Electronic Supplementary Material (ESI) for Molecular BioSystems.

Supporting Information:

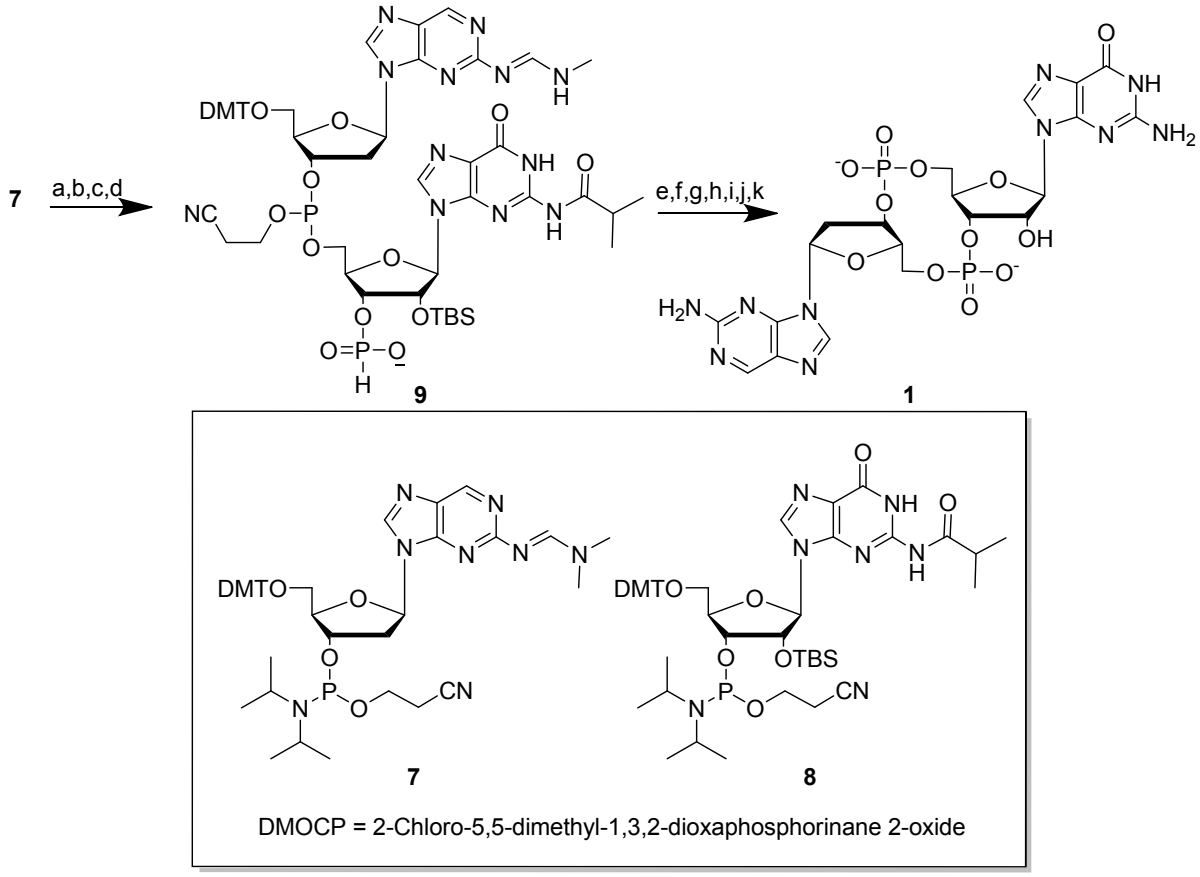
A cyclic dinucleotide containing 2-aminopurine is a general fluorescent sensor for c-di-GMP and 3'3'-cGAMP

Benjamin Roembke, Jie Zhou, Yue Zheng, David Sayre, Allan Lizardo, Laurentee Bernard and Herman O. Sintim*

Department of Chemistry and Biochemistry, University of Maryland, College Park, MD 20742, USA. Email: hsintim@umd.edu

Table of contents:

a) **General notes:** All reagents were purchased from commercial sources and used without further purification. Pyridine and CH_3CN were distilled over CaH_2 to remove water.



Scheme S1: Synthesis of compound **1**. a) **7**, pyridinium-TFA, H_2O, CH_3CN; b) *t*-$BuNH_2$; c) *i*) 6% DCA in CH_2Cl_2, H_2O, CH_2Cl_2; *ii*) pyridine; d) **8**; e) *i*) 5.5 M *t*-BuOOH; *ii*) $Na_2S_2O_3$, H_2O; f) *i*) 6% DCA in CH_2Cl_2, H_2O, CH_2Cl_2; *ii*) pyridine; g) 2-chloro-5,5-dimethyl-1,3,2-dioxaphosphorinane 2-oxide; h) *i*) H_2O, I_2; *ii*) $Na_2S_2O_3$; i) *t*-$BuNH_2$; j) CH_3NH_2; k) TEA·3HF, TEA, pyridine.

b) **Synthesis:** 0.45 g 5'-Dimethoxytrityl-N-isobutyryl-Guanosine,2'-O-TBDMS-3'-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite (**8**) was placed in a 100 mL round bottom flask and dissolved in CH_3CN (10 mL). To this flask was added several 4 Å molecular sieves, and then the flask was capped and placed under an argon atmosphere. To another 250 mL round bottom flask was added 0.25 g 5'-Dimethoxytrityl-N2-(dimethylaminomethylidene)-2'-deoxypurine riboside,3'-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite (**7**), which was dissolved in CH_3CN (10 mL). To this flask, 0.65 g pyridinium trifluoroacetate and H_2O (90 µL) were added. This

was allowed to stir for 1 minute. Then 10 mL *t*-$BuNH_2$ was added and allowed to stir for 10 minutes. This reaction mixture was concentrated to a foam. After concentration, CH_3CN (10 mL) was added and then the mixture was concentrated again. This was repeated one more time. The concentrate was then dissolved in CH_2Cl_2 (25 mL) and H_2O (180 µL). 28 mL of 6% (v/v) DCA in CH_2Cl_2 was then added to this solution. After 10 min of stirring the acid was quenched with pyridine (20 mL). The solution was the concentrated under vacuum and then redissolved in CH_3CN (20 mL). This was repeated three more times. After the last addition of CH_3CN, the flask was capped with a septum and put under an Argon atmosphere. The dry solution of **8** was then transferred by cannula into reaction flask. The flask containing **8** was rinsed twice with CH_3CN (1 mL), which was also transferred into the flask by cannula. The linear coupling was allowed to react for 2 min. Then 1.3 mL anhydrous 5.5 M *t*-BuOOH was added and allowed to stir for 30 min. The reaction was then quenched by addition of 0.6 g $Na_2S_2O_3$ dissolved in H_2O (1.2 mL). This was allowed to stir for 2 min after which it was concentrated. The reaction mixture was then dissolved in CH_2Cl_2 (30 mL) and H_2O (500 µL) and then 30 mL 6% (v/v) DCA in CH_2Cl_2 was added. After 10 min the reaction was quenched by addition of pyridine (20 mL) and then concentrated. This addition and concentration was repeated two more times. After the last concentration the flask was capped with a septum and put under an argon atmosphere. This concentrate was dissolved in pyridine (10 mL) and 1.6 g 2-chloro-5,5-dimethyl-1,3,2-dioxaphosphorinane 2-oxide was added. After 10 min H_2O (1.5 mL) was added which was immediately followed by 0.8 g I_2. This was stirred for 5 min. The reaction mixture was then poured into and an Erlenmeyer flask containing 0.5 g $Na_2S_2O_3$ in H_2O (350 mL). This was allowed to stir for 5 min. To neutralize the aqueous solution, 9.8 g $NaHCO_3$ was slowly added. The aqueous solution was then extracted with 1:1 EtOAC:Et_2O (400 mL), EtOAC (400 mL) and 10:1 EtOAC:*i*-PrOH (400 mL). Each organic layer was kept separate, concentrated and allowed to dry in a vacuum desiccator overnight. Each organic extract was then independently subjected to the following deprotection protocol. To remove excess pyridine, EtOAC (30 mL) was added to the concentrated organic extract and subsequently concentrated under vacuum. This was repeated three times in total. The concentrate was then dissolved in CH_3CN (10 mL) and to which was added 10 mL *t*-$BuNH_2$. This was stirred for 10 minutes and then concentrated. To the resulting solid was then added CH_3CN (10 mL) and the suspension was sonicated. The suspension was then concentrated, and this process was repeated two more times. The solid was then dissolved in a minimal amount of CH_3OH and filtered to remove and remaining solids. The filtrate was collected and concentrated. The resulting concentrate was then subjected to 20 mL CH_3NH_2. This was allowed to stir for 90 min. The CH_3NH_2 was then removed under vacuum being careful to prevent bumping of the volatile amine. The concentrate was then dissolved in a 2:1 mixture of pyridine:TEA (6 mL) and concentrated. This was repeated two more times. The concentrate was then dissolved in 2 mL pyridine. The flask was capped with a septum and placed in a preheated 50 °C oil bath. Then, by syringe, TEA (3 mL) and 2 mL TEA·3HF was slowly added over a period of about 1 minute (Note: anything that came in contact with TEA·3HF was washed with a saturated aqueous solution of K_2CO_3). After 90 minutes of stirring the product was precipitated by addition of copious amounts of

acetone. The solid was then collected and dried in a vacuum desiccator overnight. The solid was then subjected to purification by standard RP-HPLC (see below)

c) **Purification:** RP-HPLC was performed on a Varian model 325 equipped with a Varian model 210 binary solvent delivery system. A Merck Purospher STAR RP-18 (250 mm x 10 mm; 5 µm) HPLC column was used. The method utilized 0.1 M TEAA buffer (solvent A) and CH_3CN (solvent B). The solvent gradient was as follows:

Table S1: RP-HPLC methodology

Time	Flow (ml/min)	% A	% B
0	2	95	5
30	2	90	10
35	2	80	20
40	2	0	100

The peak corresponding to an elution time of 20 minutes was collected and characterized. See Figure S1 for HPLC trace.

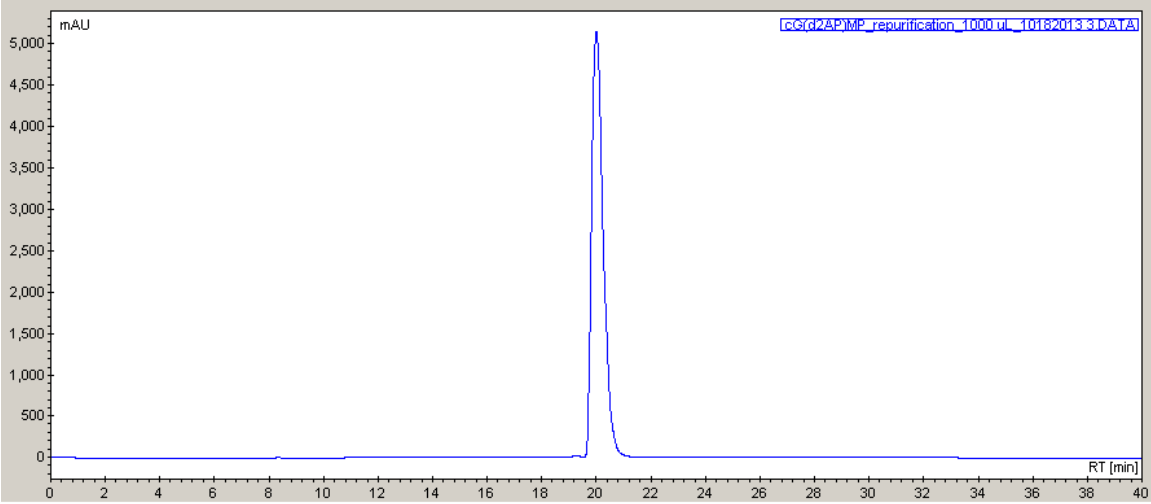


Figure S1: RP-HPLC trace of compound **1**.

d) **Characterization:**

^{1}H-NMR (600 MHz, D_2O) δ 8.42 (s, 1H), 8.19 (s, 1H), 7.95 (s, 1H), 6.28 (dd, *J*=7.6, 3.6 Hz, 1H), 5.85 (s, 1H), 4.95 (dd, *J*=10.4, 4.4 Hz, 1H), 4.79 (td, *J*=8.5, 5.0 Hz), 4.29 (d, 8.5 Hz, 1H), 4.25-4.19 (m, 1H), 4.17 (d, *J*=5.3 Hz, 1 H), 4.08 (d, *J*= 12.0 Hz, 1H), 3.99-3.94 (m, 1H), 2.92 (ddd, *J*=11.2, 7.1, 3.4 Hz, 1H), 2.64 (dt, *J*=14.4, 7.7 Hz, 1H)

^{13}C-NMR (151 MHz, D_2O) δ 181.41, 159,73, 158.91, 153.91, 152.15, 150.73, 149.04, 142.15, 136.98, 127.15, 116.36, 89.13, 82.93, 82.37, 79.86, 73.35, 71.36, 70.59, 62.39, 62.12, 37.82

^{31}P-NMR (243 MHz, D_2O) δ 0.36, 0.13

ESI-MS: Negative mode, predicted: 657.0978, observed: 657.0990

e) **CD of other cyclic dinucleotides:**

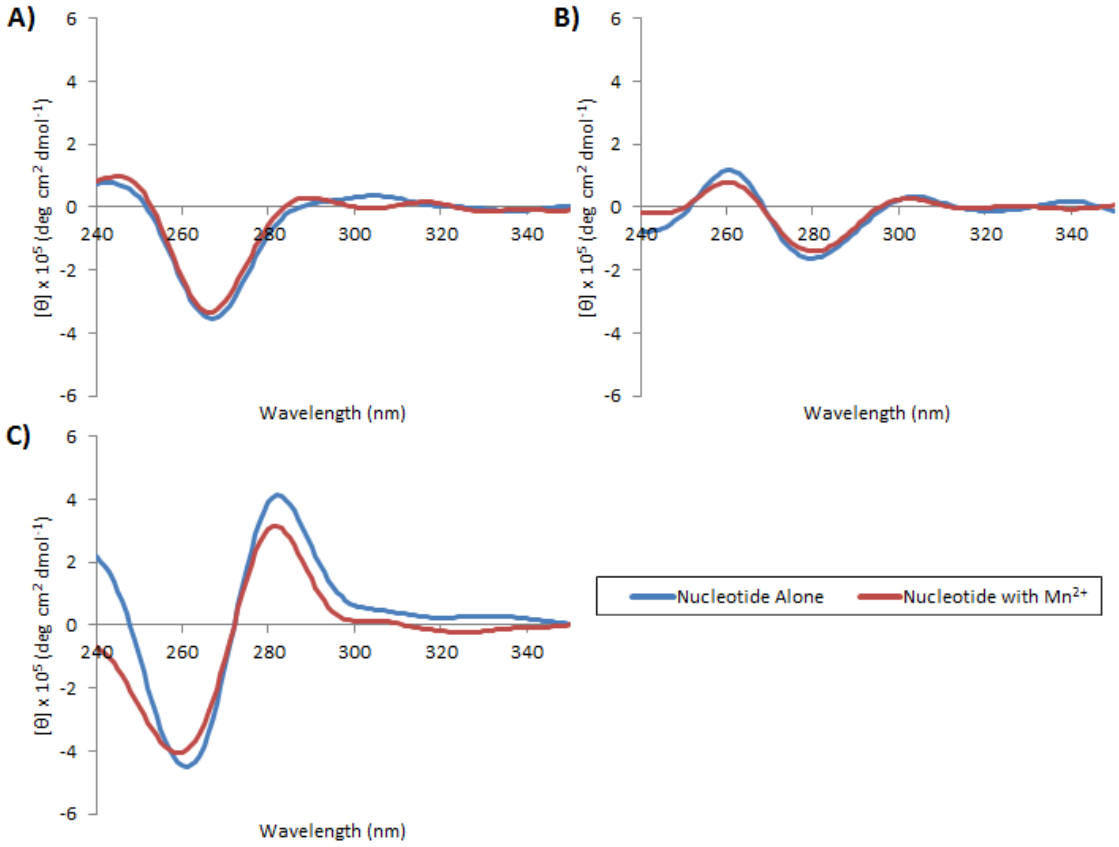


Figure S2: Various cyclic nucleotides with and without Mn. **A)** c-di-AMP **B)** 2',3'-cAGMP **C)** 2',3'-cGAMP **Conditions:** 40 µM cyclic nucleotide, 15 mM Mn (where applicable) in 50 mM Tris-HCl (pH = 7.5)

f) **Protein overexpression and purification:** WspR, WspR D70E and RocR overexpression were induced by adding 1 mM IPTG at an OD_{600} of 0.6. After 6-hour induction at 30°C, bacteria cells were collected by centrifugation and resuspended in lysis buffer (10 mM Tris-HCl, pH = 8.0, 100 mM NaCl). Cells were lysed by sonication and lysate was centrifuged at 22,000 rpm for 25 min. Supernatant was collected and passed through a Nickel affinity column (GE HisTrap HP). The purified proteins were then dialyzed in 10 mM Tris-HCl (pH = 8.0) with 100 mM NaCl overnight.

g) **Fluorescence measurements:** All measurements were done on either a Varian Cary Eclipse spectrophotometer or a Molecular Devices SpectraMax M5e plate reader. Before measurement all samples were annealed, with the exception of enzymatic samples, at 95 °C for 5 min and then allowed to slowly cool to room temperature for 15 min. All samples were then incubated at 4 °C for at least 2 h.

h) **c-di-GMP dimer crystal structure[1]:**

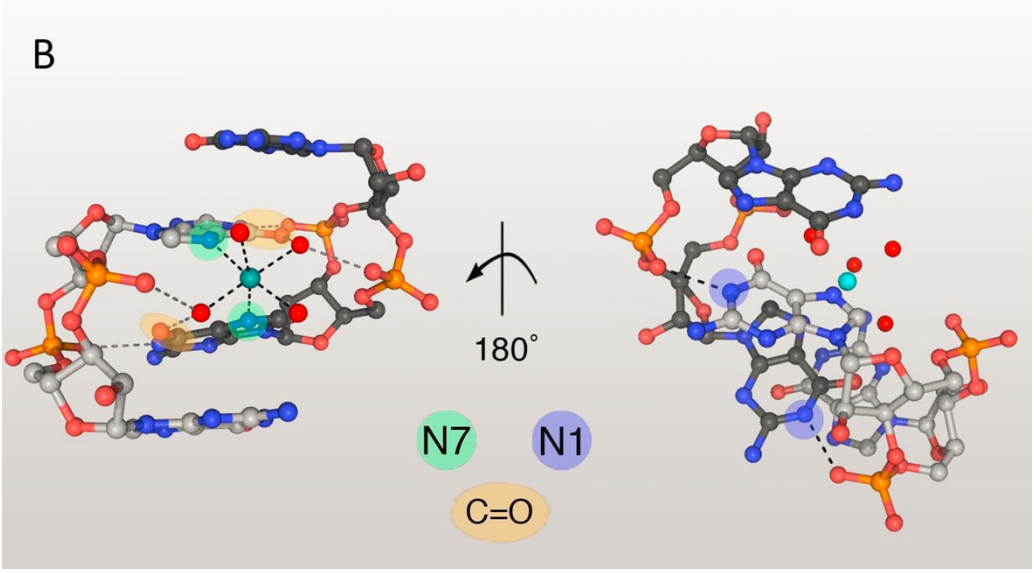


Figure S3: Crystal structure of c-di-GMP dimer with Mn^{2+} shows coordination of the O6 of the carbonyl and the N7 coordinating the Mn^{2+}. In c-di-AMP the O6 interaction could not be established because the carbonyl is replaced by an amino functionality. Copied with permission from ref. [1] (Oxford Journals).

i) **Time course measurement of cG(d2AP)MP binding to c-di-GMP**

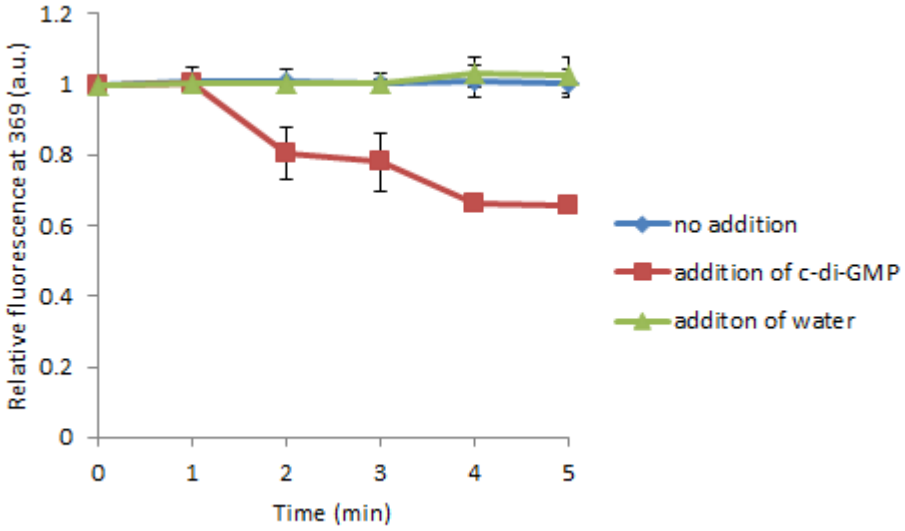


Figure S4: Time course measurement of cG(d2AP)MP incubating with c-di-GMP. 1 µL of 2 mM c-di-GMP was added at 1.5 min and 2.5 min. A decrease in absorbance is immediately observed (red squares). As controls 1 µL of water was added at the same time points (green triangles) and no additions were made as well (blue diamonds). No decrease in fluorescence is observed in these time course measurements. **Conditions:** 1 µM 3',3'-cG(d2AP)MP, 15 mM $MnCl_2$ in 50 mM Tris-HCl (pH=7.5). 1 µL of 2 mM c-di-GMP or water was added at 1.5 min and 2.5 min.

j) **HPLC enzymatic cleavage assays:**

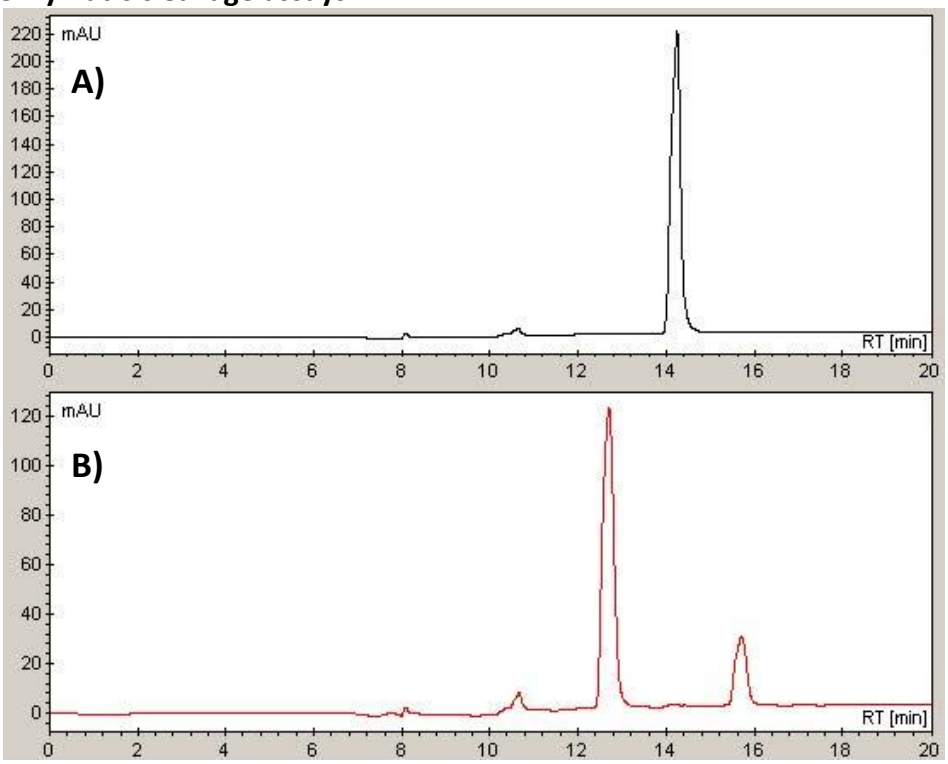


Figure S5: HPLC enzymatic cleavage assays. **A)** 3',3'-cG(d2AP)MP alone and **B)** 3',3'-cG(d2AP)MP with PDE SVPD. **Conditions: A)**100 µM 3',3'-cG(d2AP)MP, 15 mM $MgCl_2$ in 50 mM Tris-HCl (pH = 8,5). **B)** 100 µM 3',3'-cG(d2AP)MP, 15 mM $MgCl_2$ and 0.001 units/mL SVPD in 50 mM Tris-HCl (pH = 8,5). **HPLC Conditions:** Total sample volume was 100 µL. Column was a Merck Purospher STAR RP-18 (250 mm x 10 mm; 5 µm). 0.1 M TEAA buffer (solvent A) and CH_3CN (solvent B) were used as the mobile phase with a flow rate of 2 mL/min. Samples containing PDE's were filtered through a 5000 kDa centrifugal filter to remove enzymes.

Table S2: RP-HPLC methodology for HPLC enzymatic cleavage assay.

Time	Flow (ml/min)	% A	% B
0	2	95	5
16	2	80	20
18.5	2	10	90
20	2	10	90

k) **Derivation of Equation 5:**

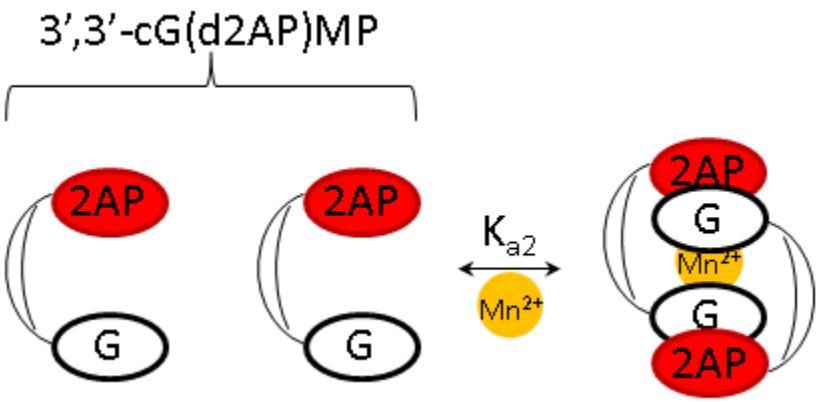


Figure S6: Schematic representation of homodimerization of 3',3'-cG(d2AP)MP

Because of mass balance,

$$[2AP] + 2[2AP_2] = [2AP]_T \qquad \textbf{Eqn. S1}$$

Then we can describe [2AP] as a fraction of $[2AP]_T$.

$$\alpha = \frac{[2AP]}{[2AP]_T} \qquad \textbf{Eqn. S2}$$

Substitution of Eq. S2 into Eq. S1 gives,

$$1 - \alpha = \frac{2[2AP_2]}{[2AP]_T} \qquad \textbf{Eqn. S3}$$

Total fluorescence is given by Eq. 4,

$$F_T = f_1[2AP] + f_2[2AP_2] \qquad \textbf{Eqn. 2}$$

Substituting in Eq. S2 and S3 into Eq. 4 and rearranging gives,

$$\alpha = \frac{F_T - \frac{[2AP]_T f_2}{2}}{[2AP]_T f_1 - \frac{[2AP]_T f_2}{2}} \qquad \textbf{Eqn. S4}$$

The homodimerization constant, K_{a3} is given by,

$$K_{a2} = \frac{[2AP_2]}{[2AP]^2} \qquad \textbf{Eqn. 1}$$

Substituting Eq. S1 into Eq. 2 gives,

$$K_{a2} = \frac{[2AP]_T - [2AP]}{2[2AP]^2}$$ **Eqn. S5**

Expanding this equation and solving using the quadratic equation gives,

$$[2AP] = \frac{-1 + \sqrt{1 + 8K_{a2}[2AP]_T}}{4K_{a2}}$$ **Eqn. S6**

Substituting Eq. S2 into Eq. S6 gives,

$$\alpha = \frac{-1 + \sqrt{1 + 8K_{a2}[2AP]_T}}{4K_{a2}[2AP]_T}$$ **Eqn. S7**

Setting Eq. S7 equal to Eq. S4 and rearranging yields,

$$F_T = \left(\frac{-1 + \sqrt{1 + 8K_{a2}[2AP]_T}}{4K_{a2}[2AP]_T}\right)\left([2AP]_T f_1 - \frac{[2AP]_T f_2}{2}\right) + \frac{[2AP]_T f_2}{2}$$ **Eqn. 3**

I) **Determination of IC_{50}'s of cyclic dinucleotides with 3',3'-cG(d2AP)MP**

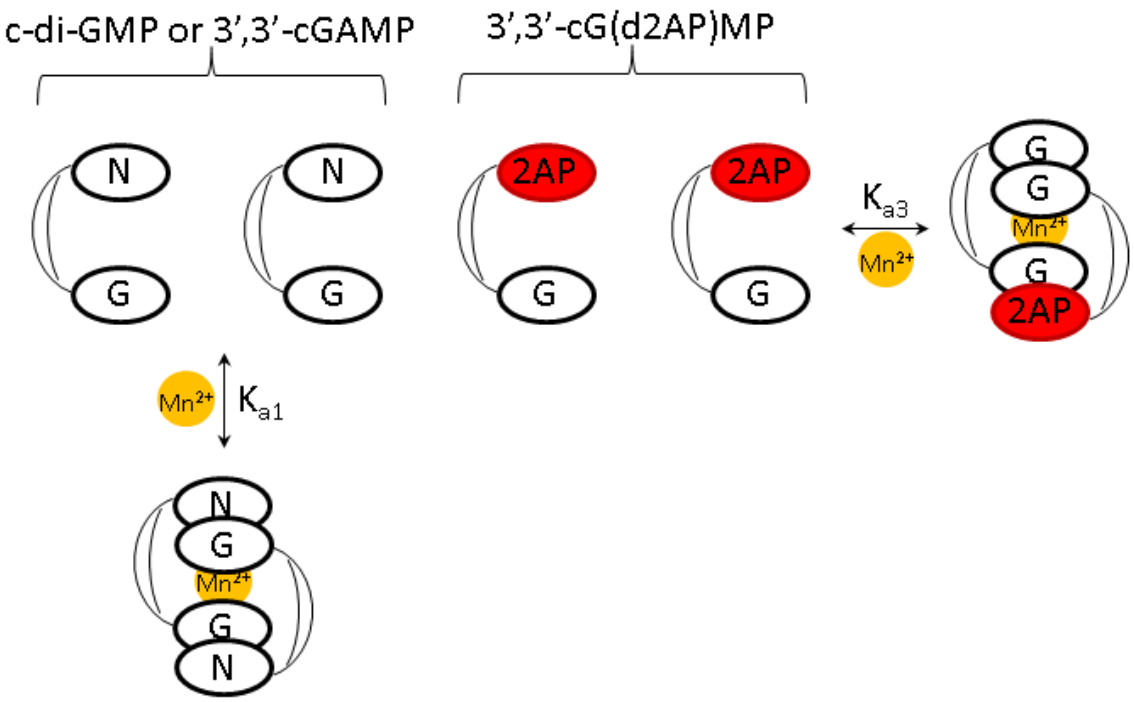


Figure S7: Schematic representation of c-di-GMP or 3',3'-cGAMP homodimerizing and binding to 3',3'-cG(d2AP)MP

To fit titration data the Wang method was used which used the following model[2]:

$$[cNMP]_T = IC_{50}\frac{f - f_0}{f_\infty - f} + [2AP]_T\frac{f - f_0}{f_\infty - f_0} \quad \textbf{Eqn. S9}$$

Where $[cNMP]_T$ is the total concentration of added c-di-GMP or 3',3'-cGAMP, IC_{50} is the IC_{50} of binding of c-di-GMP or 3',3'-cGAMP to 3',3'-cG(d2AP)MP, f is the fluorescence of a sample with n concentration of cyclic dinucleotide, f_0 is the fluorescence of 3',3'-cG(d2AP)MP with no additional cyclic dinucleotides, f_∞ is the fluorescence when 100% of 3',3'-cG(d2AP)MP is bound and $[2AP]_T$ is the total concentration of 3',3'-cG(d2AP)MP.

References

1. V. Stelitano, A. Brandt, S. Fernicola, S. Franceschini, G. Giardina, A. Pica, S. Rinaldo, F. Sica and F. Cutruzzola, *Nucleic Acids Res*, 2013, **41**.
2. Y. Wang, K. Hamasaki and R. R. Rando, *Biochemistry*, 1997, **36**, 768-779.