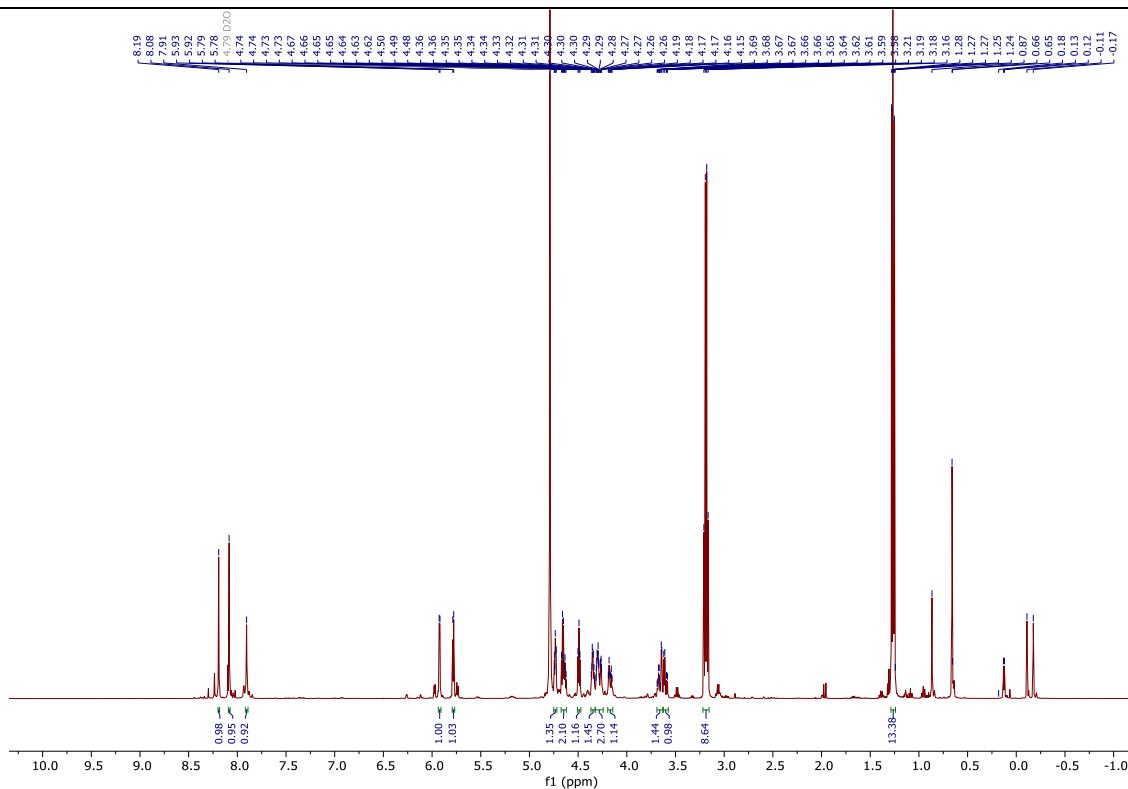
HPLC Profile



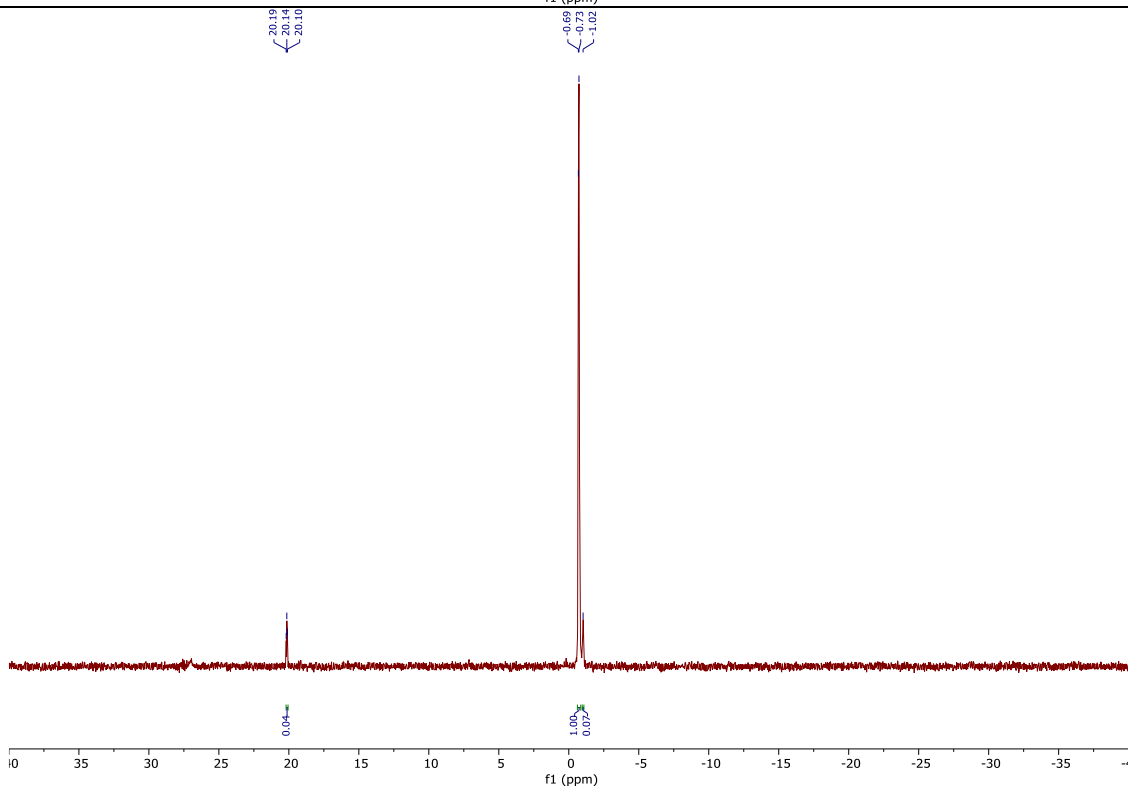
HR MS



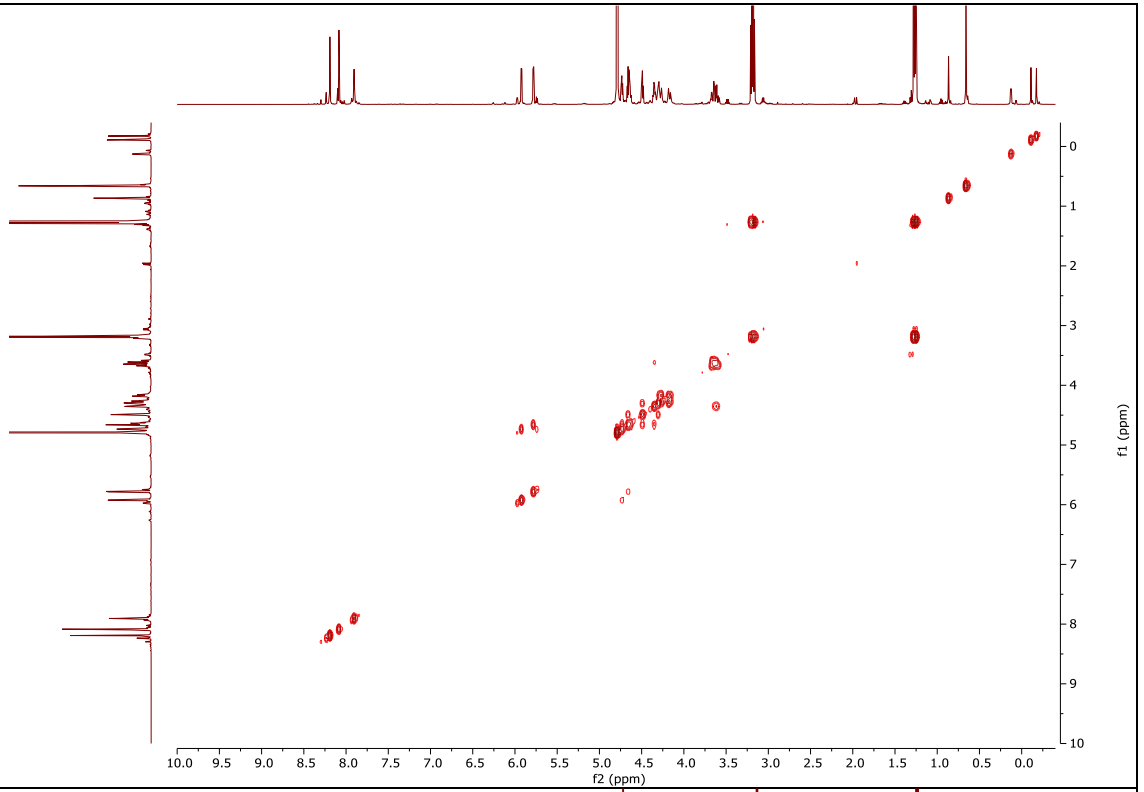
¹H NMR



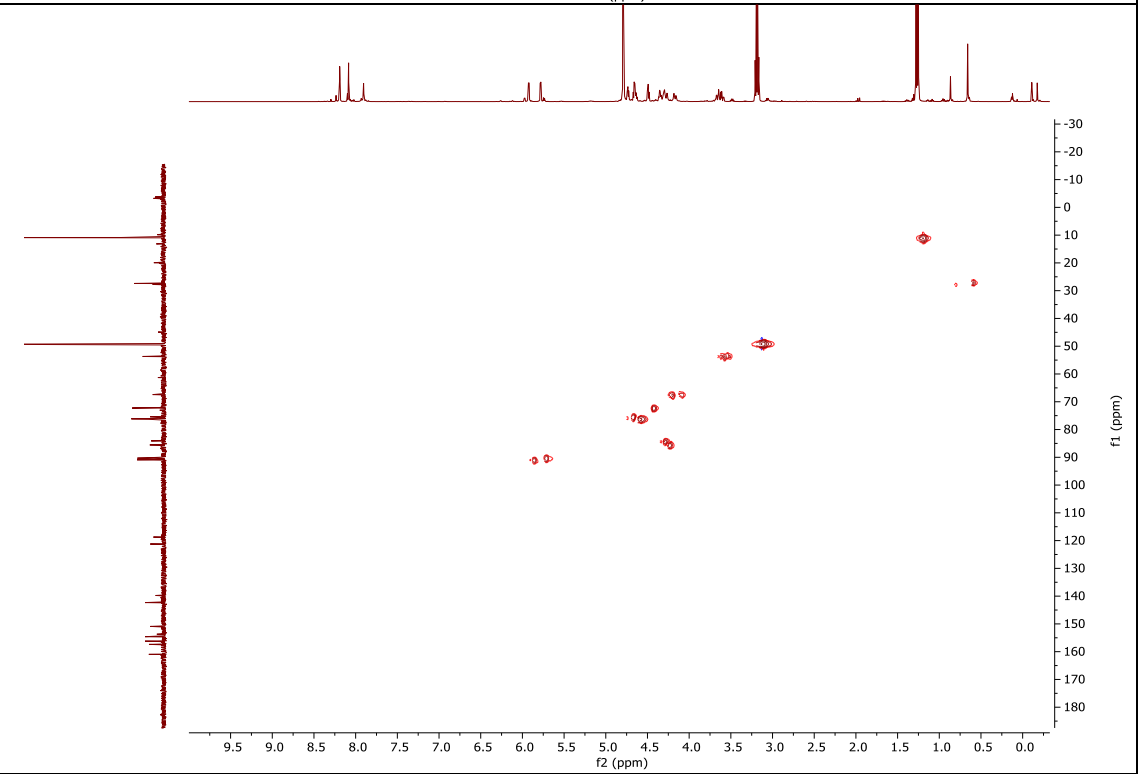
³¹P NMR



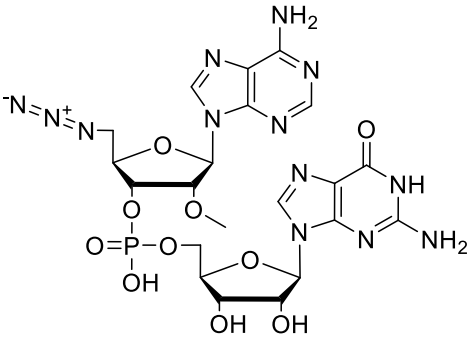
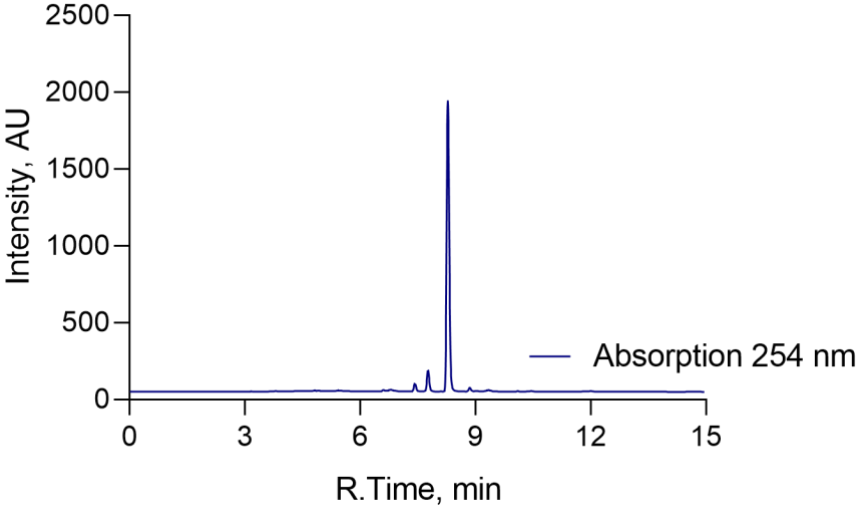
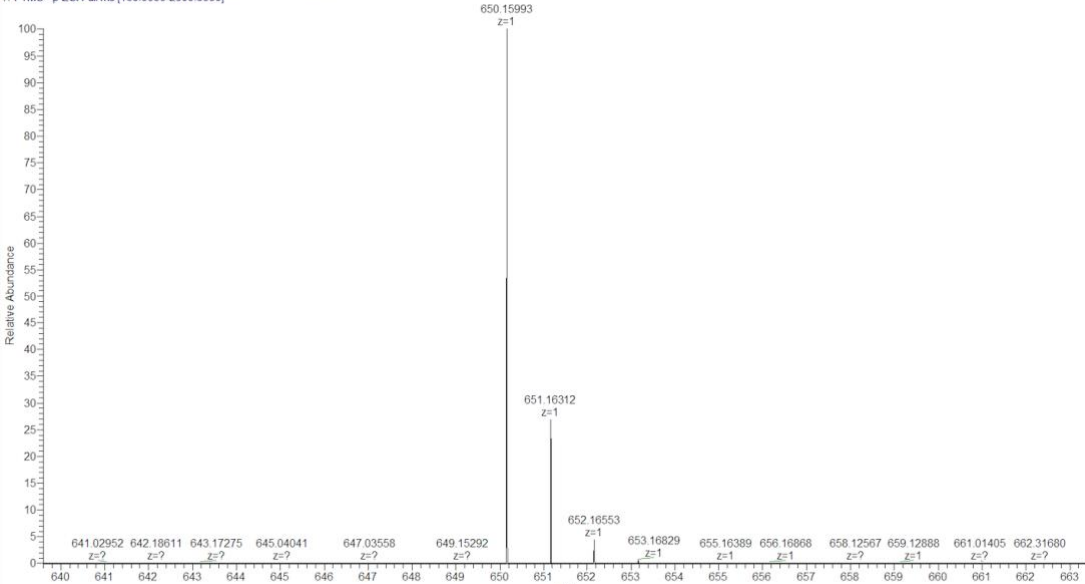
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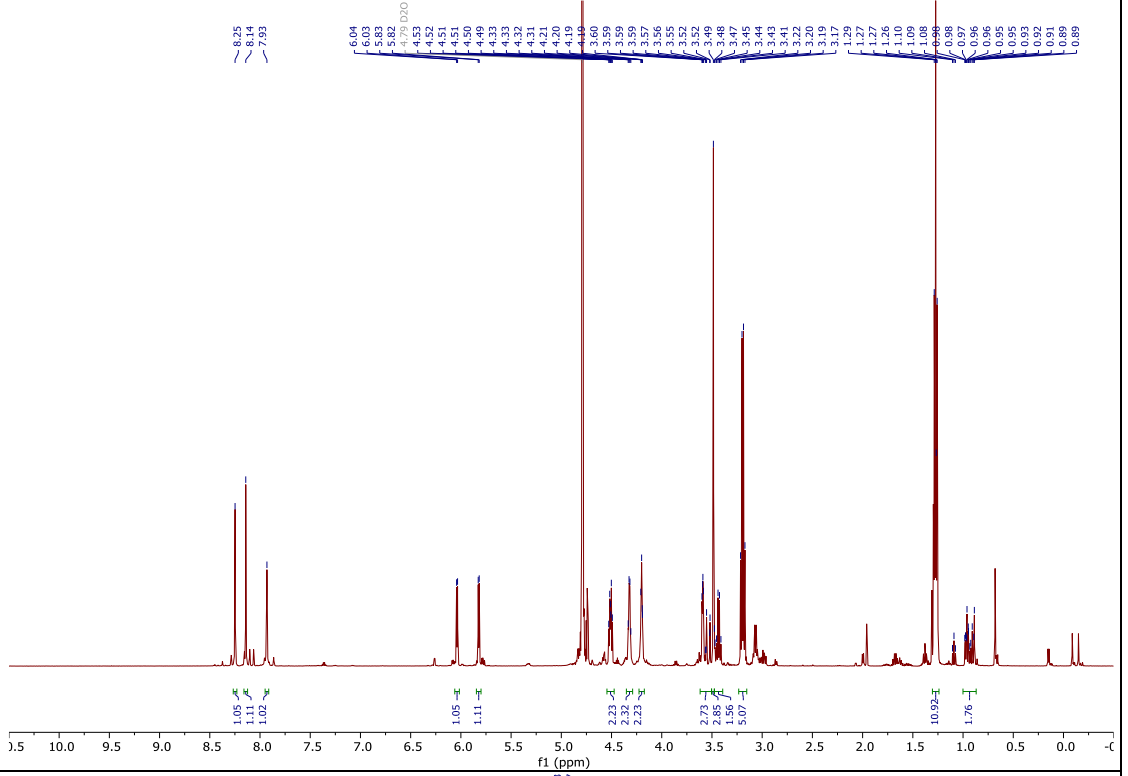
¹H-¹³C HSQC NMR



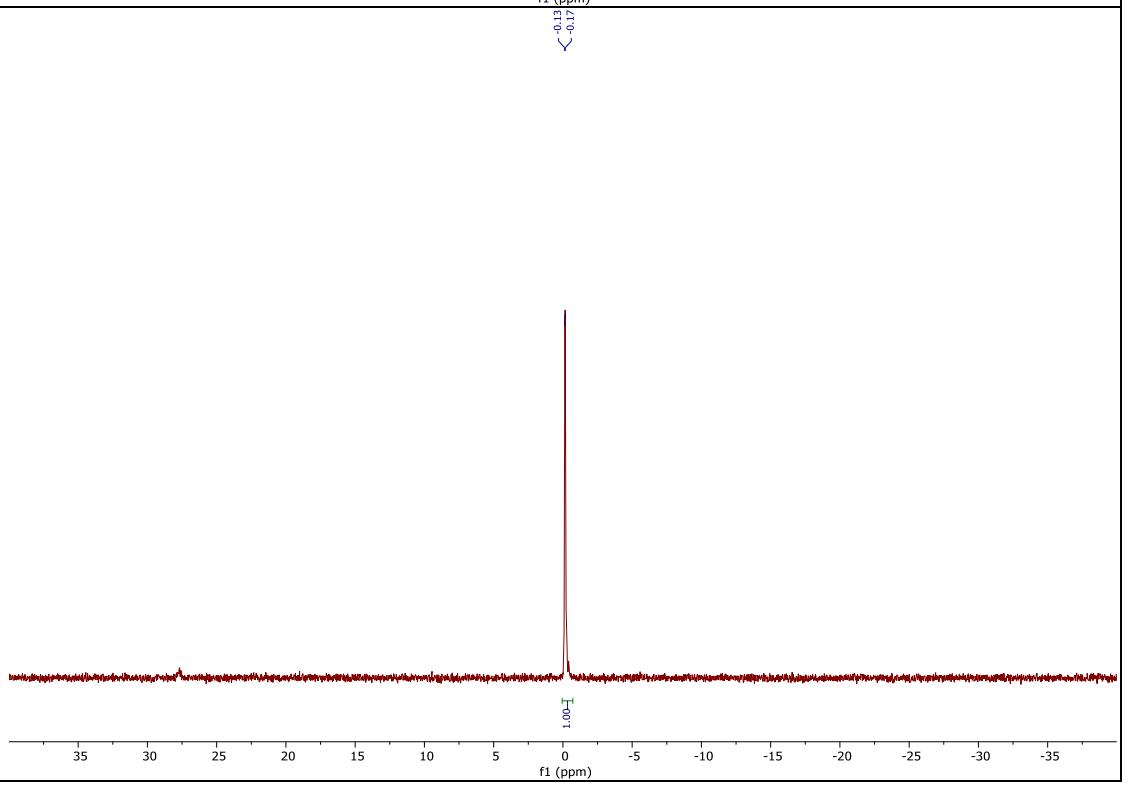
5'-N₃AmpG 12b

Structure	 <p>The chemical structure shows a nucleotide derivative. It features a ribose sugar with a phosphate group at the 5' position and an adenine base at the 1' position. The adenine base is substituted with an azide group (-N₃) at the 6-position. The ribose sugar is also substituted with a phosphate group at the 3' position, which is linked to another ribose sugar. This second ribose sugar is substituted with a phosphate group at the 5' position and a guanine base at the 1' position. The guanine base is substituted with an amino group (-NH₂) at the 2-position.</p>																																		
HPLC Profile	 <p>The HPLC profile shows a single sharp peak at approximately 8.5 minutes. The y-axis is labeled 'Intensity, AU' and ranges from 0 to 2500. The x-axis is labeled 'R.Time, min' and ranges from 0 to 15. A legend indicates 'Absorption 254 nm'.</p>																																		
HR MS	 <p>The HR MS spectrum shows relative abundance versus m/z. The y-axis is labeled 'Relative Abundance' and ranges from 0 to 100. The x-axis is labeled 'm/z' and ranges from 640 to 683. The base peak is at m/z 650.15993 (z=1). Other significant peaks are labeled with their m/z values and charge states (z).</p> <table border="1"><thead><tr><th>m/z</th><th>z</th></tr></thead><tbody><tr><td>641.02952</td><td>z=?</td></tr><tr><td>642.18611</td><td>z=?</td></tr><tr><td>643.17275</td><td>z=?</td></tr><tr><td>645.04041</td><td>z=?</td></tr><tr><td>647.03558</td><td>z=?</td></tr><tr><td>649.15292</td><td>z=?</td></tr><tr><td>650.15993</td><td>z=1</td></tr><tr><td>651.16312</td><td>z=1</td></tr><tr><td>652.16553</td><td>z=1</td></tr><tr><td>653.16829</td><td>z=1</td></tr><tr><td>655.16389</td><td>z=1</td></tr><tr><td>656.16868</td><td>z=1</td></tr><tr><td>658.12567</td><td>z=?</td></tr><tr><td>659.12888</td><td>z=1</td></tr><tr><td>661.01405</td><td>z=?</td></tr><tr><td>662.31680</td><td>z=?</td></tr></tbody></table>	m/z	z	641.02952	z=?	642.18611	z=?	643.17275	z=?	645.04041	z=?	647.03558	z=?	649.15292	z=?	650.15993	z=1	651.16312	z=1	652.16553	z=1	653.16829	z=1	655.16389	z=1	656.16868	z=1	658.12567	z=?	659.12888	z=1	661.01405	z=?	662.31680	z=?
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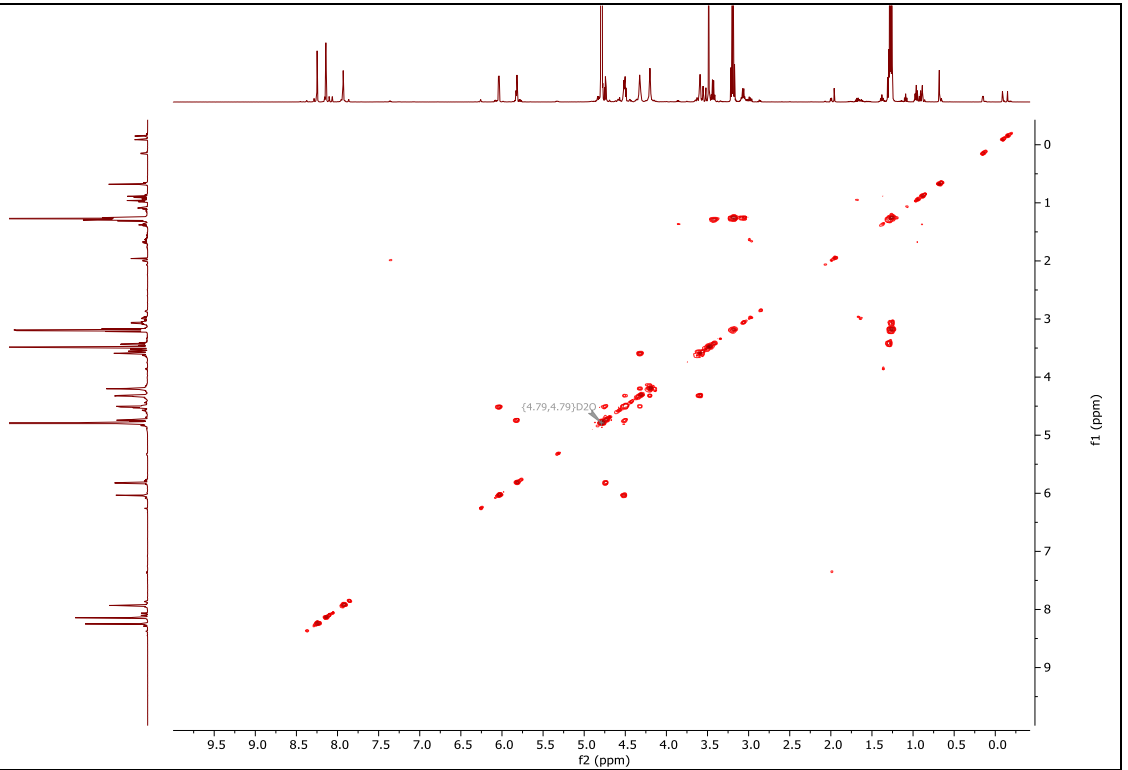
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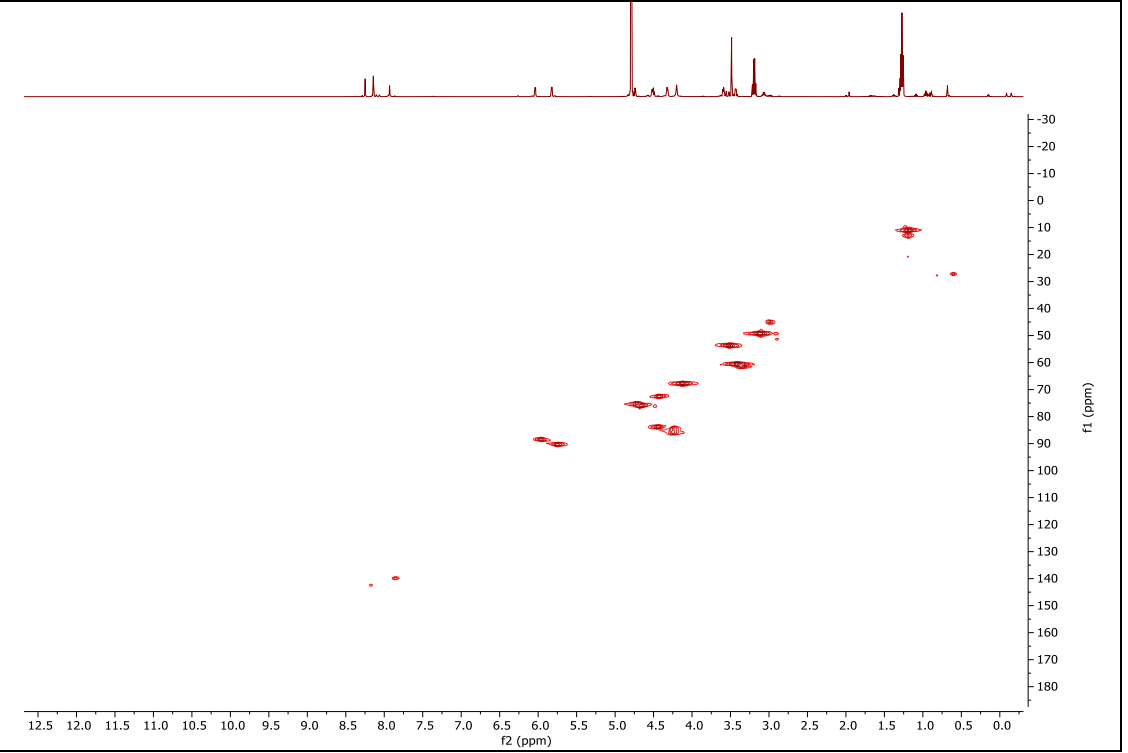
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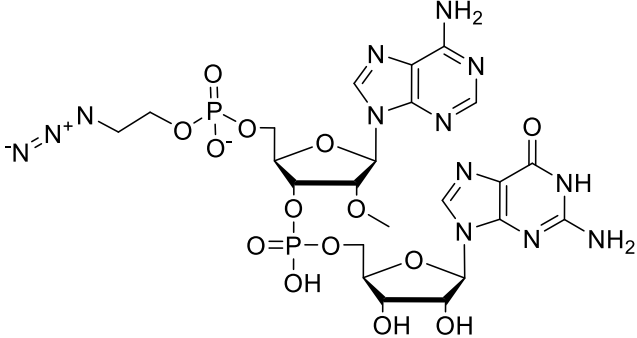
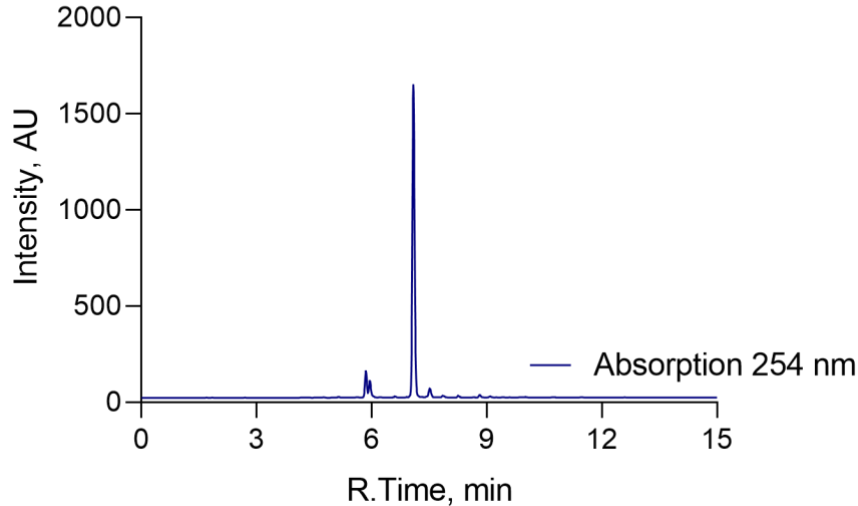
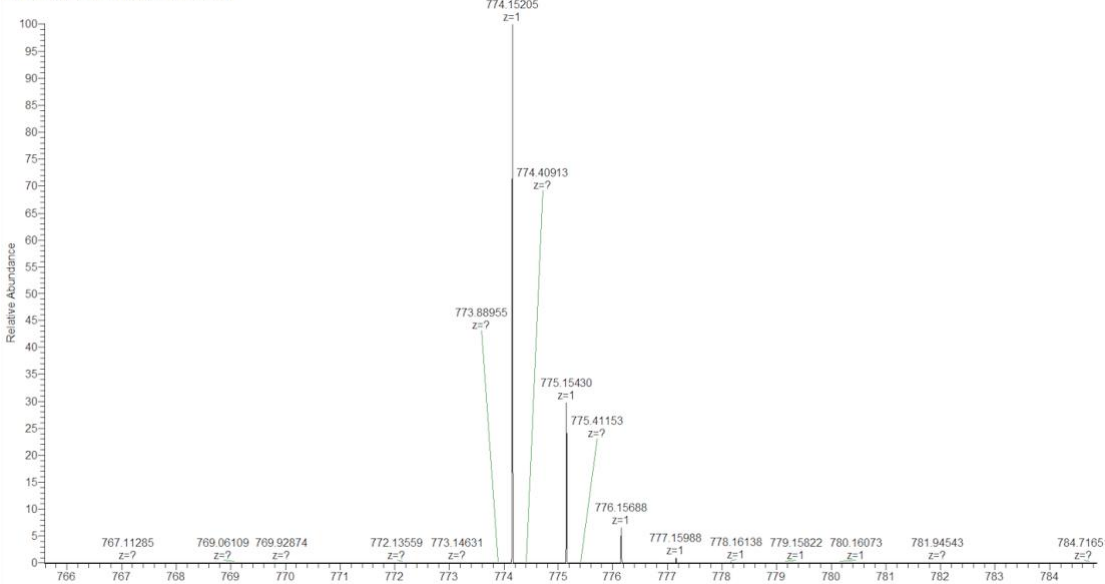
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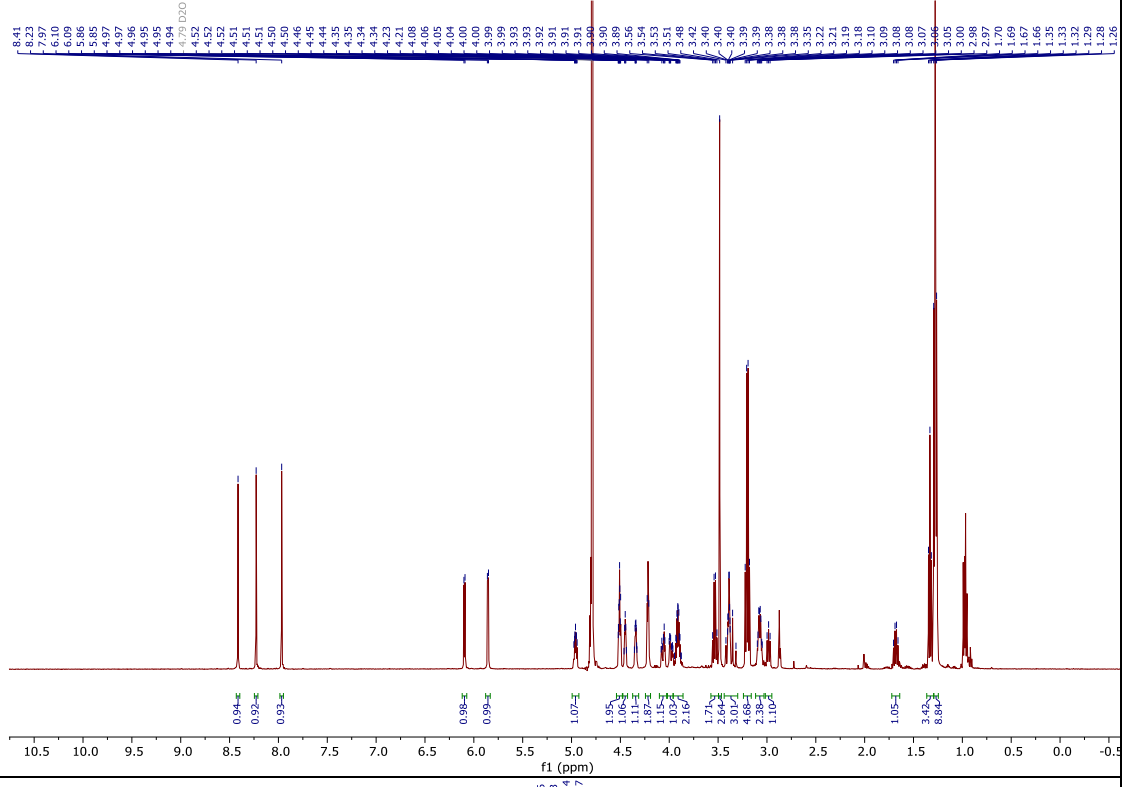
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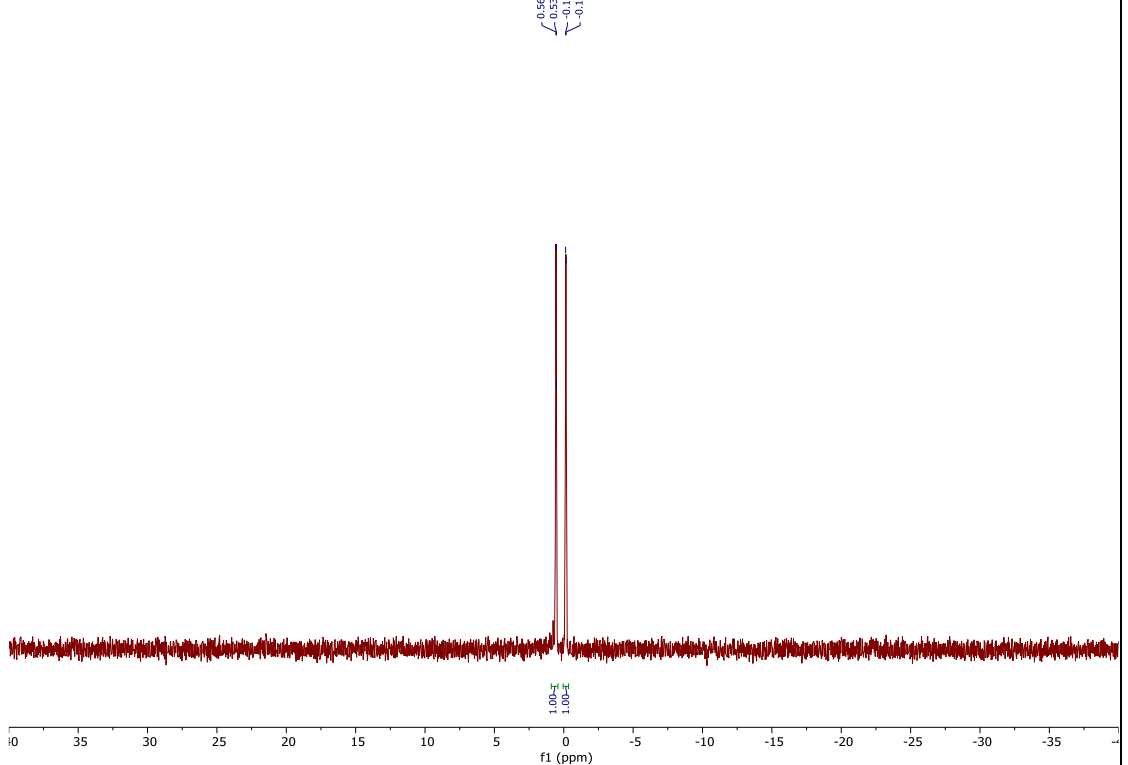
5'-N₃C₂H₄pAmpG 12c

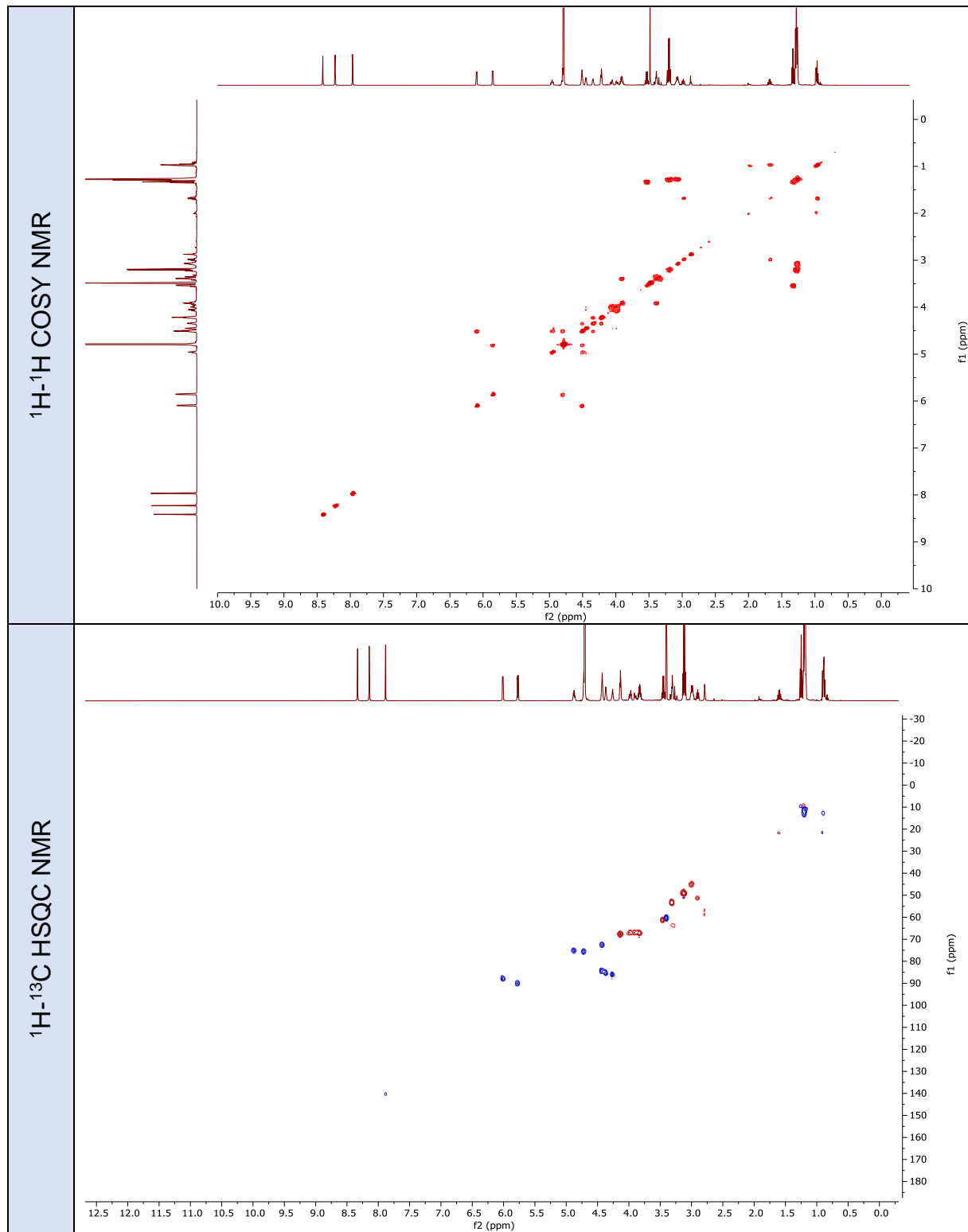
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HPLC Profile	 <p>Intensity, AU</p> <p>R.Time, min</p> <p>Absorption 254 nm</p>																																				
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¹H NMR

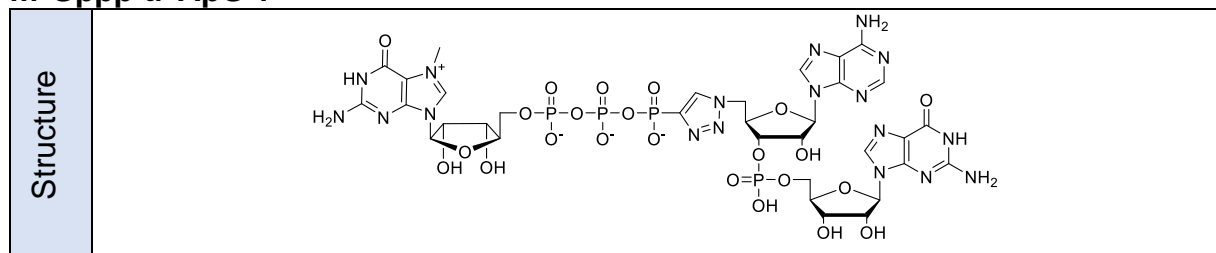


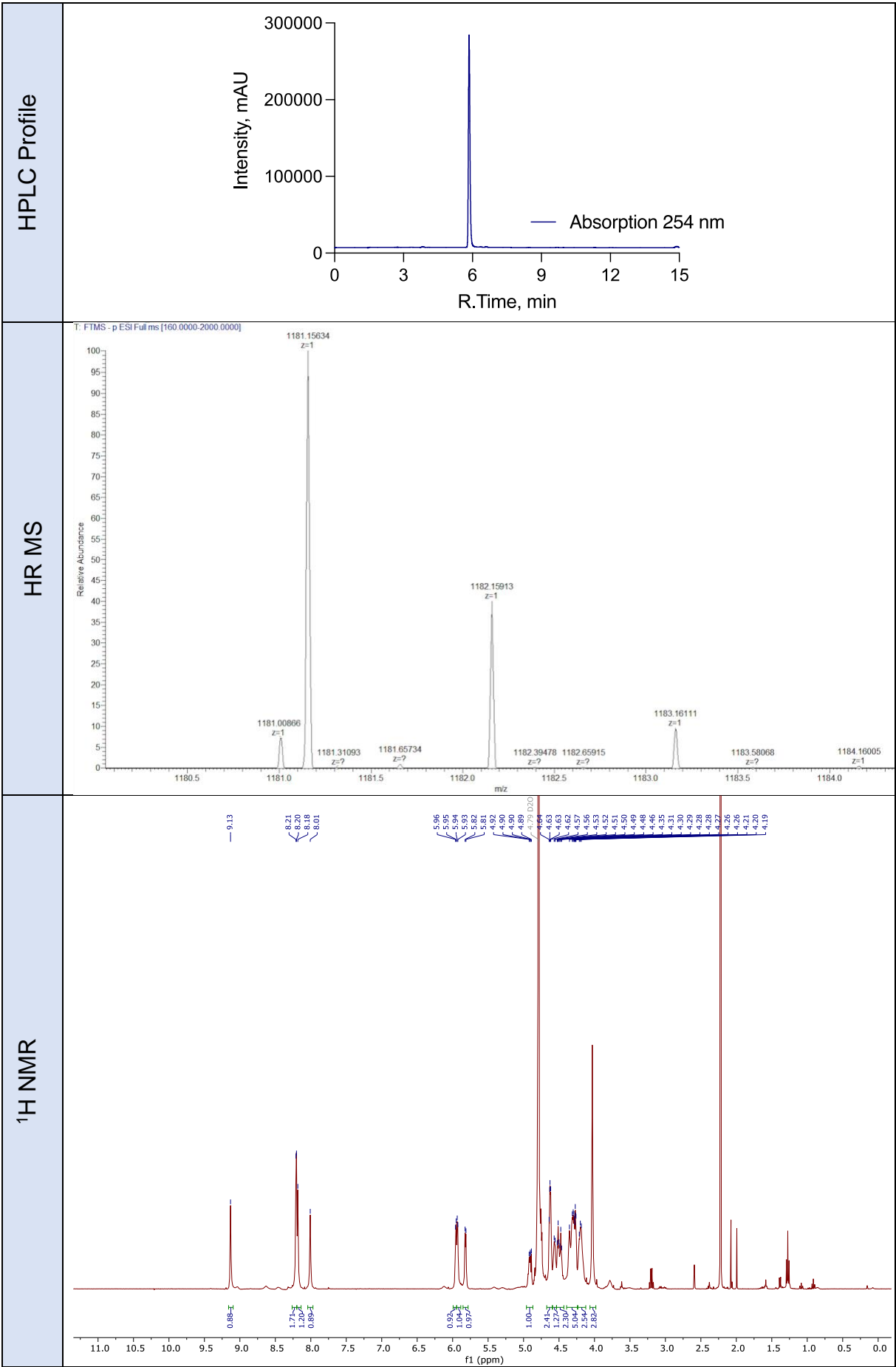
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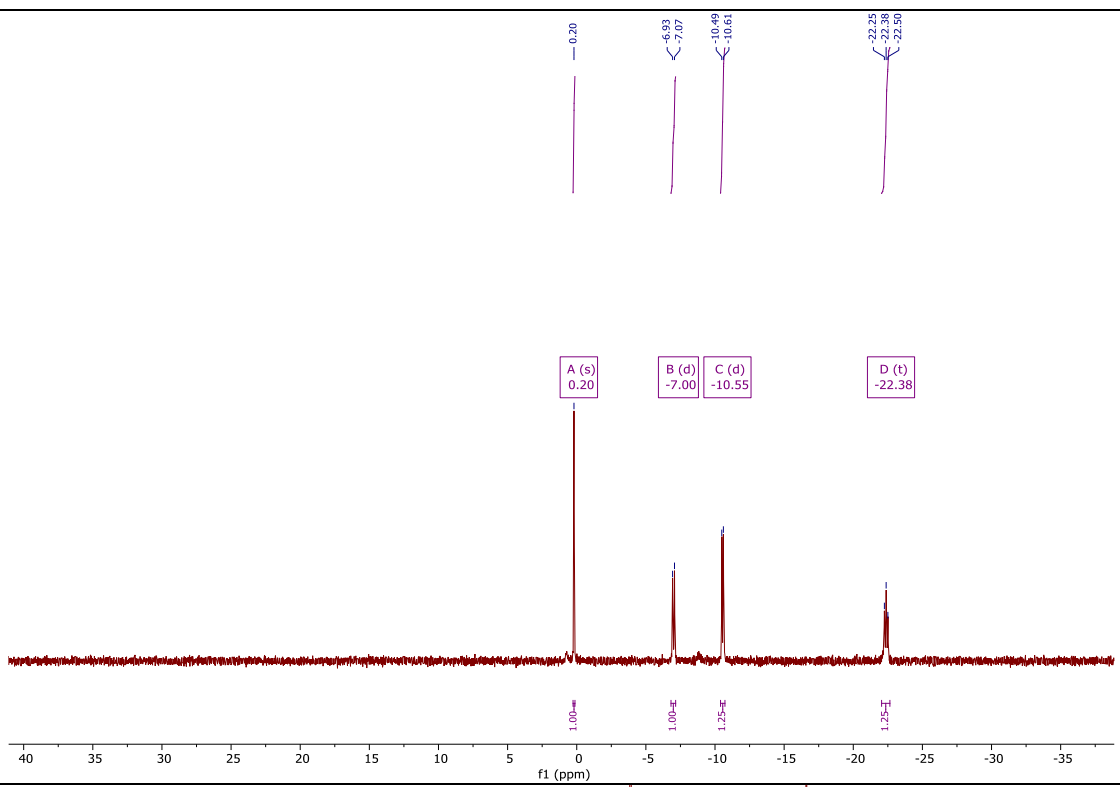


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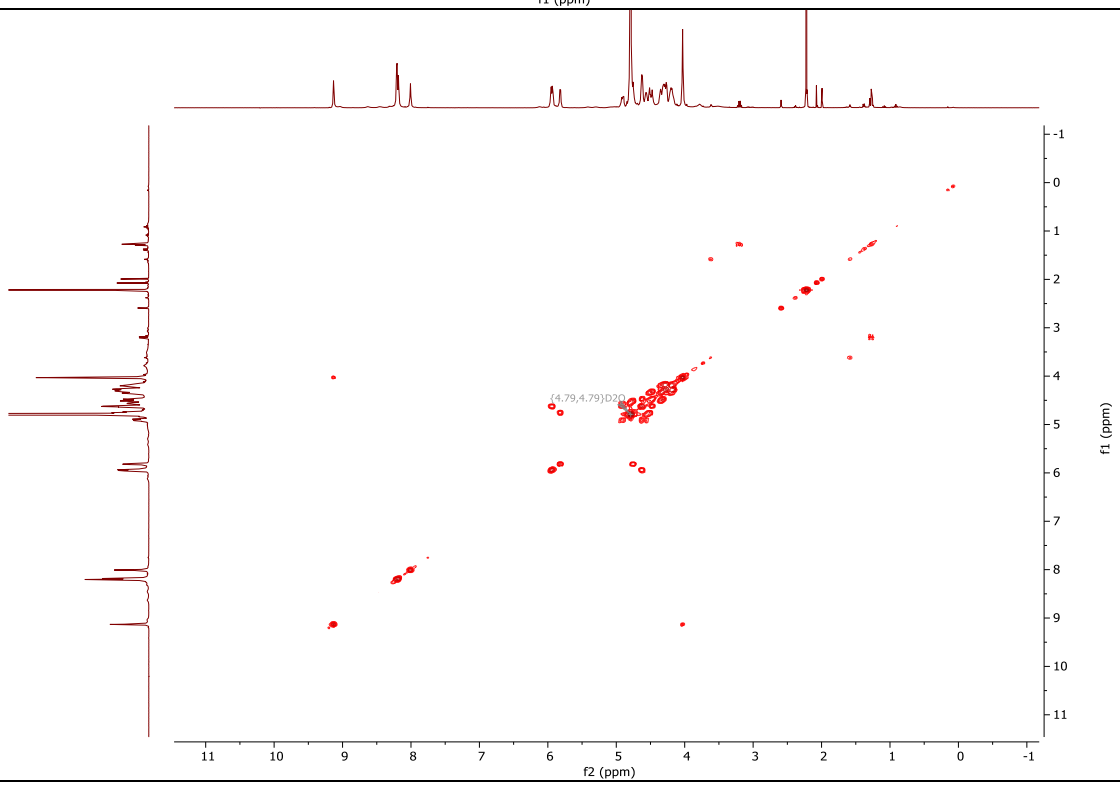




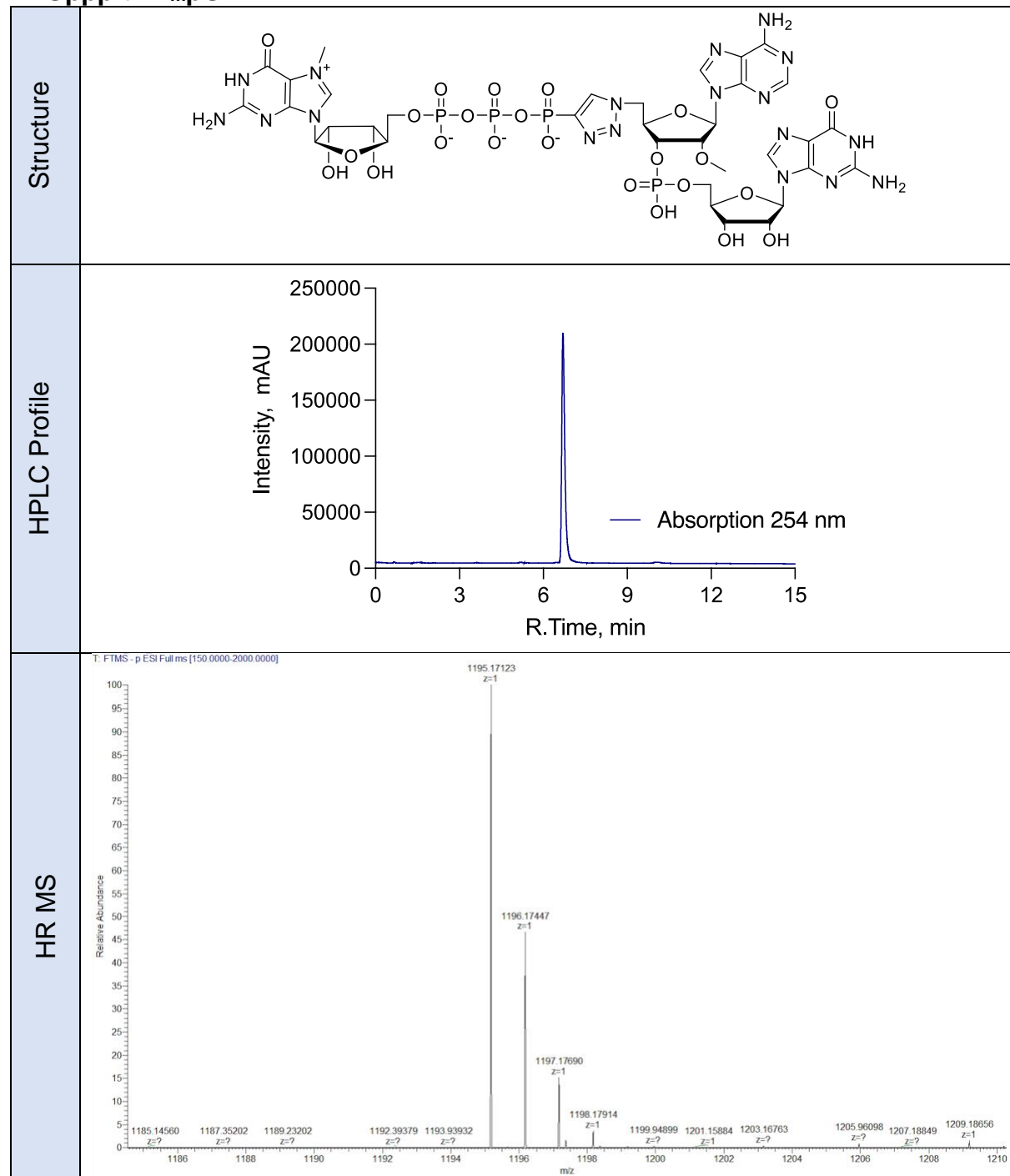
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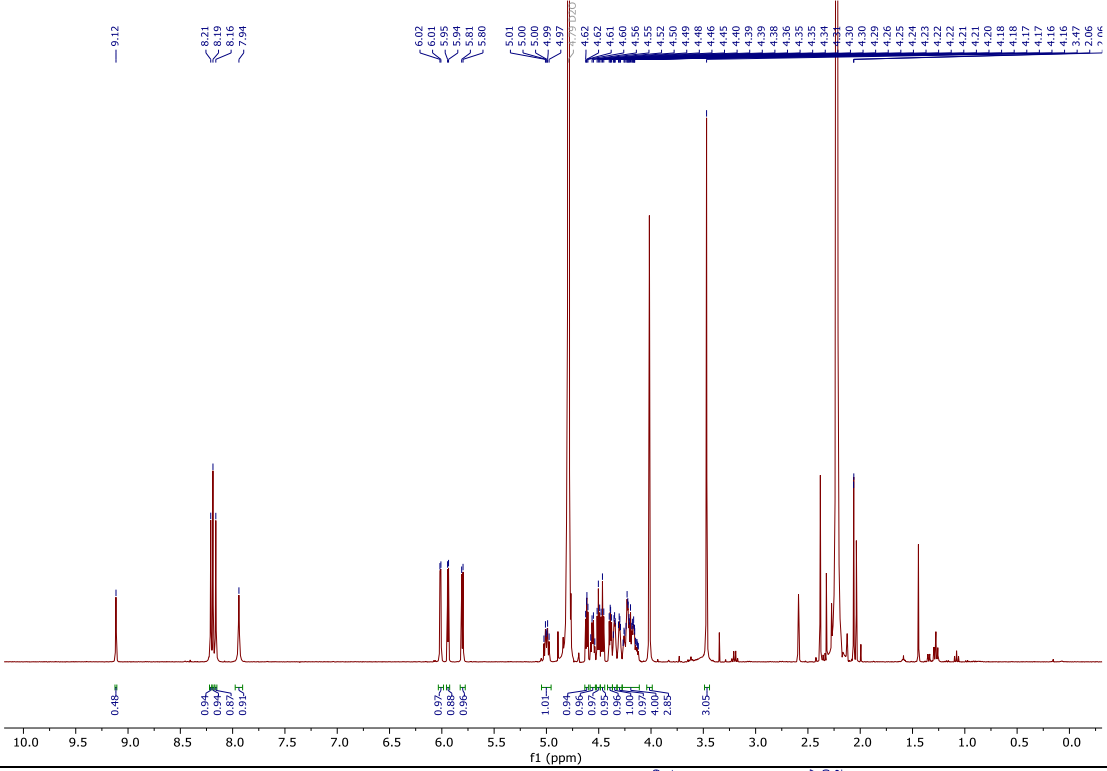
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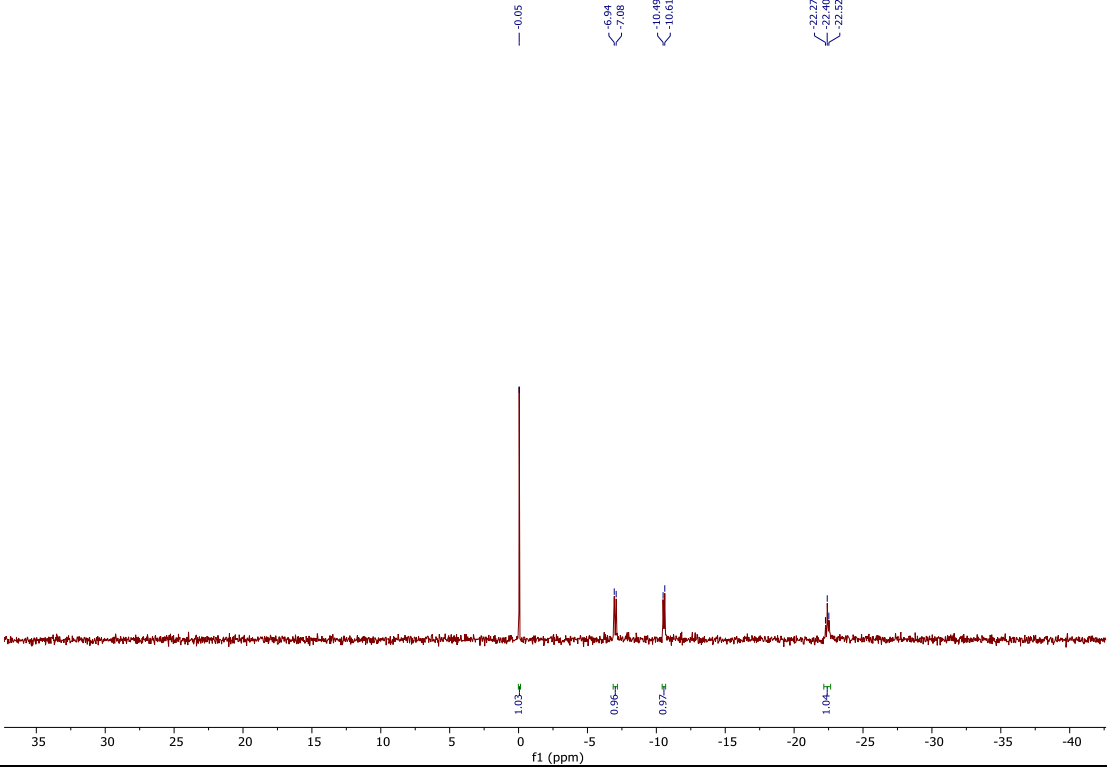
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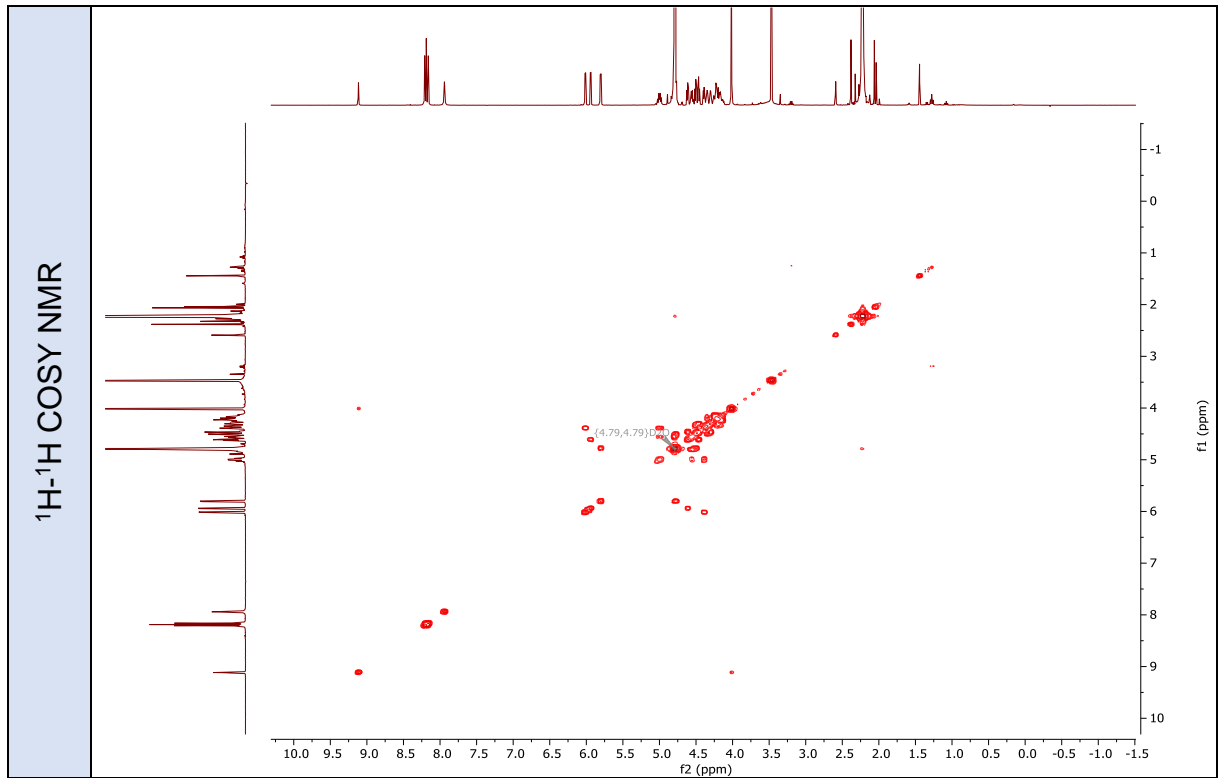


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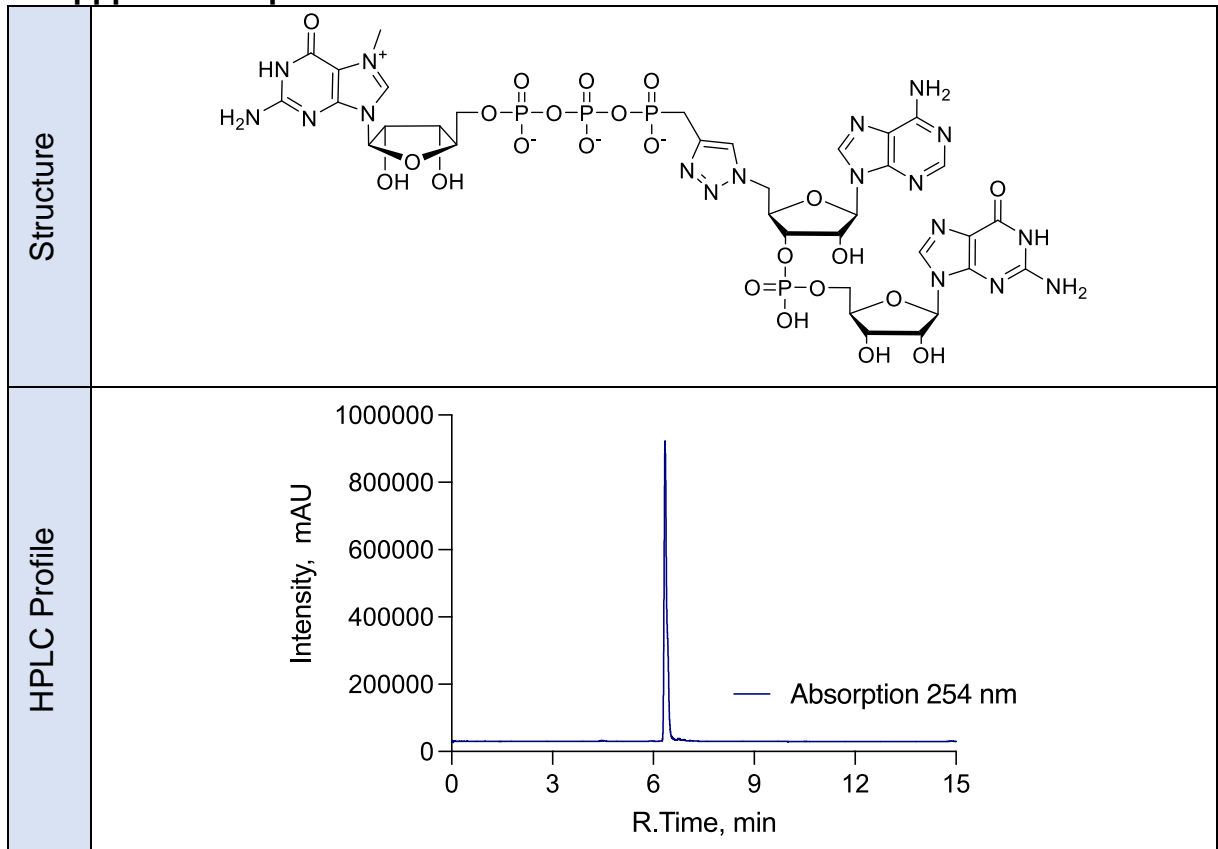


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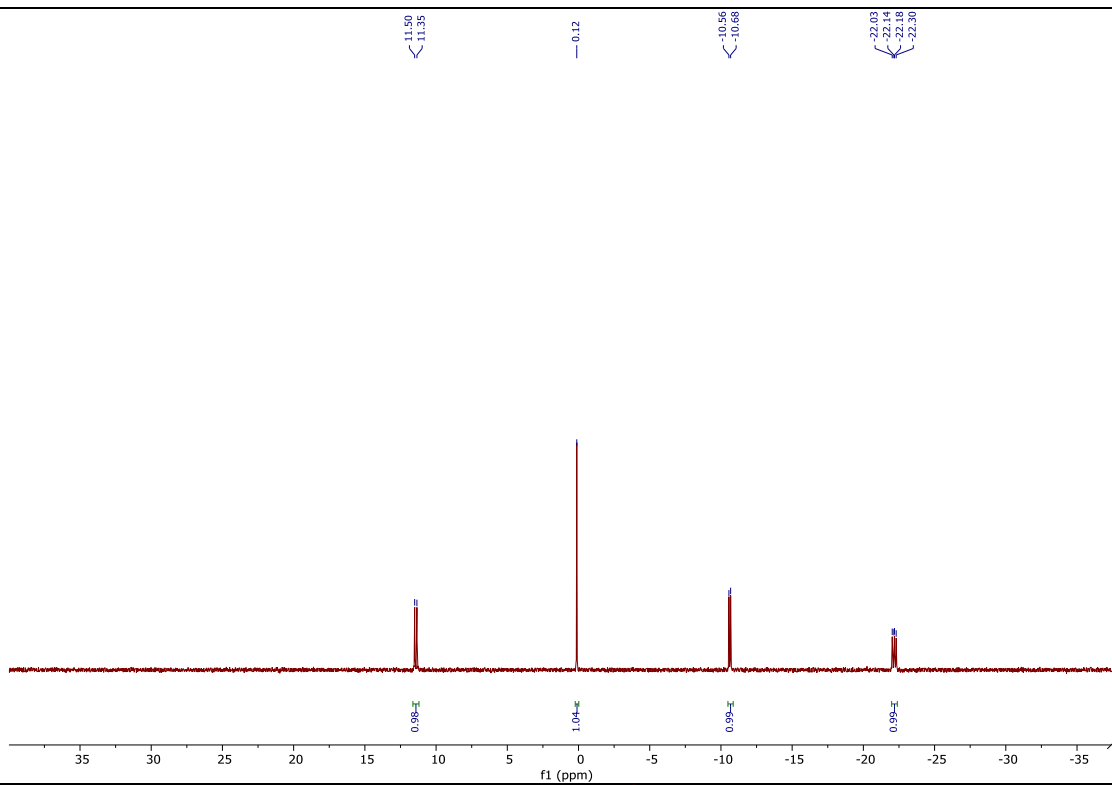




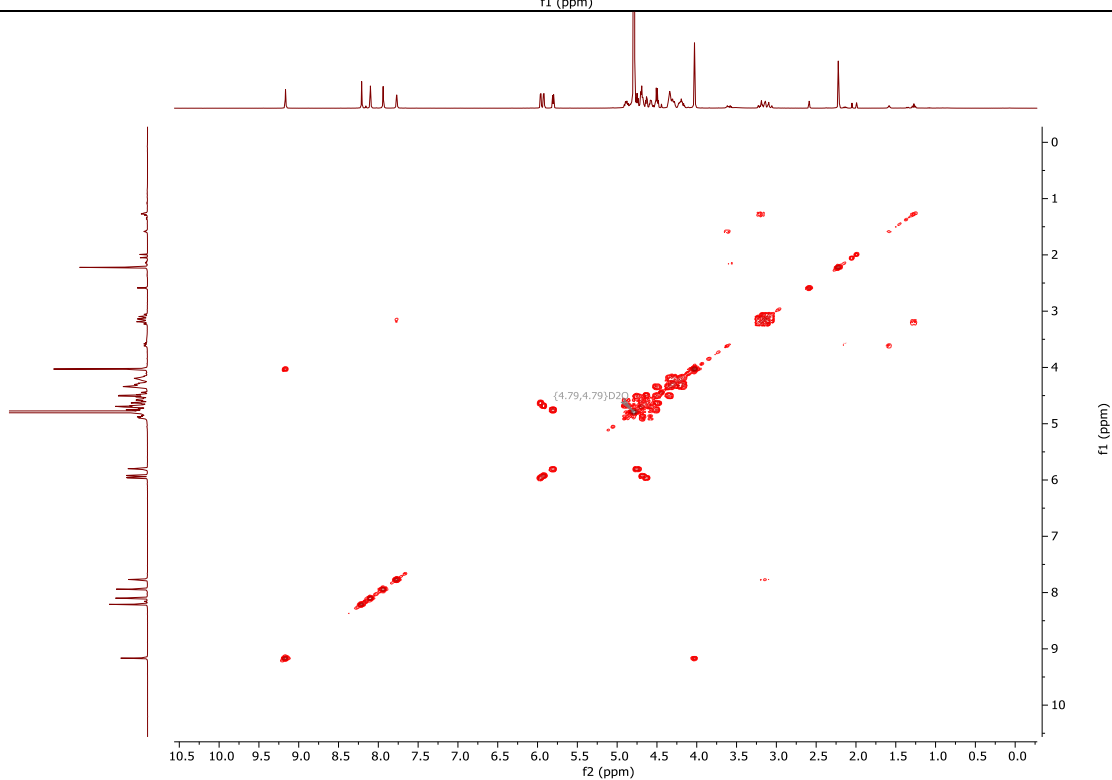
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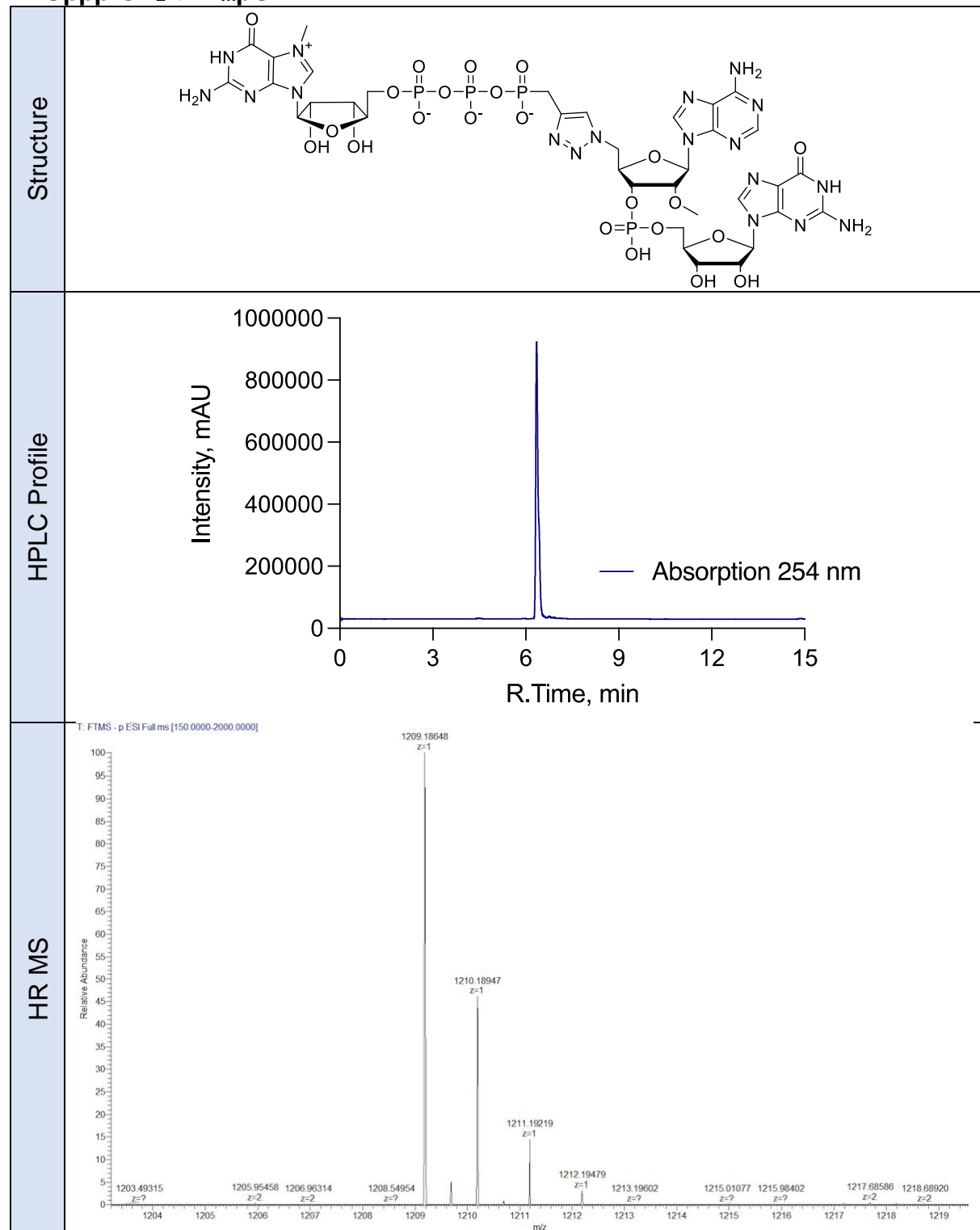
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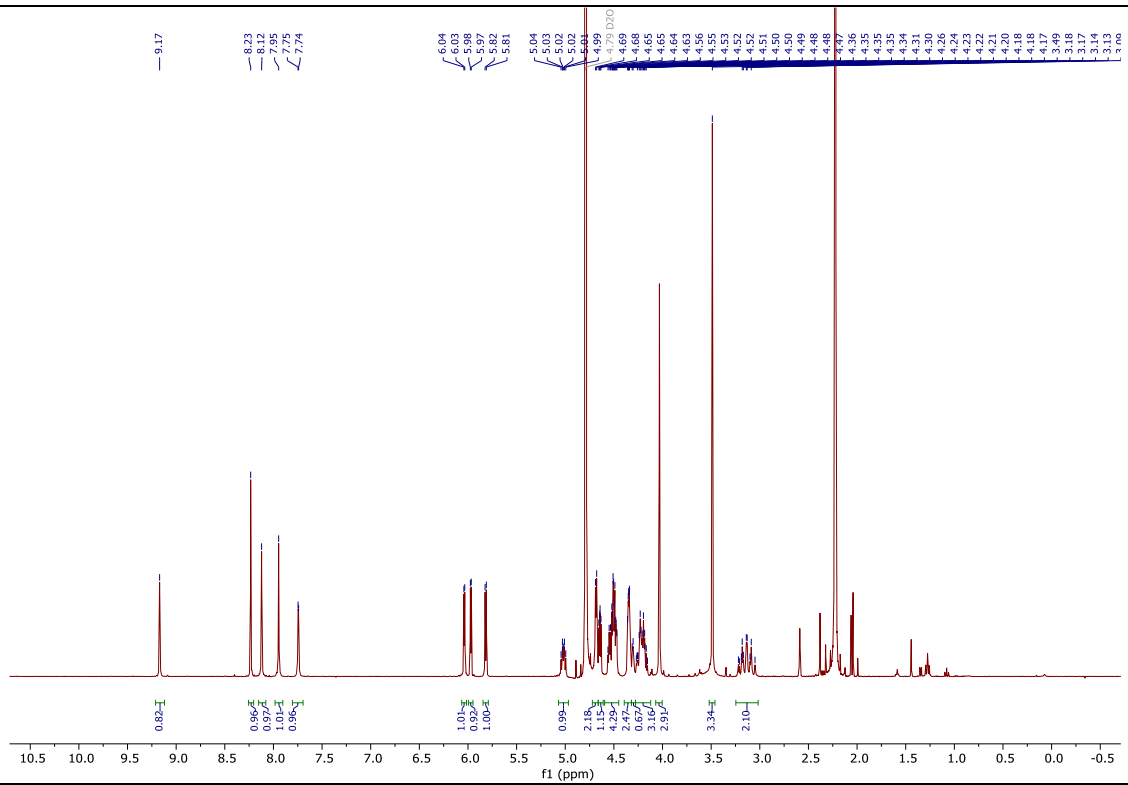
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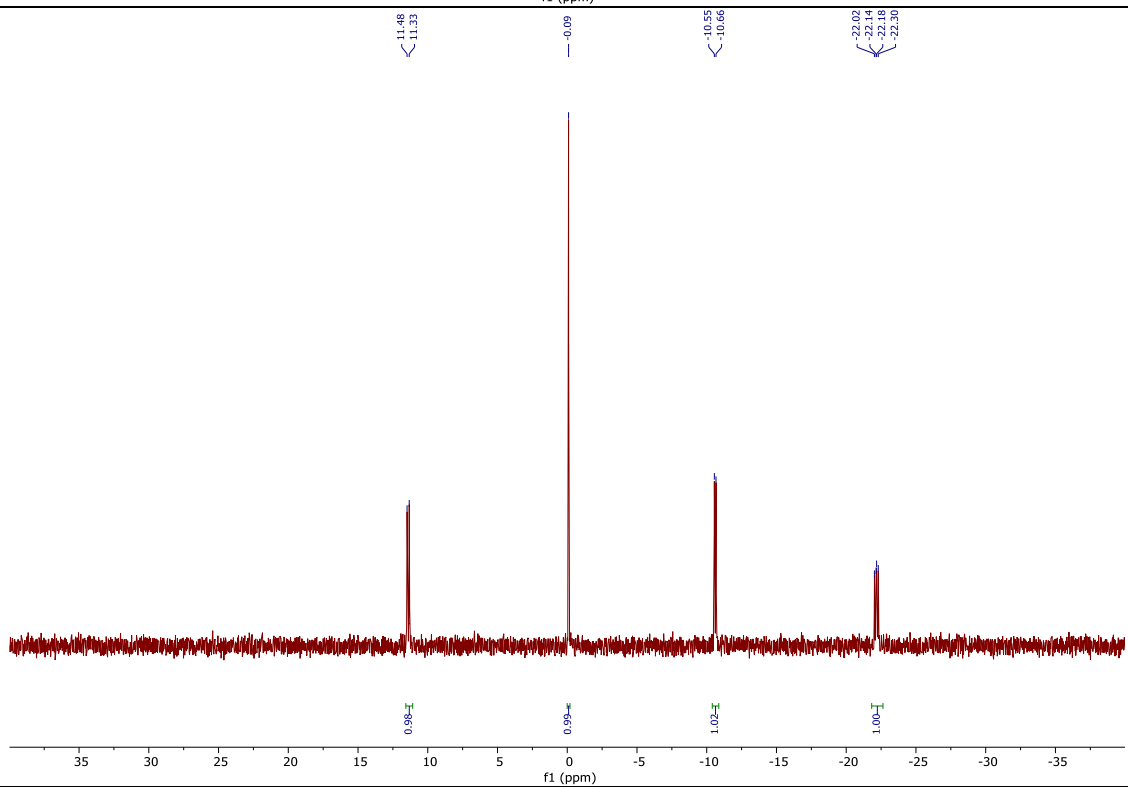
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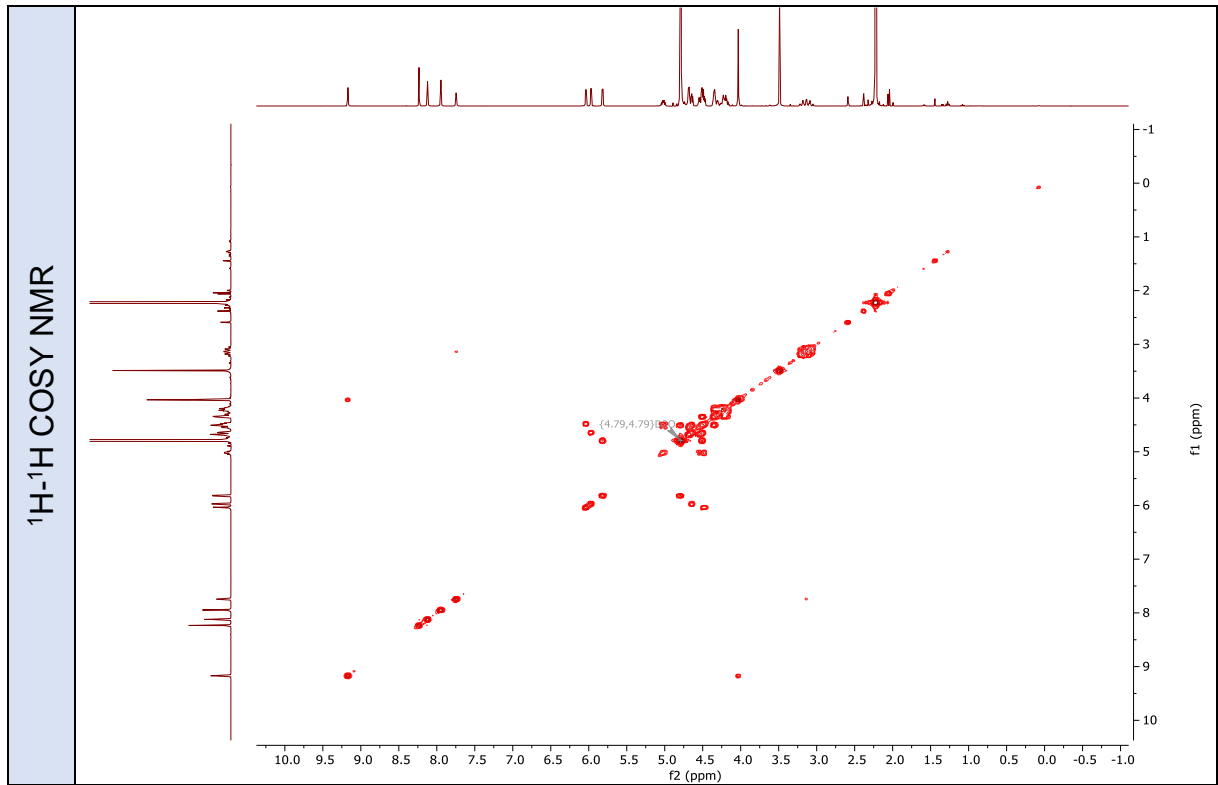


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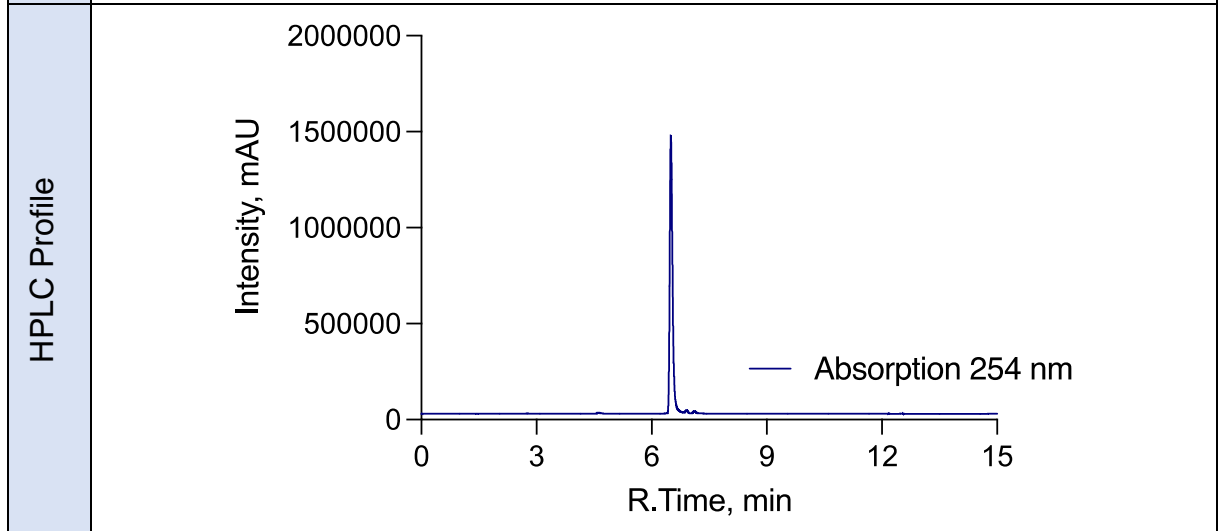
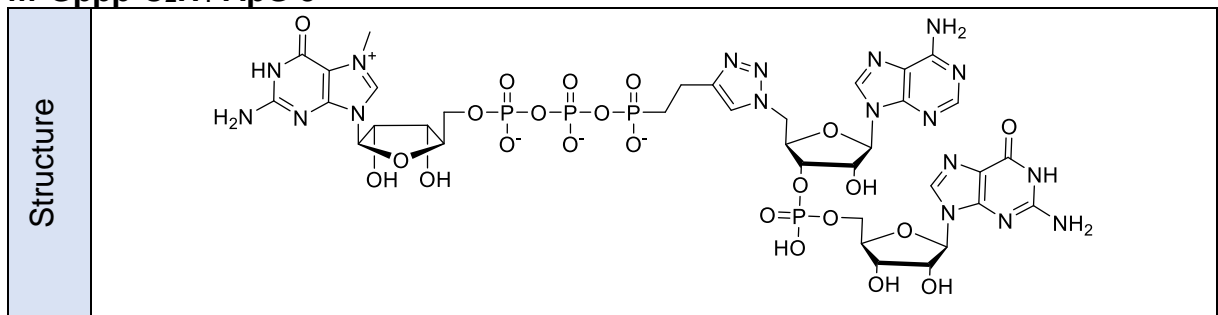


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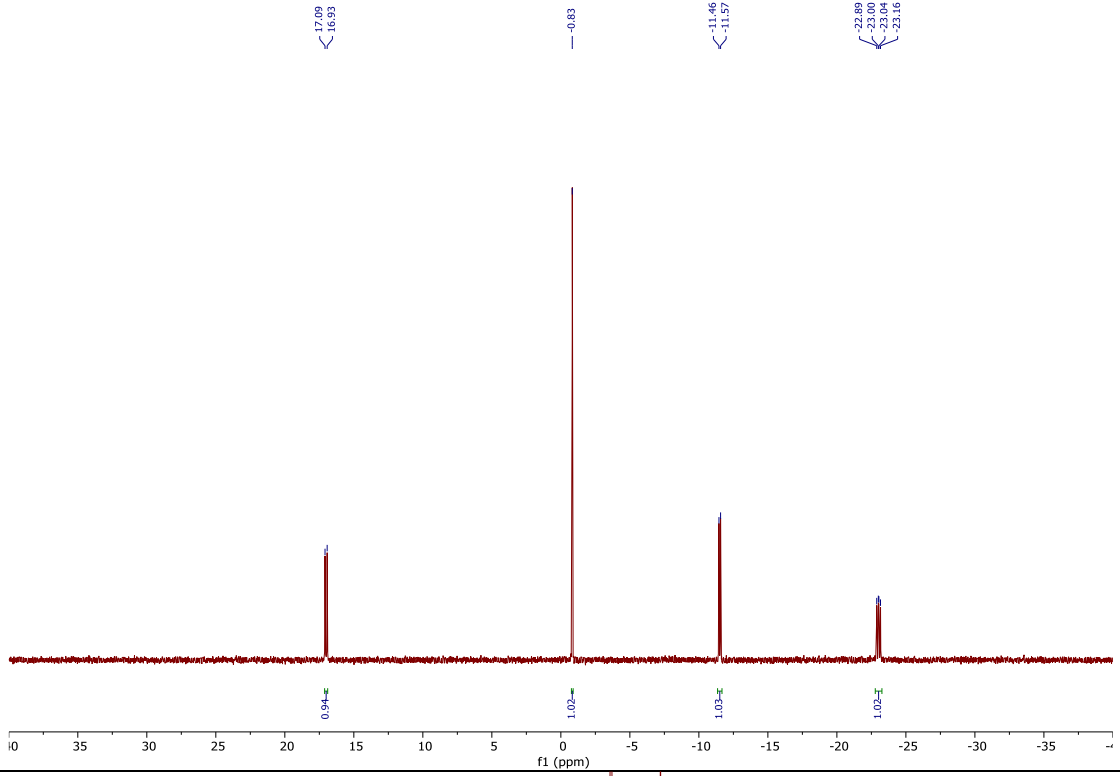




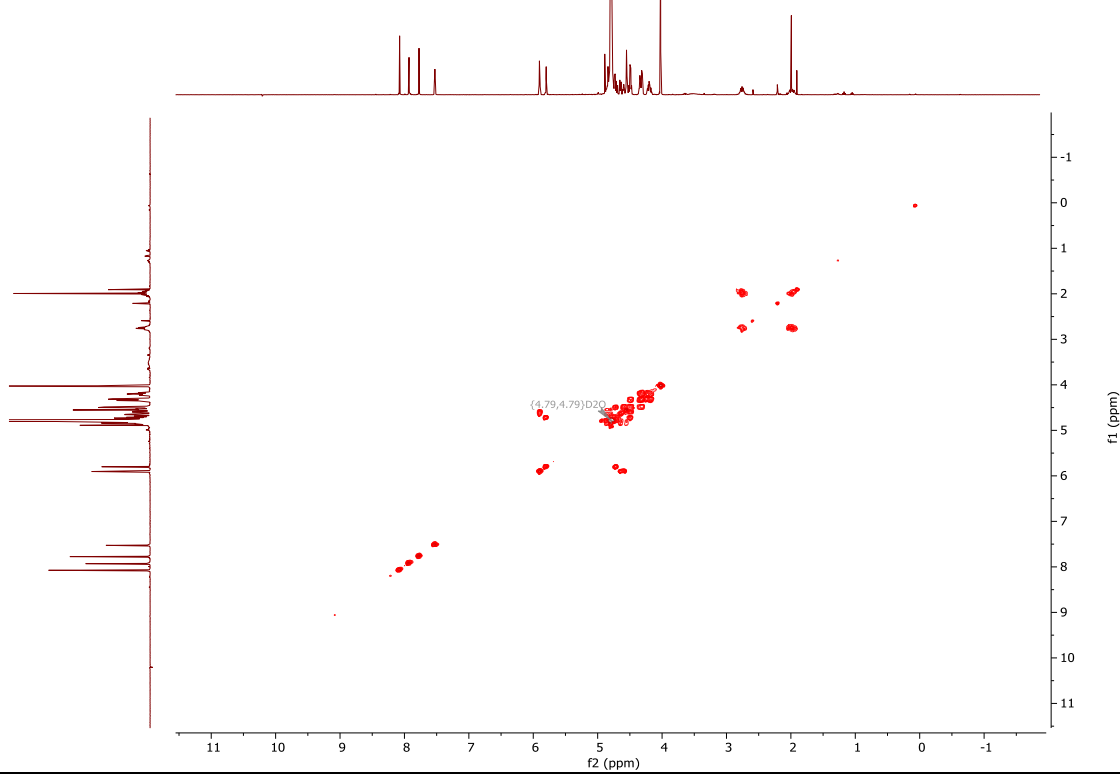
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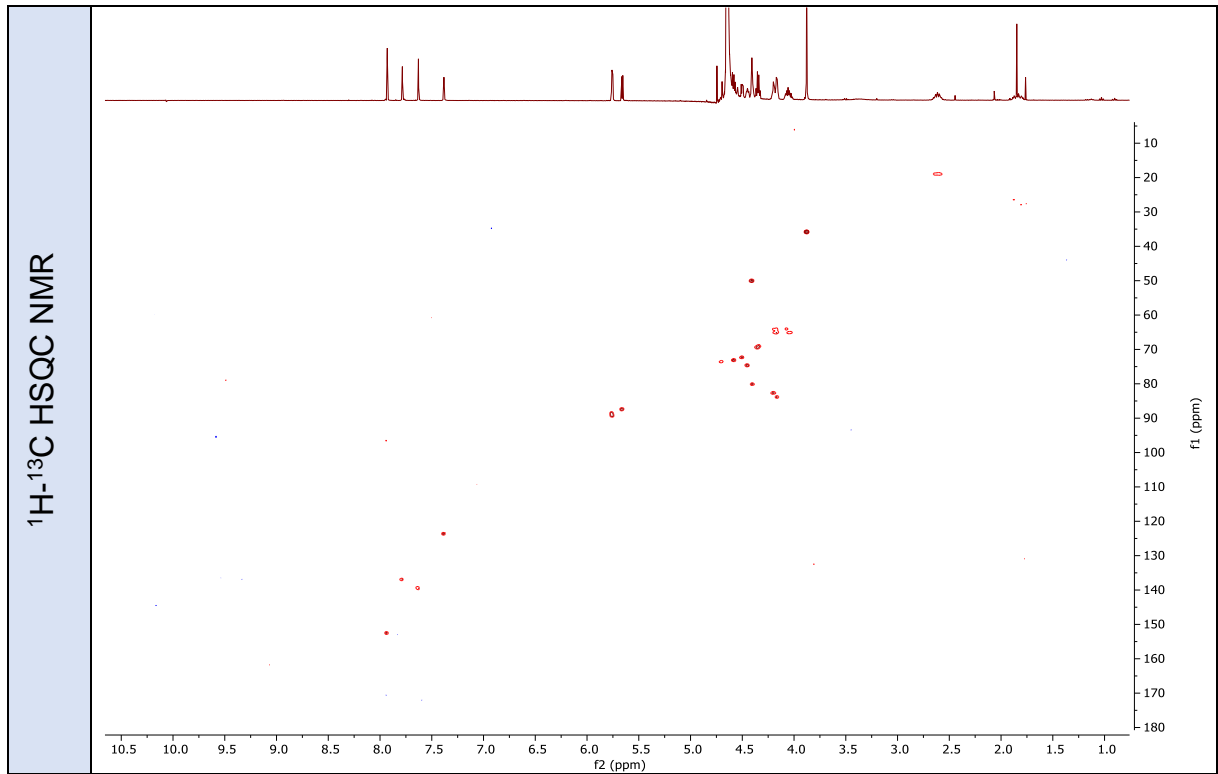


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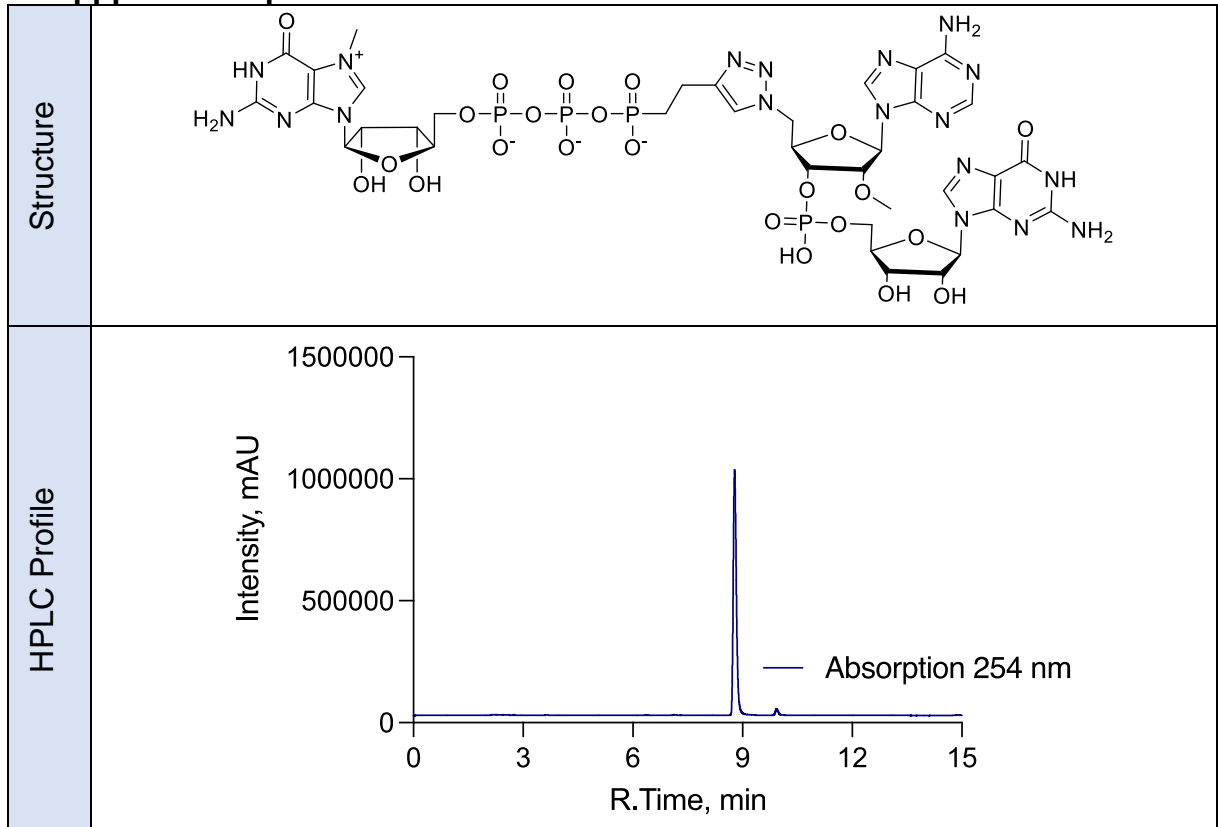


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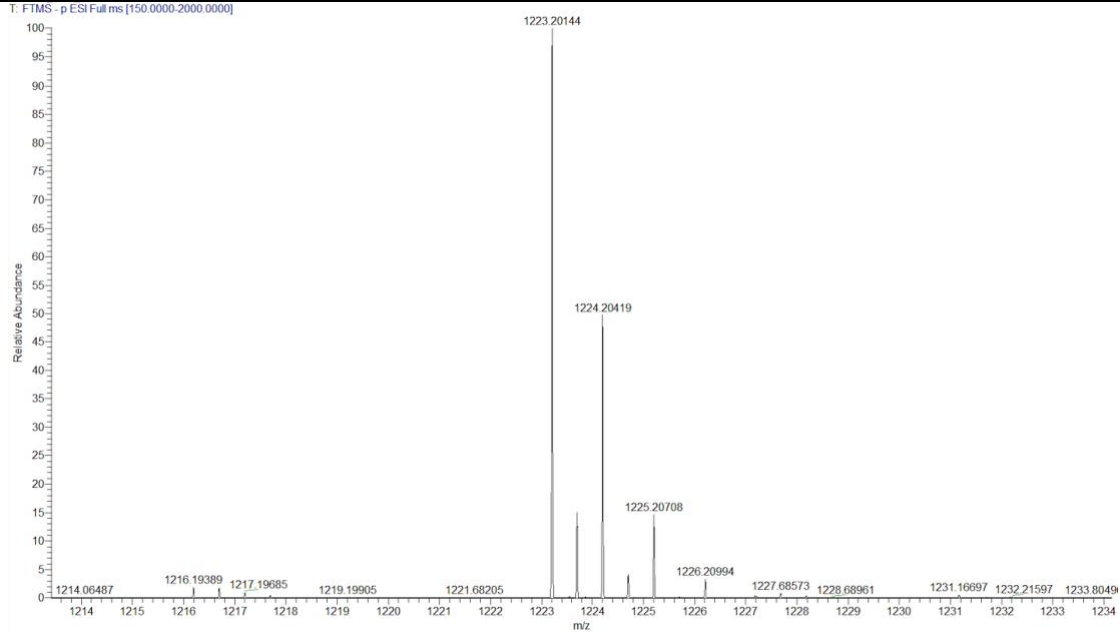




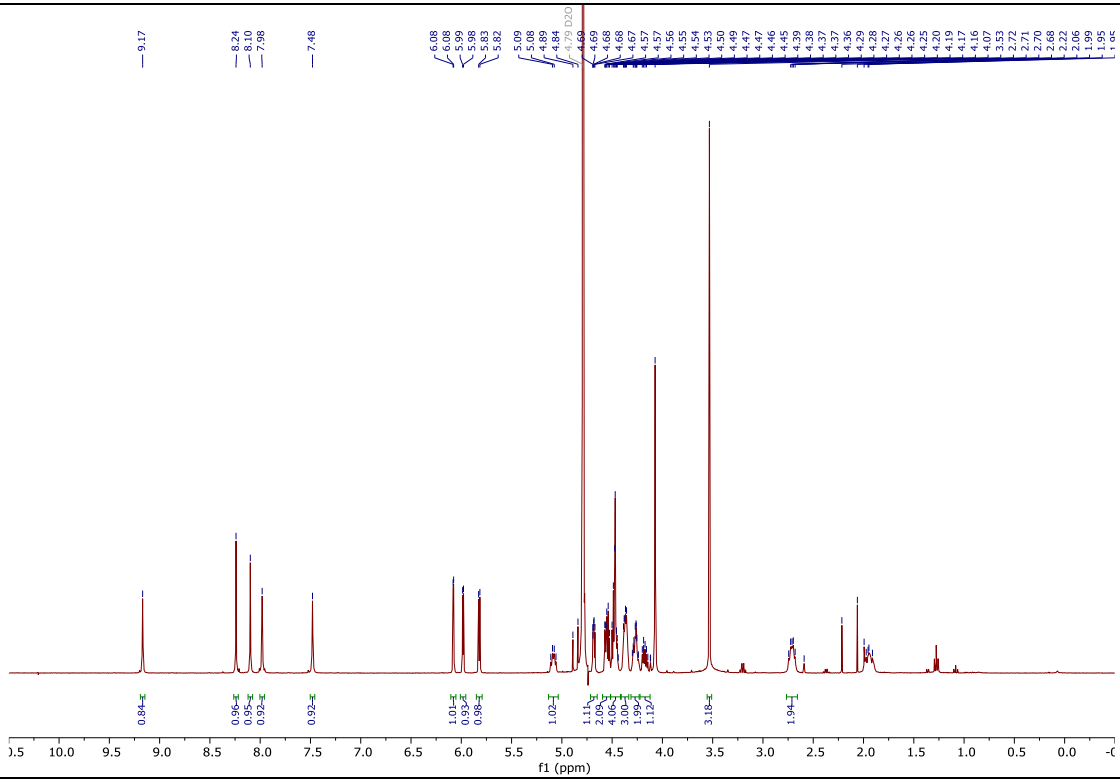
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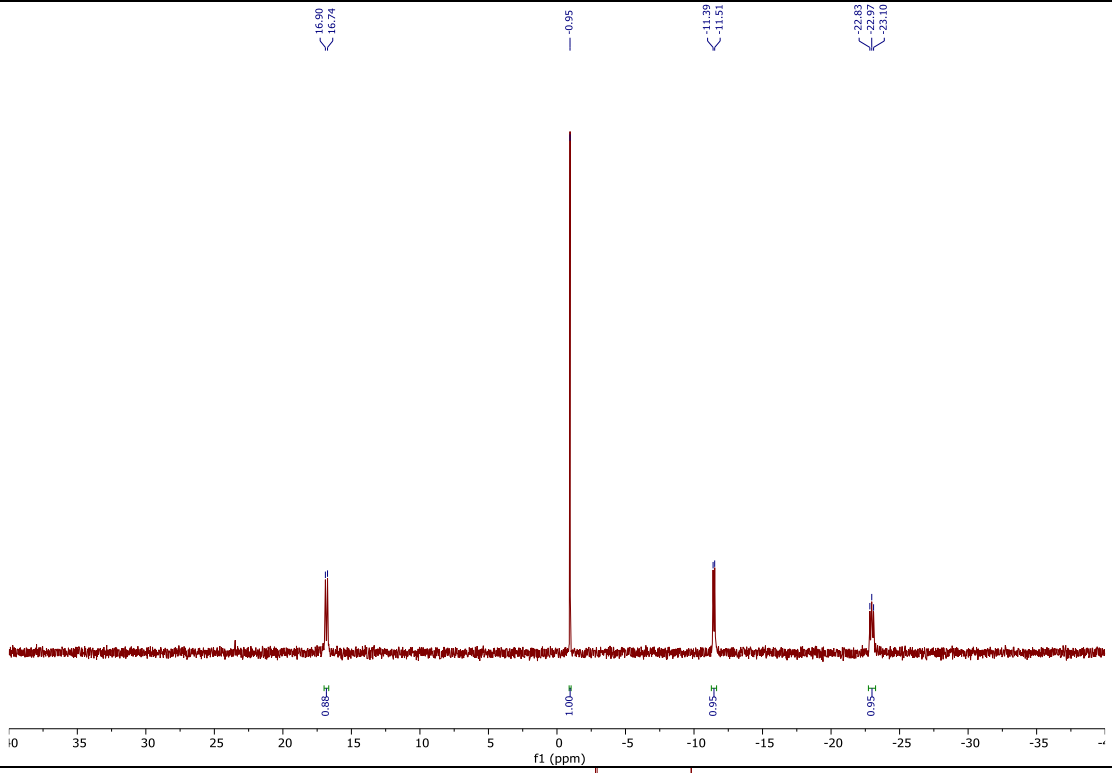
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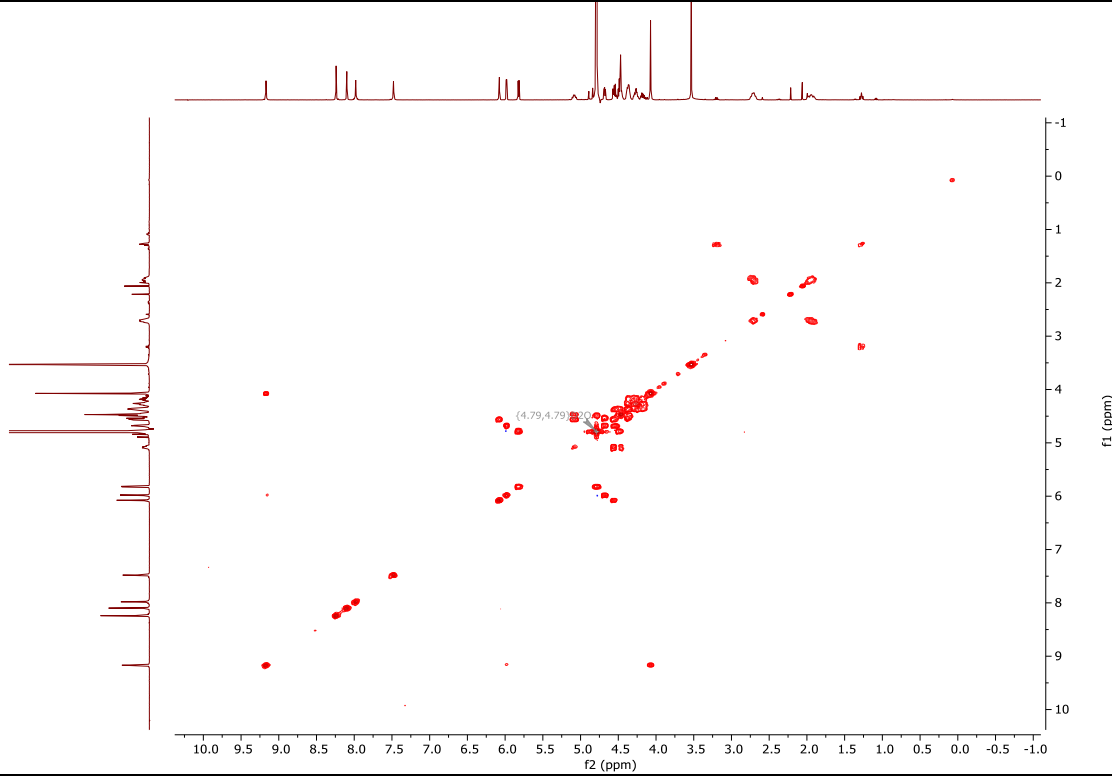
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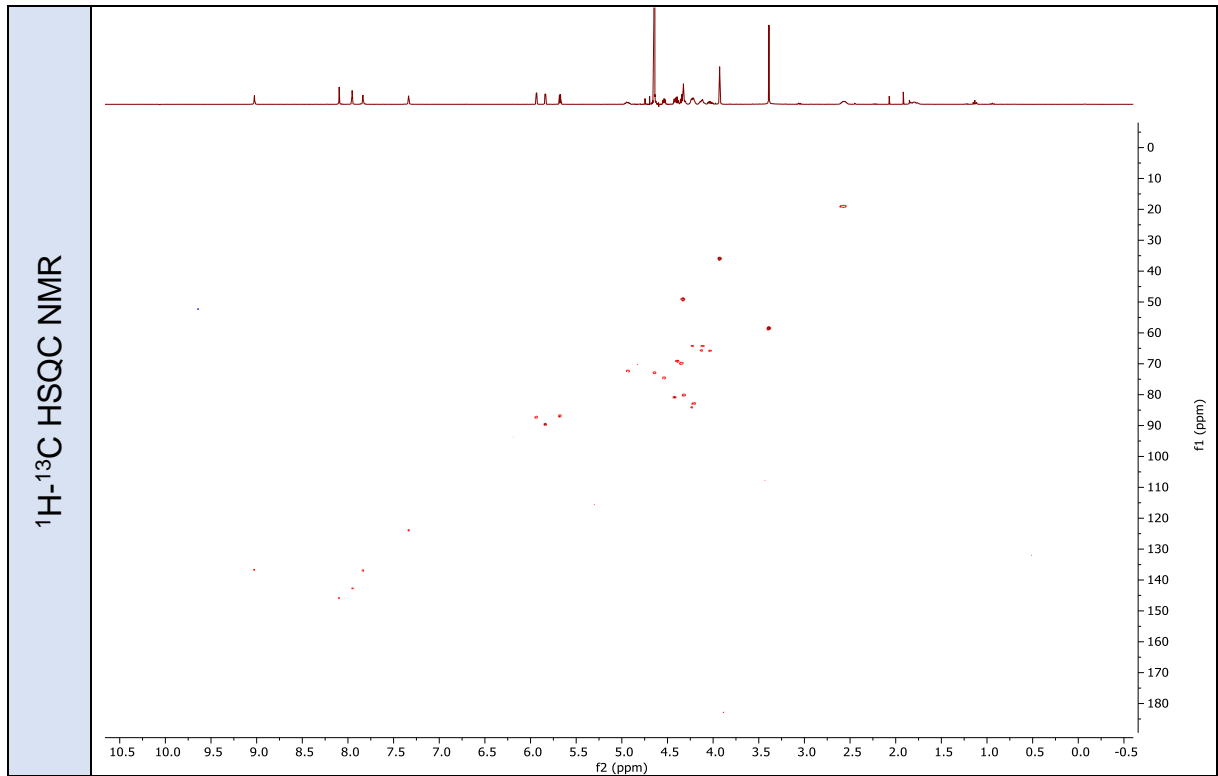


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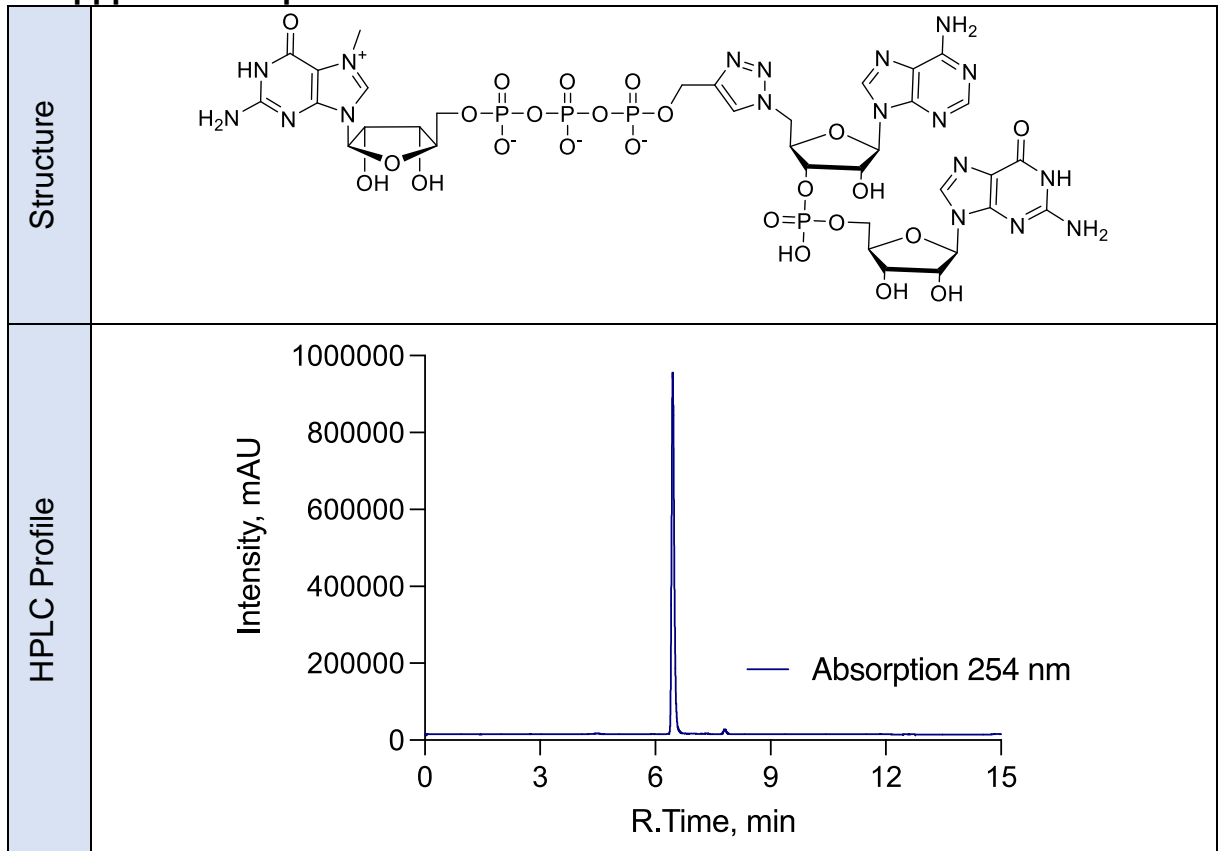


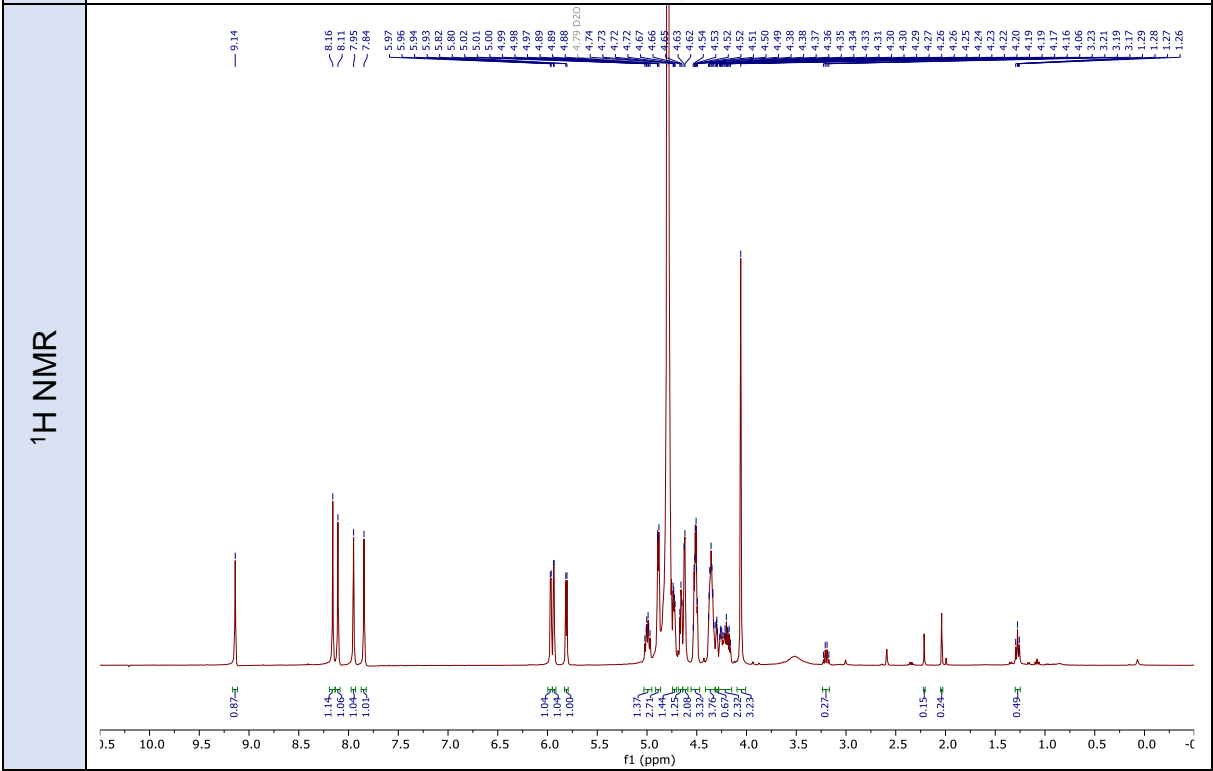
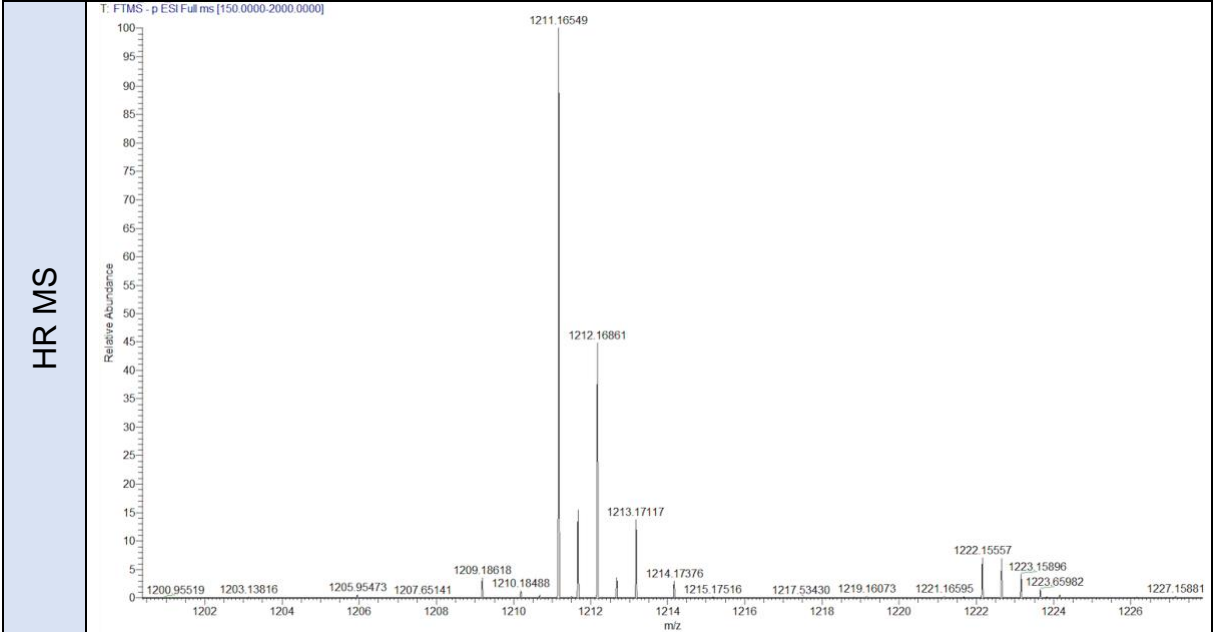
¹H-¹H COSY NMR



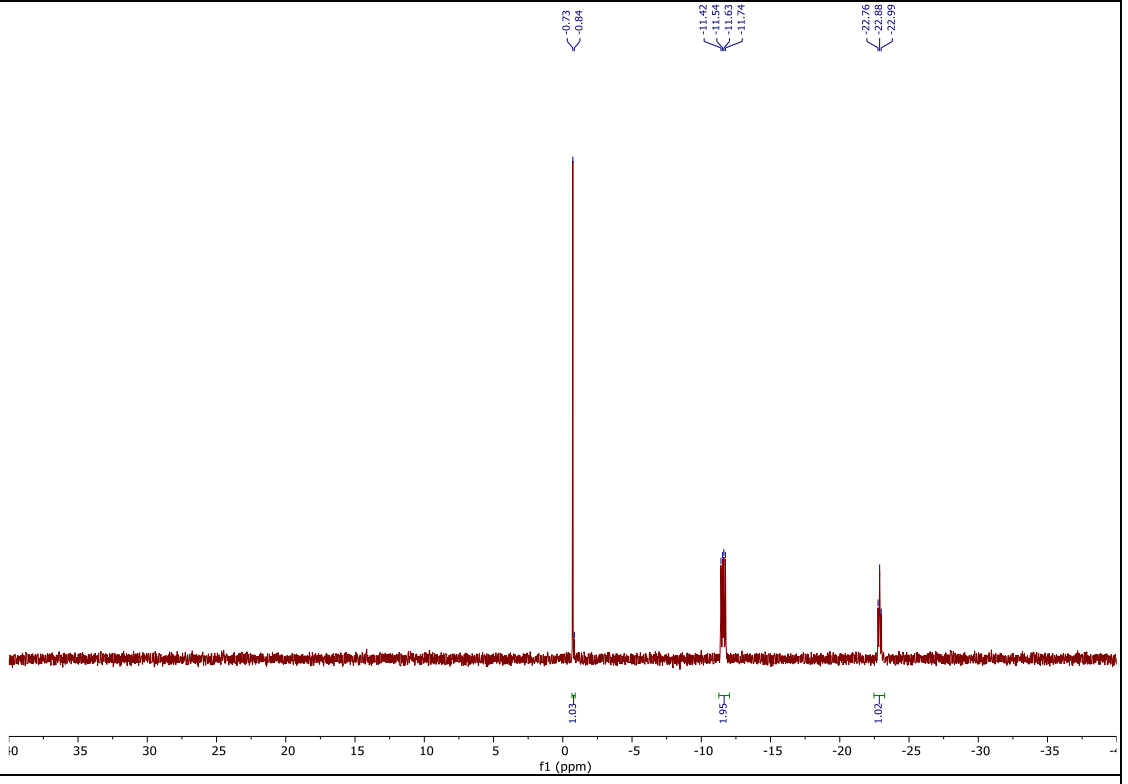


$\text{m}^7\text{GpppOCH}_2\text{-tr-ApG 7}$

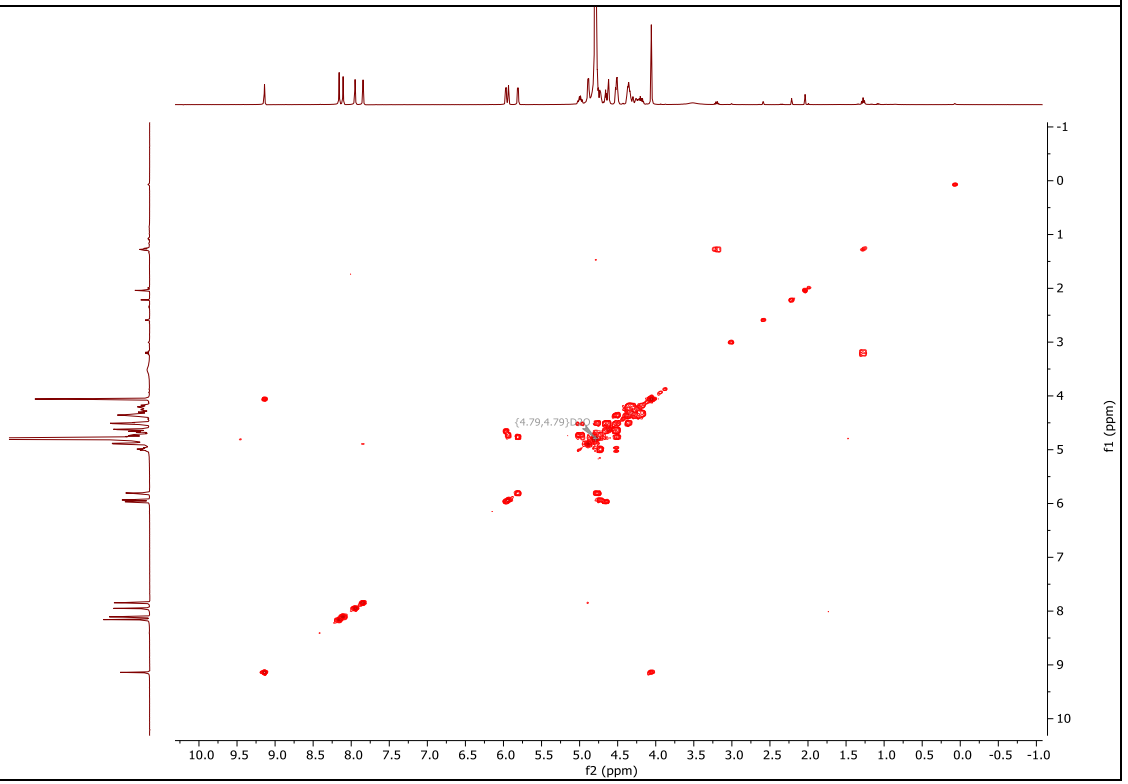


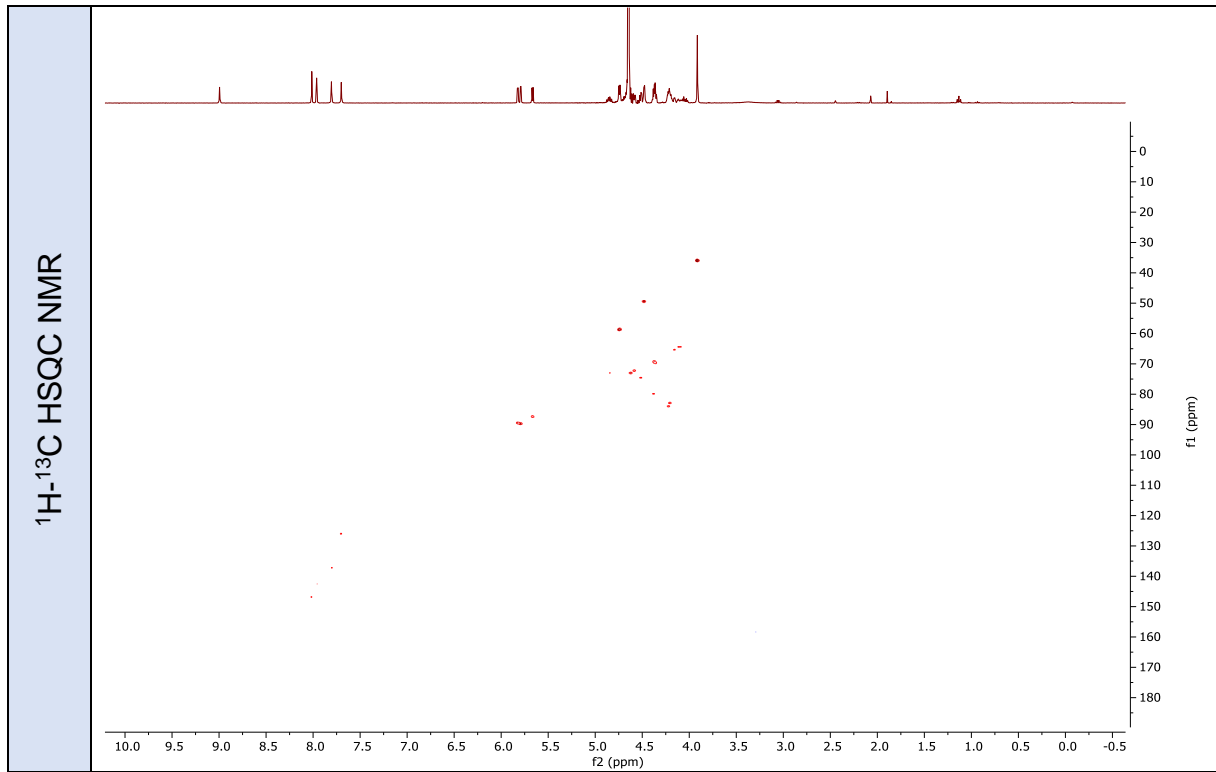


³¹P NMR

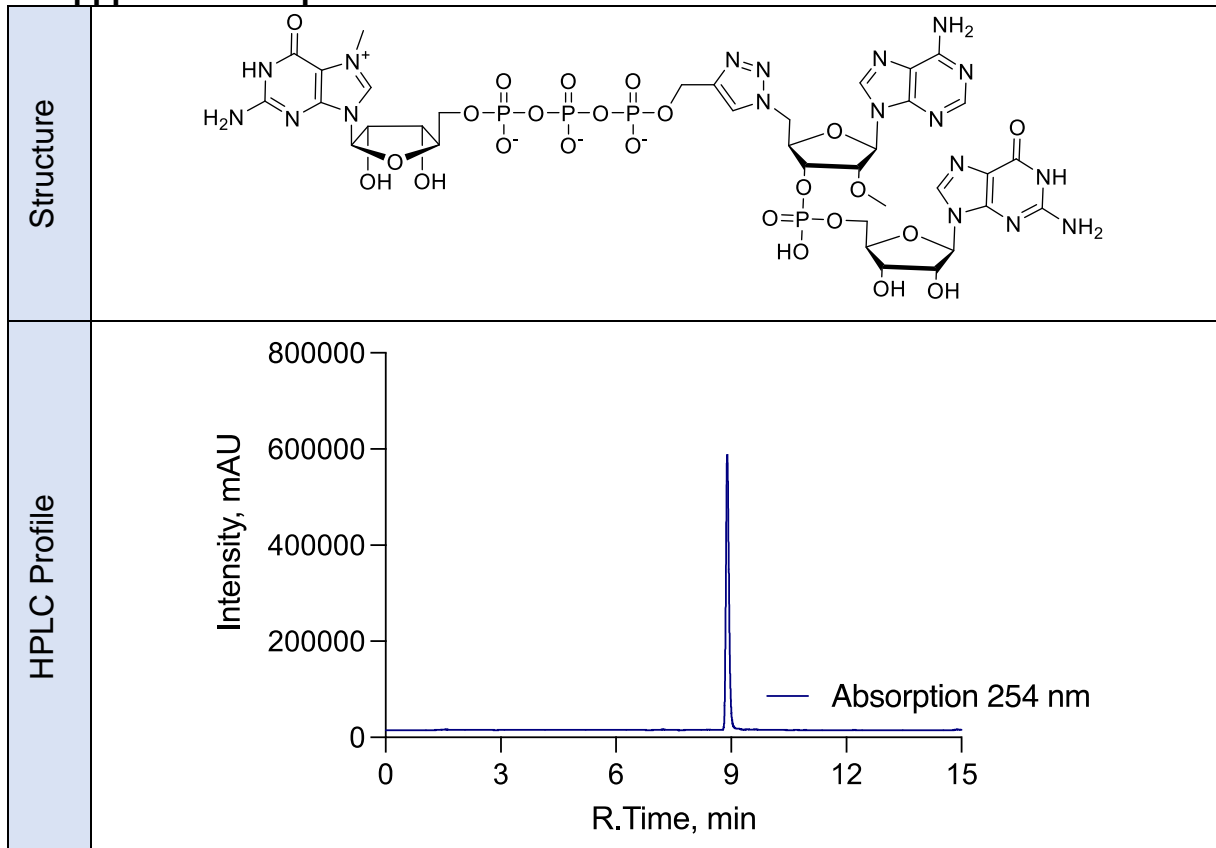


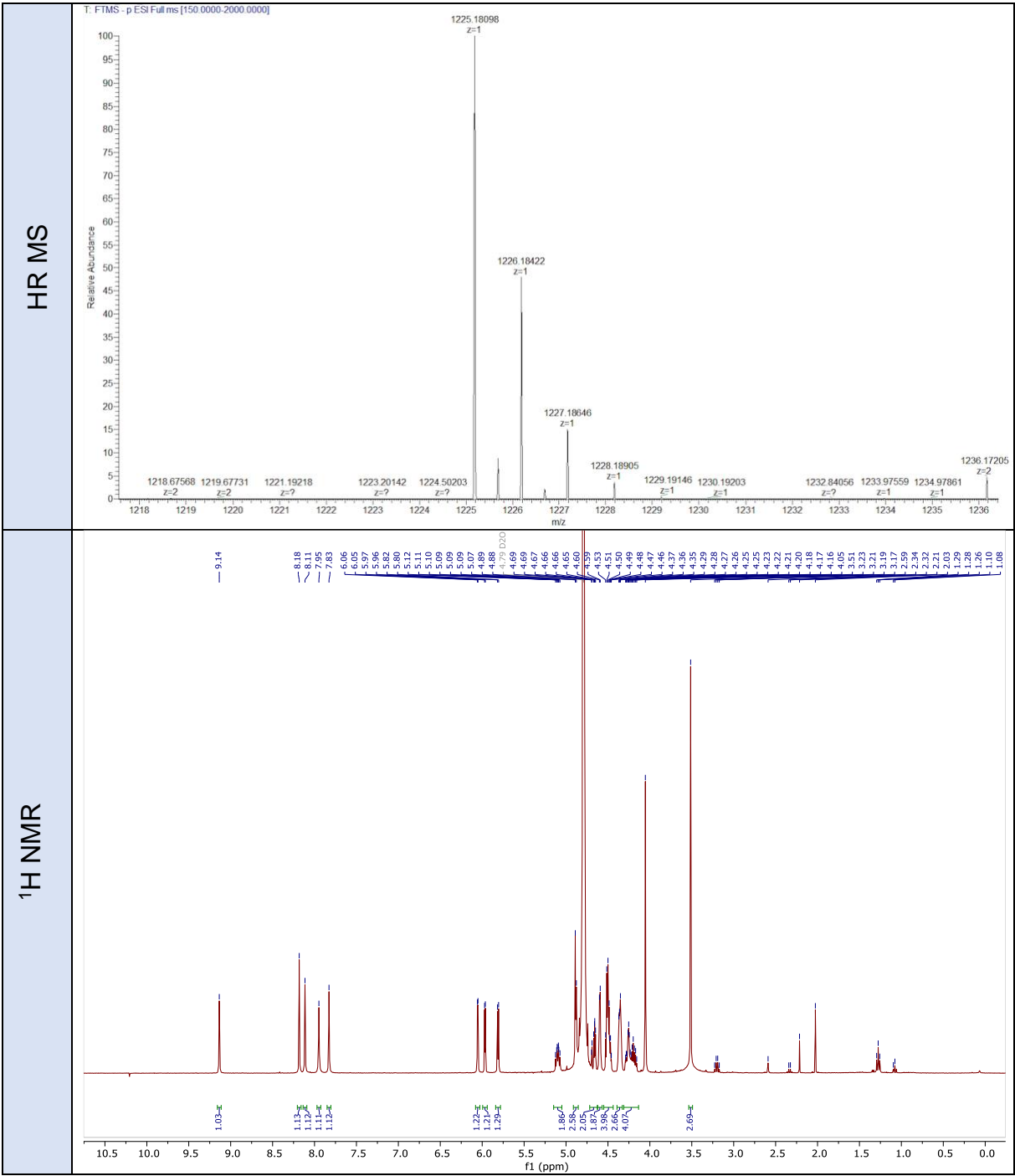
¹H-¹H COSY NMR



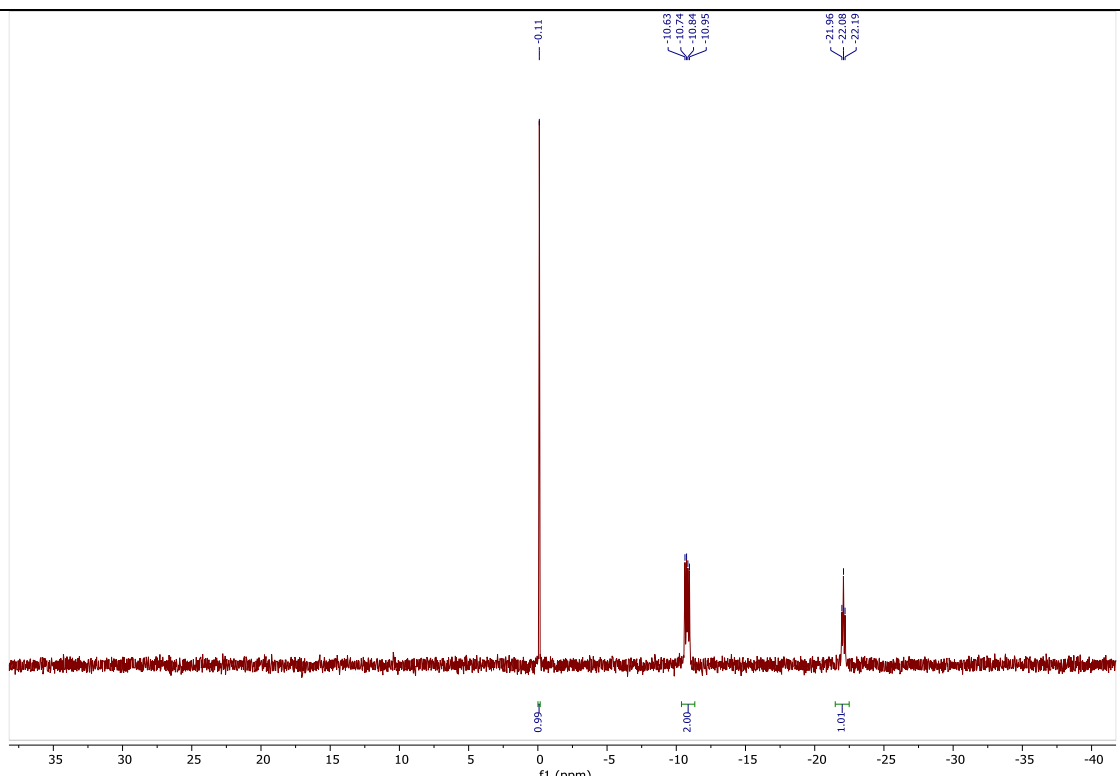


$m^7\text{GpppOCH}_2\text{-tr-AmpG 8}$

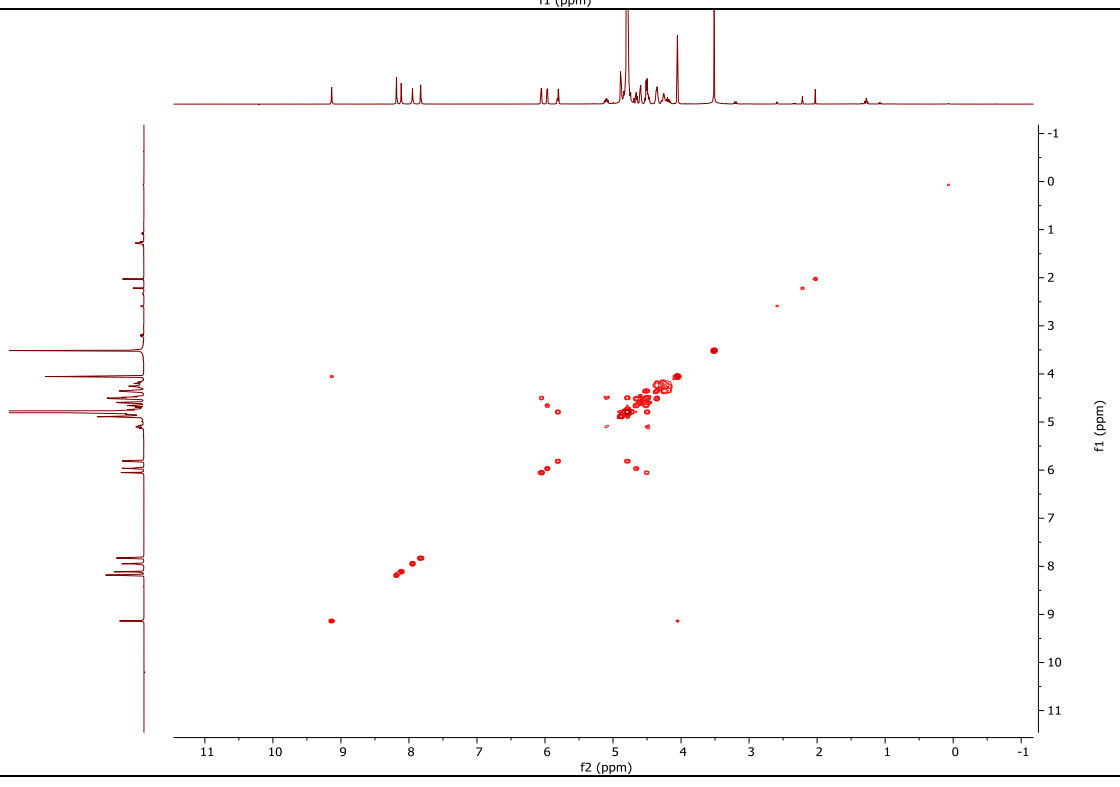




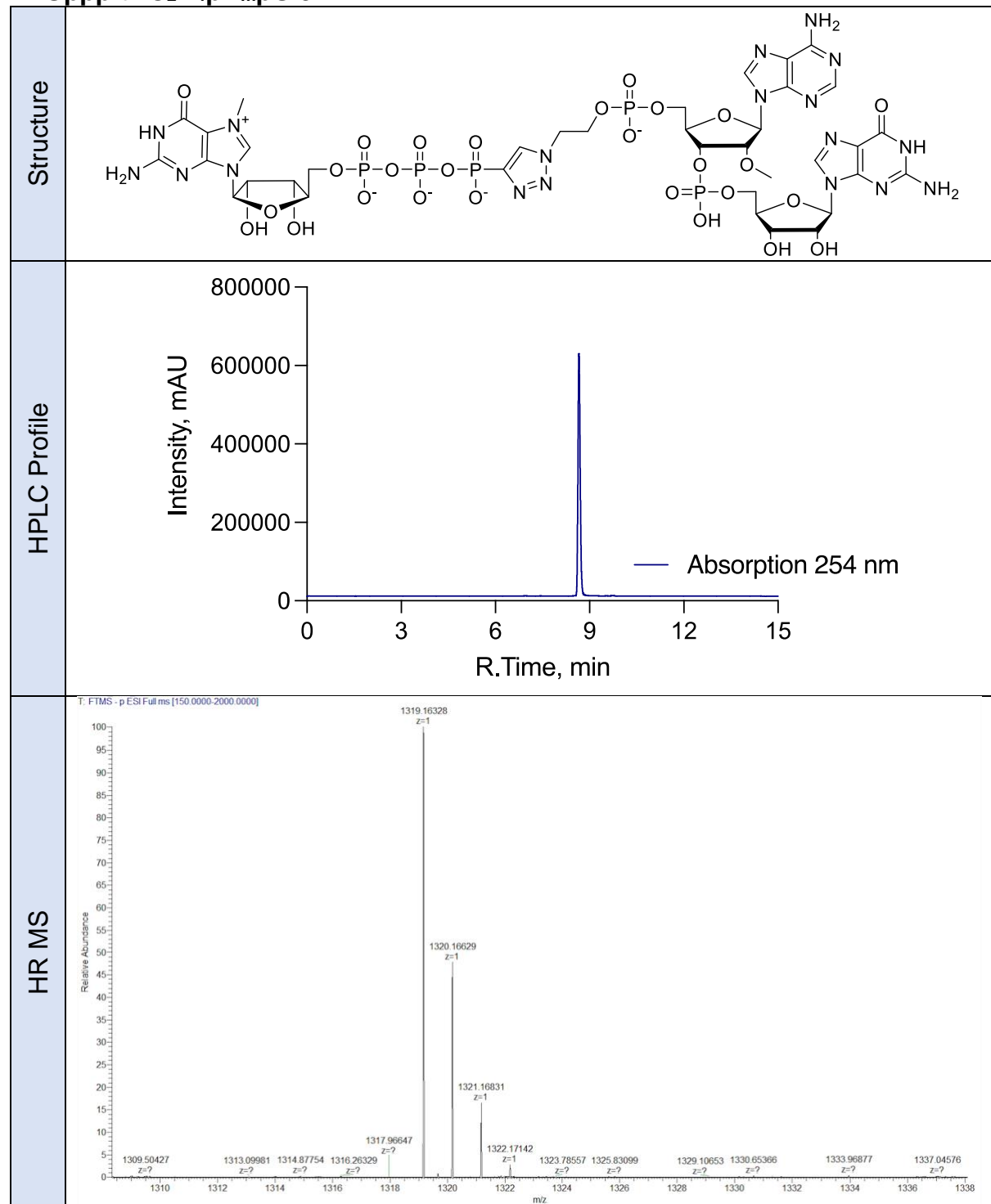
³¹P NMR



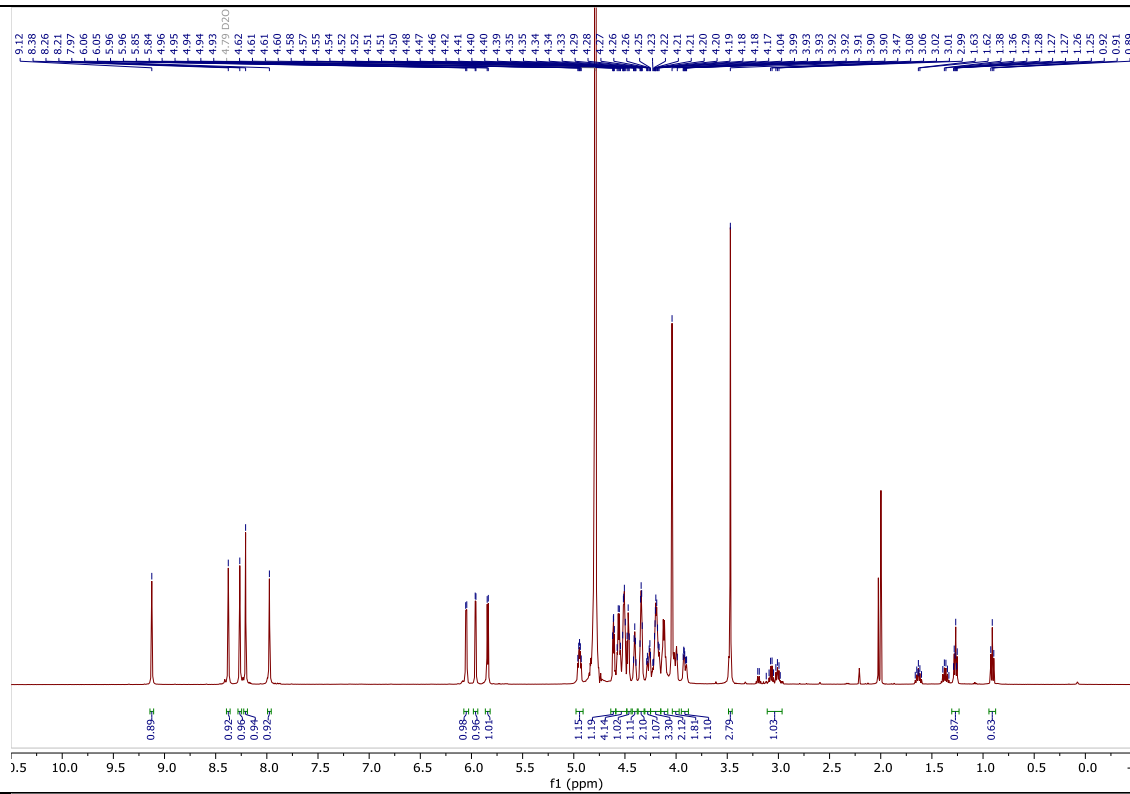
¹H-¹H COSY NMR



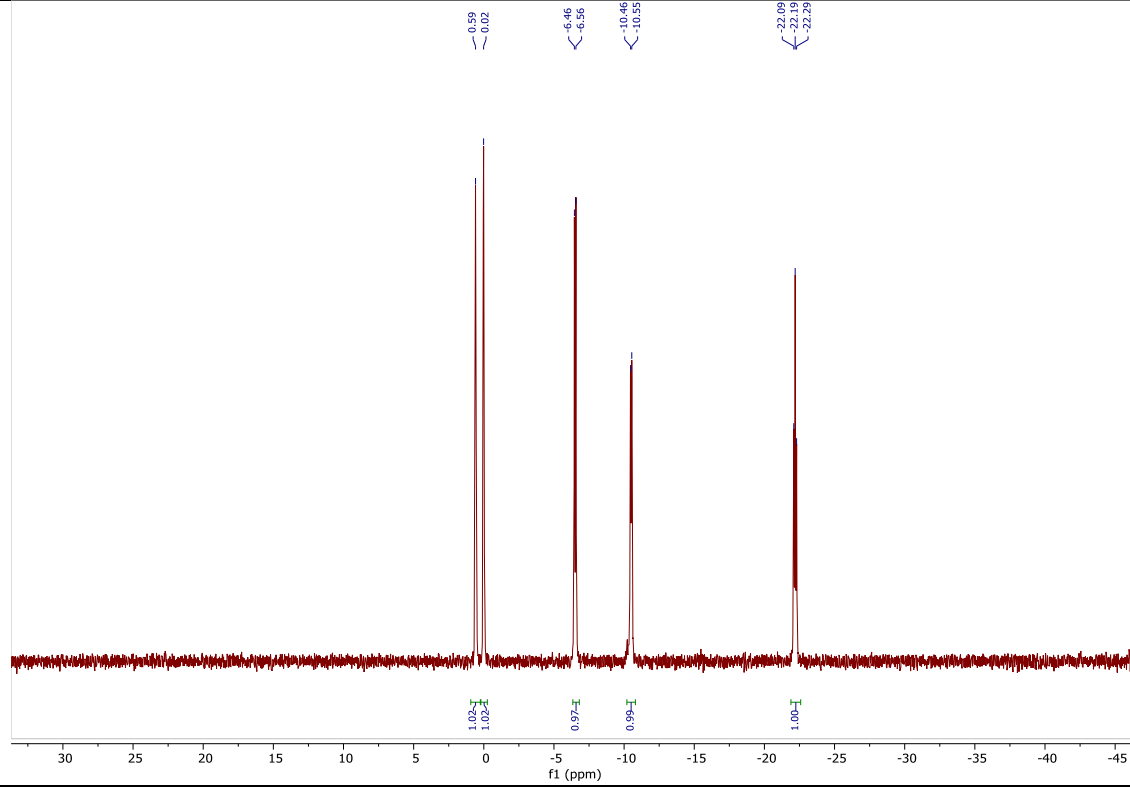
m⁷Gppp-tr-C₂H₄pAmpG 9



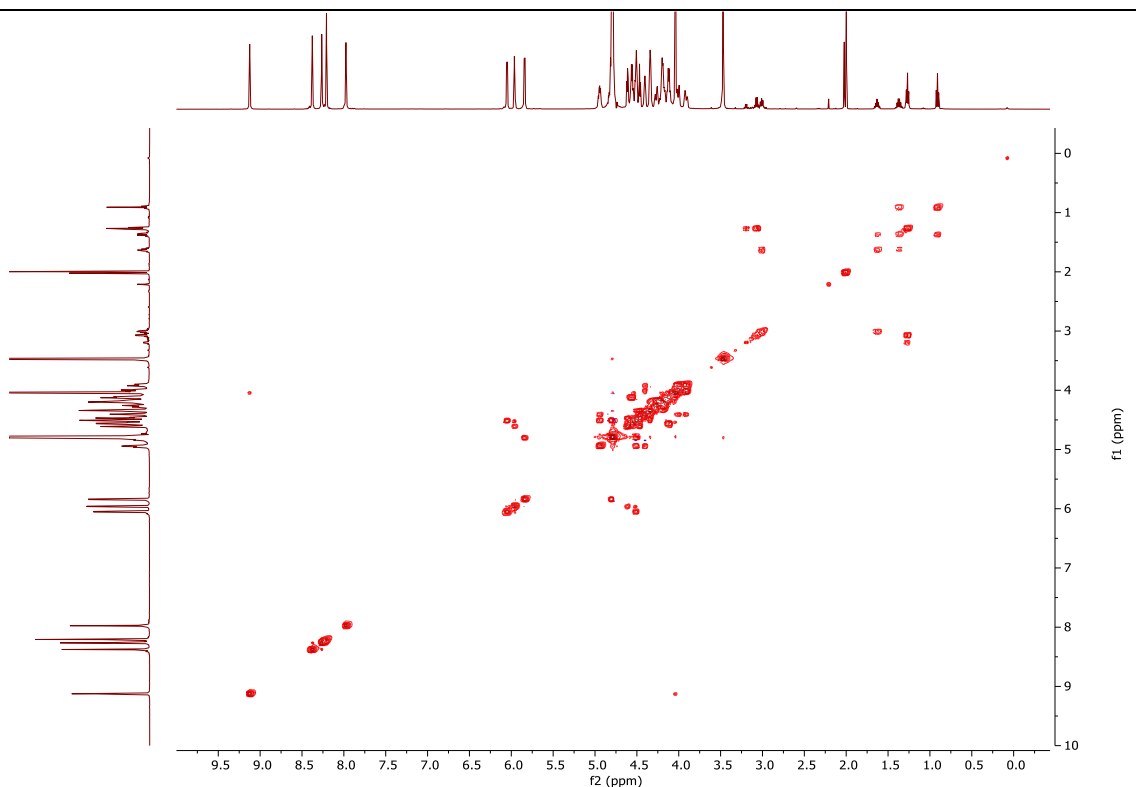
¹H NMR



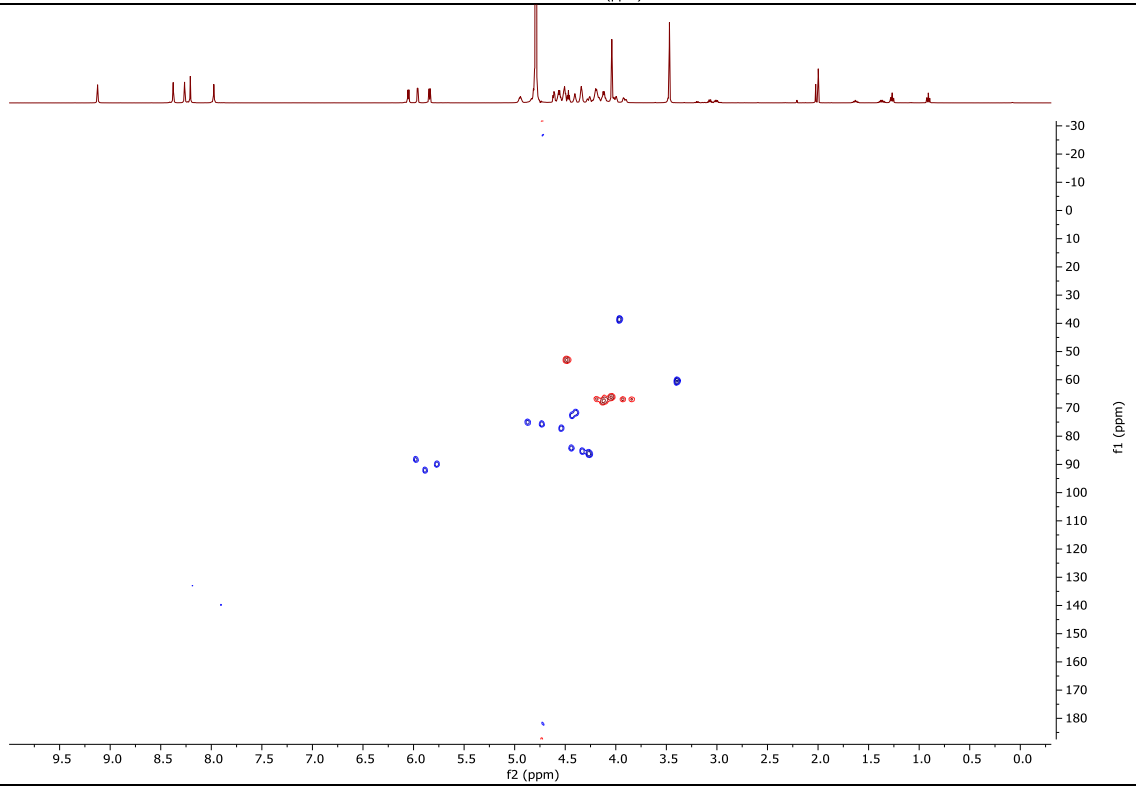
³¹P NMR



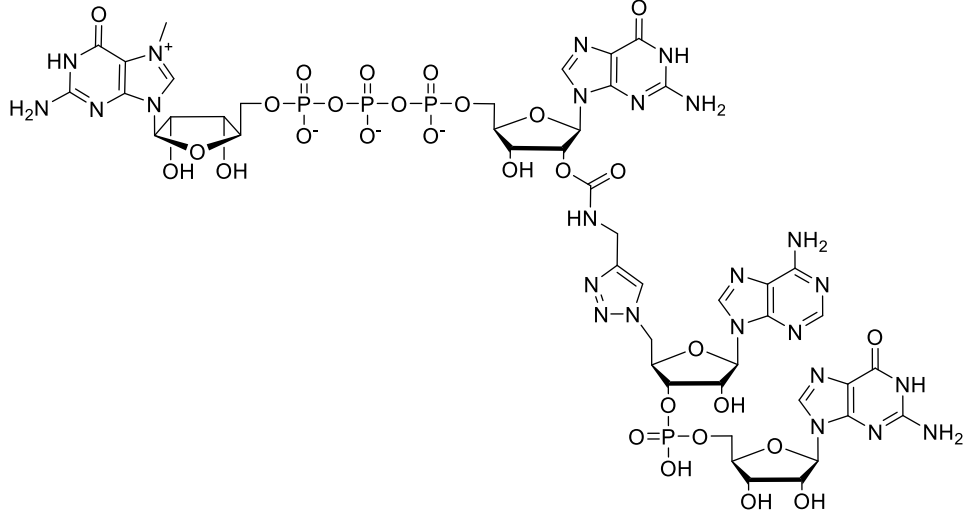
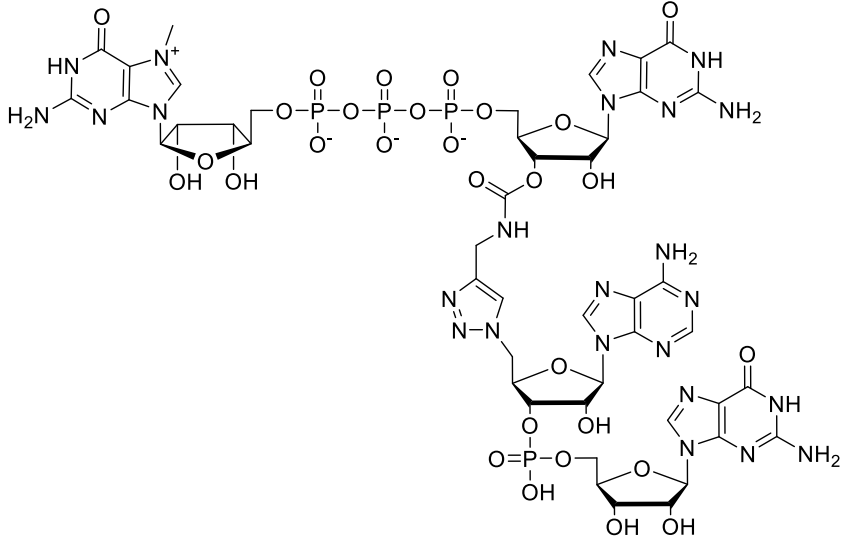
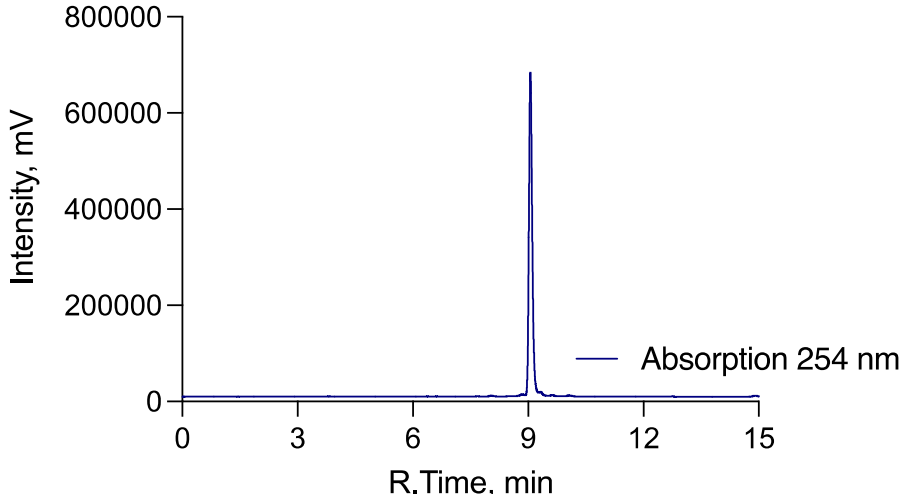
¹H-¹H COSY NMR



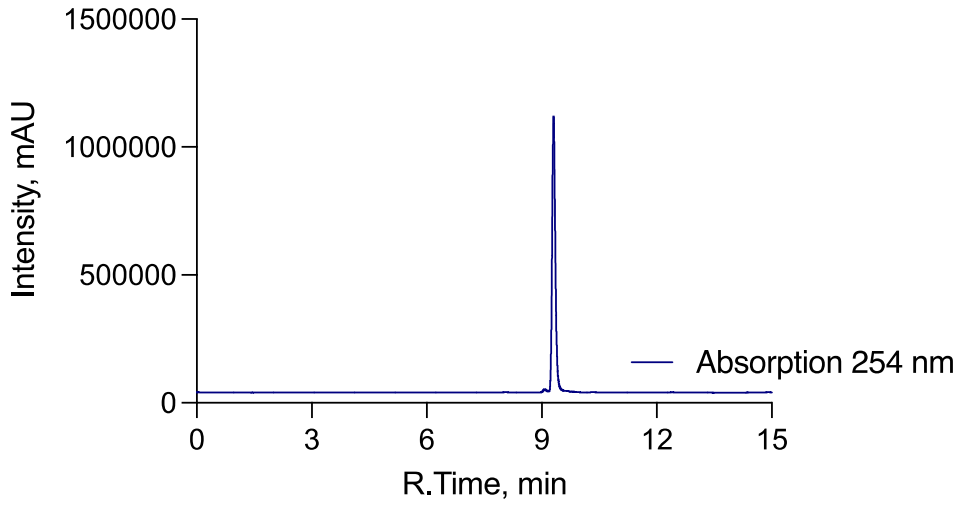
¹³C-¹H HSQC NMR



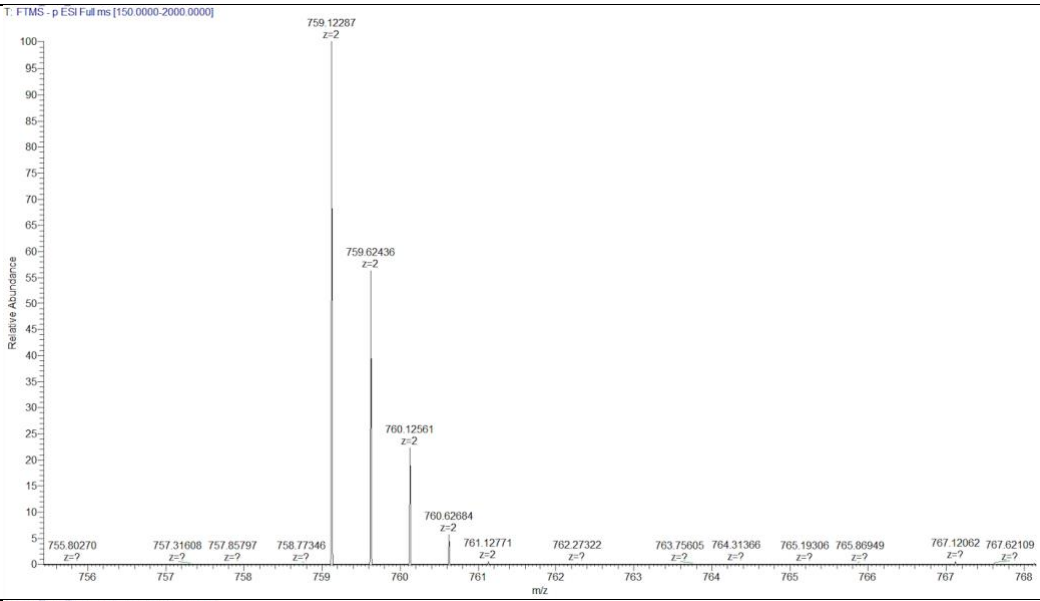
m⁷GpppG-carb-CH₂-tr-ApG (both isomers) 10a and 10b

Structure 10a	
Structure 10b	
HPLC Profile (isomer 10a)	 <p>Intensity, mV</p> <p>R.Time, min</p> <p>— Absorption 254 nm</p>

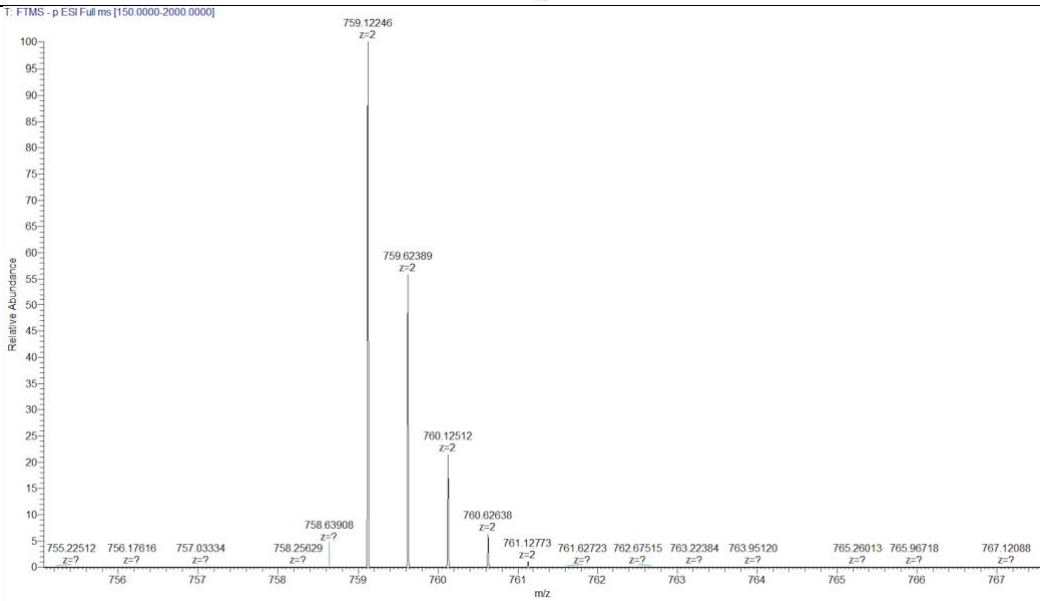
HPLC Profile (isomer 10b)



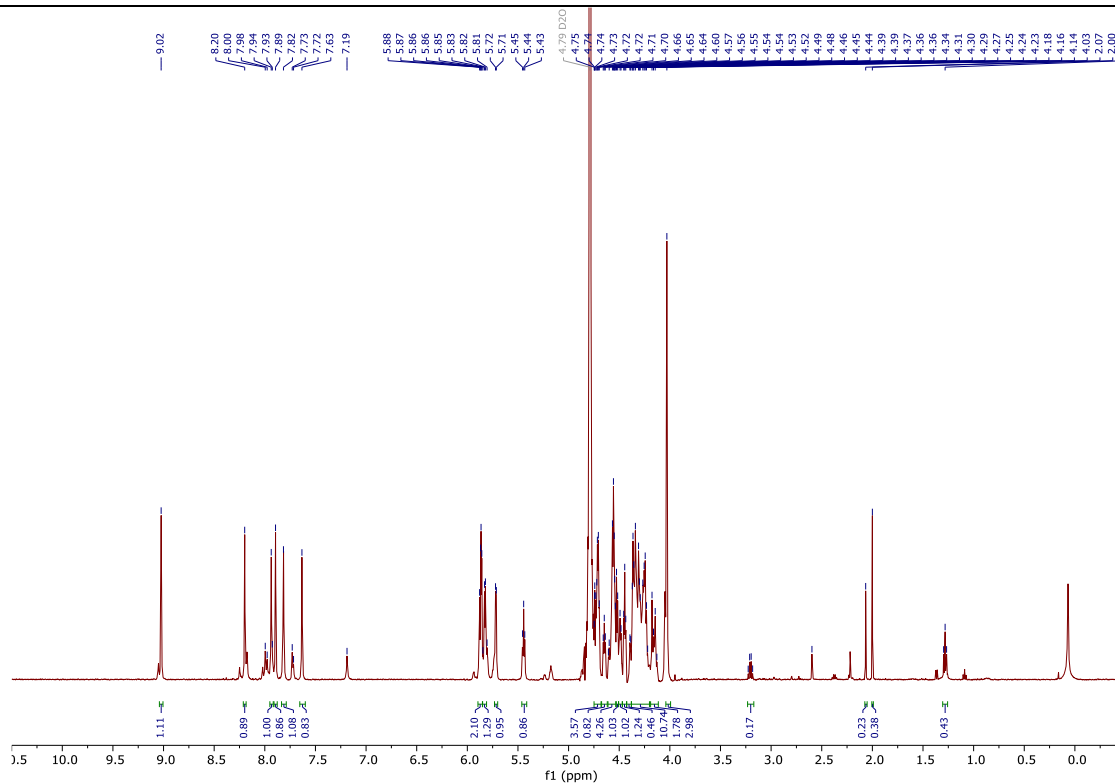
HR MS (isomer 10a)



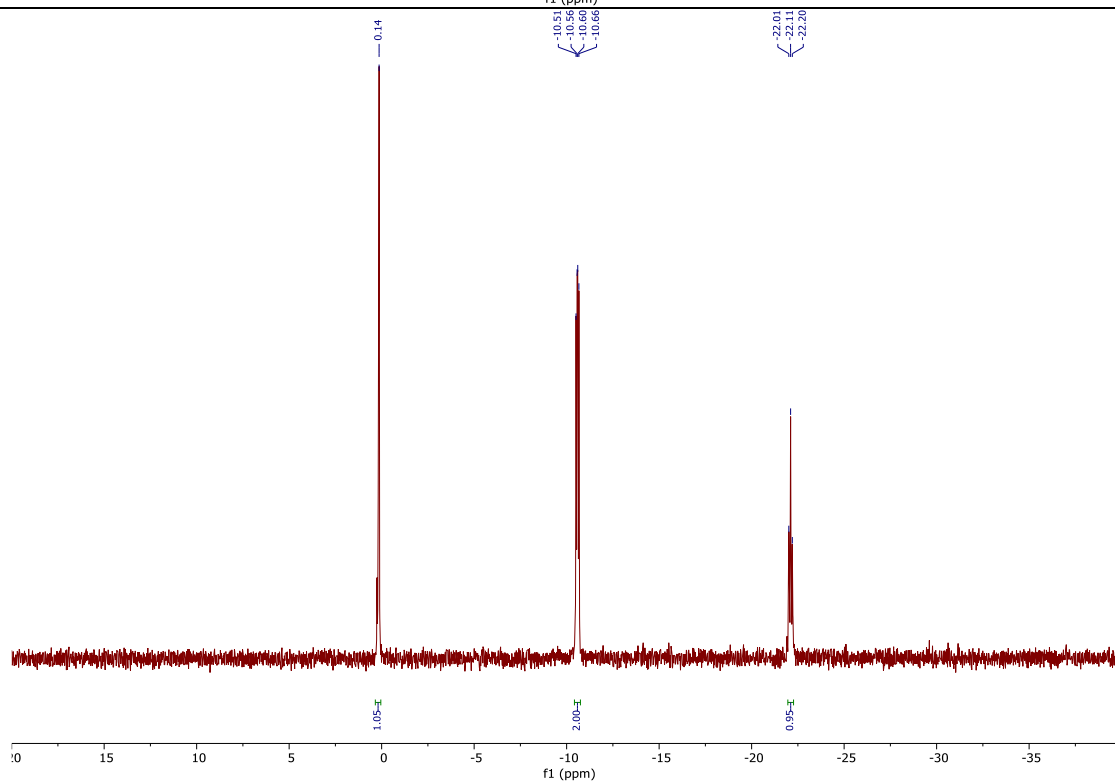
HR MS (isomer 10b)



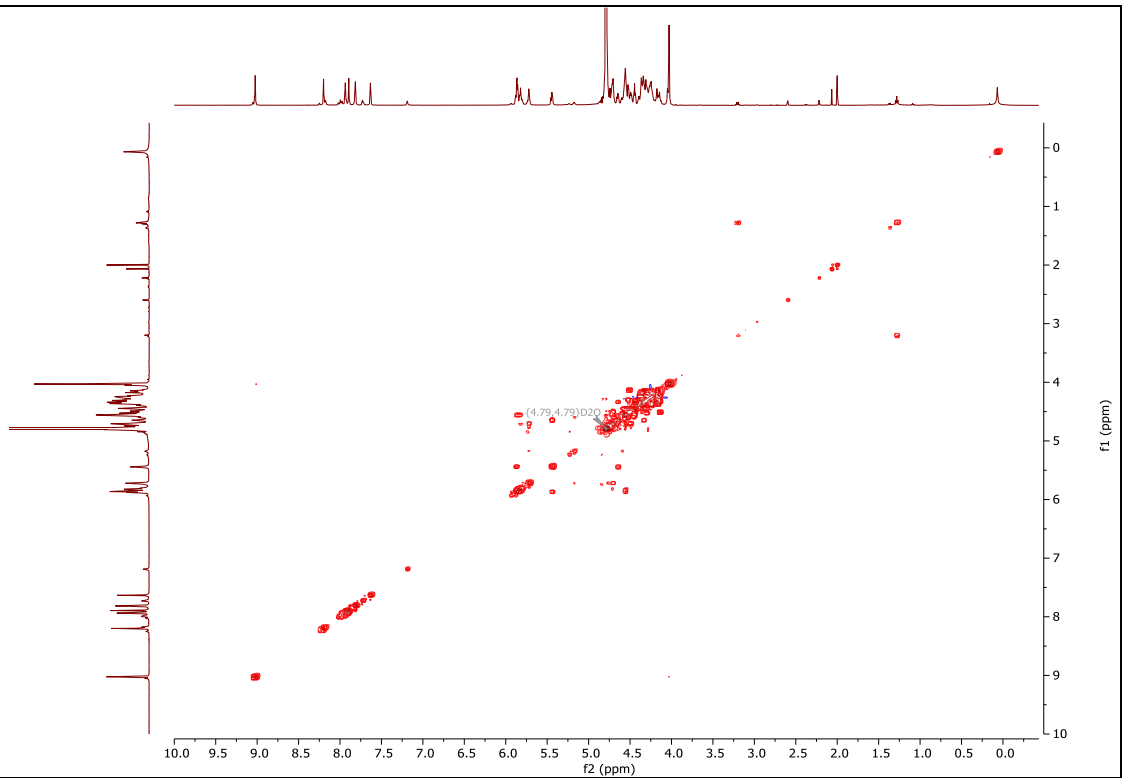
¹H NMR



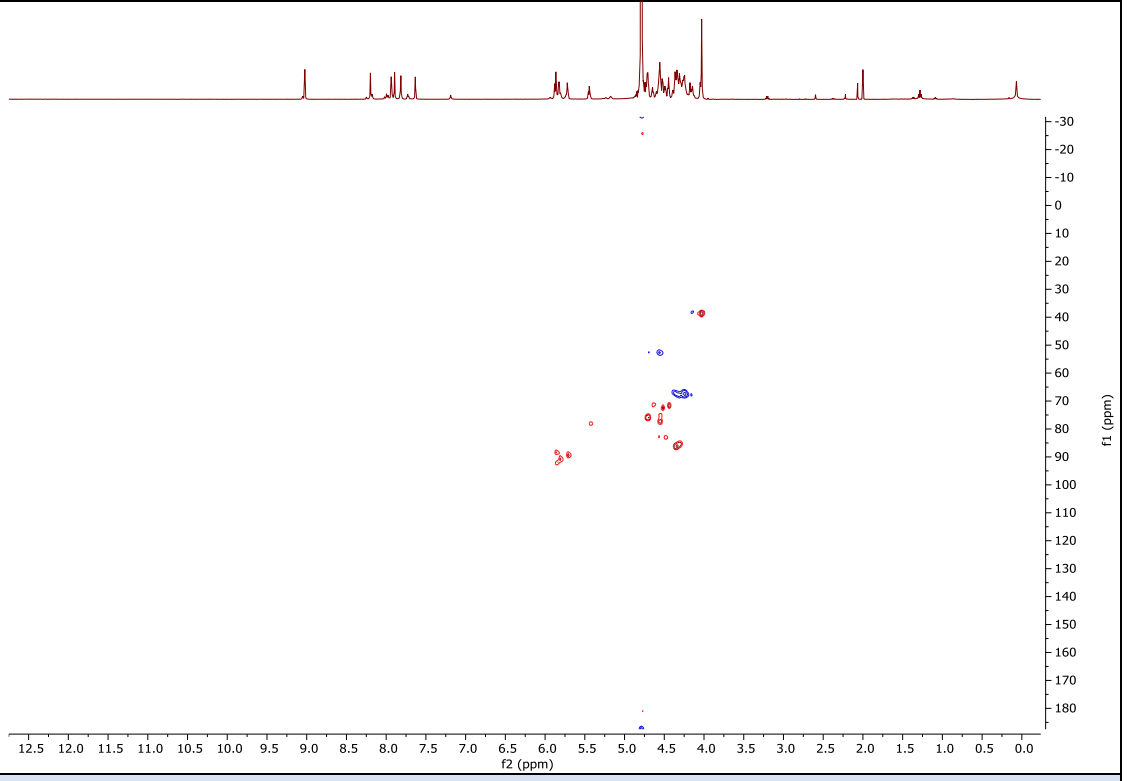
³¹P NMR



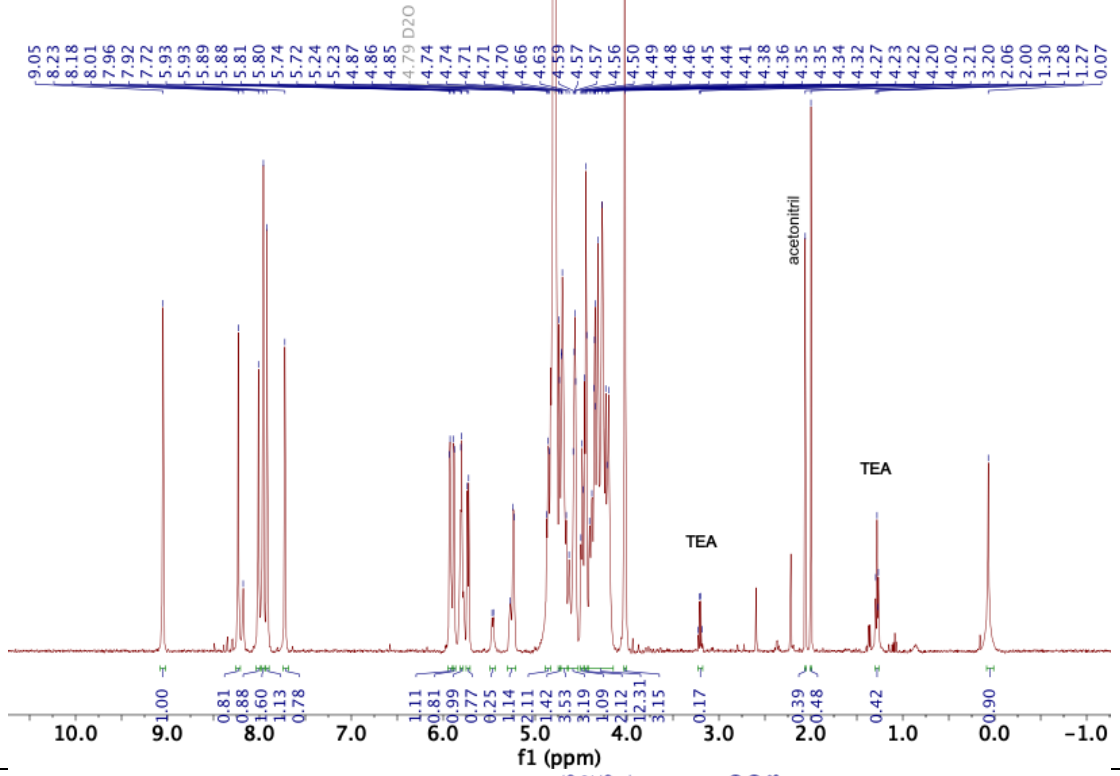
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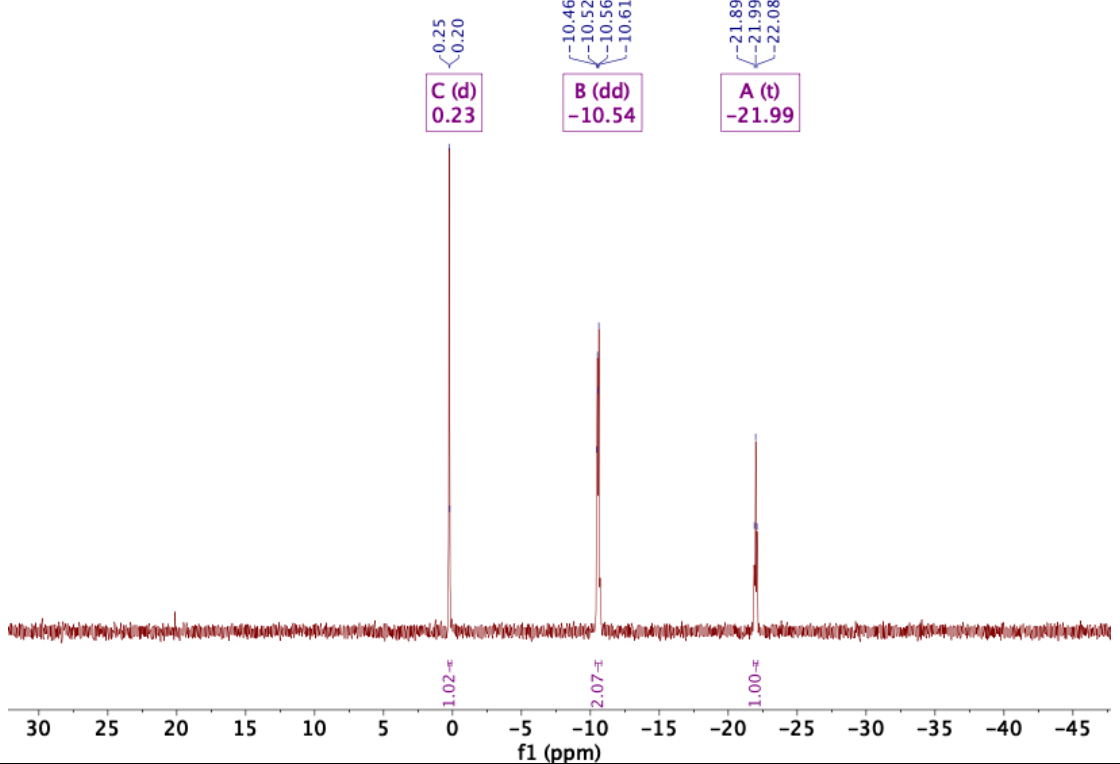
¹³C-¹H HSQC

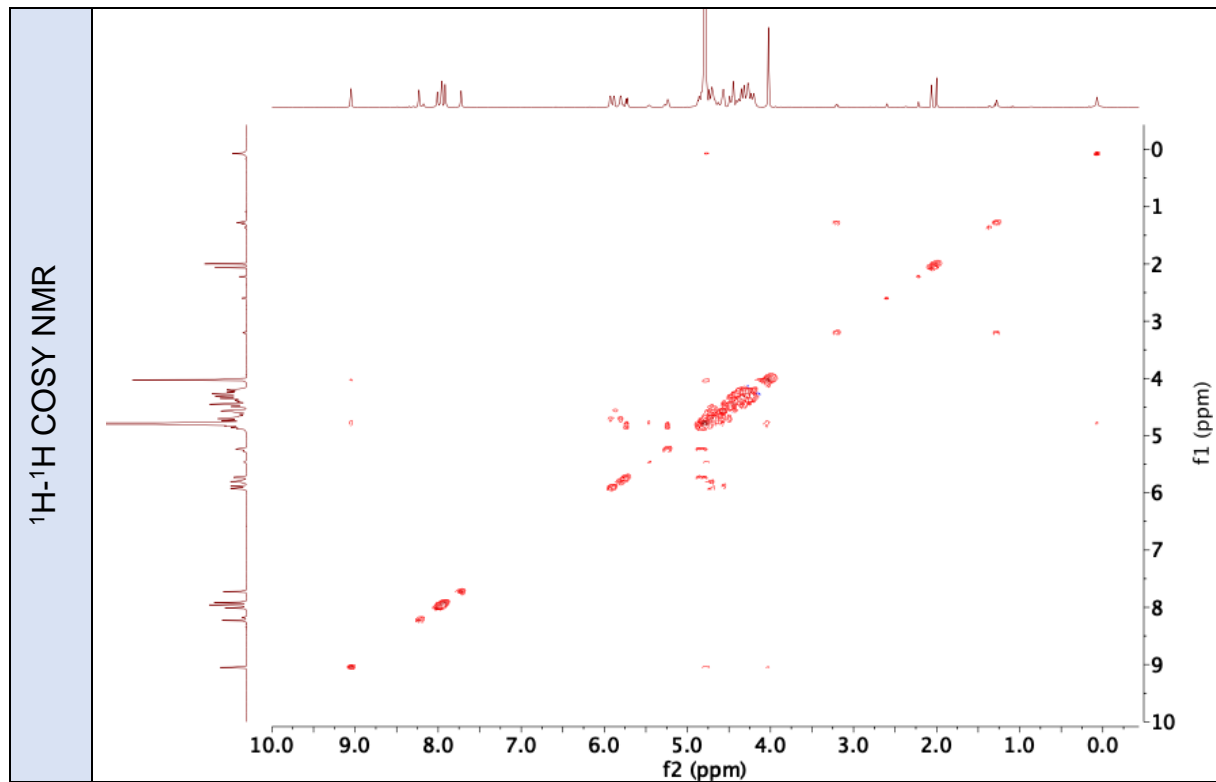


¹H NMR

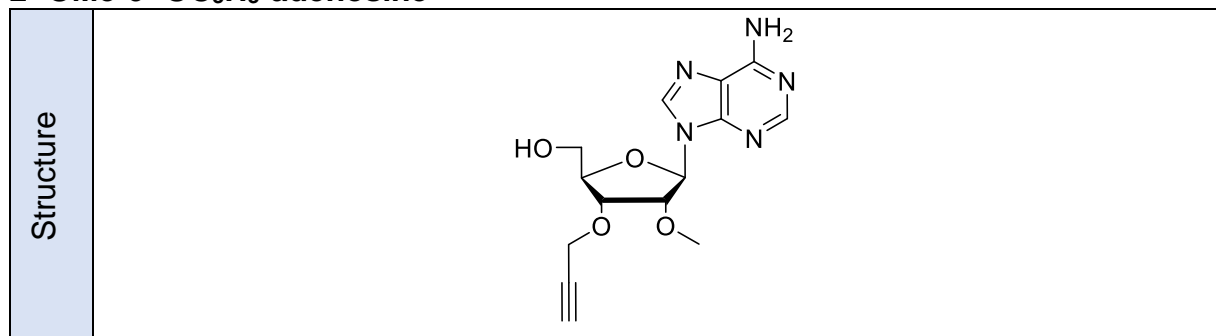


³¹P NMR

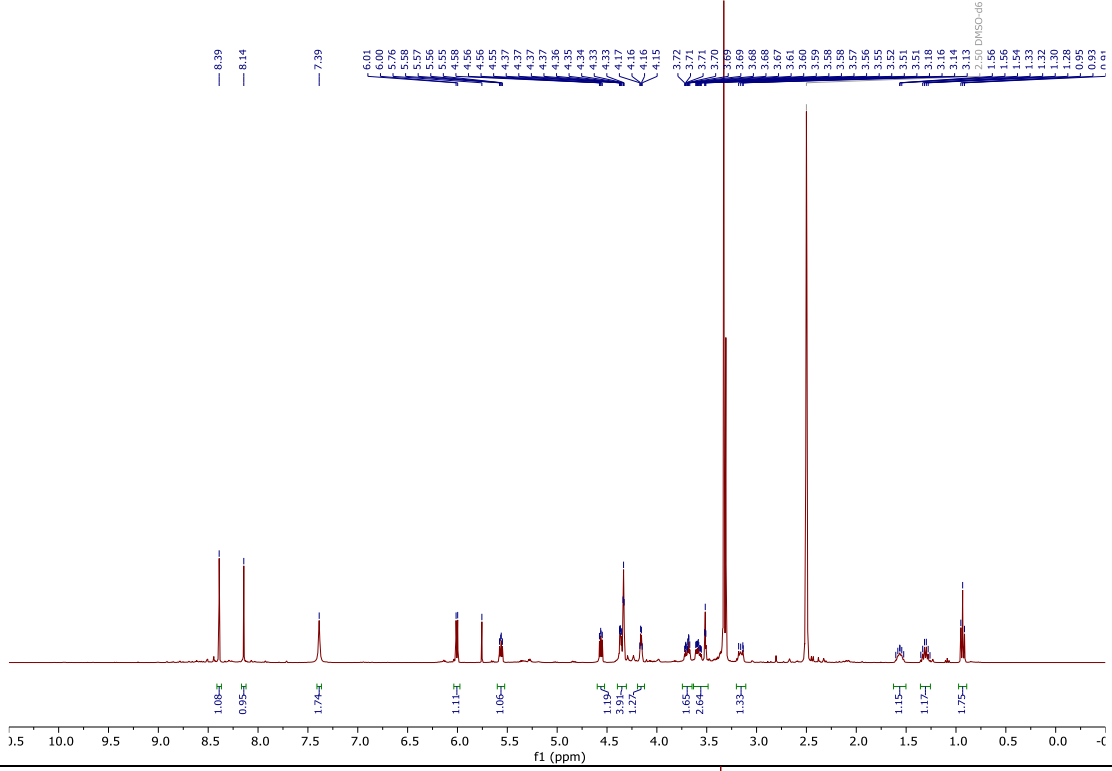




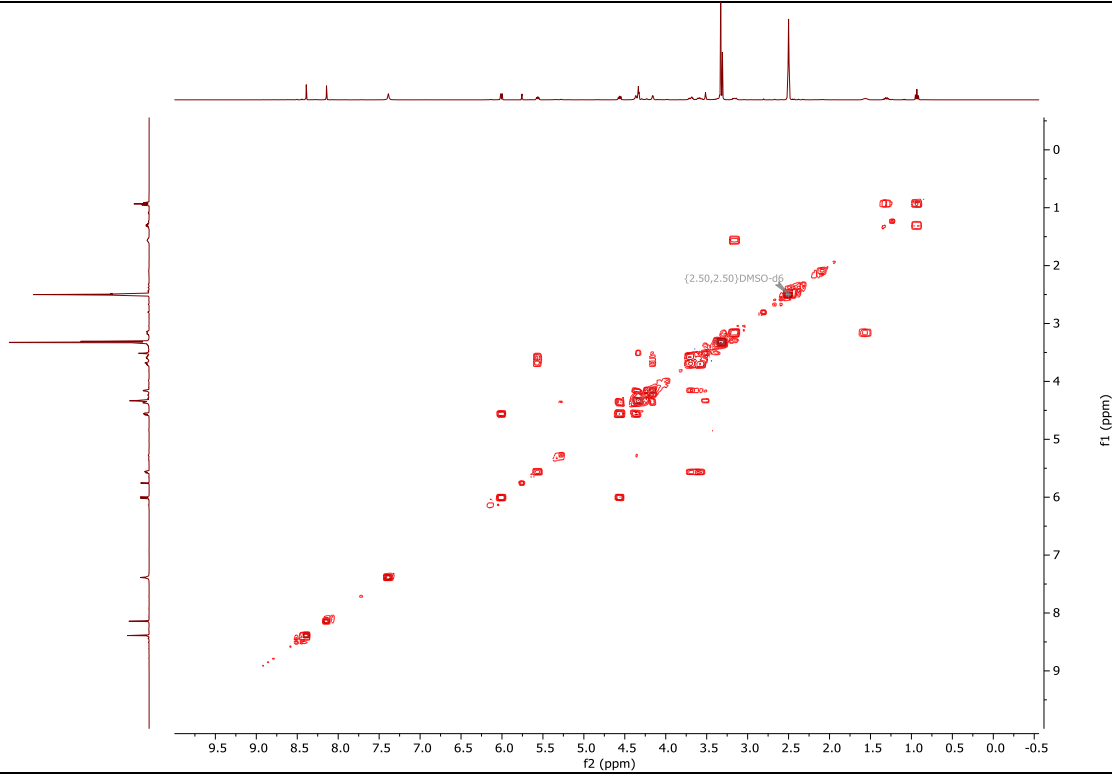
2'-OMe-3'-OC₃H₃-adenosine

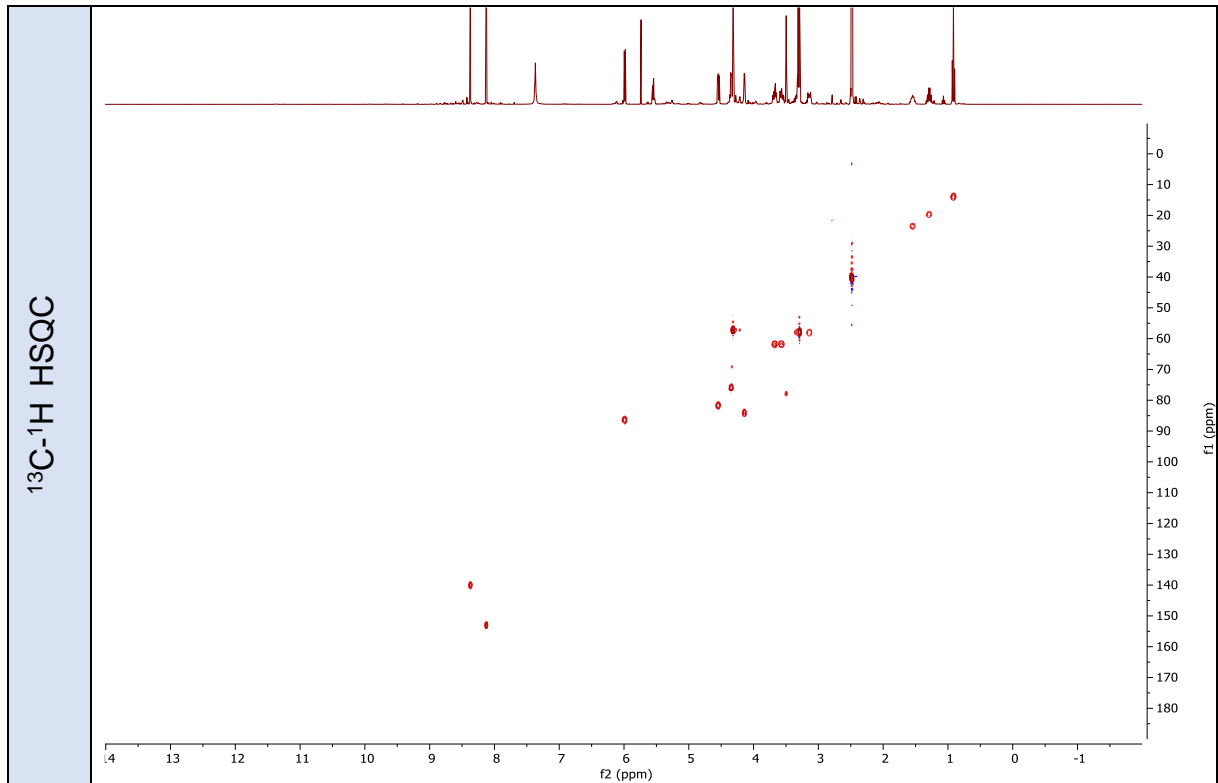


¹H NMR

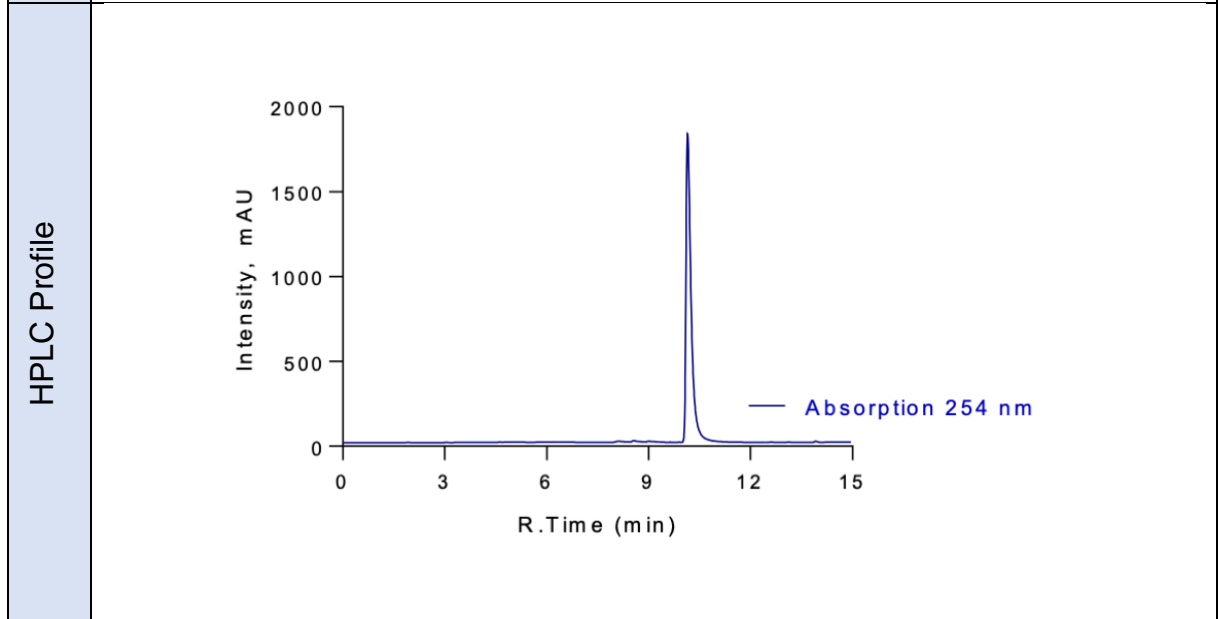
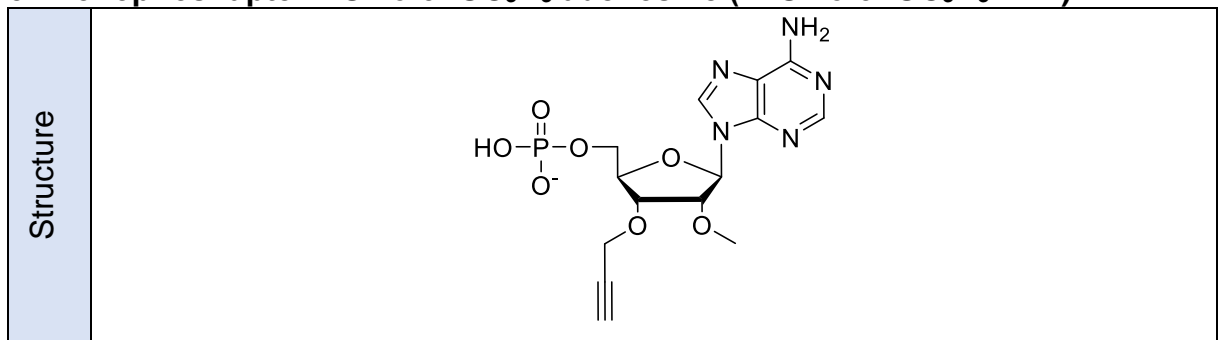


¹H-¹H COSY NMR

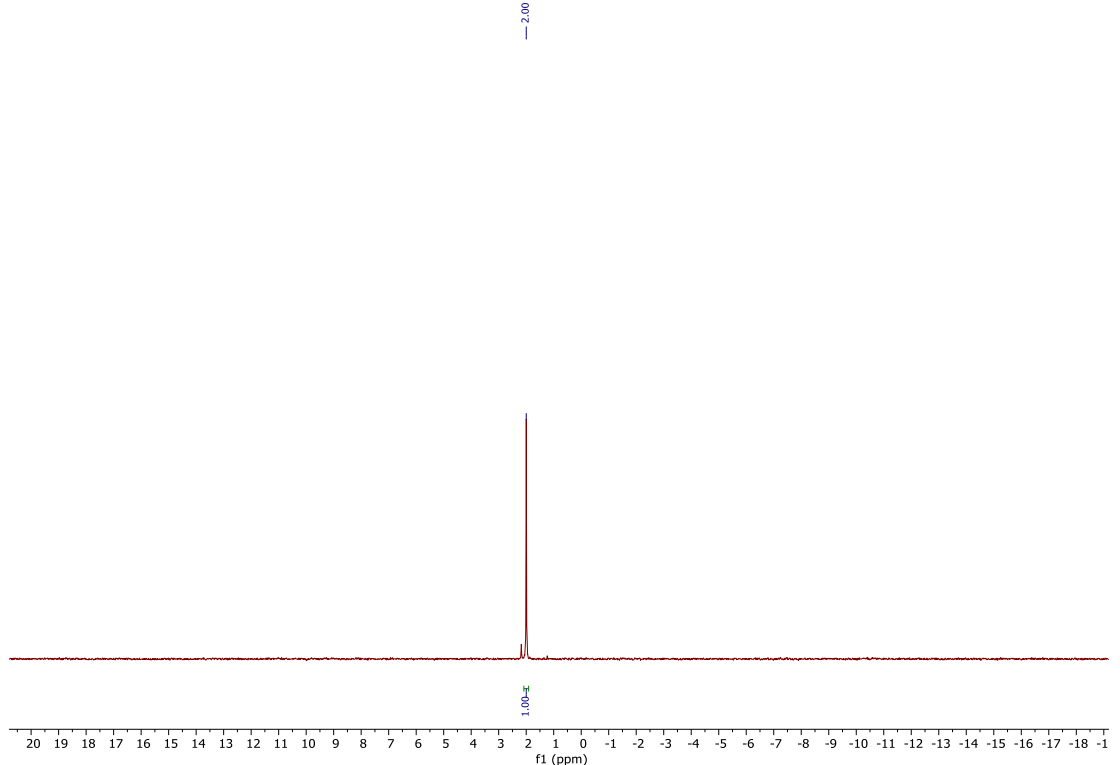




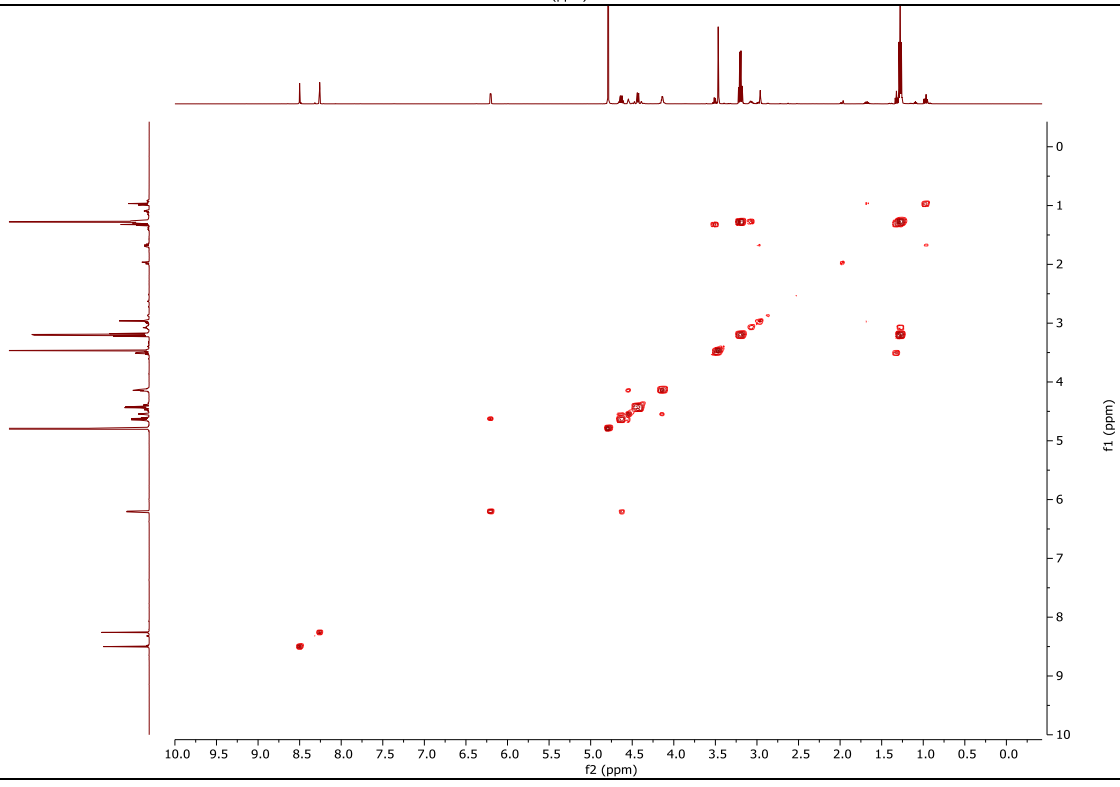
5'-monophosphate 2'-OMe-3'-OC₃H₃ adenosine (2'-OMe-3'-OC₃H₃AMP)

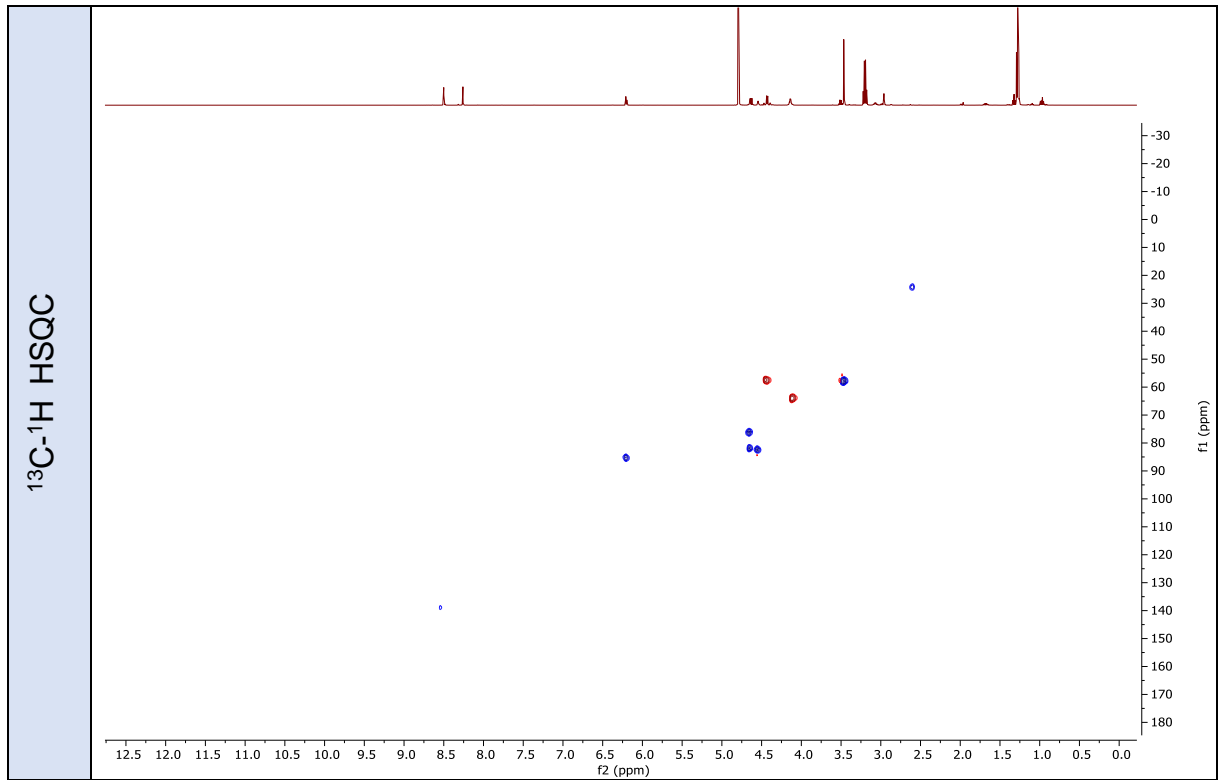


³¹P NMR

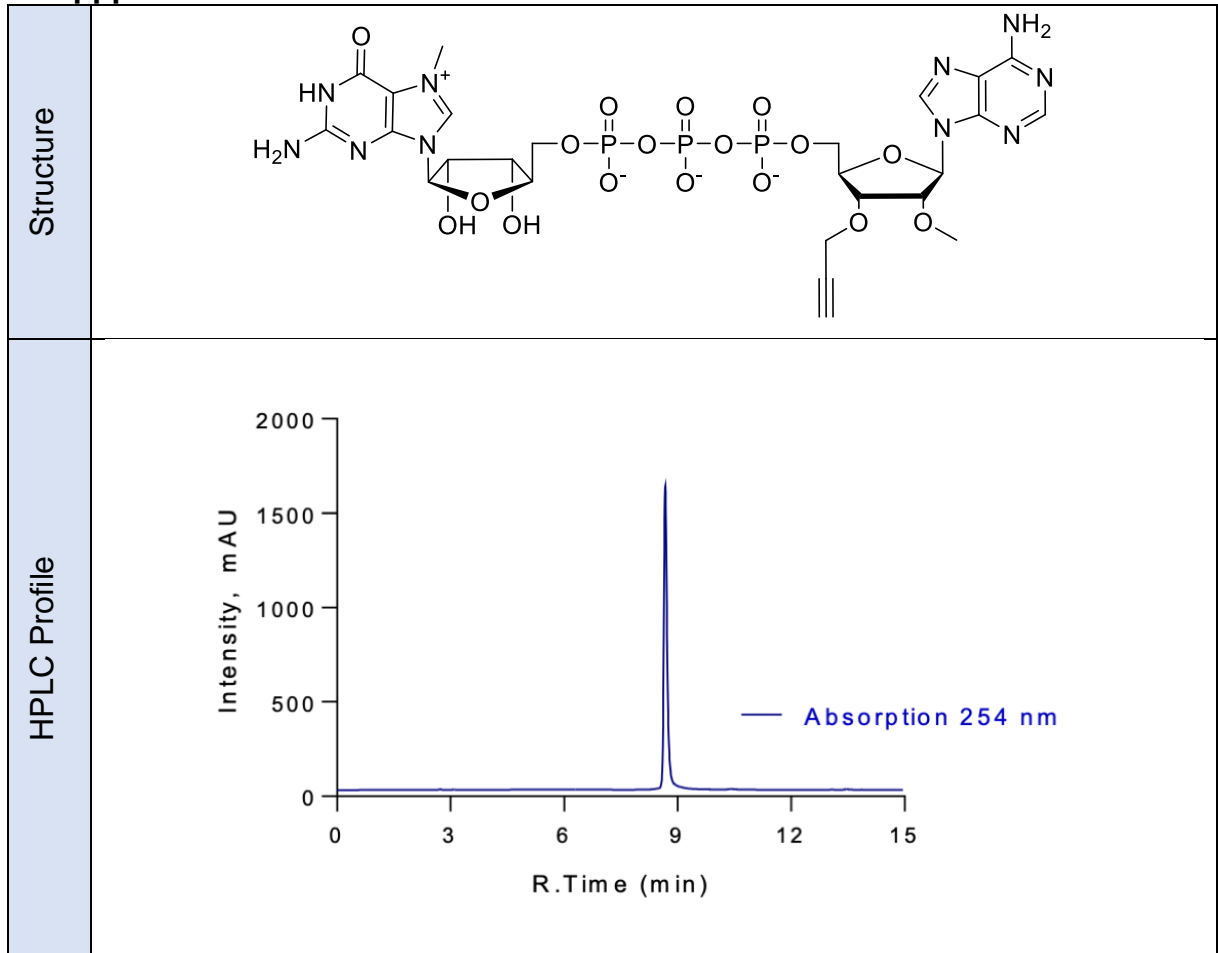


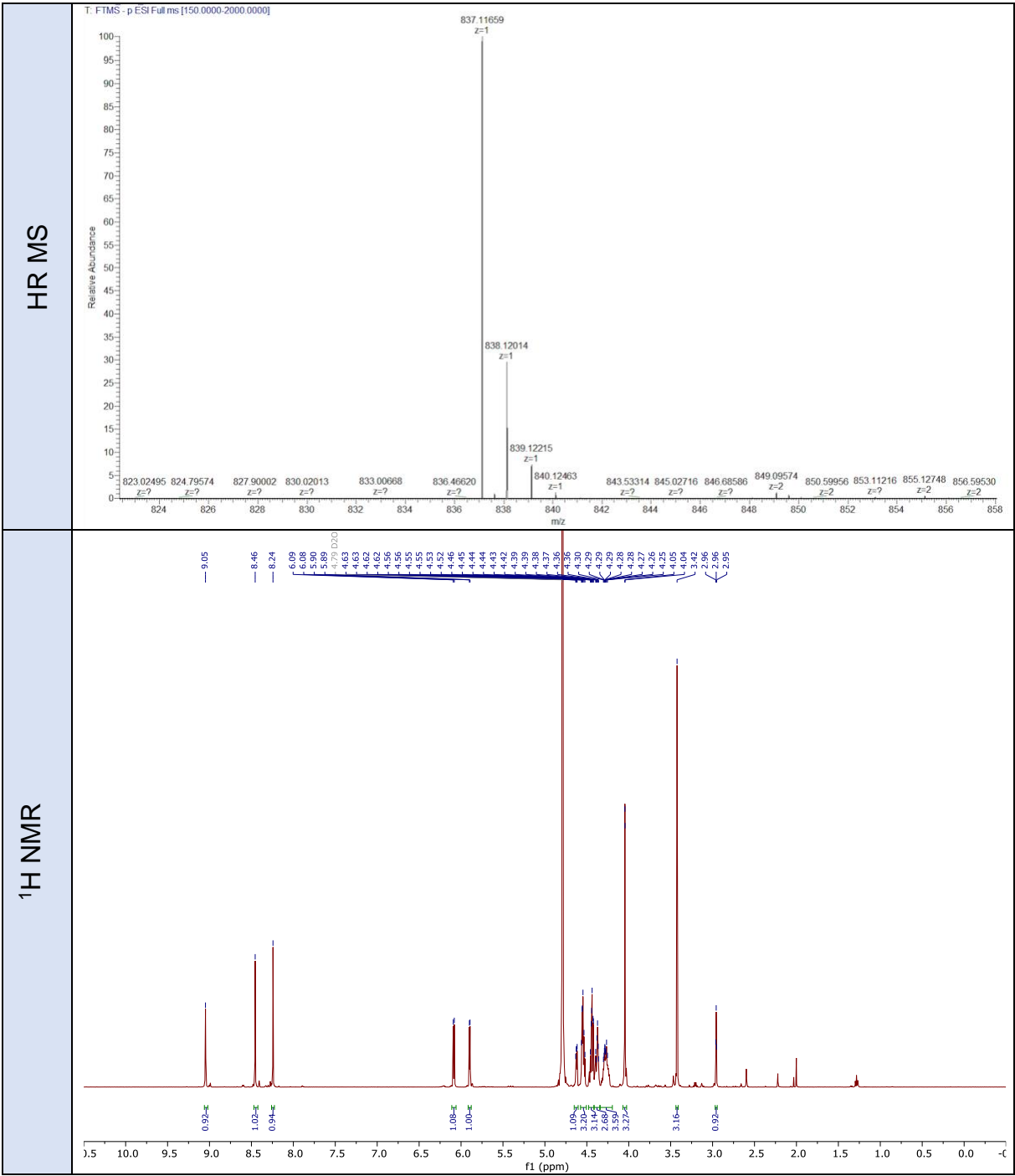
¹H-¹H COSY NMR



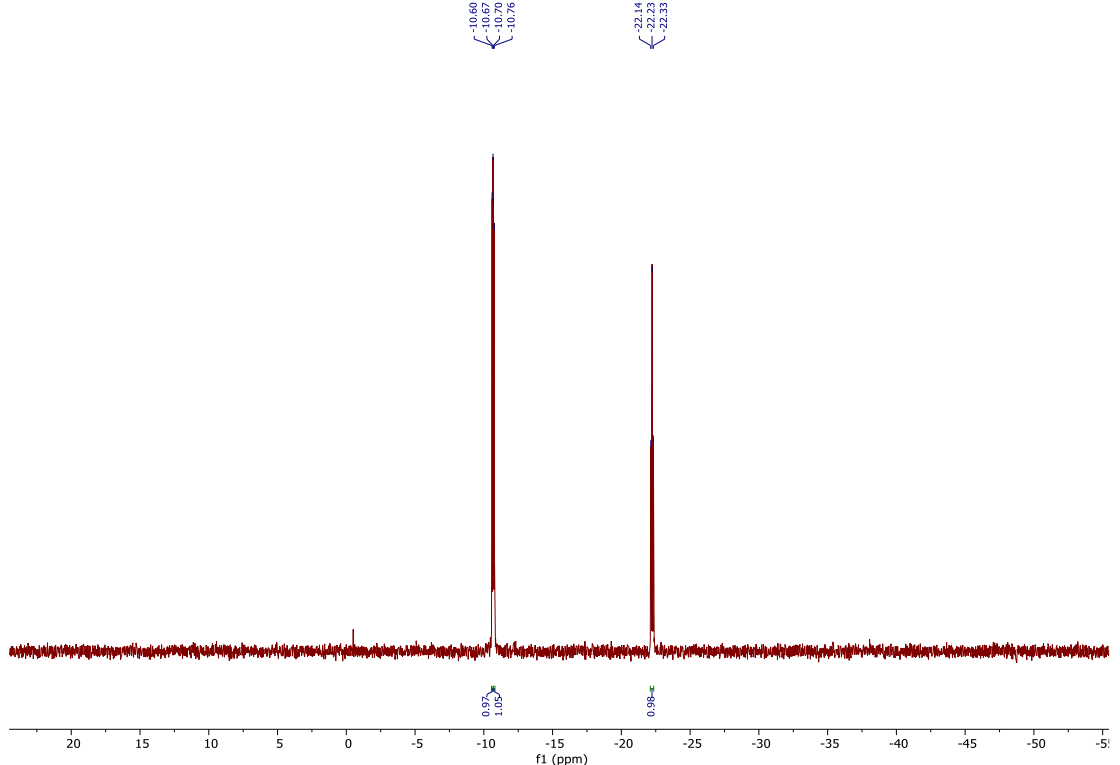


$\text{m}^7\text{GpppA-2'OMe-3'-OC}_3\text{H}_3$

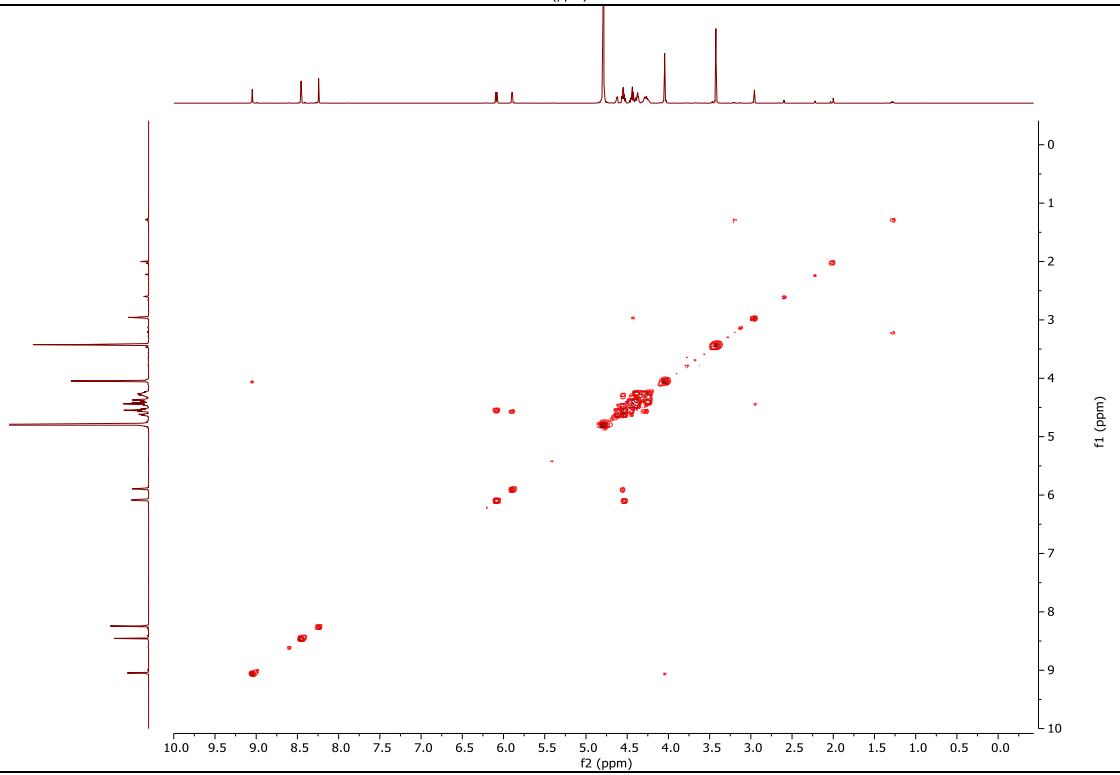


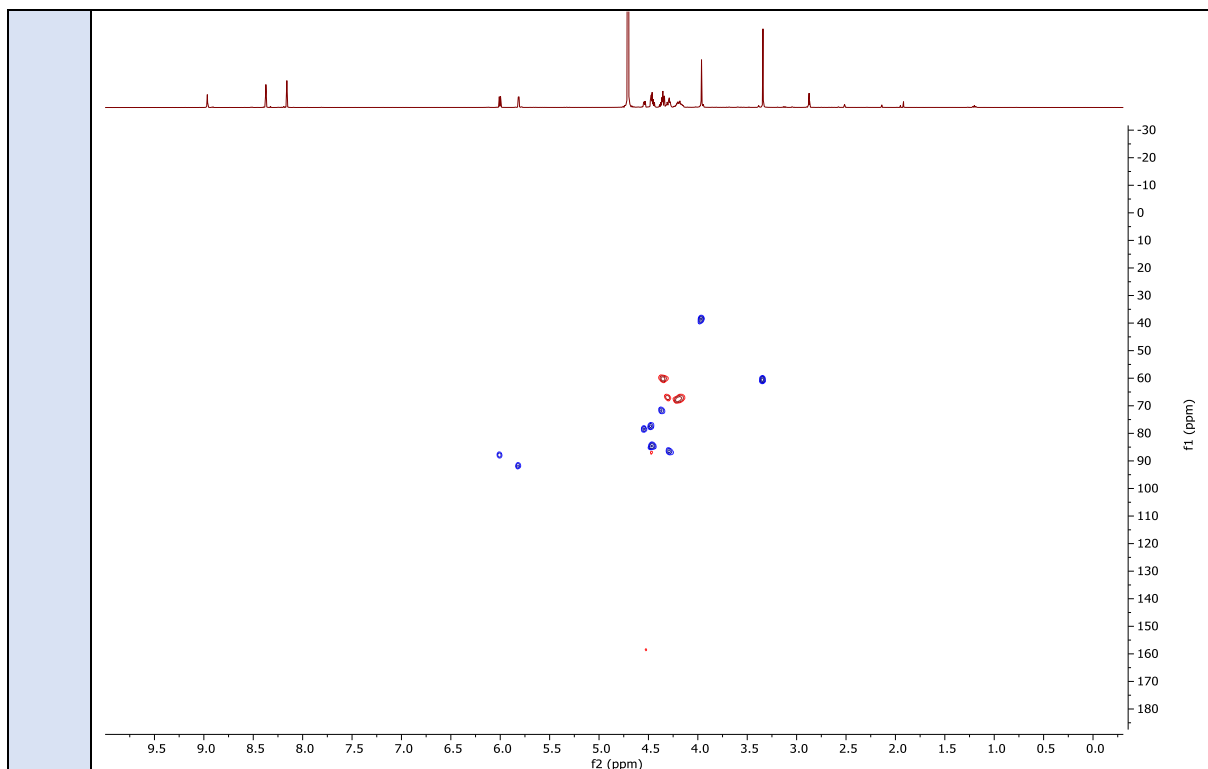


³¹P NMR

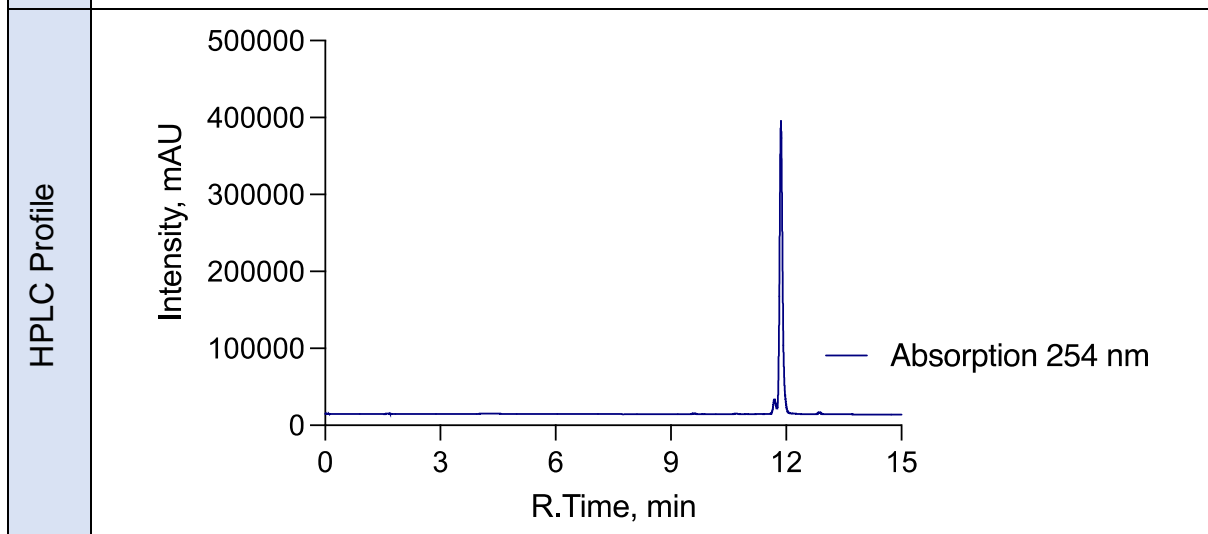
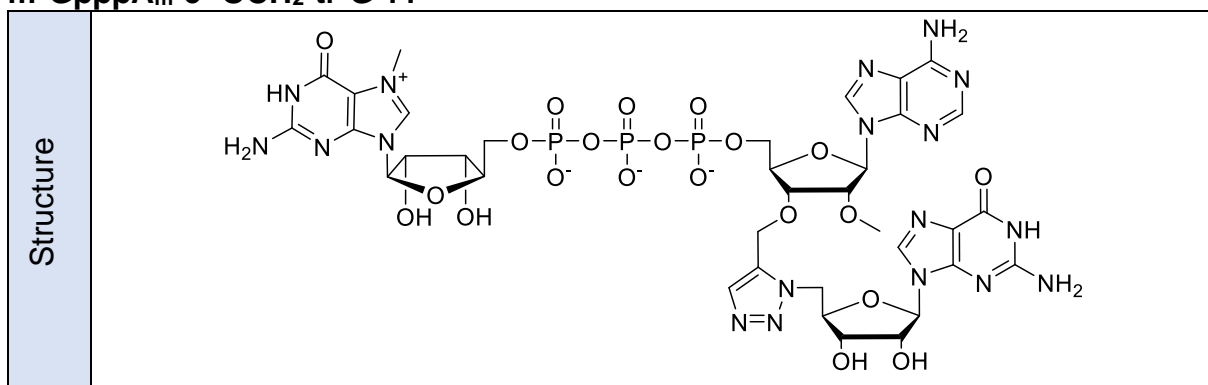


¹H-¹H COSY NMR

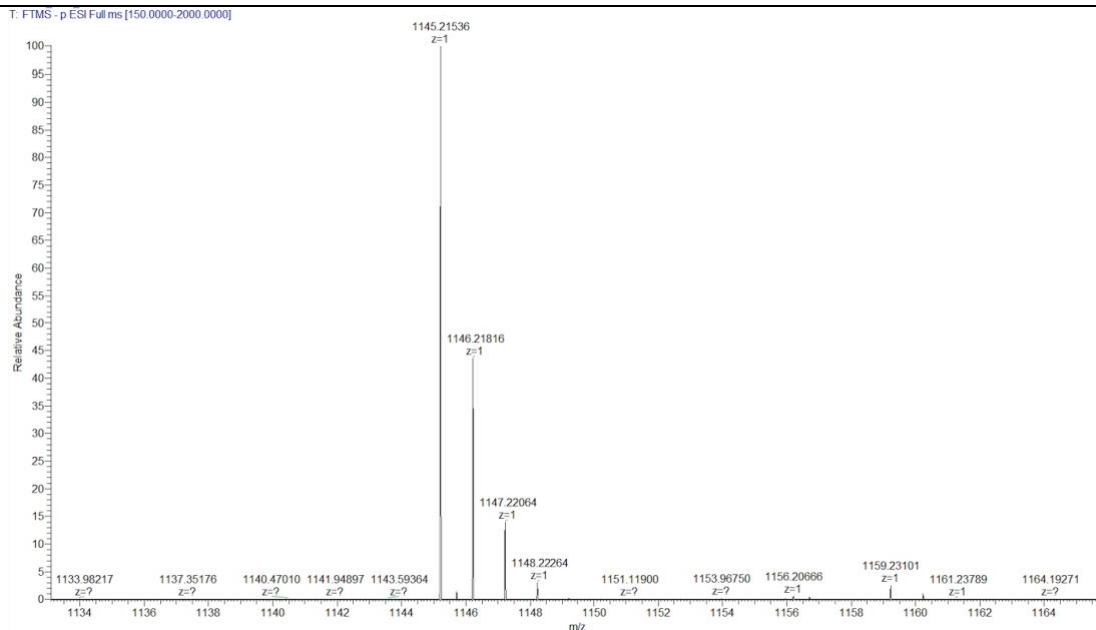




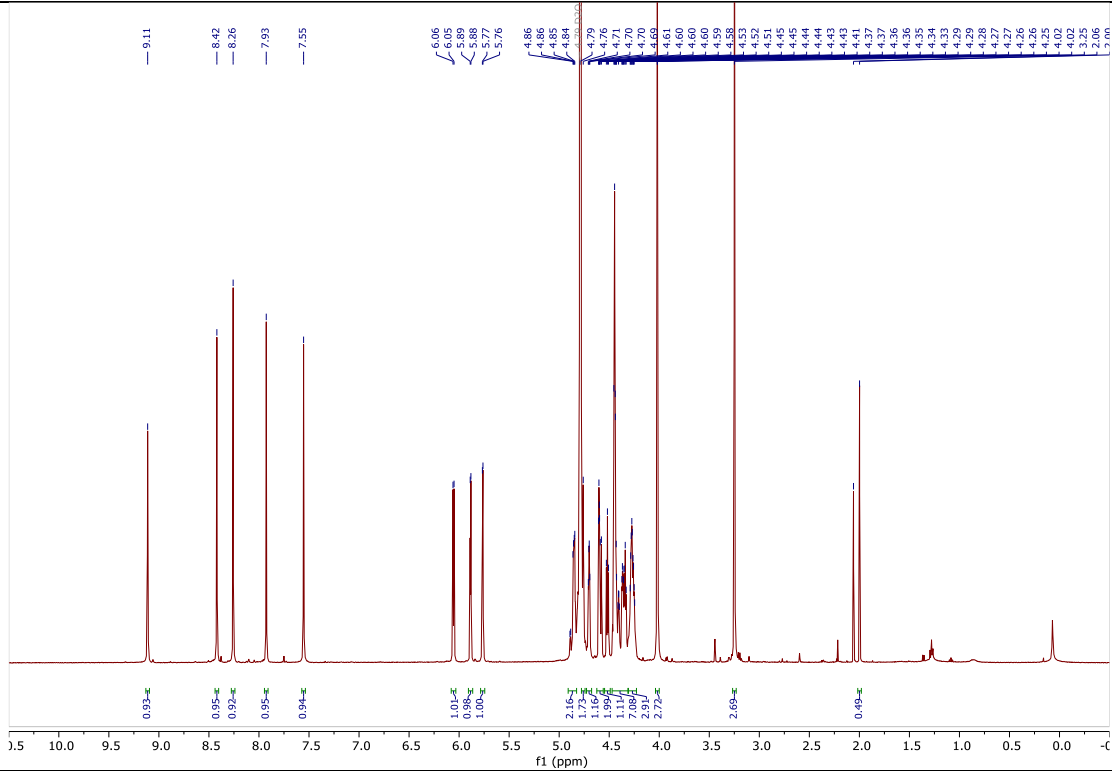
m⁷GpppA_m-3'-OCH₂-tr-G 11



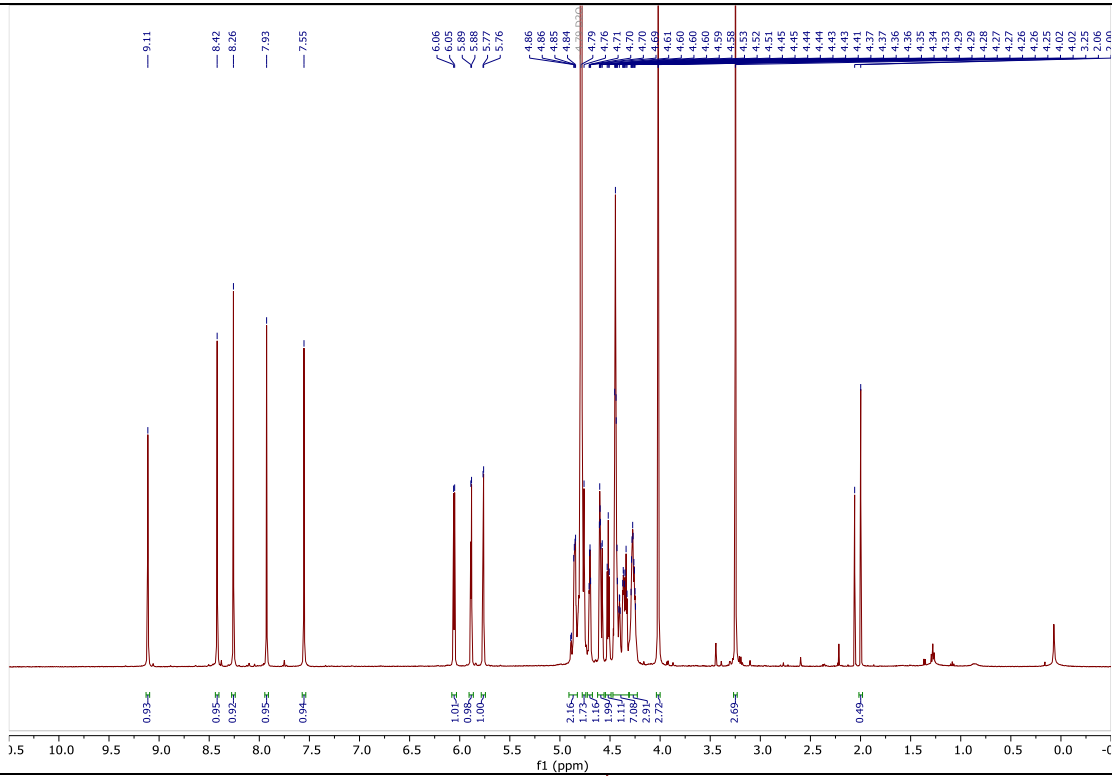
HR MS



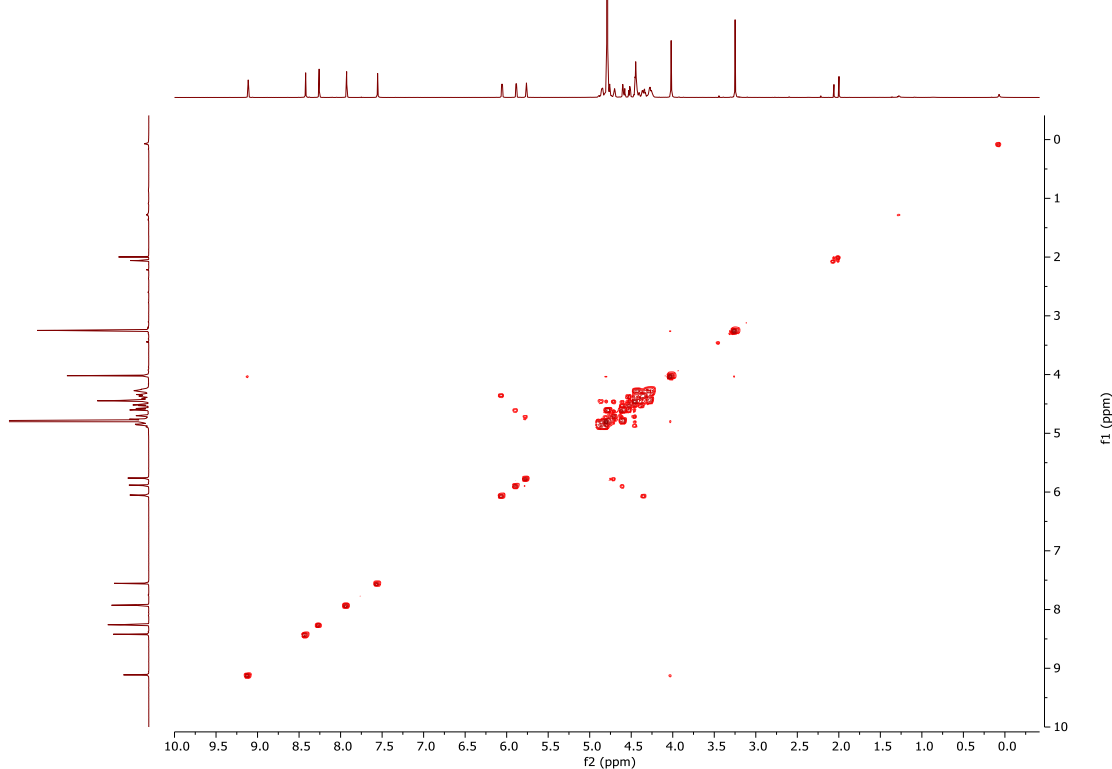
¹H NMR

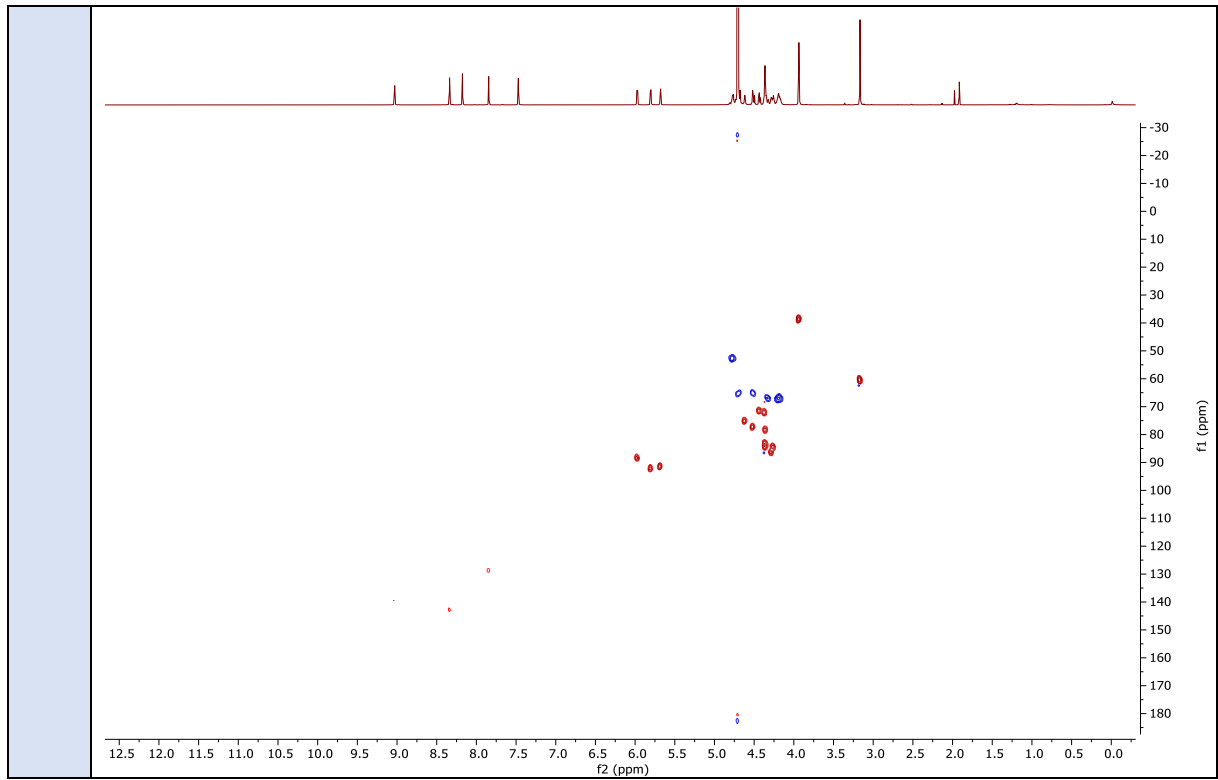


³¹P NMR



¹H-¹H COSY NMR





Figures

Figure S1. mRNA purity

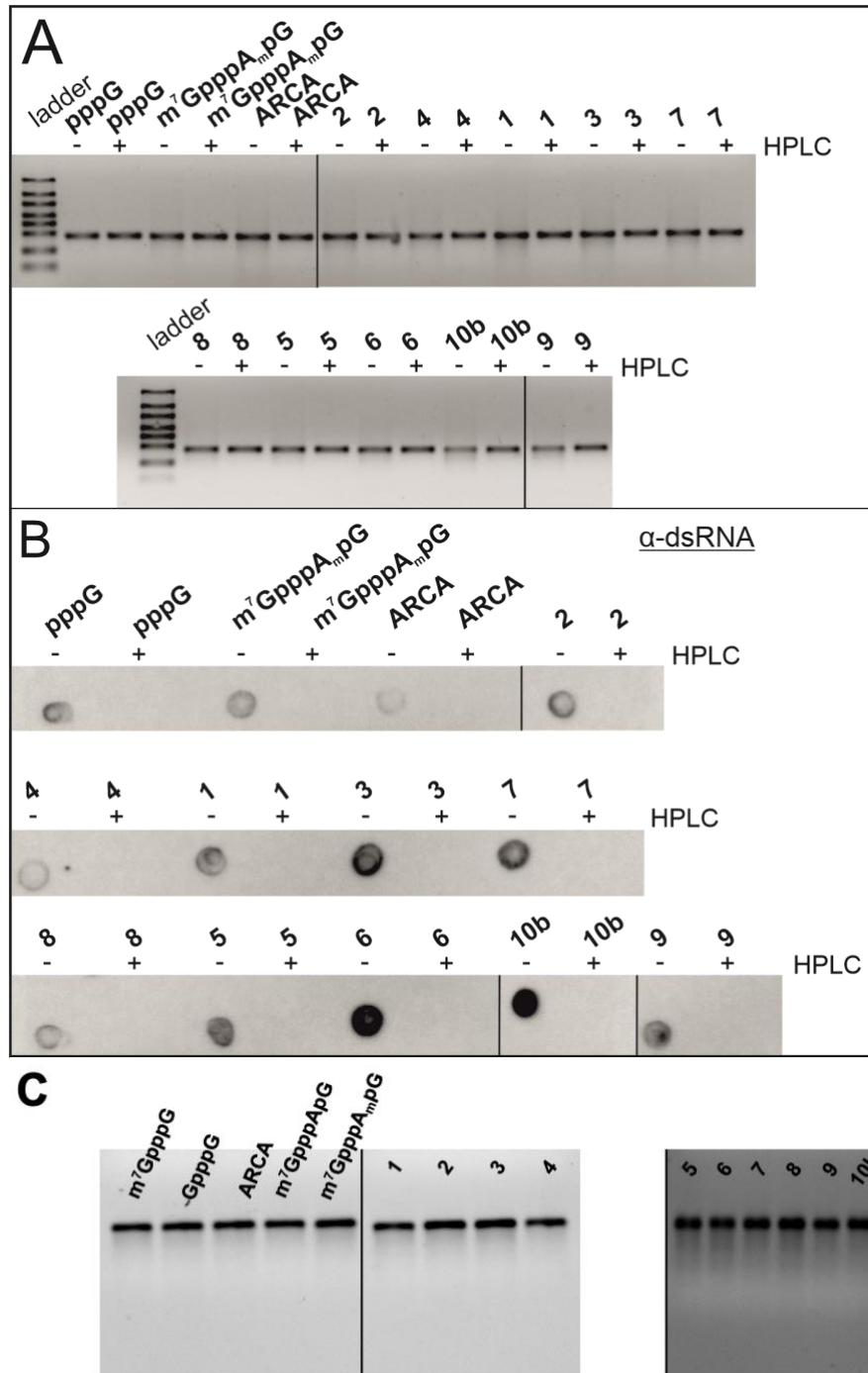


Figure S1. A. Representative 1.2% (TBE) agarose gels of capped RNA encoding *Gaussia* luciferase. B. Dot blot analysis 25 ng of mRNAs were analyzed as previously described in Sikorski et al. 2020³ with modifications. C. Representative 1.2% (TBE) agarose gels of capped RNA encoding *Gaussia* luciferase. mRNAs were purified using a commercial kit and used in RRL experiments.

Figure S2. Luciferase activity in rabbit reticulocyte lysate

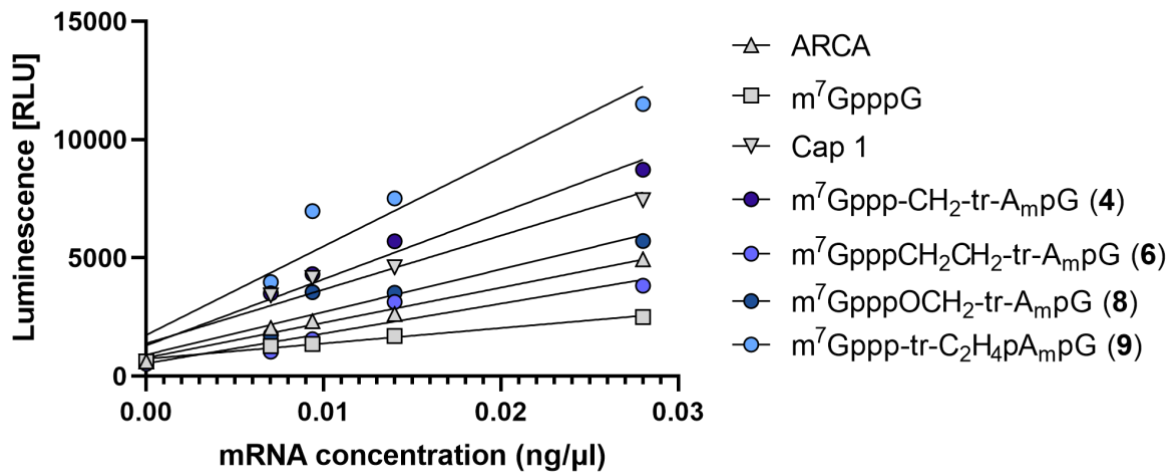


Figure S2. Luciferase activity produced by translation of mRNAs capped with various cap analogues in rabbit reticulocyte lysate – results of a single experiment.

Figure S3. Susceptibility to degradation by hDCP1/DCP2 complex.

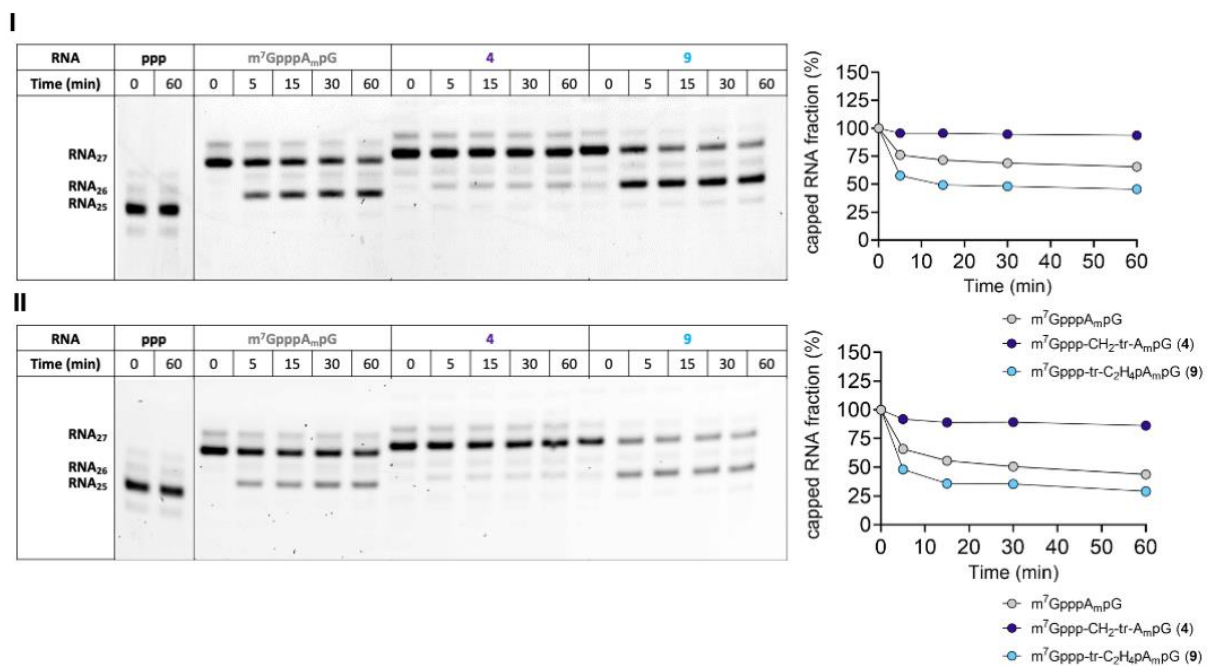


Figure S2. Susceptibility to degradation by hDCP1/DCP2 complex. Two separate repetitions. Experiment: 20 ng of capped RNA was subjected to 11 nM hDCP1/DCP2 in 50 mM Tris/HCl pH 8.0, 50 mM NH₄Cl, 0.01% Igepal with addition of 1 mM DTT, 5 mM MgCl₂ and 2 mM MnCl₂ at 37°C. Reactions were stopped at particular time points (5, 15, 30 and 60 min) and analysed by PAGE (15% acrylamide / 7 M urea / TBE gel) using SyberGold staining. Capped RNA fractions were calculated based on densitometric analysis of bands intensities.

RNA₂₅ - uncapped RNA (control).

RNA₂₇ - RNA capped with m⁷GpppAm_mP G, cap 4 or cap 9.

RNA₂₆ - RNA decapped by hDCP1/DCP2 complex.

- (1) Walczak, S.; Nowicka, A.; Kubacka, D.; Fac, K.; Wanat, P.; Mroczek, S.; Kowalska, J.; Jemielity, J. A novel route for preparing 5' cap mimics and capped RNAs: phosphate-modified cap analogues obtained via click chemistry. *Chemical Science* **2017**, *8* (1), 260-267, Article. DOI: 10.1039/c6sc02437h.
- (2) Hebert, N.; Beck, A.; Lennox, R. B.; Just, G. A NEW REAGENT FOR THE REMOVAL OF THE 4-METHOXYBENZYL ETHER - APPLICATION TO THE SYNTHESIS OF UNUSUAL MACROCYCLIC AND BOLAFORM PHOSPHATIDYLCHOLINES. *Journal of Organic Chemistry* **1992**, *57* (6), 1777-1783, Article. DOI: 10.1021/jo00032a033.
- (3) Sikorski, P. J.; Warminski, M.; Kubacka, D.; Ratajczak, T.; Nowis, D.; Kowalska, J.; Jemielity, J. The identity and methylation status of the first transcribed nucleotide in eukaryotic mRNA 5' cap modulates protein expression in living cells. *Nucleic Acids Research* **2020**, *48* (4), 1607-1626, Article. DOI: 10.1093/nar/gkaa032.