Molecular Cell, Volume *51*

Supplemental Information

Cyclic GMP-AMP Containing Mixed Phosphodiester Linkages Is An Endogenous High-Affinity Ligand for STING

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Supplemental Inventory

Supplemental Information contains five supplemental figures and Supplemental Experimental Procedures.

Figure S1 is related to Figure 1, and it describes the routes of chemical synthesis of four cGAMP isomers.

Figure S2 is related to Figure 1, and it provides additional data that validate the cGAS product as 2’3’-cGAMP.

Figure S3 is related to Figure 2, and it provides additional ITC data for the binding of cGAMP isomers to STING.

Figure S4 is related to Figure 3, and it shows the sequence alignment of STING and highlights the region that undergoes ligand-induced formation of a lid consisting of a new four-stranded β-sheet. Figure S4B shows that the formation of the lid is not due to crystal packing.

Figure S5 is related to Figure 4, and it shows the direct visualization of 2’3’-cGAMP within the crystal structure of STING as well as the extensive atomic interactions that form the cGAMP-binding pocket.

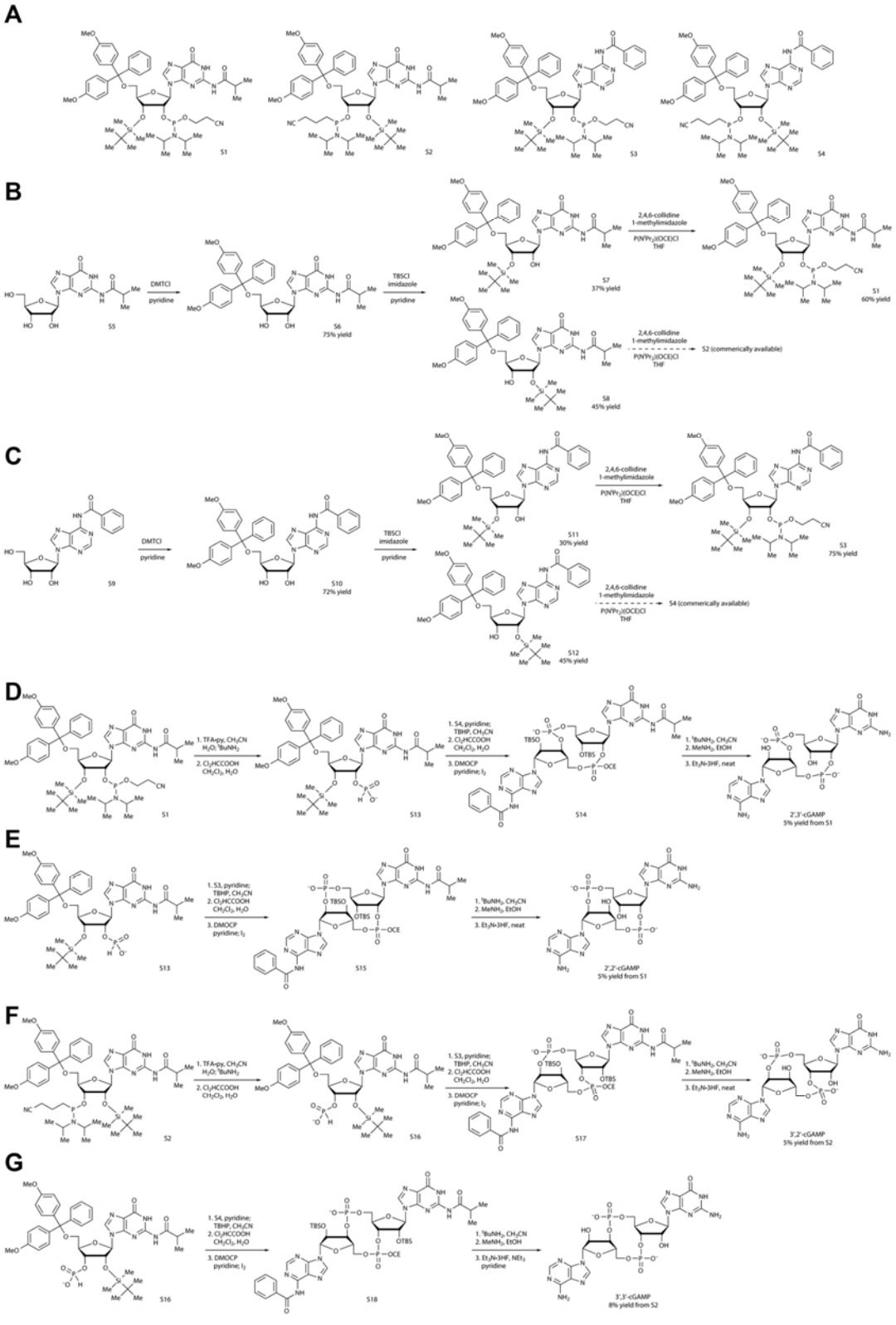


Figure S1. Chemical synthesis of cGAMPs. (A) Structure of building blocks **S1**–**S4**. **(B)** Synthesis of building block **S1**. **(C)** Synthesis of building block **S3**. **(D)** Synthesis of 2'3'-cGAMP. **(E)** Synthesis of 2'2'-cGAMP. **(F)** Synthesis of 3'2'-cGAMP. **(G)** Synthesis of 3'3'-cGAMP. Related to Figure 1.

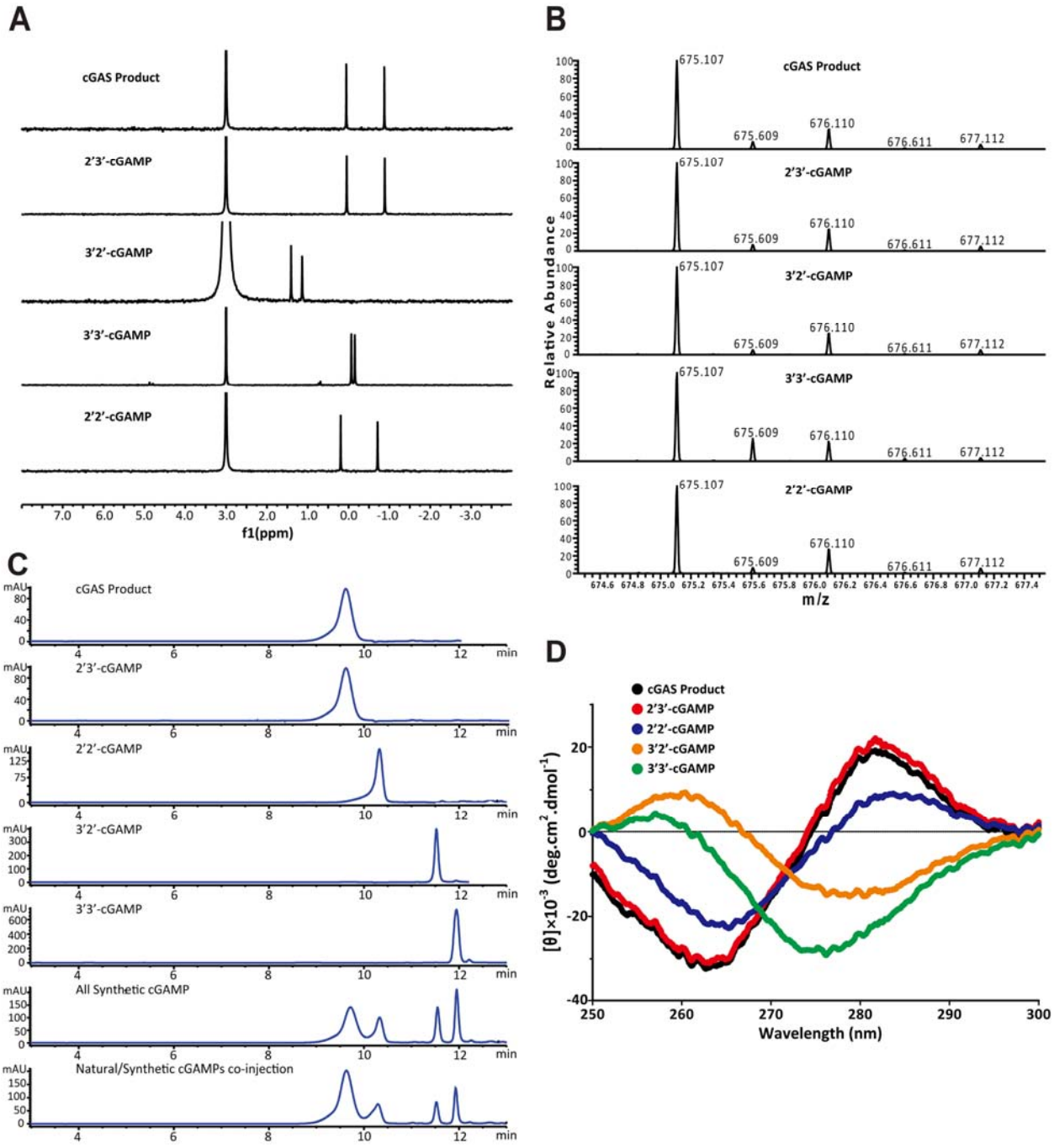


Figure S2. Structure determination of the cGAS product and synthetic cGAMPs. (**A**) ^{31}P-NMR spectra. (**B**) Isotopically resolved high resolution mass spectra of singly charged ($[M+H]^+$) ions were acquired using Q Exactive mass spectrometer. (**C**) Reverse phase HPLC elution profiles. Gradient: T = 0–5 min: 100% 0.1 M $NaH_2PO_4/Na_2HPO_4/H_2O$, T = 12.5 min: 12.5% MeCN. 1 mL/min. Retention time: natural cGAMP, 9.62 min; 2'3'-cGAMP, 9.62 min; 2'2'-cGAMP, 10.33 min; 3'2'-cGAMP, 11.52 min; 3'3'-cGAMP, 11.95 min. (**D**) CD spectra at 23 °C in 5 mM $NaH_2PO_4/Na_2HPO_4/H_2O$ buffer (pH 7.4). Compound concentrations: natural cGAMP, 175 µM; 2'3'-cGAMP, 175 µM; 2'2'-cGAMP, 250 µM; 3'2'-cGAMP, 250 µM; 3'3'-cGAMP, 250 µM. Related to Figure 1.

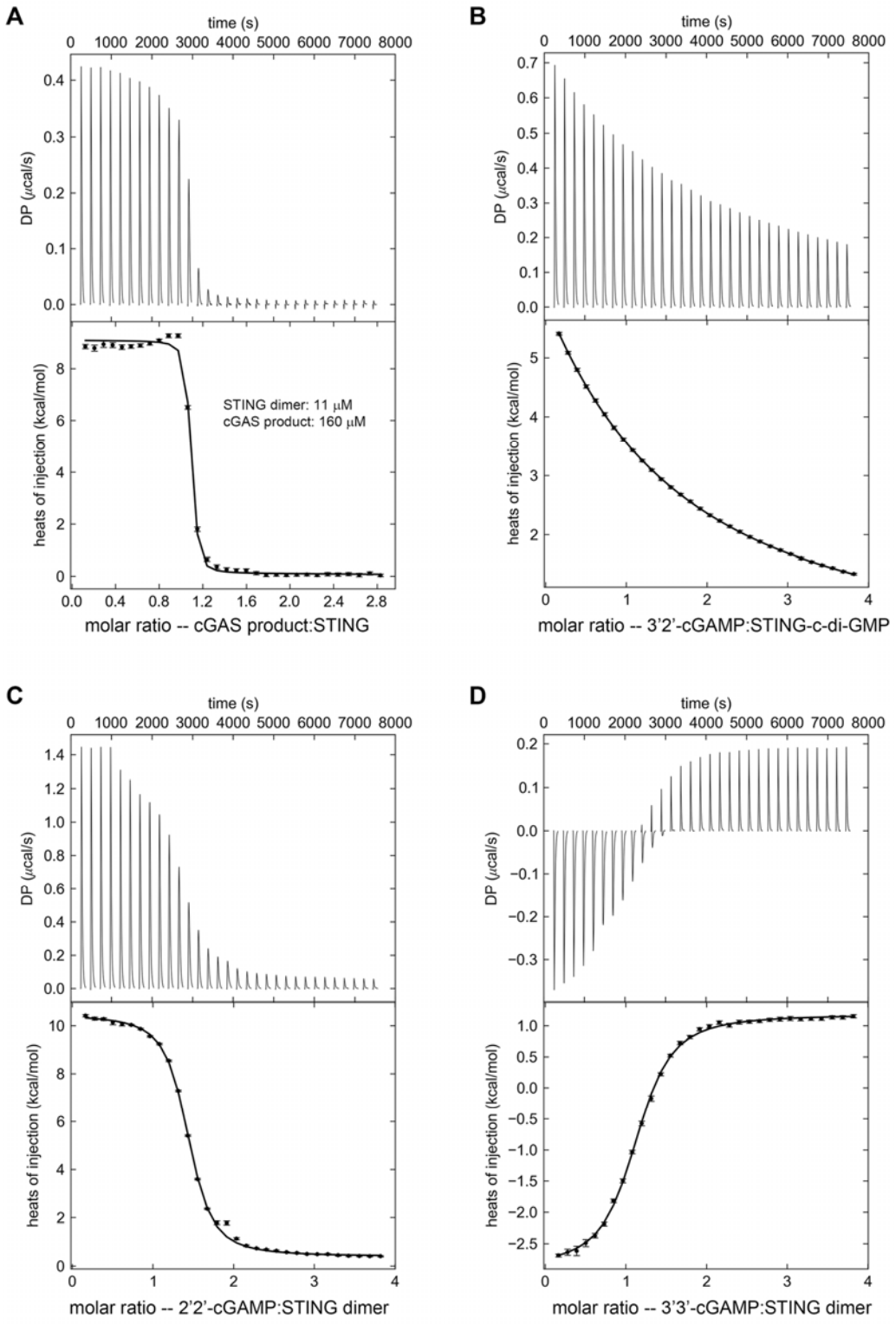


Figure S3. Measurements of cGAMP binding to STING by ITC. (**A**) The cGAS product was titrated into a solution of apo-STING dimer. The original titration traces (top) and integrated data (bottom) show that this binding is endothermic and the affinity is very high such that curve fitting may not be precise. (**B**) Synthetic 3'2'-cGAMP was titrated into a solution of c-di-GMP bound STING, because no obvious heat change was detected if it were titrated into a solution of apo-STING dimer. (**C** and **D**) Synthetic 2'2'-cGAMP (C) and 3'3'-cGAMP (D) were titrated to a solution of apo-STING dimer. Related to Figure 2.

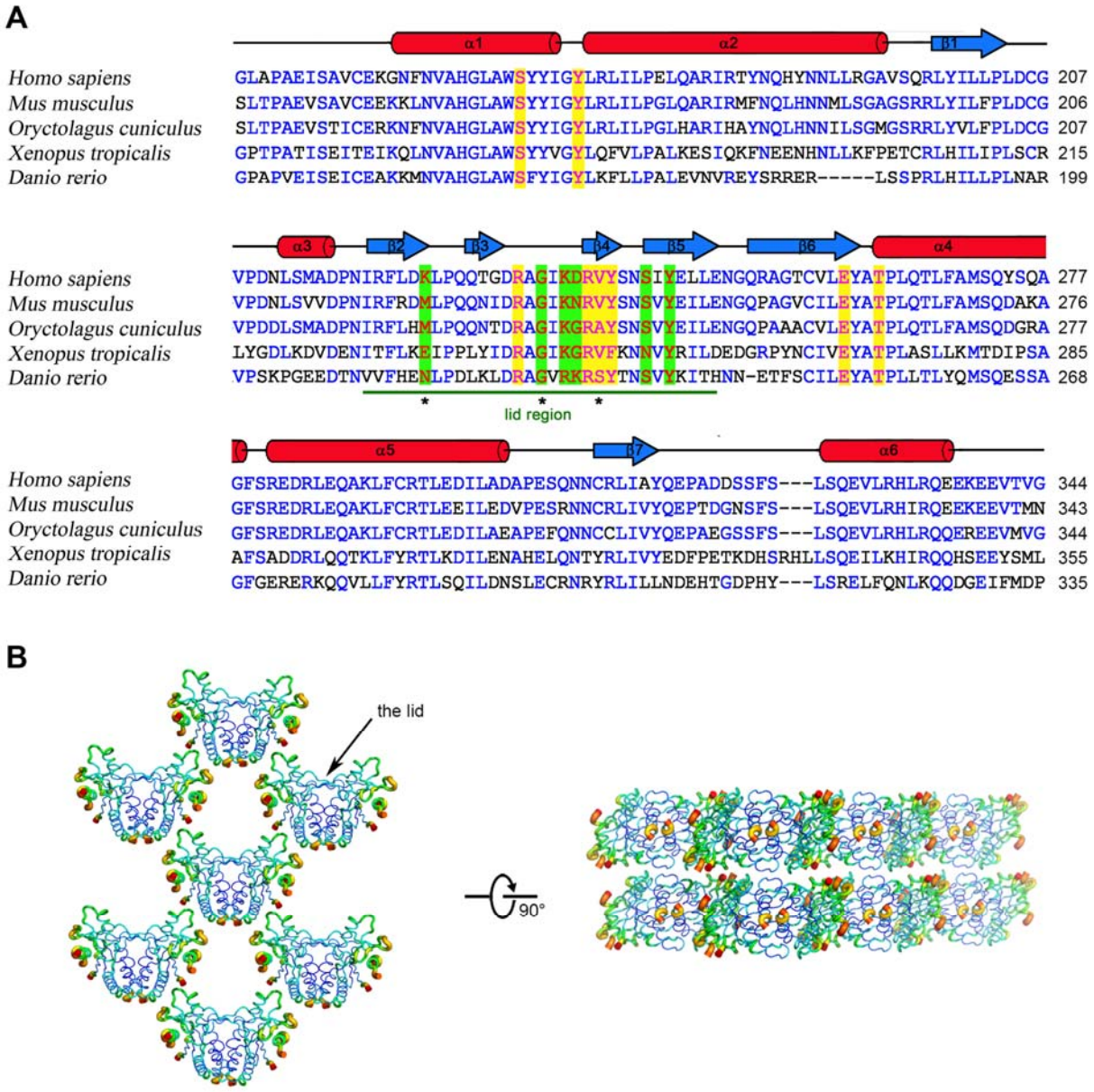


Figure S4. Sequence alignment of STING and crystallography of cGAMP bound STING. (**A**) Secondary structural elements of STING are indicated above the sequence alignment. Residues involved in the interdomain interactions on the new β sheet induced by 2'3'-cGAMP are shaded in green and colored in red. Residues involved in 2'3'-cGAMP binding are shaded in yellow and colored in magenta. The asterisk indicates the residue in which the atom in the main chain interacts with another residue. The accession numbers for STING are: *Homo sapiens*, GI: 119582502; *Mus musculus*, GI: 254692993; *Oryctolagus cuniculus*, GI: 291387441; *Xenopus (Silurana) tropicalis*, GI: 350529313; *Danio rerio*, GI: 410810119. The sequences are aligned with ClustalW. (**B**) Crystal packing manner of cGAMP bound STING in the space group of C2. The structure is colored according to crystallographic temperature factors (blue<16.79 $Å^2$ to red>110.34 $Å^2$) by PyMol. Related to Figure 3.

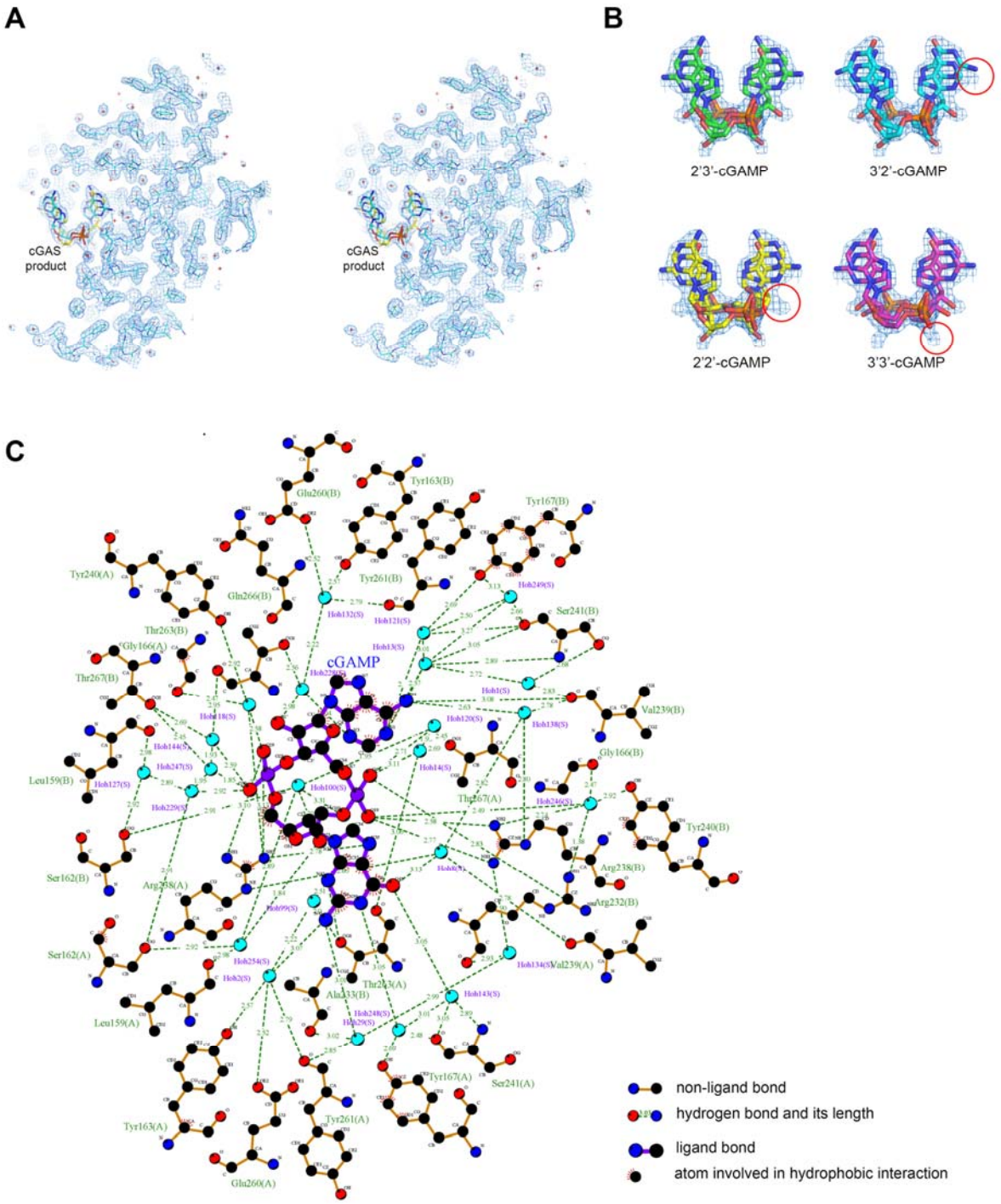


Figure S5. Direct visualization of the cGAS product and its extensive interactions with STING. (**A)** Representative electron density maps of the cGAS product within the STING structure. The stereo view of a representative slab of the $2F_o$-F_c electron density of the cGAS product bound to STING. The electron density, shown in blue mesh, is contoured at 1.0 σ. Two alternative conformations of cGAMP are colored in yellow and cyan, respectively. (**B)** Only 2'3'-cGAMP, not other cGAMP isomers, perfectly fits the electron density. (**C**) The cGAMP binding pocket plotted by LIGPLOT (Laskowski and Swindells, 2011)**.** Carbon atoms (black), oxygen atoms (red), nitrogen atoms (blue), phosphorus atoms (purple) and water molecules (magenta) are shown in solid circles. Hydrophobic interactions are shown in radial lines, while polar contacts are shown in dashed lines. The distances are in Å. Related to Figure 4.

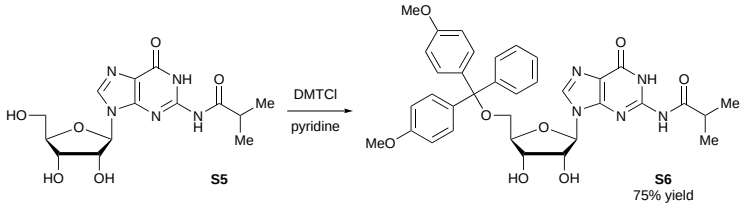
SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Chemical Synthesis of cGAMP

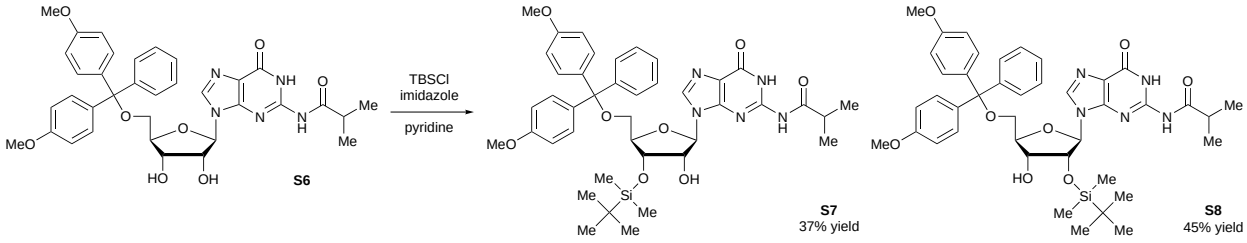
General Methods. All reactions were performed in glassware under a positive pressure of argon. The normal-phase flash column chromatography was performed with EMD silica gel 60 (230–400 mesh ASTM). TLC analyses were performed on EMD 250 μm Silica Gel 60 F254 plates and visualized by quenching of UV fluorescence (λ_{max}= 254 nm), or by staining ceric ammonium molybdate. ^{1}H, ^{13}C, and ^{31}P NMR spectra were recorded on Varian Inova-500, or Inova-400. ^{1}H NMR chemical shifts are referenced to the solvent residual peak (CD_3CN: δ 1.94 ppm, CD_3OD: δ 3.31 ppm, DMSO-*d*6: δ 2.50 ppm, D_2O: δ 4.79 ppm) and ^{31}P NMR chemical shifts to pH 7.4 phosphate buffer (δ 3.00 ppm). The multiplicities are presented as follows: brs = broad singlet, s = singlet, d = doublet, t = triplet, m = multiplet. Mass spectra were acquired on Agilent 6120 Single Quadrupole LC/MS. Analytical HPLC was performed using ACE 5 C18-AR column with dimension 4.6×150 mm. Preparative HPLC was performed using ACE 5 C18-AR column with dimension 10×150 mm. The CD spectrum was recorded on a JACSO J-815 CD Spectrometer.

cGAMP synthesis was performed using procedures modified from published methods (Gaffney et al., 2010; Zhang et al., 2006), as detailed below.

Synthesis of Building Block S1

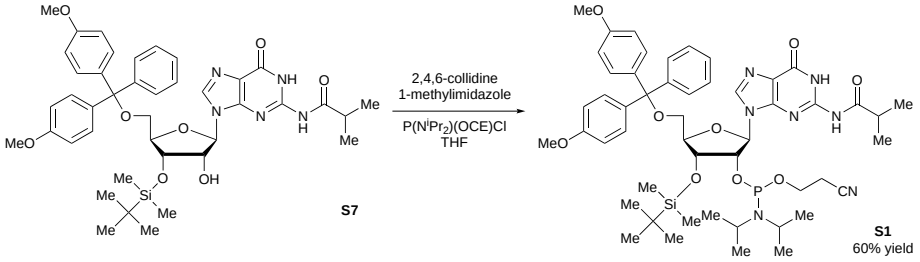


To a solution of **S5** (0.088 g, 0.25 mmol) in dry pyridine (2.5 mL) was added a solution of 4, 4'-dimethoxytrityl chloride (0.094 g, 0.26 mmol) in dry pyridine (0.5 mL) under argon. The mixture was stirred for 3 h at room temperature. The solvent was then removed by vacuum and the residue was purified by flash column chromatography (5→10% methanol/methylene chloride) to afford **S6** (0.123 g, 0.19 mmol, 75%) as a white foam. ^{1}H NMR (400 MHz, CD_3CN) δ 7.83 (s, 1H), 7.40–7.37 (m, 2H), 7.29–7.18 (m, 7H), 6.81–6.75 (m, 4H), 5.82 (d, *J* = 4.3 Hz, 1H), 4.68 (t, *J* = 4.8 Hz, 1H), 4.46 (t, *J* = 5.1 Hz, 1H), 4.12–4.08 (m, 1H), 3.742 (s, 3H), 3.735 (s, 3H), 3.38 (dd, *J* = 10.5, 5.9 Hz, 1H), 3.24 (dd, *J* = 8.4, 2.4 Hz, 1H), 2.61–2.52 (m, 1H), 1.15 (d, *J* = 6.8 Hz, 3H), 1.13 (d, *J* = 6.9 Hz, 3H).



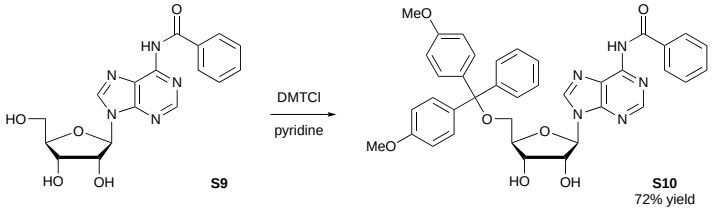
To a solution of compound **S6** (0.18 g, 0.28 mmol) and imidazole (0.056 g, 0.83 mmol) in dry pyridine (3.0 mL) was added *tert*-butyldimethylsilyl chloride (0.062 g, 0.41 mmol). The mixture was stirred for 4 h at room temperature. After removing the volatiles, methylene chloride (50 mL) was added and the solution was washed by saturated sodium bicarbonate solution and brine, dried over anhydrous sodium sulfate, evaporated, and purification by flash column chromatography (30→60% ethyl acetate/toluene) to afford **S7** (0.077 g, 0.10 mmol, 37%) and **S8**

(0.096 g, 0.13 mmol, 45%) each as a white solid. **S7**: ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.14 (s, 1H), 7.35–7.30 (m, 2H), 7.29–7.18 (m, 7H), 6.86–6.81 (m, 4H), 5.78 (d, J = 5.9 Hz, 1H), 5.59–5.54 (m, 1H), 4.64–4.56 (m, 1H), 4.24–4.18 (m, 1H), 3.93–3.89 (m, 1H), 3.71 (s, 6H) , 3.30–3.24 (m, 1H), 2.78–2.68 (m, 1H), 1.11 (d, J = 6.8 Hz, 6H), 0.80 (s, 9H), 0.04(s, 3H), 0.00 (s, 3H). **S8**: ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.11 (s, 1H), 7.36–7.31 (m, 2H), 7.29–7.17 (m, 7H), 6.87–6.80 (m, 4H), 5.87 (d, J = 5.7 Hz, 1H), 5.16 (d, J = 5.4 Hz, 1H), 4.61 (t, J = 5.5 Hz, 1H), 4.17–4.12 (m, 1H), 4.08–4.04 (m, 1H), 3.71 (s, 6H), 2.78–2.68 (m, 1H), 1.10 (d, J = 6.5 Hz, 3H), 1.09 (d, J = 6.5 Hz, 3H), 0.74 (s, 9H), –0.03 (s, 3H), –0.14 (s, 3H).

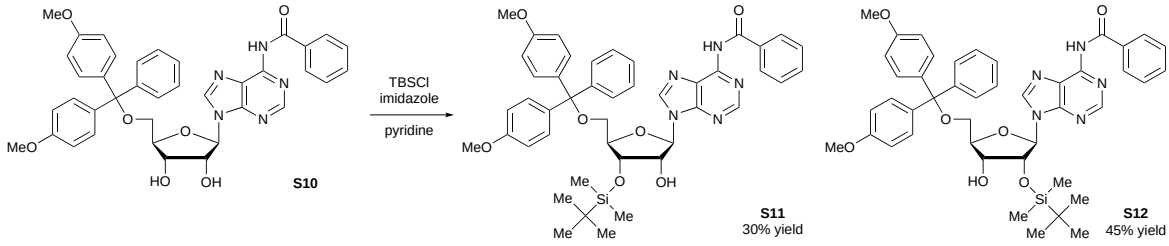


To a solution of compound **S7** (0.103 g, 0.13 mmol) in dry tetrahydrofuran (2.0 mL) was added 2,4,6-collidine (0.121 mL, 0.93 mmol) and *N*-methylimidazole (0.005mL, 0.067 mmol). 2-Cyanoethyl diisopropylphosphoramidochloridite (0.074 mL, 0.33 mmol) was then added dropwise over 5 min. After stirring for 1 h at room temperature, the mixture was diluted with ethyl acetate (30 mL) and washed with saturated sodium bicarbonate solution and brine, dried over anhydrous sodium sulfate, evaporated, and purified by flash column chromatography (50% ethyl acetate/hexanes→1% methanol/methylene chloride containing 1% triethylamine) to afford **S1** (0.077 g, 0.079 mmol, 60%) as a white solid. ^{1}H NMR (400 MHz, CD_3CN) δ 7.86 (s, 1H), 7.45–7.41 (m, 2H), 7.33–7.22 (m, 7H), 6.86–6.81 (m, 4H), 6.01 (d, J = 5.7 Hz, 1H), 4.81–4.74 (m, 1H), 4.34–4.30 (m, 1H), 4.15–4.08 (m, 2H), 3.76 (s, 6H), 3.65–3.25 (m, 4H), 2.60–2.41 (m, 2H), 1.25–0.95 (m, 12H), 0.87 (s, 9H), 0.10 (s, 3H), 0.025 (s, 3H).

Synthesis of Building Block S3

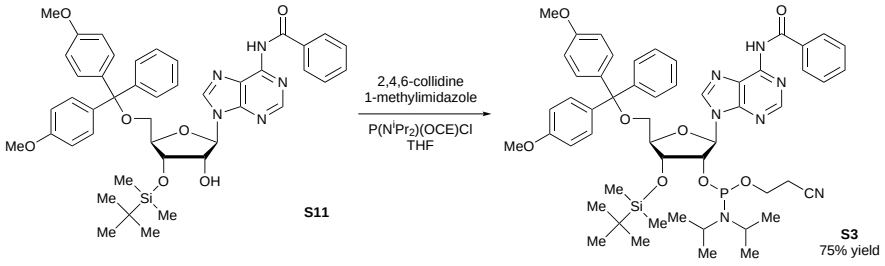


To a solution of **S9** (0.372 g, 1.0 mmol) in dry pyridine (5.0 mL) was added a solution of 4,4'-dimethoxytrityl chloride (0.542 g, 1.5 mmol) in dry pyridine (1.0 mL) under argon. The mixture was stirred for 3 h at room temperature. The solvent was then removed by vacuum and the residue was purified by flash column chromatography (5→10% methanol/methylene chloride) to afford **S10** (0.485 g, 0.72 mmol, 72%) as a white foam. ^{1}H NMR (400 MHz, CD_3OD) δ 8.64 (s, 1H), 8.53 (s, 1H), 8.08 (d, *J* = 7.2 Hz, 2H), 7.66 (t, *J* = 7.4 Hz, 1H), 7.60–7.54 (m, 2H), 7.43–7.38 (m, 2H), 7.31–7.15 (m, 7H), 6.83–6.77 (m, 4H), 6.16 (d, *J* = 4.6 Hz, 1H), 4.97 (t, *J* = 4.8 Hz, 1H), 4.55 (t, *J* = 5.0 Hz, 1H), 3.76 (s, 3H), 3.75 (s, 3H), 3.45–3.41 (m, 2H).



To a solution of compound **S10** (0.48 g, 0.71 mmol) and imidazole (0.145 g, 2.1 mmol) in dry pyridine (9.0 mL) was added *tert*-butyldimethylsilyl chloride (0.16 g, 1.1 mmol). The mixture was stirred for 4 h at room temperature. After removing the volatiles, methylene chloride (100 mL) was added and the solution was washed by saturated sodium bicarbonate solution and brine, dried over anhydrous sodium sulfate, evaporated, and purification by flash column chromatography (20→50% ethyl acetate/toluene) to afford **S11** (0.167 g, 0.21 mmol, 30%) and

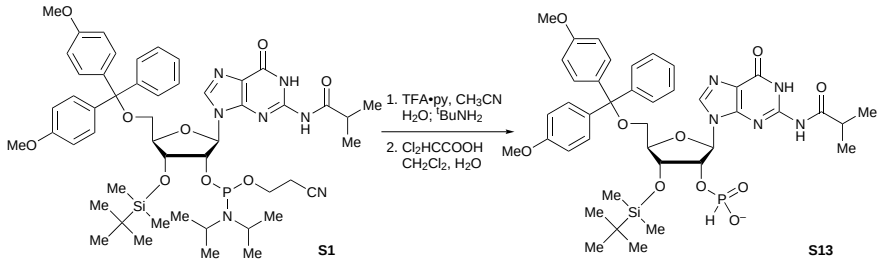
S12 (0.251 g, 0.32 mmol, 45%) each as a white solid. **S11**: ^{1}H NMR (400 MHz, CD_3CN) δ 9.29 (brs, 1H), 8.62 (s, 1H), 8.28 (s, 1H), 7.95 (d, *J* = 9.5 Hz, 2H), 7.60 (t, *J* = 7.4 Hz, 1H), 7.53–7.47 (m, 2H), 7.35–7.31 (m, 2H), 7.23–7.14 (m, 7H), 6.76 (dd, *J* = 9.0, 2.9 Hz, 4H), 6.02 (d, *J* = 3.7 Hz, 1H), 4.80 (q, *J* = 4.0 Hz, 1H), 4.70 (t, *J* = 3.2 Hz, 1H), 4.09 (q, *J* = 4.8 Hz, 1H), 3.701 (s, 3H), 3.699 (s, 3H), 3.44 (d, *J* = 5.2 Hz, 1H), 3.39 (dd, *J* = 10.7, 3.2 Hz, 1H), 3.15 (dd, *J* = 10.8, 4.6 Hz, 1H), 0.83 (s, 9H), 0.06 (s, 3H), –0.01 (s, 3H). **S12**: ^{1}H NMR (400 MHz, CD_3CN) δ 9.27 (brs, 1H), 8.59 (s, 1H), 8.27 (s, 1H), 8.00 (d, *J* = 7.7 Hz, 2H), 7.65 (t, *J* = 7.4 Hz, 1H), 7.58–7.52 (m, 2H), 7.47–7.43 (m, 2H), 7.35–7.19 (m, 7H), 6.84 (dd, *J* = 8.9, 1.8 Hz, 4H), 6.04 (d, *J* = 4.7 Hz, 1H), 4.97 (t, *J* = 4.8 Hz, 1H), 4.39 (q, *J* = 5.0 Hz, 1H), 4.19 (q, *J* = 4.3 Hz, 1H), 3.75 (s, 6H), 3.42–3.33 (m, 2H), 3.14 (d, *J* = 5.6 Hz, 1H), 0.83 (s, 9H), 0.00 (s, 3H), –0.10 (s, 3H).



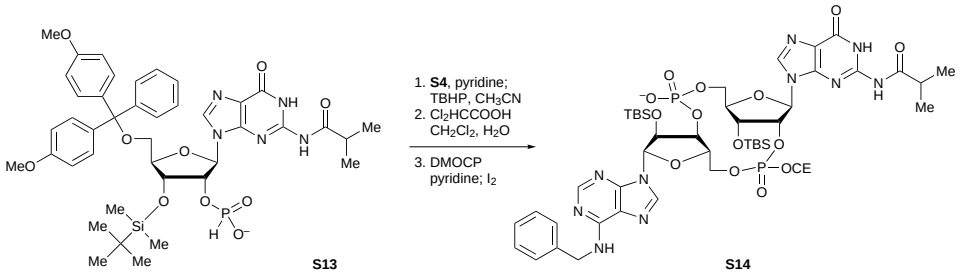
To a solution of compound **S11** (0.485 g, 0.62 mmol) in dry tetrahydrofuran (10 mL) was added 2,4,6-collidine (0.560 mL, 0.93 mmol) and *N*-methylimidazole (0.024 mL, 0.31 mmol). 2-Cyanoethyl diisopropylphosphoramidochloridite (0.353 mL, 1.57 mmol) was then added dropwise over 5 min. After stirring for 1 h at room temperature, the mixture was diluted with ethyl acetate (150 mL) and washed with saturated sodium bicarbonate solution and brine, dried over anhydrous sodium sulfate, evaporated, and purified by flash column chromatography (50% ethyl acetate/hexanes containing 1% triethylamine) to afford **S3** (0.46 g, 0.47 mmol, 75%) as a white solid. ^{1}H NMR (400 MHz, CD_3CN) δ 9.25 (brs, 1H), 8.65 (s, 1H), 8.33 (s, 1H), 8.00 (d, *J* = 7.8 Hz, 2H), 7.70–7.61 (m, 2H), 7.59–7.52 (m, 2H), 7.44–7.35 (m, 2H), 7.32–7.16 (m, 7H), 6.82

(dd, *J* = 8.9, 4.2 Hz, 4H), 6.25 (d, *J* = 4.0 Hz, 1H), 5.05–4.98 (m, 1H), 4.72 (t, *J* = 4.9 Hz, 1H), 4.18 (q, *J* = 4.8 Hz, 1H), 3.76 (s, 3H), 3.75 (s, 3H), 3.71–3.61 (m, 1H), 3.61–3.53 (m, 3H), 3.49 (dd, *J* = 10.8, 4.0 Hz, 1H), 3.24 (dd, *J* = 10.7, 4.6 Hz, 1H), 2.46 (t, *J* = 6.1 Hz, 2H), 1.13 (d, *J* = 6.8 Hz, 3H), 1.10 (d, *J* = 6.7 Hz, 3H), 0.86 (s, 9H), 0.12 (s, 3H), 0.07 (s, 3H).

Synthesis of 2',3'-cGAMP

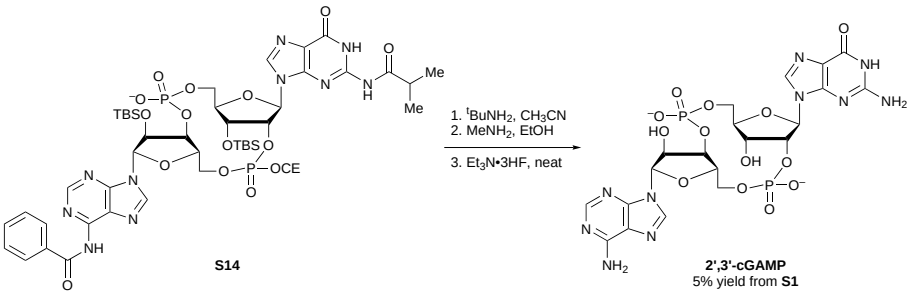


To a solution of **S1** (0.049 g, 0.05 mmol) in aectonitrile (0.25 mL) and water (0.0018 mL, 0.1 mmol) was added pyridinium trifluoroacetate (0.012 g, 0.06 mmol). After stirring for 1 min, *tert*-butylamine (0.25 mL) was added. After stirring for 10 min, the solvent was removed and the residue was dissolved in methylene chloride (0.6 mL), and water (0.009 mL, 0.5 mmol) and dichloroacetic acid (6% in methylene chloride, 0.6 mL, 0.44 mmol) was added. After stirring for 10 min, the reaction was quenched by pyridine (0.07 mL, 0.87 mmol). The solution was concentrated to give **S13**, which was used directly for next step.



To a solution of crude **S13** obtained above in acetonitrile (0.12 mL) was added a solution of **S4** (0.064 g, 0.065 mmol) in acetonitrile (0.20 mL) under argon. After stirring for 2 min, anhydrous

t-butyl hydroperoxide (5.5 M in decane, 0.028 mL, 0.15 mmol) was added. After stirring for 30 min, the reaction was quenched by 33% sodium bisulfite solution (0.025 mL) at 0 °C. The mixture was then concentrated to a yellow oil. The residual yellow oil was dissolved in methylene chloride (0.8 mL) and water (0.009 mL, 0.5 mmol) and dichloroacetic acid (6% in methylene chloride, 0.8 mL, 0.6 mmol) was added. After stirring for 10 min, the reaction was quenched by pyridine (2.5 mL). The solution was concentrated to 1.0 mL before adding 2-chloro-5,5-dimethyl-1,3,2-dioxaphosphorinane-2-oxide (0.034 g, 0.17 mmol). After stirring for 10 min, the reaction was quenched by water (0.032 mL, 1.8 mmol) followed by iodine (0.017 g, 0.065 mmol) immediately. After stirring for 5 min, the mixture was poured into a 0.14 % sodium bisulfite solution (7 mL). After stirring for 5 min, sodium bicarbonate (0.20 g) was added slowly. The mixture was extracted by ethyl acetate/diethyl ether (1:1, 5 mL). The organic layer was concentrated to an oil and used directly for the next step.



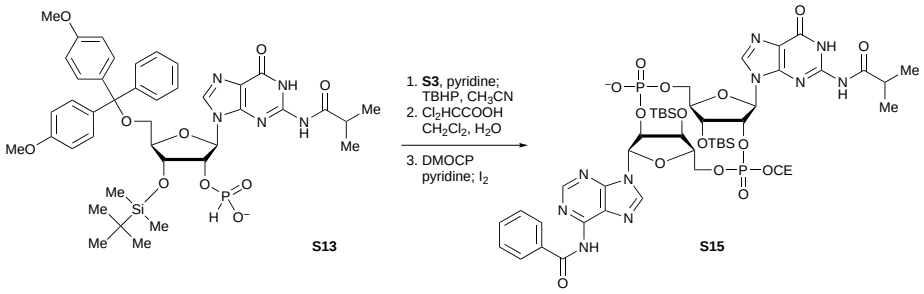
S14

2',3'-cGAMP
5% yield from **S1**

To a solution of crude **S14** obtained above in acetonitrile (0.25 mL) was added *tert*-butylamine (0.5 mL). After stirring for 10 min, the yellow solution was concentrated to a yellow foam and methylamine (33% in anhydrous ethanol, 1.79 mL, 15 mmol) was added. After stirring for 90 min, the mixture was concentrated to an oil and treated with triethylamine trihydrofluoride (0.20 mL) in a plastic vial. After stirring for 5 h, the reaction was quenched by 1.0 M ammonium acetate (2.5 mL). The mixture was then stirred vigorously at 35 °C for 30 min. After cooling to room temperature, the solution was filtered. The yellow filtrate was concentrated to a small

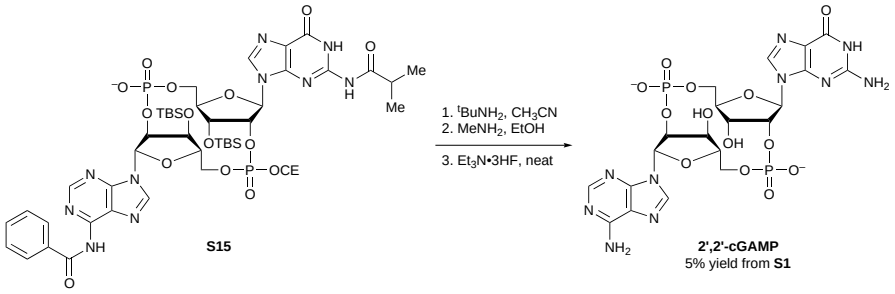
volume and purified by HPLC (eluent A: water with 0.1% trifluoroacetic acid, eluent B: acetonitrile with 0.1% trifluoroacetic acid, gradient: T = 0 min: 0% B, T = 7 min: 0 % B, T = 20 min: 10 % B, 4.8 mL/min) to give 2',3'-cGAMP as a white powder (1.7 mg, 5% from **S1**). ^{1}H NMR (500 MHz, 30 mM $NaH_2PO_4/Na_2HPO_4/D_2O$, *c* = 1.05 mM, 50 °C) δ 8.58 (s, 1H), 8.56 (s, 1H), 8.16 (s, 1H), 6.45 (s, 1H), 6.22 (d, *J* = 8.4 Hz, 1H), 5.91–5.86 (m, 1H), 5.33–5.29 (m, 1H), 5.07 (d, *J* = 4.1 Hz, 1H), 4.88 (d, *J* = 4.1 Hz, 1H), 4.76–4.74 (m, 1H), 4.73–4.70 (m, 1H), 4.70–4.67 (m, 1H), 4.55–4.49 (m, 1H), 4.48–4.40 (m, 2H); ^{31}P NMR (162 MHz, 30 mM $NaH_2PO_4/Na_2HPO_4/D_2O$, *c* = 1.05 mM, 25°C) δ 0.05, –0.89; MS (ES^+) calcd for $C_{20}H_{24}N_{10}O_{13}P_2$ $[M+H]^+$ 675.1, found 675.1.

Synthesis of 2',2'-cGAMP



To a solution of crude **S13** prepared from **S1** (0.049 g) in acetonitrile (0.12 mL) was added a solution of **S3** (0.064 g, 0.065 mmol) in acetonitrile (0.20 mL) under argon. After stirring for 2 min, anhydrous *t*-butyl hydroperoxide (5.5 M in decane, 0.028 mL, 0.15 mmol) was added. After stirring for 30 min, the reaction was quenched by 33% sodium bisulfite solution (0.025 mL) at 0 °C. The mixture was then concentrated to a yellow oil. The residual yellow oil was dissolved in methylene chloride (0.8 mL) and water (0.009 mL, 0.5 mmol) and dichloroacetic acid (6% in methylene chloride, 0.8 mL, 0.6 mmol) was added. After stirring for 10 min, the reaction was quenched by pyridine (2.5 mL). The solution was concentrated to 1.0 mL before adding 2-chloro-

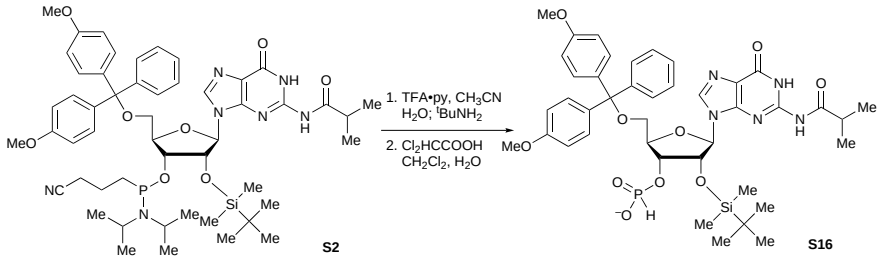
5,5-dimethyl-1,3,2-dioxaphosphorinane-2-oxide (0.034 g, 0.17 mmol). After stirring for 10 min, the reaction was quenched by water (0.032 mL, 1.8 mmol) followed by iodine (0.017 g, 0.065 mmol) immediately. After stirring for 5 min, the mixture was poured into a 0.14 % sodium bisulfite solution (7 mL). After stirring for 5 min, sodium bicarbonate (0.20 g) was added slowly. The mixture was extracted by ethyl acetate/diethyl ether (1:1, 5 mL). The organic layer was concentrated to an oil and used directly for the next step.



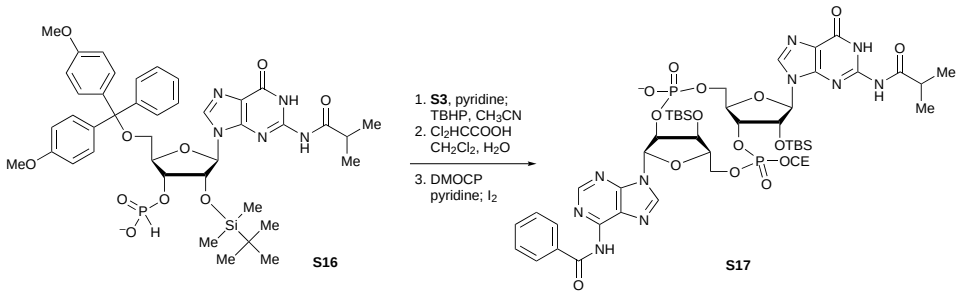
To a solution of crude **S15** obtained above in acetonitrile (0.25 mL) was added *tert*-butylamine (0.5 mL). After stirring for 10 min, the yellow solution was concentrated to a yellow foam and methylamine (33% in anhydrous ethanol, 1.79 mL, 15 mmol) was added. After stirring for 90 min, the mixture was concentrated to an oil and treated with triethylamine trihydrofluoride (0.20 mL) in a plastic vial. After stirring for 5 h, the reaction was quenched by 1.0 M ammonium acetate (2.5 mL). The mixture was then stirred vigorously at 35 °C for 30 min. After cooling to room temperature, the solution was filtered. The yellow filtrate was concentrated to a small volume and purified by HPLC (eluent A: water with 0.1% trifluoroacetic acid, eluent B: acetonitrile with 0.1% trifluoroacetic acid, gradient: T = 0 min: 0% B, T = 7 min: 0 % B, T = 20 min: 10 % B, 4.8 mL/min) to give 2',2'-cGAMP as a white powder (1.6 mg, 5% from **S1**). ^{1}H NMR (500 MHz, 30 mM $NaH_2PO_4/Na_2HPO_4/D_2O$, *c* = 1.50 mM, 50 °C) δ 8.48 (s, 1H), 8.40 (s, 1H), 8.13 (s, 1H), 6.58 (d, *J* = 8.0 Hz, 1H), 6.27 (d, *J* = 8.5 Hz, 1H), 5.62–5.57 (m, 1H), 5.52–5.48(m, 1H), 4.97 (d, *J* = 4.4 Hz, 1H), 4.95 (d, *J* = 4.3 Hz, 1H), 4.75–4.72 (m, 1H), 4.56–4.48 (m,

2H), 4.47–4.41 (m, 2H); ^{31}P NMR (162 MHz, 30 mM $NaH_2PO_4/Na_2HPO_4/D_2O$, *c* = 1.05 mM, 25°C) δ 0.19, –0.71; MS (ES^+) calcd for $C_{20}H_{24}N_{10}O_{13}P_2$ $[M+H]^+$ 675.1, found 675.1.

Synthesis of 3',2'-cGAMP

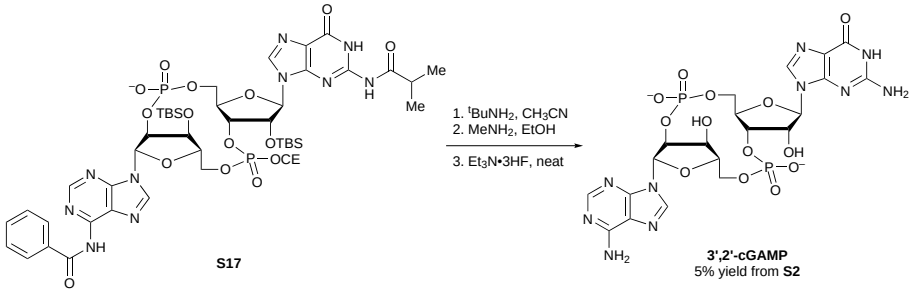


To a solution of **S2** (0.049 g, 0.05 mmol) in aectonitrile (0.25 mL) and water (0.0018 mL, 0.1 mmol) was added pyridinium trifluoroacetate (0.012 g, 0.06 mmol). After stirring for 1 min, *tert*-butylamine (0.25 mL) was added. After stirring for 10 min, the solvent was removed and the residue was dissolved in methylene chloride (0.6 mL), and water (0.009 mL, 0.5 mmol) and dichloroacetic acid (6% in methylene chloride, 0.6 mL, 0.44 mmol) was added. After stirring for 10 min, the reaction was quenched by pyridine (0.07 mL, 0.87 mmol). The solution was concentrated to give **S16**, which was used directly for next step.



To a solution of crude **S16** obtained above in acetonitrile (0.12 mL) was added a solution of **S3** (0.064 g, 0.065 mmol) in acetonitrile (0.20 mL) under argon. After stirring for 2 min, anhydrous *t*-butyl hydroperoxide (5.5 M in decane, 0.028 mL, 0.15 mmol) was added. After stirring for 30 min, the reaction was quenched by 33% sodium bisulfite solution (0.025 mL) at 0 °C. The

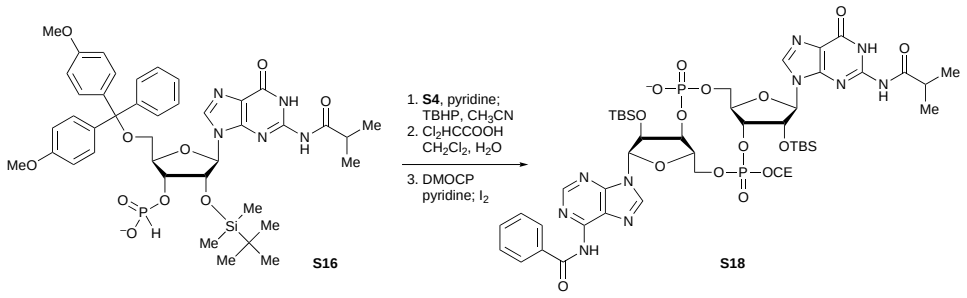
mixture was then concentrated to a yellow oil. The residual yellow oil was dissolved in methylene chloride (0.8 mL) and water (0.009 mL, 0.5 mmol) and dichloroacetic acid (6% in methylene chloride, 0.8 mL, 0.6 mmol) was added. After stirring for 10 min, the reaction was quenched by pyridine (2.5 mL). The solution was concentrated to 1.0 mL before adding 2-chloro-5,5-dimethyl-1,3,2-dioxaphosphorinane-2-oxide (0.034 g, 0.17 mmol). After stirring for 10 min, the reaction was quenched by water (0.032 mL, 1.8 mmol) followed by iodine (0.017 g, 0.065 mmol) immediately. After stirring for 5 min, the mixture was poured into a 0.14 % sodium bisulfite solution (7 mL). After stirring for 5 min, sodium bicarbonate (0.20 g) was added slowly. The mixture was extracted by ethyl acetate/diethyl ether (1:1, 5 mL). The organic layer was concentrated to an oil and used directly for the next step.



To a solution of crude **S17** obtained above in acetonitrile (0.25 mL) was added *tert*-butylamine (0.5 mL). After stirring for 10 min, the yellow solution was concentrated to a yellow foam and methylamine (33% in anhydrous ethanol, 1.79 mL, 15 mmol) was added. After stirring for 90 min, the mixture was concentrated to an oil and treated with triethylamine trihydrofluoride (0.20 mL) in a plastic vial. After stirring for 5 h, the reaction was quenched by 1.0 M ammonium acetate (2.5 mL). The mixture was then stirred vigorously at 35 °C for 30 min. After cooling to room temperature, the solution was filtered. The yellow filtrate was concentrated to a small volume and purified by HPLC (eluent A: water with 0.1% trifluoroacetic acid, eluent B: acetonitrile with 0.1% trifluoroacetic acid, gradient: T = 0 min: 0% B, T = 7 min: 0 % B, T = 20

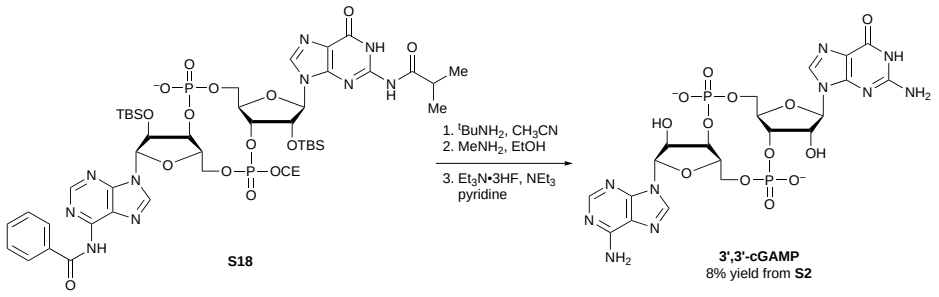
min: 7 % B, 4.8 mL/min) to give 3',2'-cGAMP as a white powder (1.7 mg, 5% from **S2**). ^{1}H NMR (500 MHz, 30 mM $NaH_2PO_4/Na_2HPO_4/D_2O$, *c* = 1.50 mM, 50°C) δ 8.75 (s, 1H), 8.43 (s, 1H), 8.11 (s, 1H), 6.53 (d, *J* = 7.5 Hz, 1H), 6.19 (s, 1H), 5.53–5.47 (m, 1H), 5.29–5.23(m, 1H), 5.17–5.13 (m, 1H), 4.90–4.87 (m, 1H), 4.73–4.67 (m, 2H), 4.58–4.50 (m, 2H), 4.44–4.34 (m, 2H); ^{31}P NMR (162 MHz, 30 mM $NaH_2PO_4/Na_2HPO_4/D_2O$, *c* = 1.05 mM, 25°C) δ 1.41, 1.14; MS (ES^+) calcd for $C_{20}H_{24}N_{10}O_{13}P_2$ $[M+H]^+$ 675.1, found 675.1.

Synthesis of 3',3'-cGAMP



To a solution of crude **S16** prepared from **S2** (0.097 g) in acetonitrile (0.12 mL) was added a solution of **S4** (0.128 g, 0.13 mmol) in acetonitrile (0.40 mL) under argon. After stirring for 2 min, anhydrous *t*-butyl hydroperoxide (5.5 M in decane, 0.055 mL, 0.3 mmol) was added. After stirring for 30 min, the reaction was quenched by 33% sodium bisulfite solution (0.05 mL) at 0 °C. The mixture was then concentrated to a yellow oil. The residual yellow oil was dissolved in methylene chloride (1.6 mL) and water (0.018 mL, 1.0 mmol) and dichloroacetic acid (6% in methylene chloride, 1.6 mL, 1.2 mmol) was added. After stirring for 10 min, the reaction was quenched by pyridine (5.0 mL). The solution was concentrated to 2.0 mL before adding 2-chloro-5,5-dimethyl-1,3,2-dioxaphosphorinane-2-oxide (0.068 g, 0.35 mmol). After stirring for 10 min, the reaction was quenched by water (0.064 mL, 3.5 mmol) followed by iodine (0.033 g, 0.13 mmol) immediately. After stirring for 5 min, the mixture was poured into a 0.14 % sodium

bisulfite solution (14 mL). After stirring for 5 min, sodium bicarbonate (0.40 g) was added slowly. The mixture was extracted by ethyl acetate/diethyl ether (1:1, 10 mL). The organic layer was concentrated to an oil and used directly for the next step.



To a solution of crude **S18** obtained above in acetonitrile (0.50 mL) was added *tert*-butylamine (0.5 mL). After stirring for 10 min, the yellow solution was concentrated to a yellow foam and washed with methylene chloride (0.6 mL). Methylamine (33% in anhydrous ethanol, 3.58 mL, 29 mmol) was then added to the resulting white solid. After stirring for 90 min, the mixture was concentrated to an oil. The residue was azeotroped with pyridine/triethylamine (1.0 mL/0.4 mL) for three times and then dissolved in pyridine (0.08 mL) in a plastic vial. To this solution at 50°C, triethylamine (0.50 mL, 3.6 mmol) and triethylamine trihydrofluoride (0.30 mL) were added simultaneously. After stirring for 1 h, the reaction solution was quenched by methoxytrimethylsilane (1.5 mL) at room temperature. The mixture was then concentrated to a yellow solid and purified by HPLC (eluent A: water with 0.1% trifluoroacetic acid, eluent B: acetonitrile with 0.1% trifluoroacetic acid, gradient: T = 0 min: 0% B, T = 7 min: 0 % B, T = 20 min: 7 % B, 4.8 mL/min) to give 3',3'-cGAMP as a white powder (5.5 mg, 8% from **S2**). ^{1}H NMR (500 MHz, 30 mM $NaH_2PO_4/Na_2HPO_4/D_2O$, c = 1.50 mM, 50 °C) δ 8.67 (s, 1H), 8.48 (s, 1H), 8.28 (s, 1H), 6.44 (s, 1H), 6.24 (s, 1H), 5.19–5.13 (m, 2H), 5.02 (d, *J* = 4.5 Hz, 1H), 5.00 (d, *J* = 4.8 Hz, 1H), 4.75–4.70 (m, 1H), 4.69–4.64 (m, 2H), 4.64–4.58 (m, 1H), 4.41–4.34 (m, 2H);

^{31}P NMR (162 MHz, 30 mM $NaH_2PO_4/Na_2HPO_4/D_2O$, *c* = 1.50 mM, 25°C) δ 0.07, 0.15; MS (ES+) calcd for $C_{20}H_{24}N_{10}O_{13}P_2$ $[M+H]^+$ 675.1, found 675.1.

Characterization of natural cGAMP

^{1}H NMR (500 MHz, 30 mM $NaH_2PO_4/Na_2HPO_4/D_2O$, *c* = 1.05 mM, 50°C) δ 8.58 (s, 1H), 8.56 (s, 1H), 8.16 (s, 1H), 6.45 (s, 1H), 6.22 (d, *J* = 8.5 Hz, 1H), 5.91–5.86 (m, 1H), 5.33–5.29(m, 1H), 5.07 (d, *J* = 4.0 Hz, 1H), 4.87 (d, *J* = 4.1 Hz, 1H), 4.76–4.73 (m, 1H), 4.73–4.70 (m, 1H), 4.70–4.67 (m, 1H), 4.55–4.49 (m, 1H), 4.48–4.40 (m, 2H); ^{31}P NMR (162 MHz, 30 mM $NaH_2PO_4/Na_2HPO_4/D_2O$, *c* = 1.05 mM, 25°C) δ 0.06, –0.88; MS (ES^+) calcd for $C_{20}H_{24}N_{10}O_{13}P_2$ $[M+H]^+$ 675.1, found 675.1.

SUPPLEMENTAL REFERENCES

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Zhang, Z., Kim, S., Gaffney, B.L., and Jones, R.A. (2006). Polymorphism of the signaling molecule c-di-GMP. J Am Chem Soc *128*, 7015-7024.