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# Florescent analogs of cyclic and linear dinucleotides as phosphodiesterase and oligoribonuclease activity probes

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**Experimental Section**:

1) General experimental methods

Phosphoramidites were purchased from Chemgenes and/or Glen Research. Column used in HPLC was obtained from Nacalai. Solvents used in HPLC were purchased from VWR and/or Fisher Scientific. Other chemicals were all purchased from Sigma-Aldrich unless indicated otherwise. Dry acetonitrile and pyridine were obtained from distillation over $CaH_2$ and dried overnight with drying kit containing molecular sieves (4Å) from Chemegenes prior to use. HPLC was performed with an Agilent system, equipped with a UV detector. All samples were filtered with a 0.2 μm syringe filter prior to the injection. NMR spectra were measured on Bruker 400 or 500 MHz. Mass spectra (MS) were recorded by JEOL AccuTOF-CS (ESI negative mode) or Agilent MSD/TOF. UV absorbance spectra were obtained on a JASCO V-630 spectrophotometer with 1 cm path length cuvette. Fluorescence spectra were carried out on a Cary Eclipse fluorescence spectrophotometer or BioTek Cytation 5 Cell Imaging Multi-Mode Reader. The concentration of a stock solution of c-di-GMP was determined by the measuring of absorbance at 253 nm using 28,600 $M^{-1}cm^{-1}$ as a molar extinction coefficient. The concentration of c-di-AMP was determined by the measuring of absorbance at 259 nm using 27,000 $M^{-1}cm^{-1}$ as a molar extinction coefficient. The concentration of stock solutions of compound **1** and **5** were determined from the absorbance at 260 nm using 15,300 $M^{-1}cm^{-1}$ as molar extinction coefficients. The concentration of stock solution of compound **2** was determined from the absorbance at 260 nm using 19,000 $M^{-1}cm^{-1}$ as molar extinction coefficients. The concentration of stock solutions of compound **3** and **6** were determined from the absorbance at 260 nm using 14,500 as molar extinction coefficients. The concentration of stock solutions of compound **4** was determined from the absorbance at 260 nm using 18,200 $M^{-1}cm^{-1}$ as molar extinction coefficients.

2) Synthesis of compounds **2** to **4**.

Literature procedure was used but with a slight modification.[1] DMT-protected guanine or adenine phosphoramidite (450 mg) was dissolved in dry acetonitrile (5 mL) and to this was added water (18 μL) and pyridinium trifluoroacetate (100 mg) and the mixture was reacted for 1 min. The cyanoethyl protecting group was removed by adding *tert*–butyl

amine (5 mL) and the reaction stirred for 10 min. Solvent was removed and dichloromethane (6 mL), water (90 µL) and 6% dichloroacetic acid in dichloromethane (6 mL) were added. The reaction was stirred for 10 min to deprotect the DMT group. Dry pyridine (0.7 mL) was added to the reaction mixture and concentrated to dryness using a rotary evaporator. Any adventitious water was removed with an oil pump. To this dried sample was added DMT-protected fluorescent base phophoramidite (250 mg, etheno or 2-AP) dissolved in 5 mL dry acetonitrile containing 3 Å Molecular sieves. The coupled dinucleotide was immediately oxidized with 0.25 mL of 5.5 M *tert*-butyl hydroperoxide in decane for 30 min. After quenching with sodium thiosulfate solution, the reaction mixture was concentrated again to dryness. Deprotection was carried out again by adding dichloromethane (8 mL), water (90 µL) and 6% dichloroacetic acid in dichloromethane (8 mL) and reacted for 10 min. The reaction was quenched with dry pyridine (5 mL). The cyclization into cyclic dinucleotides was done with 95% 5,5-dimethyl-2-oxo-2-chloro-1,3,2-dioxaphosphinane, DMOCP, (400 mg) for 10 min. Water (0.3 mL) was added to the cyclization reaction to quench the reaction, followed by addition of iodine (100 mg) and stirring for 5 min. Nucleobase protecting group was removed with 33% methylamine in anhydrous ethanol (10 mL), stirring for 90 min. TBS protecting group at the 2'-position was removed by adding triethylamine trihydrogen fluoride (0.5 mL) and stirred at 55 $^{o}$C for 90 min. Acetone was then added to the reaction mixture and white precipitates, which formed, were collected after centrifugation. The crude product was re-dissolved in water (5 mL), purified by HPLC (Nacalai tesque 5C18-PAQ column), HPLC condition: 1→13% B, 0→16 min (A: 0.1 M triethylammonium acetate (TEAA) in water, pH 7.0; B: acetonitrile), concentrated at a reduced pressure, and washed with acetone (2 mL × 5) to remove the excess of TEAA buffer.

**3'3'-cA(d2AP)MP** (Compound **2**). $^{1}$H NMR (400 MHz, $D_2O$, with water suppresion) δ 8.49 (s, 1H), 8.37 (s, 1H), 8.23 (s, 1H), 8.13 (s, 1H), 6.41-6.27 (m, 1H), 6.10 (s, 1H), 4.42-3.92 (m, 4H), 3.19-3.06 (m, 1H), 3.00-2.84 (m, 1H). $^{31}$P NMR (162 MHz, $D_2O$) δ -0.30, -0.46. HRMS (ESI-) m/z calcd for $C_{20}H_{23}N_{10}O_{11}P_2^-$ $[M-H]^-$ 641.1028, found 641.1051.

**3'3'-cG(etheno)MP** (Compound **3**). $^{1}$H NMR (400 MHz, $D_2O$, with water suppression) δ 8.86 (s, 1H), 8.31 (s, 1H), 7.74 (d, $J$ = 1.4 Hz, 1H), 7.59 (s, 1H), 7.35 (d, $J$ = 1.3 Hz, 1H), 6.07 (s, 1H), 5.72 (s, 1H), 4.42-4.23 (m, 1H), 4.05-3.92 (m, 2H). $^{31}$P NMR (162 MHz, $D_2O$) δ -0.73, -1.02. HRMS ($ESI^-$) *m/z* calcd for $C_{22}H_{23}N_{10}O_{13}P_2^-$ $[M-H]^-$ 697.0927, found 697. 0903.

**3'3'-cA(etheno)MP** (Compound **4**). $^{1}$H NMR (500 MHz, $D_2O$, with water suppression) δ 8.88 (s, 1H), 8.38 (s, 1H), 8.19 (s, 1H), 8.05 (s, 1H), 7.78 (s, 1H), 7.44 (s, 1H), 6.18 (s, 1H), 6.03 (s, 1H), 4.41-4.33 ( 4H), 4.12-3.97 (m, 3H). $^{31}$P NMR (203 MHz, $D_2O$) δ -1.64, -1.67. HRMS (ESI-) m/z calcd for $C_{22}H_{23}N_{10}O_{10}P_2^-$ $[M-H]^-$ 681.0978, found 681.0969.

3) Synthesis of compounds **5** and **6**

On Applied Biosystem 392 DNA/RNA synthesizer, 2-AP or etheno phosphoramidite was coupled to rA or rG-controlled pore glass (CPG), followed by cleavage from CPG with 30% $NH_4OH$ at room temperature for 12 hours. The TBS group was then deprotected using $Et_3N{\cdot}3HF$ at 55 $^{o}$C for 1 hour. The reaction mixture was washed with acetone (50 mL X3) and subjected to HPLC purification.

**(d2AP)pG** (Compound **5**). $^{1}$H NMR (500 MHz, D$^{6}$-DMSO) δ 8.58 (s, 1H), 8.24 (s, 1H), 7.96 (s, 1H), 6.57 (s, 4H), 6.29-6.20 (m, 1H), 5.69 (d, *J* = 5.8 Hz, 1H), 5.39-5.34 (m, 3H), 4.71 (s, 1H), 4.52 (s, 1H), 4.21 (s, 1H), 4.01-3.83 (m, 5H). $^{31}$P NMR (203 MHz, $D_2O$) δ -1.18. HRMS (ESI-) m/z calcd for $C_{20}H_{24}N_{10}O_{10}P^-$ 595.1420, found 595.1399.
**(etheno)pG** (Compound **6**). $^{1}$H NMR (500 MHz, $D_2O$, with water suppression) δ 8.90 (s, 1H), 8.25 (s, 1H), 7.82 (s, 1H), 7.71 (s, 1H), 7.44 (s, 1H), 5.92 (d, *J* = 3.6 Hz, 1H), 5.59 (d, *J* = 4.3 Hz, 1H), 4.36 (d, *J* = 4.6 Hz, 1H), 4.29 (t, *J* = 5.1 Hz, 1H), 4.25-4.11 (m, 3H), 4.01-3.99 (m, 1H), 3.79-3.64 (m, 3H), 2.92-2.87 (m, 1H). $^{31}$P NMR (203 MHz, $D_2O$) δ -0.83. HRMS (ESI-) m/z calcd for $C_{22}H_{24}N_{10}O_{11}P^-$ 635.1369, found 635.1358.

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4) Protein purification

BL21(DE3) cells containing protein expression plasmids were grown at 37 °C until $OD_{600}$ reached 0.6 and expression was induced by addition of 1 mM IPTG. After overnight induction at 16 °C, bacterial cells were harvested by centrifugation (Sorvall LYNX 6000 Superspeed Centrifuge) at 5,000 rpm for 20 min. Cells were resuspended in lysis buffer and lysed by sonication. Lysis buffer for RocR contains 50 mM Tris–HCl pH 7.5, 250 mM NaCl and 10 mM imidazole. Lysis buffer for YybT contains 50 mM Tris, pH 8.0 and 150 mM NaCl. Lysis buffer for Orns (*E. coli*, *P. aeruginosa* and *M. smegmatis* Orns) contains 10 mM Tris-HCl, pH 8.0 and 100 mM NaCl. After centrifuge at 22,000 rpm for 25 min, proteins were purified from supernatant using a GE Hitrap Nickel column and dialyzed into lysis buffer overnight.

5) Enzymatic assays

PDE cleavage assays were conducted in corresponding enzyme reactions buffers. RocR reaction buffer contained 100 mM Tris-HCl, pH 8.0, 20 mM KCl and 25 mM $MgCl_2$. YybT buffer contained 100 mM Tris-HCl, pH 8.3, 20 mM KCl, 0.5 mM $MnCl_2$ and 1 mM dithiothreitol (DTT). Orns (*E. coli*, *P. aeruginosa* and *M. smegmatis* Orns) buffer contained 10 mM Tris-HCl, pH 8.0, 100 mM NaCl and 5 mM $MgCl_2$.

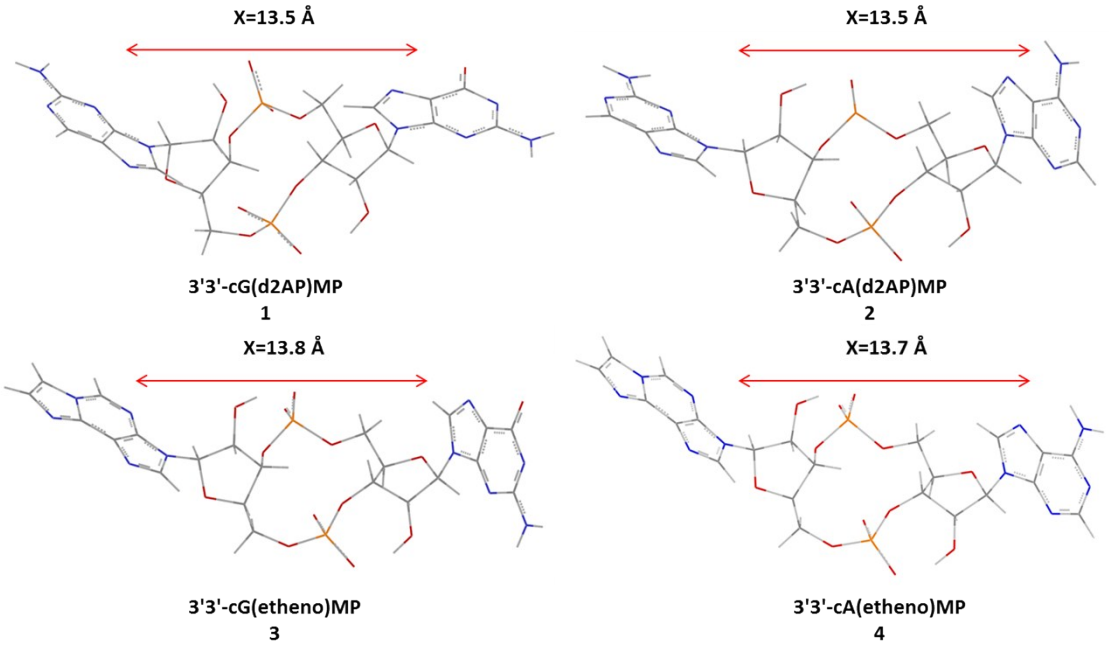



**Figure S1**: Optimized structures of compound **1** to **4**, computed with Gaussian 09 software with HF/6-31G basis set.

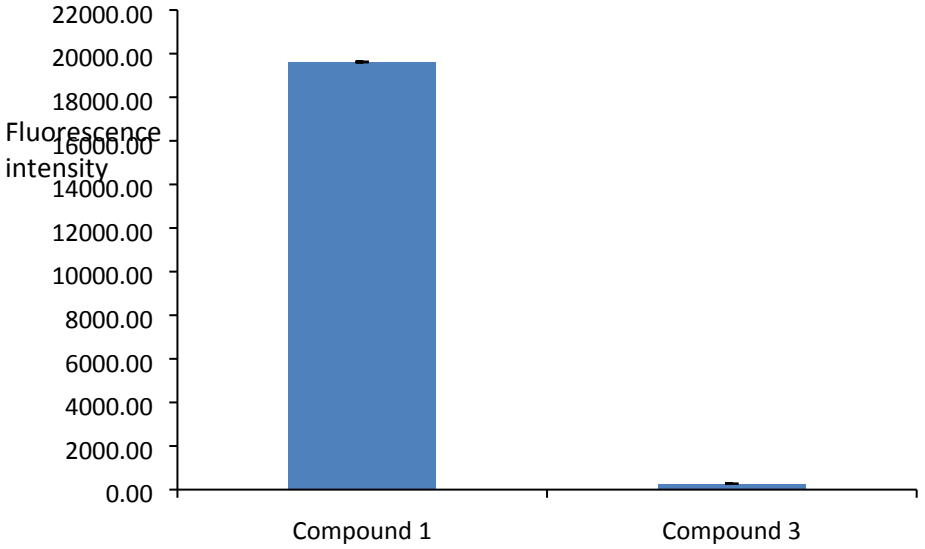



**Figure S2**: Absolute fluorescence intensities at $\lambda_{em}$ for cyclic probes (compound **1** and **3**). Fluorescence of compound **1** and **3** (1 μM) was measured by BioTek Cytation 5 Cell Imaging Multi-Mode Reader with λex=310 nm and λem=375 nm for compound **1**, and $\lambda_{ex}$=345 nm and $\lambda_{em}$=435 nm for compound **3**.

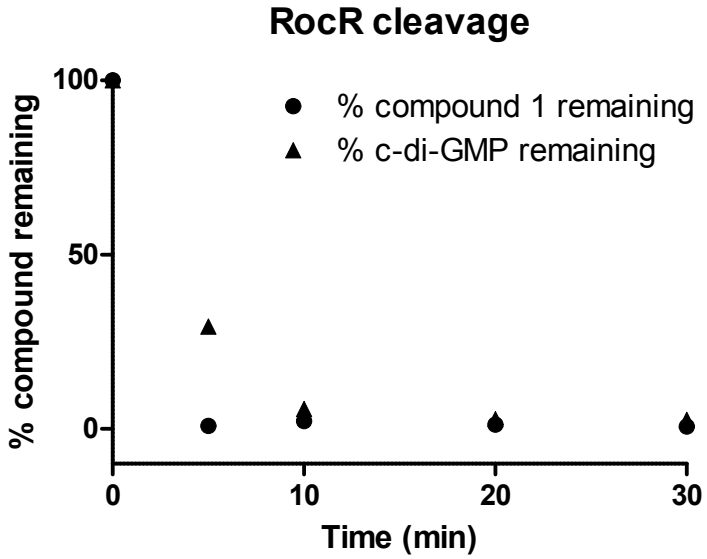



**Figure S3**: % of compound remaining of compound **1** and c-di-GMP upon cleavage of RocR, analyzed by HPLC. [RocR] = 1 μM, [Compound **1** or c-di-GMP] = 10 μM. Enzymatic assays were carried out at 37 °C, stopped at 0 min, 5 min, 10 min, 20 min and 30 min, and analyzed by HPLC. HPLC condition: 0→16 min, 99%→87% A (A: 0.1 M triethylammonium acetate, B: acetonitrile), 55 °C, COSMOSIL 5C18-MS-II, 4.6 X 250 mm, 5 μm.

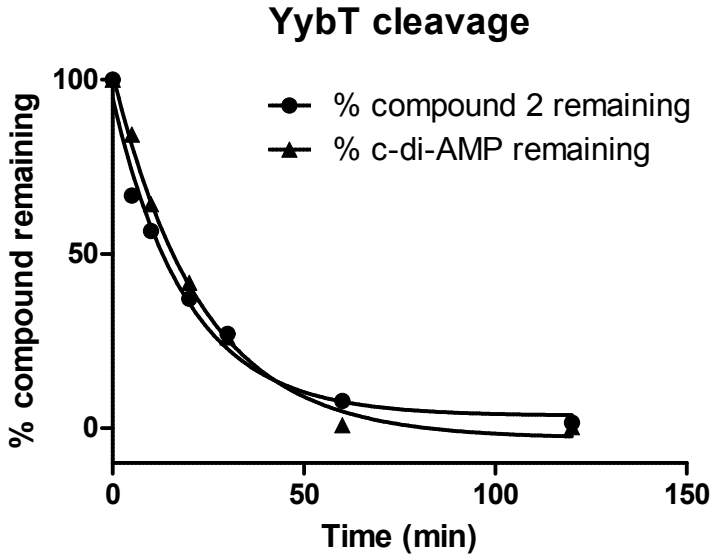



**Figure S4**: % of compound remaining of compound **2** and c-di-AMP upon cleavage of YybT, analyzed by HPLC. [YybT] = 1 μM, [Compound **2** or c-di-AMP] = 10 μM. Enzymatic assays were carried out at 37 °C, stopped at 0 min, 5 min, 10 min, 20 min, 30 min, 60 min and 120 min and analyzed by HPLC. HPLC condition: 0→16 min, 99%→87% A (A: 0.1 M triethylammonium acetate, B: acetonitrile), 55 °C, COSMOSIL 5C18-MS-II, 4.6 X 250 mm, 5 μm.

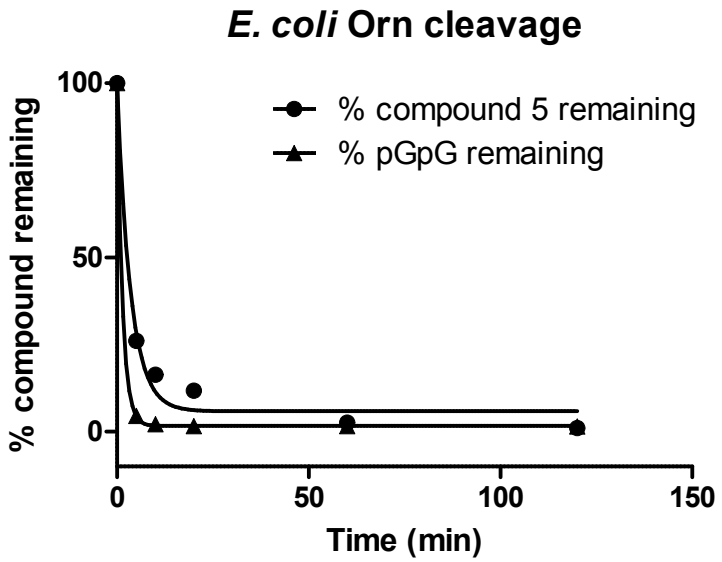
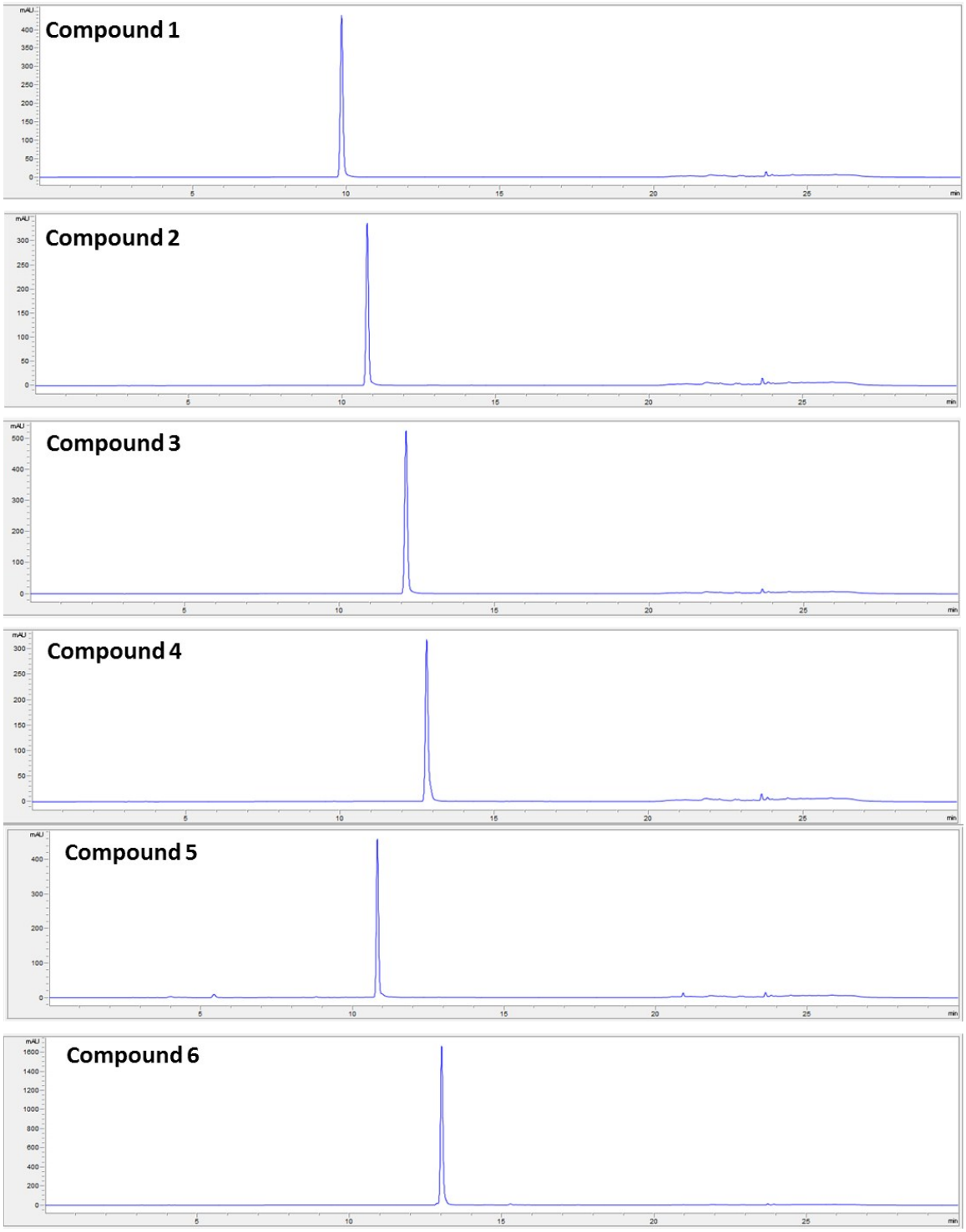


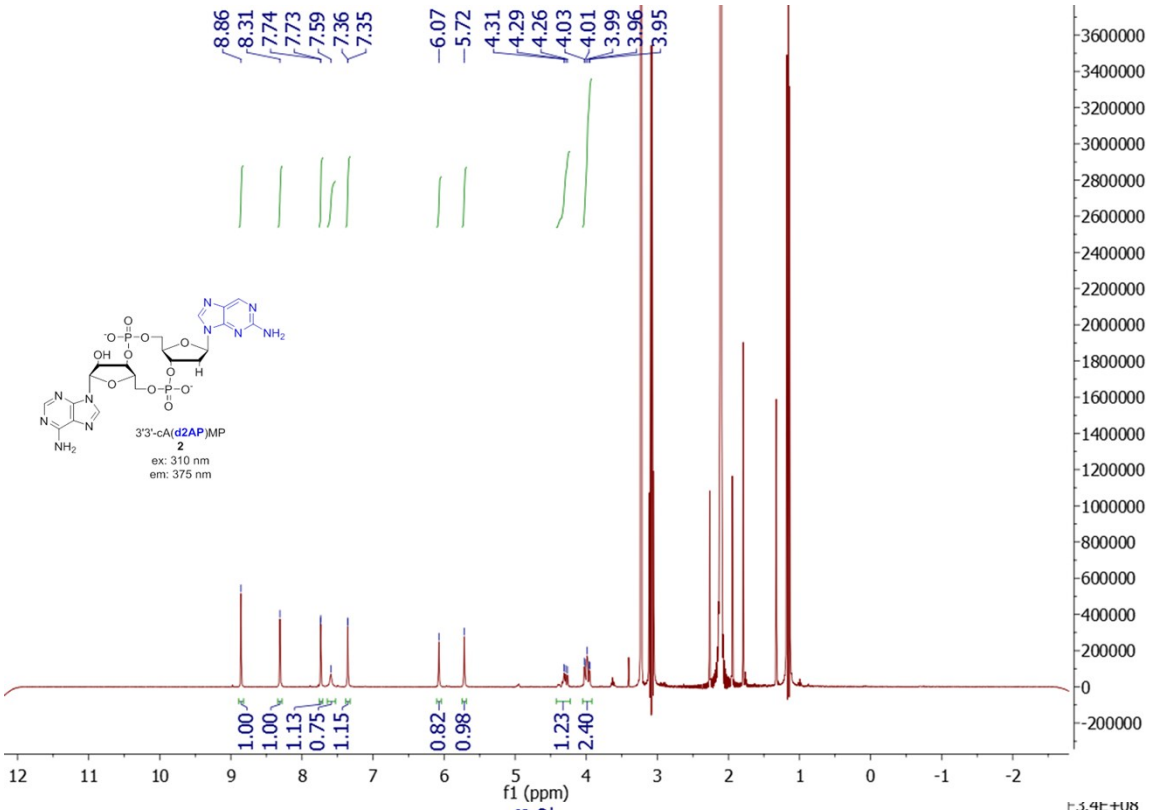

**Figure S5**: % of compound remaining of compound **5** and pGpG upon cleavage of *E. coli* Orn, analyzed by HPLC. [Orn] = 1 μM, [Compound **5** or pGpG] = 10 μM. Enzymatic assays were carried out at 37 °C, stopped at 0 min, 5 min, 10 min, 20 min, 60 min and 120 min and analyzed by HPLC. HPLC condition: 0→16 min, 99%→87% A (A: 0.1 M triethylammonium acetate, B: acetonitrile), 55 °C, COSMOSIL 5C18-MS-II, 4.6 X 250 mm, 5 μm.

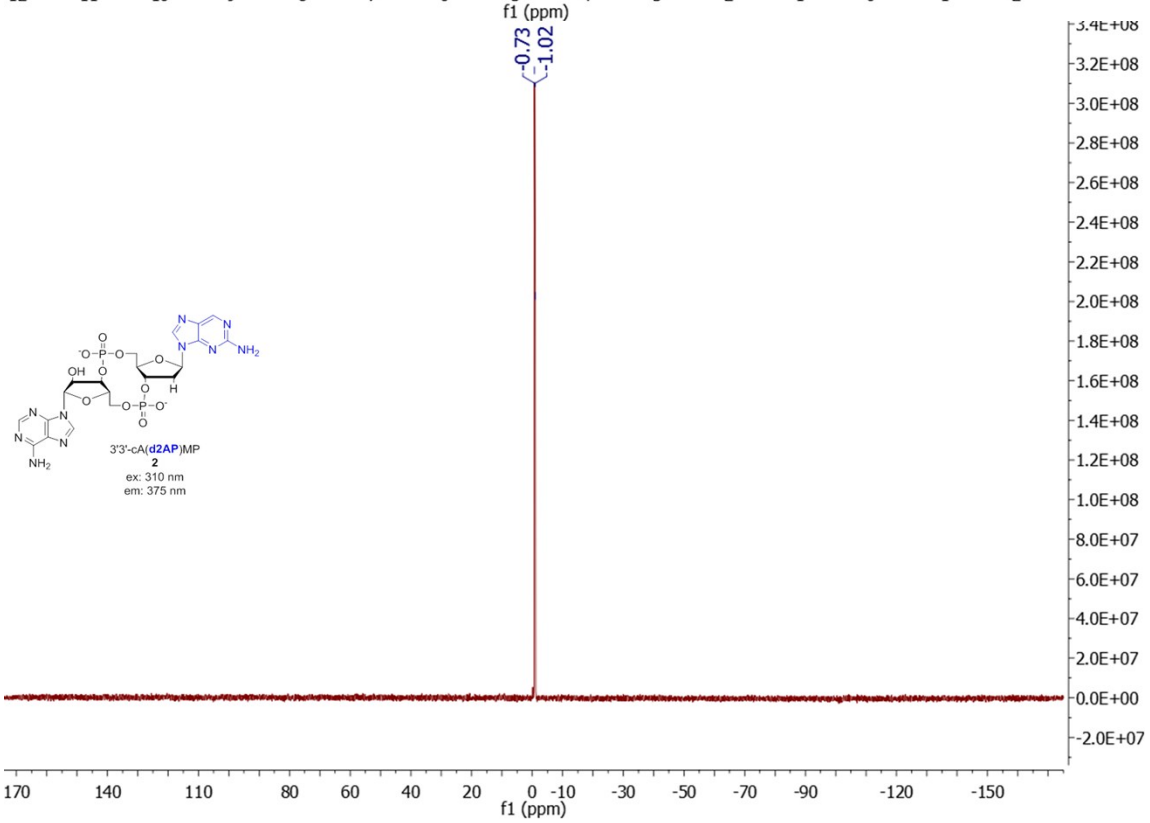
HPLC analyses of compounds **1** to **6**.




HPLC condition: 0→16 min, 99%→87% A (A: 0.1 M triethylammonium acetate, B: acetonitrile), 55 °C, COSMOSIL 5C18-MS-II, 4.6 X 250 mm, 5 μm.

Spectra of compound **2** to **6**


8.86
8.31
7.74
7.73
7.59
7.36
7.35
6.07
5.72
4.31
4.29
4.26
4.03
4.01
3.99
3.96
3.95
1.00
1.00
1.13
0.75
1.15
0.82
0.98
1.23
2.40
f1 (ppm)
3'3'-cA(d2AP)MP
2
ex: 310 nm
em: 375 nm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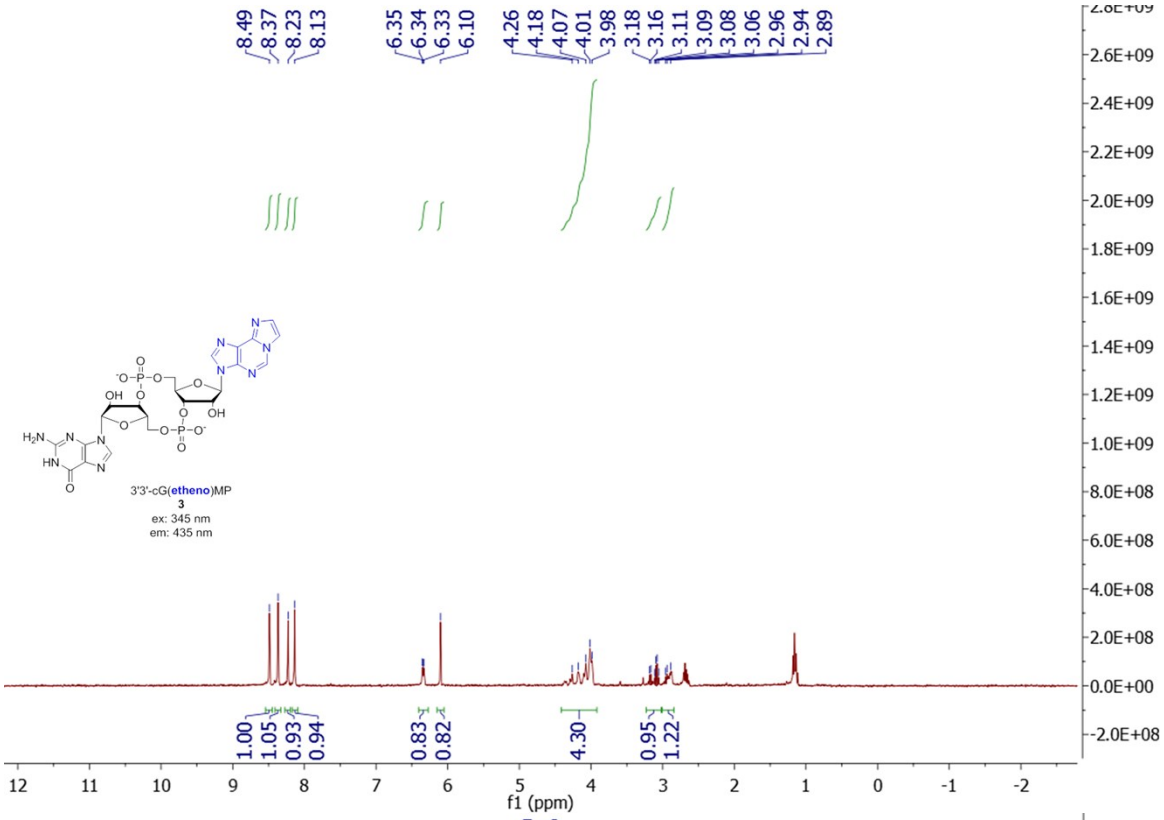
2
ex: 310 nm
em: 375 nm


8.49
8.37
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8.13
6.35
6.34
6.33
6.10
4.26
4.18
4.07
4.01
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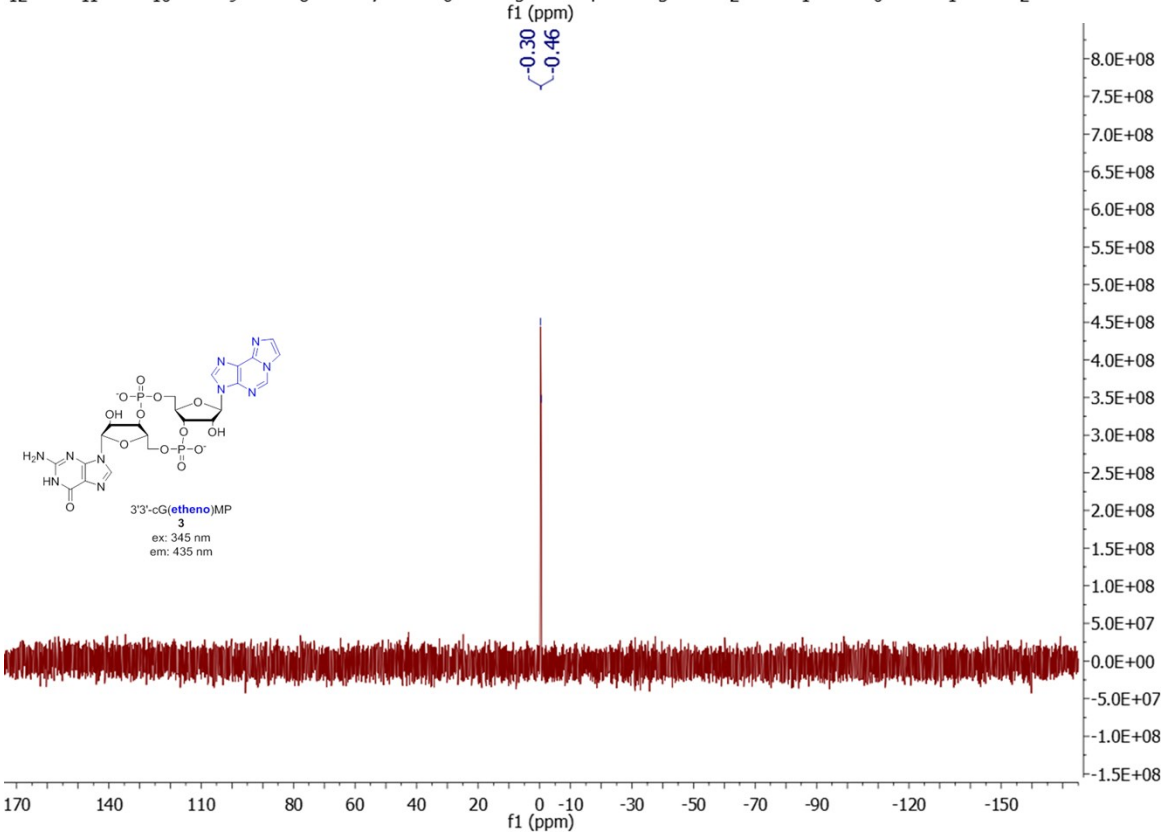
3.18
3.16
3.11
3.09
3.08
3.06
2.96
2.94
2.89
3'3'-cG(etheno)MP
3
ex: 345 nm
em: 435 nm
1.00
1.05
0.93
0.94
0.83
0.82
4.30
0.95
1.22
f1 (ppm)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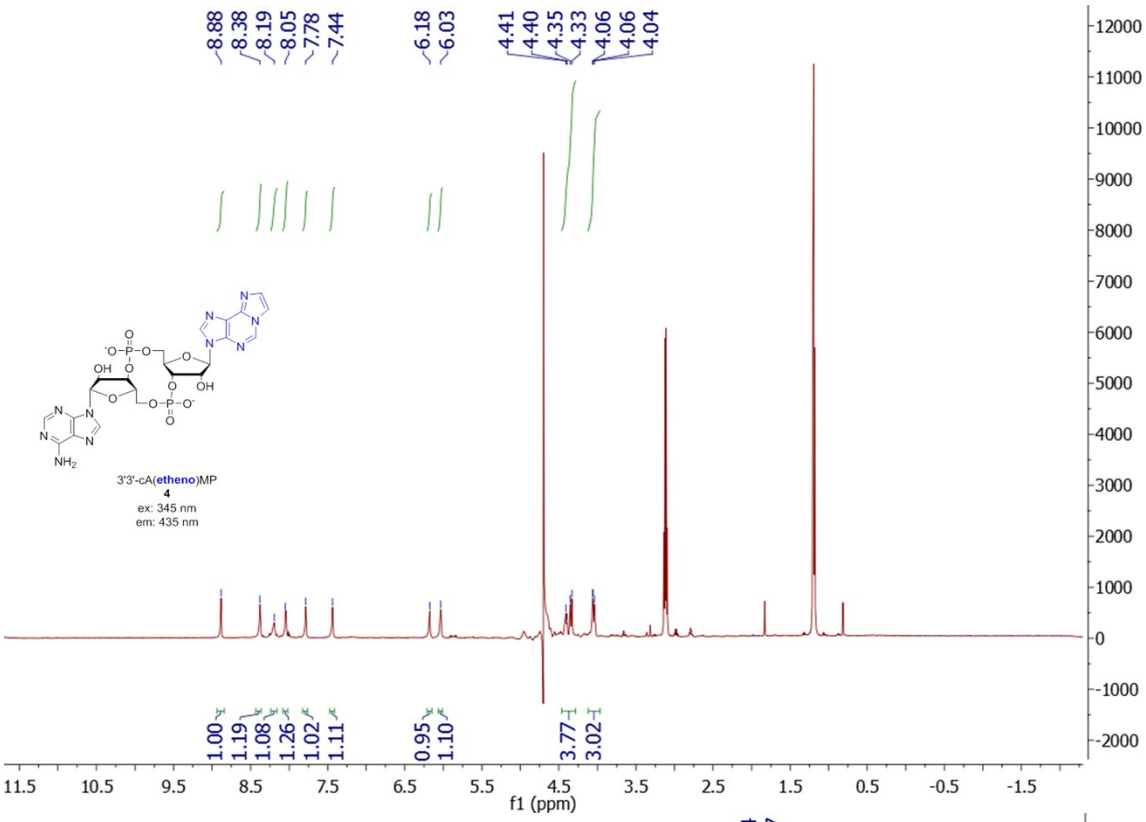
ex: 345 nm
em: 435 nm
f1 (ppm)


8.88
8.38
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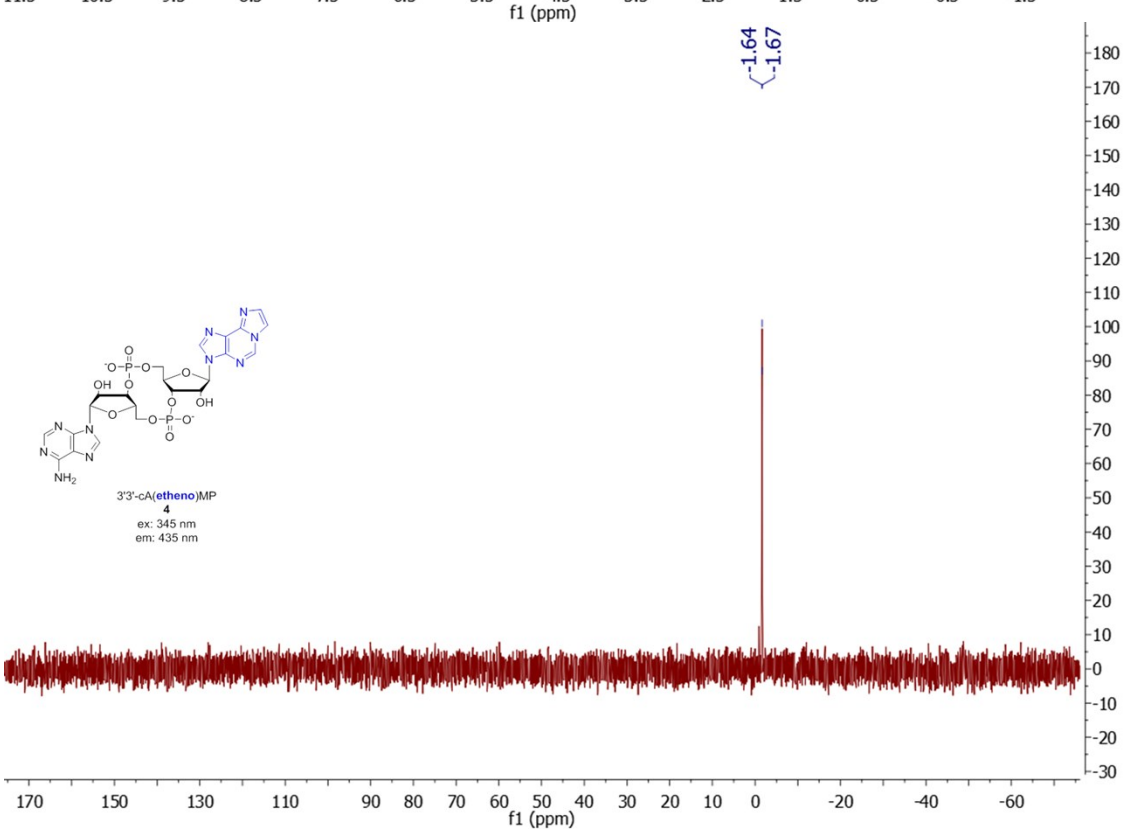
7.44
6.18
6.03
4.41
4.40
4.35
4.33
4.06
4.06
4.04
1.00
1.19
1.08
1.26
1.02
1.11
0.95
1.10
3.77
3.02
3'3'-cA(etheno)MP
4
ex: 345 nm
em: 435 nm
f1 (ppm)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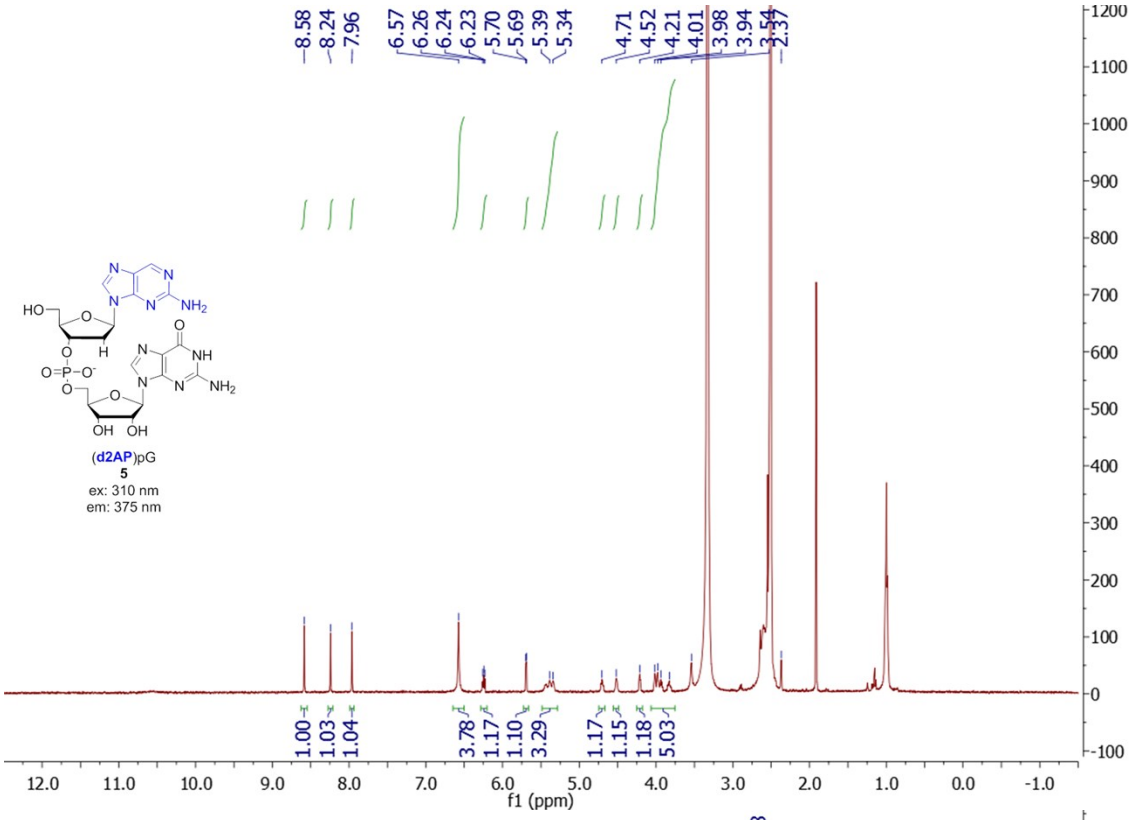
f1 (ppm)


8.58
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6.24
6.23
5.70
5.69
5.39
5.34
4.71
4.52
4.21
4.01
3.98
3.94
3.54
2.37
1.00
1.03
1.04
3.78
1.17
1.10
3.29
1.17
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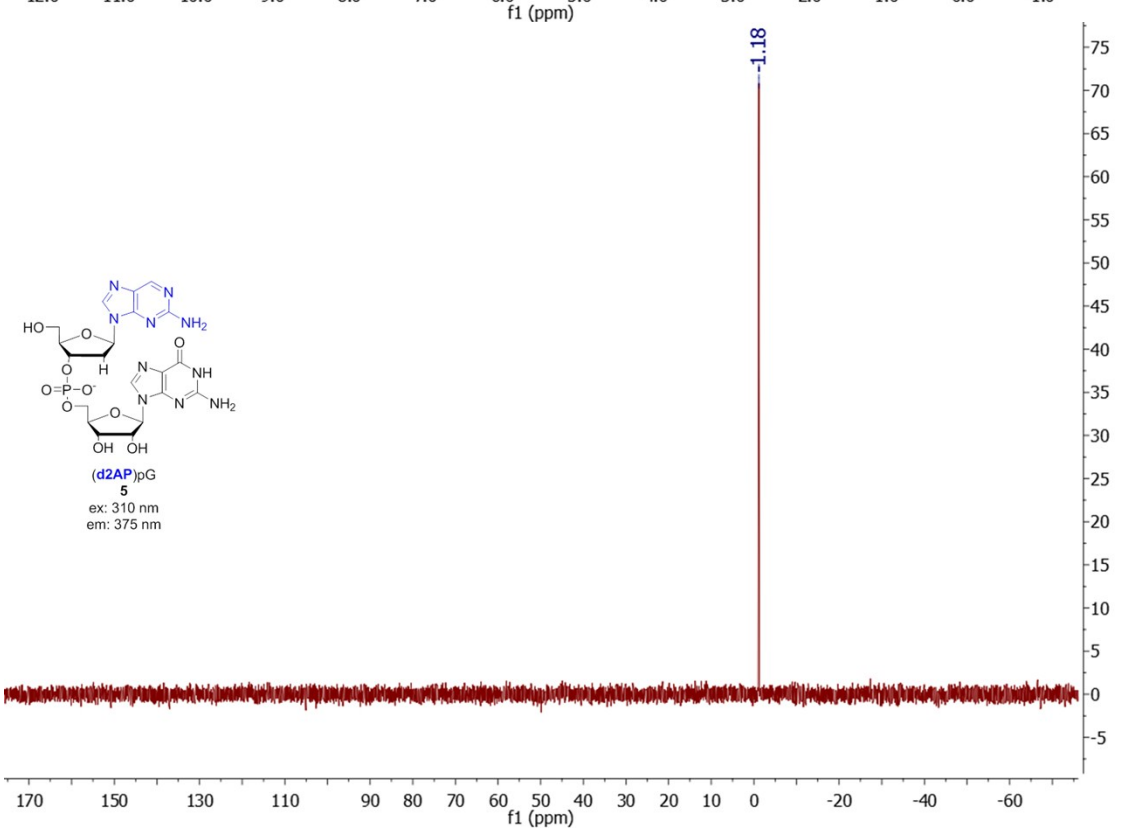
1.18
5.03
f1 (ppm)
(d2AP)pG
5
ex: 310 nm
em: 375 nm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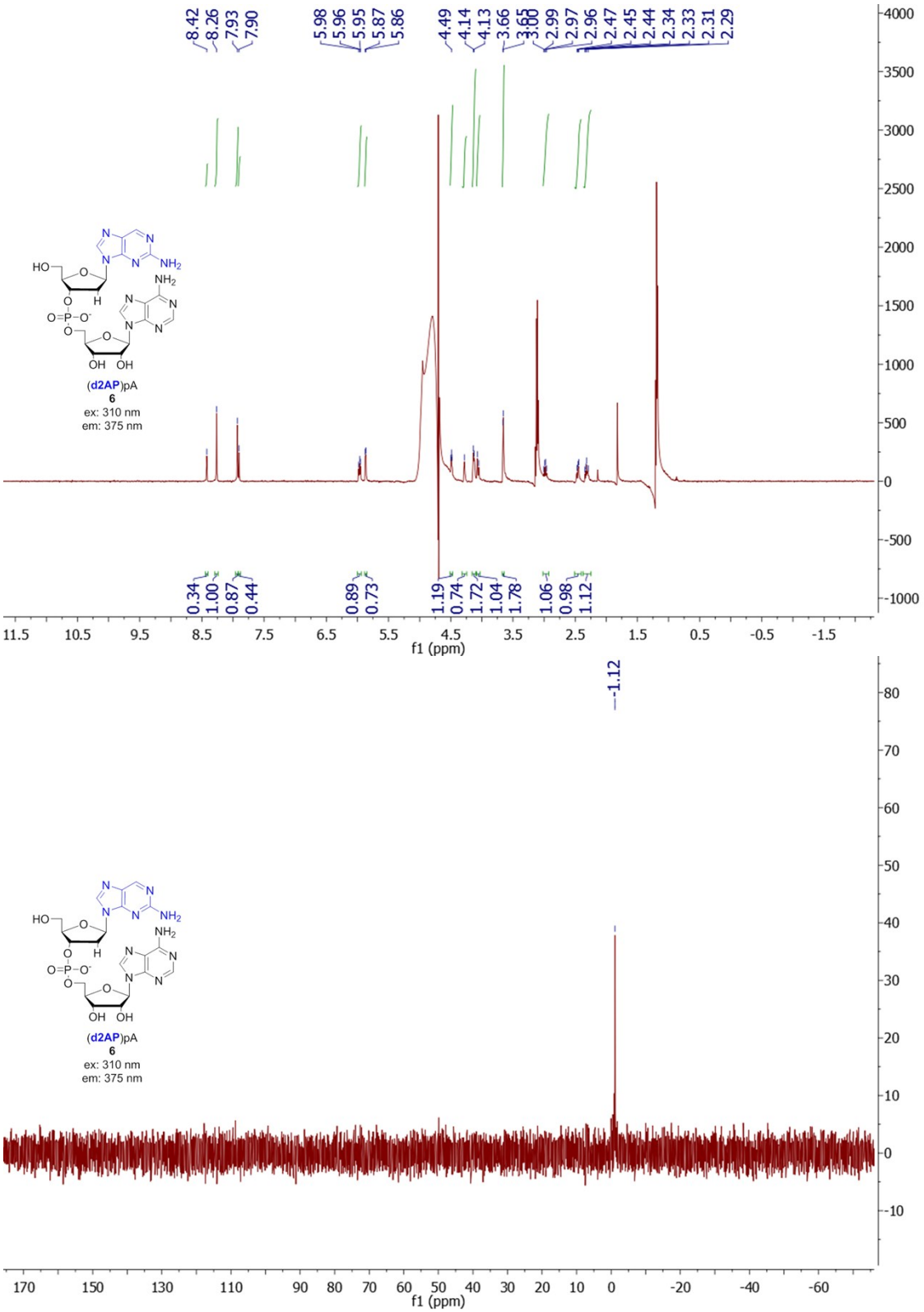


Reference:

1. B. L. Gaffney, E. Veliath, J. Zhao and R. A. Jones, *Org. Lett.*, 2010, **12**, 3269-3271.