

Supporting Information

Enzymatic Preparation of 2'-5', 3'-5' Cyclic Dinucleotides, Their Binding Properties to Stimulator of Interferon Genes Adaptor Protein and Structure/Activity Correlations

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1. SUPPLEMENTAL MATERIALS

pUNO1-hSTING-WT, pUNO1-hSTING-HAQ, 3'3'-c-di-GMP, 2'3'-cGAMP, 3'3'-cGAMP, 3'3'-c-di-AMP, 2'2'-cGAMP, and blasticidin were purchased from InvivoGen (San Diego, USA). Lipofectamine 2000, OptiMEM medium, SYPRO Orange and hygromycin were purchased from ThermoFisher (Waltham, USA). STING antibody (cat. #. MAB7169) and Mouse IgG HRP-conjugated Antibody (cat. #. HAF007) were supplied by R&D Systems (Minneapolis, USA). DMEM with high glucose, FBS, poly-D-lysine, Digitonin A, sucrose, DTT, HEPES, BSA, ATP, GTP and other common chemicals were purchased from Sigma Aldrich (Prague, Czech Republic). 293T cells were obtained from ATCC (Manassas, USA). Phusion® High-Fidelity DNA Polymerase, Q5® Site-Directed Mutagenesis Kit, HindIII, BglII, NdeI, NotI and BamHI were delivered by NEB (Ipswich, USA). Luciferase Assay System reagent was purchased from Promega (Madison, USA). Rosetta-gami B (DE3) competent cells, Amicon® Ultra-15 10 K, were from Merck Millipore (Billerica, USA). Ni-NTA resin (cat. # 745400.25 Macherey-Nagel, Düren, Germany).

2. SUPPLEMENTAL METHODS

Cloning of pUNO1- hSTING-AQ, pUNO1- hSTING-Q and pUNO1- hSTING-REF plasmids

pUNO1-hSTING-Q and pUNO1-hSTING-REF were prepared by site-directed mutagenesis of pUNO1-hSTING-WT with NEB Q5® Site-Directed Mutagenesis Kit and primers STING_R293Q_F (AGACACTTGAGGACATCCTG) and STING_R293Q_R (GGCAGAAGAGTTTGGCCTGC) and STING_R232H_F (ATGCTGGCATCAAGGATCGG) and STING_R232H_R (GGTCACCGGTCTGCTGGGGC). Allelic version AQ was prepared by restriction of plasmids pUNO1-hSTING-WT and pUNO1-hSTING-HAQ with enzymes BglII and EcoRI (NEB). The smaller fragment from pUNO1-hSTING-HAQ, carrying only the STING AQ mutations, was ligated into pUNO1-hSTING-WT plasmid backbone. This subcloning resulted in plasmid pUNO1-hSTING-AQ. All the constructs were sequenced for verification.

Cloning of pGL64.27-4xISRE plasmid

Two complementary oligonucleotides of the sequence AAAGATCTTGAAAGTGAAACCTTGAAAAACGAAACTGGACAAAGGGAAACTGCA GAAACTGAAACAAAGCTTAA and TTAAGCTTTGTTTCAGTTTCTGCAGTTTCCCTTTGTCCAGTTTCGTTTTCCAAGGTTTC ACTTTCCAAGATCTTT containing four interferon-sensitive response elements (ISRE) were synthesized by Sigma Aldrich (Prague, Czech Republic). The oligonucleotides were mixed in equal molar amounts, hybridized, and cleaved by restriction endonucleases HindIII and BglII. Ultimately, they were ligated into plasmid pGL4.27 (Promega, Madison, USA) linearized with the same enzymes. As result the sequence with four ISRE sites was placed upstream of the minimum promoter of firefly luciferase reporter gene.

Development of 293T reporter cells

293T cells were seeded a day before transfection at density 125,000 cells per cm² onto poly-D-lysine coated six well plates in antibiotic free DMEM medium with high glucose supplemented with 10% heat inactivated FBS. On the day of transfection, 2.5 µg of the plasmid pUNO1-hSTING-WT, pUNO1- hSTING-HAQ, pUNO1- hSTING-AQ, pUNO1-hSTING-Q or pUNO1-hSTING-REF encoding human WT, HAQ (R71H, G230A and R293Q), AQ (G230A and R293Q), Q (R293Q) or REF STING (R232H)¹ were diluted in 125 µL OptiMEM medium and mixed with 125 µL of the same medium containing 12.5 µL of Lipofectamine 2000. After 5 min incubation at room temperature (RT), 250 µL of the mixture was added dropwise to the cells in one well. Cells were incubated 36 hours at 37 °C with 5% CO₂, and then detached with 0.05% Trypsin and 0.22 g/L EDTA. Transfected cells were seeded onto poly-D-lysine coated six well plates at density 50,000 cells per 1 cm² in DMEM medium with high glucose containing 10% heat inactivated FBS, 30µg/mL blasticidin, 0.06 mg/ml Penicillin G and 0.1mg/ml Streptomycin Sulfate. The medium was replenished every 3 – 4 days until visible colonies of cells resistant to blasticidin were formed. Blasticidin resistant cells stably expressing different variants of STING protein were further transfected with pGL64.27-4xISRE plasmid following the same procedure as described above. The transfected cells were selected for the resistance to 300 µg/mL hygromycin in DMEM with high glucose containing 10% heat inactivated FBS, 30µg/mL blasticidin, 0.06 mg/ml Penicillin G and 0.1 mg/ml Streptomycin Sulfate. Homogeneous culture of stably double transfected cells was prepared by limiting dilution of cells in 96 well plates and wells with cells were selected that originated from a single cell. These cells were expanded, and expression of *wt* STING was confirmed by western blot using monoclonal mouse anti STING antibodies (cat. #. MAB7169, 1:1000 dilution; 2° antibody cat. #. HAF007, 1:2000 dilution, both from R&D Systems,

Minneapolis, USA), and by induction of firefly luciferase expression in the presence of 50 μ M STING agonist 2'3' cGAMP. Genomic DNA from the transfected cells was amplified with primers pUNO1_Seq_F (TGCTTGCTCAACTCTACGTC) and pUNO1_Seq_R (GTGGTTTGTCCAAACTCATC) that were complementary to pUNO1 plasmids and the presence of correct STING gene variant in the transfected cells was confirmed by DNA sequencing.

Cloning, expression and purification of *wt* STING protein

Human *wt* STING cDNA was amplified by the use of PCR by employing Phusion® High-Fidelity DNA Polymerase and oligonucleotides hSTING140-BamH-For (GTGGGATCCGCCCCAGCTGAGATCTCTGCAG) and hSTING379-Not-Rev3 (TATGCGGCCGCCTATTACACAGTAACCTCTTCCTTTTC) from pUNO1-hSTING-WT. Purified PCR products were cleaved with restriction enzymes BamHI and NotI and cloned into the pSUMO vector linearized with the identical enzymes. Plasmid pSUMO was created by introducing 8-His-SUMO sequence between NdeI and BamHI sites of pHis-parallel2 plasmid. pSUMO- *wt* STING thus encoded truncated human *wt* STING (amino acid residues 140-343) with N-terminal 8xHis and SUMO tag. The recombinant *wt* STING protein was overexpressed in Rosetta-gami B (DE3) competent cells. Bacterial pellets were re-suspended in ice-cold lysis buffer containing 50 mM Tris-Cl pH 8.0, 300 mM NaCl, 3 mM β -mercaptoethanol, 10% (v/v) glycerol, and 20 mM imidazole using Dounce homogenizer. DNase I and RNase A were added (final concentration 50 μ g/ml) together with MgCl₂ (final concentration 5 mM) to the homogenate and bacteria were lysed using French Press G-M™ High-Pressure Cell Press Homogenizer (1500 psi, 3 cycles). Lysates were spun 30,000 g for 20 min and supernatant was gently stirred with Ni-NTA resin for 30 min. The resin was poured into a chromatography column, washed with 50 ml buffer A (50 mM Tris-Cl (pH 8.0), 800 mM NaCl, 3 mM β -mercaptoethanol; 10% glycerol; 20 mM imidazole) and 8-His-SUMO tagged STING proteins were eluted with 15 ml buffer A containing 300 mM imidazole. The eluted protein was cleaved with recombinant SUMO protease (80 μ g/ml of protein solution). STING protein

was further purified by size exclusion chromatography using HiLoad 16/60 Superdex 75 (GE Healthcare Bio-Sciences, Pittsburgh, USA) in 50 mM Tris-Cl buffer pH 7.4 containing 150 mM NaCl, and 10% glycerol. For crystallography experiment, the protein was also passed through ionex chromatography on HiTrap™ Q HP (GE Healthcare, Bio-Sciences, Pittsburgh, USA) column (Buffer A: 50 mM NaCl, 50 mM Tris-HCl pH 7.4; buffer B: 1 M NaCl, 50 mM Tris-HCl pH 7.4). Protein was concentrated with Amicon® Ultra-15 10 K device and flash frozen in liquid N₂.

Cloning of cGAS Enzymes

Exons encoding chicken cGAS were amplified using Q5 polymerase from chicken (*Gallus gallus*) genomic DNA using oligonucleotides:

pET22b_gcGAS_Rec_F (TTTAAGAAGGAGATATACATATGGAGGAGACCGCGGCGGG),
gcGAS_E1R (CTTGACGTGCTCGTAGTAGC), gcGAS_E2F
(GCTACTACGAGCACGTCAAGATATCTGAACCAAATGAGTT), gcGAS_E2R
(TTTTAATGTGTTTTAGTTCT), gcGAS_E3F
(AGAACTAAAACACATTAAAAATGTGGAAGTAACTGTGAAA), gcGAS_E3R
(CTCTTGGGAGCTTTTCTTTT), gcGAS_E4F
(AAAAGAAAAGCTCCCAAGAGGAAACACCTGGCGACTCTCT), gcGAS_E4R
(CTGCAACACTTCACTCCATC), gcGAS_E5F
(GATGGAGTGAAGTGTTGCAGGAAAGATTGTCTCAAACCTTC) and gcGAS_6His_Rec_R2
(TGGTGCTCGAGTGCGGCCGCCACCTGGTGAAATACTGGGA). All the PCR products
were pooled together, mixed with NdeI+HindIII restricted plasmid pET-22b(+) and recombined
using Gibson Assembly® Master Mix (New England Biolabs) according to the manufacturer's
instructions. Positive clones were sequenced and compared to the database. This resulted in
plasmid pET-22b(+) gcGAS.

Exons encoding human cGAS were amplified using Q5 polymerase from genomic DNA of HepG2 cells using oligonucleotides pET22b_hcGAS_Rec_F (TTTAAGAAGGAGATATACATATGCAGCCTTGGCACGAAA), hcGAS_E1R (CTTCACGTGCTCATAGTAGC), hcGAS_E2F (GCTACTATGAGCACGTGAAGATTTCTGCACCTAATGAATT), hcGAS_E2R (CTTTAATGTCGTTAATTTCT), hcGAS_E3F (AGAAATTAACGACATTAAAGATACAGATGTCATCATGAAG), hcGAS_E3R (CTTGGAACCATTTTCCTTCC), hcGAS_E4F (GGAAGGAAATGGTTTCCAAGAAGAAACATGGCG,GCTATCC), hcGAS_E4R (CTGCAACATTTCTCTTCTTT), hcGAS_E5F (AAAGAAGAGAAATGTTGCAGGAAAGATTGTTTAAAACTAA) and hcGAS_6His_Rec_R (TGGTGCTCGAGTGCGGCCGCAAATTCATCAAAAACCTGGAA). All the PCR products were pooled together, mixed with NdeI+HindIII restricted plasmid pET-22b(+) and recombined using Gibson Assembly® Master Mix (New England Biolabs) according to the manufacturer's instructions. Positive clones were sequenced and compared to the database. This resulted in plasmid pET-22b(+) hcGAS.

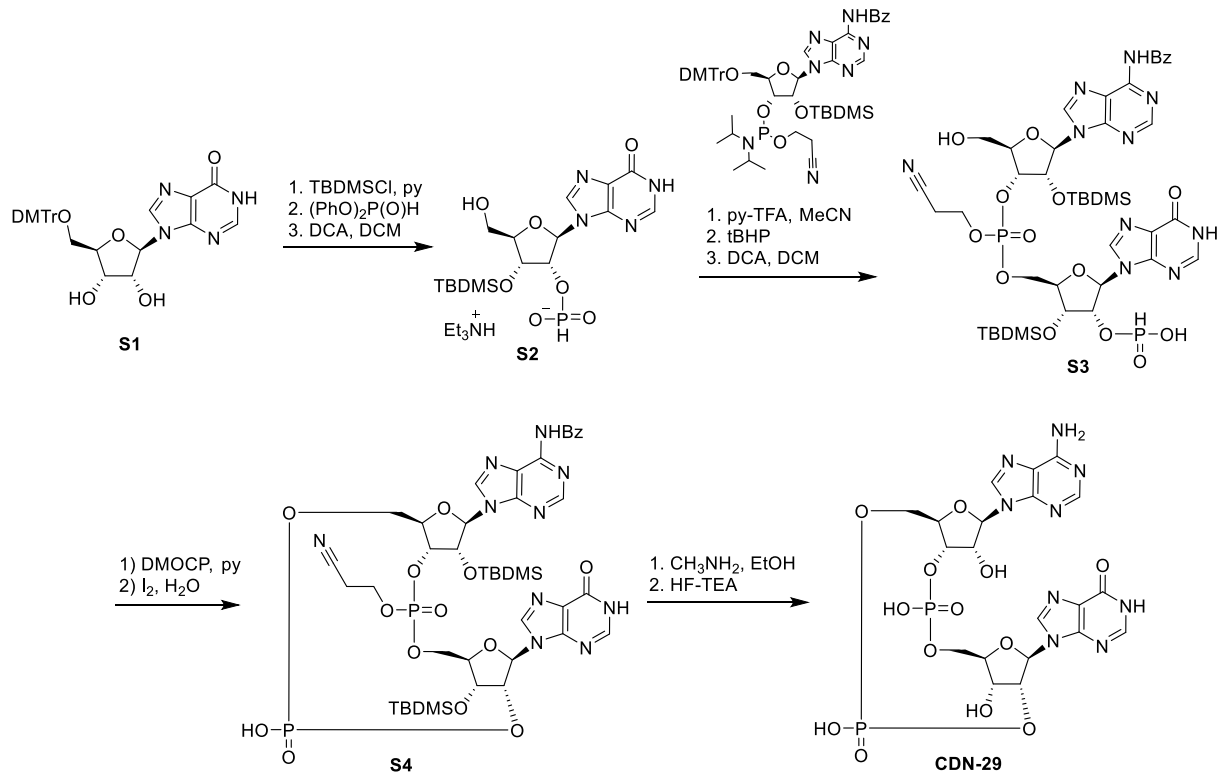
Two version of mouse cGAS cDNAs were prepared. Truncated version of *E. coli* codon optimized mouse cGAS, lacking N-terminus and encoding AA 147-507, was chemically synthesized by GenSYS company and cloned into pET-22b(+) between NdeI and XhoI sites (resulting in plasmid pET-22b(+) mcGAS-S). For full-length mouse cGAS, an additional N-terminus has been amplified from BALB/c mouse gut gDNA using Q5 polymerase and oligonucleotides mcGAS_N-term_F (TTTAAGAAGGAGATATACATATGGAAGATCCGCGTAGAAG) and mcGAS_N-term_R (ACCTTCTTCAGTTTATCCGGTTCCTTCCTGGACCCTCGCG). PCR product was

mixed with NdeI restricted plasmid pET-22b(+) mcGAS-S and recombined using Gibson Assembly® Master Mix (New England Biolabs) according to the manufacturer's instructions. Positive clones were sequenced. This resulted in plasmid pET-22b(+) mcGAS-L.

Expression and Purification of cGAS Enzymes

The protein (chicken, human or murine full-length and truncated cGAS) was overexpressed in *E. coli* BL21 (DE3) (ThermoFisher, Waltham, USA). Bacterial pellet was re-suspended in ice-cold lysis buffer containing 20 mM Phosphate Na buffer (pH 7.4), 500 mM NaCl, 10% glycerol, and 20 mM imidazole using Dounce homogenizer. DNase I and RNase A were added (final concentration 50 µg/ml) together with MgCl₂ (final concentration 5mM) to the homogenate and bacteria were lysed using MSE Soniprep 150 (3 mm Tip Solid Titanium Exponential Probe, 2 min, 50% power, amplitude 12 microns). The lysate was spun at 30,000 x g for 20 min and supernatant was loaded onto 5 mL HisTrap column (GE Healthcare BioSciences, Pittsburgh, USA). The resin was washed with 50 ml lysis buffer and protein was eluted with 15 ml 20 mM Phosphate-Na buffer (pH 7.4) buffer containing 500 mM NaCl; 10% glycerol, and 300 mM imidazole. The protein was further purified by size exclusion chromatography using HiLoad 16/60 Superdex 75 in buffer containing 150 mM NaCl; 50 mM Tris (pH 7.4), and 10% glycerol. The protein buffer was exchanged for 50% glycerol, 50 mM Tris (pH 7.6), 100 mM NaCl, 1 mM DTT, 1 mM EDTA with Amicon® Ultra-15 10 K Device (Merck Millipore Ltd.), and proteins were flash frozen in liquid N₂ and stored at -80°C for further use.

Synthesis of CDN-29



(2*R*,3*R*,4*R*,5*R*)-4-((tert-butyldimethylsilyl)oxy)-5-(hydroxymethyl)-2-(6-oxo-1,6-dihydro-9*H*-purin-9-yl)tetrahydrofuran-3-yl phosphonate, TEA⁺ salt (**S2**):

5'-DMTr inosine **S1** (Carbosynth Ltd., 2 g, 3.5 mmol) was azeotroped with pyridine (2x 10 mL), dissolved in pyridine (40 mL) and then imidazole (479 mg, 7 mmol) followed by TBDMSCl (530 mg, 3.5 mmol) were added. After stirring the reaction mixture at ambient temperature for 12 hours it was diluted with AcOEt (200 mL), washed with saturated aqueous solution of NaHCO₃ and water. Organic phase was dried over sodium sulfate and evaporated to afford a 1:1 mixture of 2' and 3' silylated products. Small amounts (< 5%) of starting material as well as double silylated compounds were also observed. Isomers were not isolated due to migration of the silyl group.

Crude mixture of silylated intermediates (3 g) was azeotroped with pyridine (2 x 15 mL), dissolved in dry pyridine (10 mL) and diphenyl phosphite (85%, 3 mL) was added in one portion. After stirring at ambient temperature for 20 min, TEA (4 mL) was added followed by water (4 mL) and the reaction was stirred for

further 20 min. Resulting solution was diluted with dichloromethane (200 mL) and washed with saturated aqueous solution of NaHCO₃ (75 mL). Water phase was extracted with dichloromethane (2 x 150 mL). Combined organic phases were dried over sodium sulfate and evaporated. Residue was adsorbed on silica and applied on a FCC column (DCM/1% Et₃N-MeOH 5 to 30%), where good separation of isomers was achieved and the desired product eluted as the second eluting isomer (1.5 g, 95% purity).

To a solution of this intermediate in dichloromethane (10 mL) was added water (360 µL, 20 mmol) followed by a solution of DCA (1.5 mL, 18 mmol) in dichloromethane (4 mL). Reaction mixture was stirred at ambient temperature for 30 min, after which it was quenched with triethylsilane (3.6 mL). Reaction mixture was then stirred for 1 hour and then pyridine (4 mL) was added and all volatiles were evaporated. Purification on reverse phase FCC (ACN in water, 0-30%) afforded **S2** (600 mg, 31% over 3 steps).

¹H NMR (401 MHz, DMSO-*d*₆) δ 8.33 (s, 1H), 8.02 (s, 1H), 6.49 (d, *J* = 591.1 Hz, 1H), 5.96 (d, *J* = 6.6 Hz, 1H), 5.24 (bs, 1H), 5.05 (ddd, *J* = 11.0, 6.6, 4.7 Hz, 1H), 4.42 (dd, *J* = 4.7, 2.6 Hz, 1H), 3.94 (td, *J* = 4.1, 2.5 Hz, 1H), 3.63 (dd, *J* = 11.9, 4.5 Hz, 1H), 3.52 (dd, *J* = 11.9, 3.9 Hz, 1H), 0.90 (s, 9H), 0.13 and 0.13 (s, 3H).

¹³C NMR (101 MHz, DMSO) δ 156.82, 148.62, 146.18, 139.19, 124.53, 86.80, 86.08 (d, *J* = 5.0 Hz), 74.83 (d, *J* = 4.2 Hz), 72.32 (d, *J* = 2.5 Hz), 61.28, 45.40, 26.06, 18.21, 8.57, -4.17, -4.81.

³¹P NMR (162 MHz, DMSO) δ 2.08.

(1R,6R,8R,9R,10S,15R,17R,18R)-8-(6-amino-9H-purin-9-yl)-3,9,12,18-tetrahydroxy-17-(6-oxo-6,9-dihydro-1H-purin-9-yl)-2,4,7,11,13,16-hexaoxa-3λ⁵,12λ⁵-diphosphatricyclo[13.2.1.0^{6,10}]octadecane-3,12-dione (**CDN-29**)

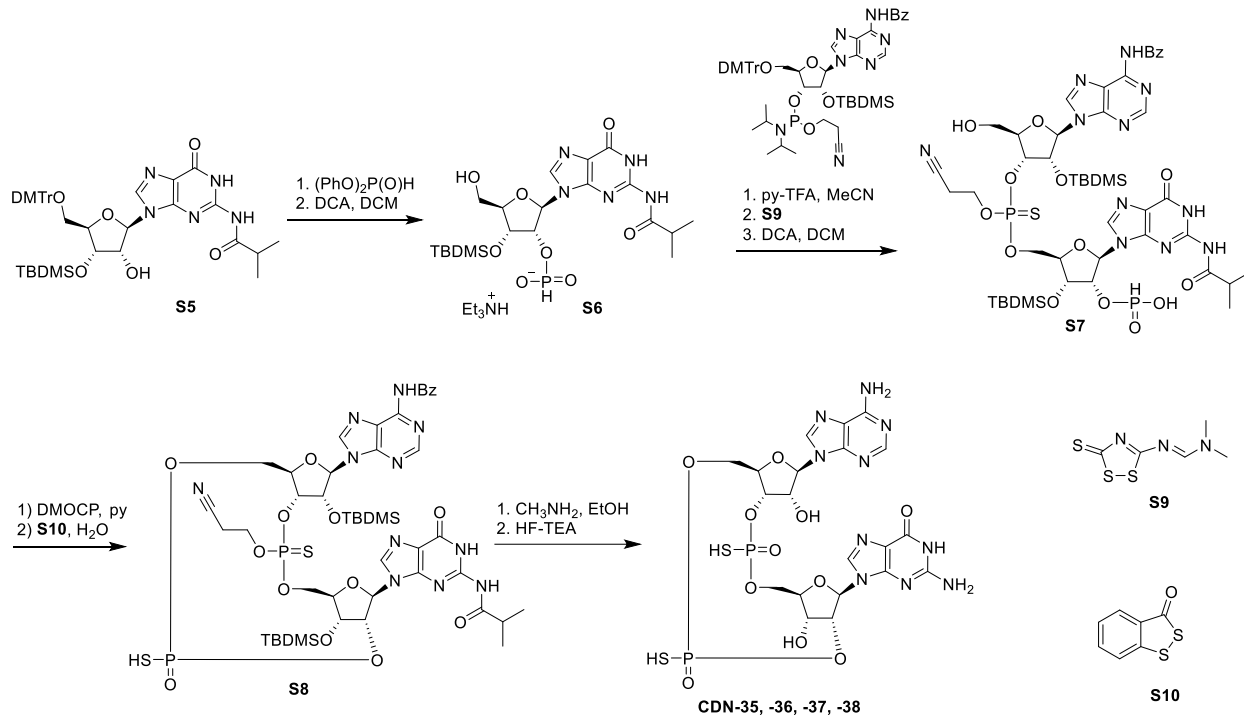
A mixture of **S2** (55 mg, 0.1 mmol) and pyridinium trifluoroacetate (py-TFA, 29 mg, 0.15 mmol) was codistilled with dry MeCN (3 x 3 mL), suspended in dry MeCN (2 mL) and stirred overnight in a sealed vessel over activated molecular sieves. In a separate flask, *N*-benzoyl-5'-*O*-[bis(4-

methoxyphenyl)phenylmethyl]-2'-O-[(1,1-dimethylethyl)dimethylsilyl]-adenosine (124 mg, 0.125 mmol, Sigma-Aldrich) was codistilled with dry MeCN (3x 3 mL), dissolved in dry MeCN (1 mL) and stirred overnight in a sealed vessel over activated molecular sieves. A solution of phosphoramidite was transferred *via syringe* to the flask with the suspension of **S2** with py-TFA and the resulting solution was stirred for 1 hour at ambient temperature. tert-Butyl hydroperoxide (tBHP, 5.5M solution in decane, 55 μ L, 0.3 mmol) was added and the reaction mixture was stirred for further 30 min. Reaction mixture was quenched with NaHSO₃ (39% soln. in water, 54 μ L, 0.27 mmol), filtered and evaporated. To a solution of the crude tritylated linear dimer in DCM (3 mL) was added water (18 μ L, 1 mmol) and a solution of DCA (74 μ L, 0.9 mmol) in DCM (3 mL) dropwise. After stirring the reaction mixture for 30 min, triethylsilane (1.5 mL) was added and the reaction mixture was stirred for further 90 min, after which it was quenched with pyridine (1.5 mL). Volatiles were evaporated and crude **3** was co-distilled with dry pyridine (3x 3 mL) and used in the next reaction without further purification.

To a solution of crude **S3** in pyridine (2 mL) was added DMOCP (65 mg, 0.35 mmol) and reaction mixture was stirred at ambient temperature for 1 hour. Water (59 μ L, 0.35 mmol) was added followed by iodine (34 mg, 0.13 mmol) and reaction mixture was stirred for 10 min, after which it was cooled down to 0 °C and quenched by the addition of NaHSO₃ (39% soln. in water, 49 μ L, 0.25 mmol). Purification on reverse phase FCC (MeCN in 50 mM aqueous NH₄HCO₃ 0-70%) afforded **S4**.

A solution of **S4** in CH₃NH₂ (33% in ethanol, 1 mL) was stirred at ambient temperature for 3 hours. Volatiles were evaporated and the residue was co-distilled with pyridine (3x 2 mL), dissolved in pyridine-TEA (1:1, v/v, 1 mL) and HF-TEA (160 μ L, 1 mmol) was added dropwise. Reaction mixture was stirred at 50 °C for 1 hour, quenched with 1M ammonium acetate (2 mL) and purified on preparative HPLC (ACN in 0.1M TEAB, 0-30%). Appropriate fractions were pooled, evaporated, co-distilled with water (3x 5 mL) and methanol (3x 5 mL), dissolved in water (10 mL) and slowly passed through a 2 mL column of Dowex 50 (Na⁺ cycle). Freeze-drying the eluent afforded the sodium salt **CDN-29**. NMR and HRMS data can be found in Table S3 and Table S5.

Synthesis of CDN-35, -36, -37



(2*R*,3*R*,4*R*,5*R*)-4-((tert-butyldimethylsilyl)oxy)-5-(hydroxymethyl)-2-(2-isobutyramido-6-oxo-1,6-dihydro-9*H*-purin-9-yl)tetrahydrofuran-3-yl phosphonate TEA^+ salt (**S6**):

Protected guanosine **S5** (ChemGenes, 2 g, 2.6 mmol) was azeotroped with pyridine (2x 10 mL), dissolved in dry pyridine (10 mL) and diphenyl phosphite (85%, 1.8 mL) was added in one portion. After stirring at ambient temperature for 20 min, TEA (3 mL) was added followed by water (3 mL) and the reaction was stirred for further 20 min. Resulting solution was diluted with dichloromethane (150 mL) and washed with saturated aqueous solution of NaHCO_3 (60 mL). Water phase was extracted with dichloromethane (2 x 150 mL). Combined organic phases were dried over sodium sulfate and evaporated.

To a solution of this intermediate in dichloromethane (10 mL) was added water (468 μL , 26 mmol) followed by a solution of DCA (2 mL, 24 mmol) in dichloromethane (10 mL). Reaction mixture was stirred at ambient temperature for 30 min, after which it was quenched with triethylsilane (3.6 mL). Reaction mixture was then stirred for 1 hour and then pyridine (4 mL) was added and all volatiles were evaporated. Purification on reverse phase FCC (ACN in water, 0-30%) afforded **S6** (1.2 g, 71% over 2 steps).

¹H NMR (501 MHz, DMSO-*d*₆) δ 12.10 (s, 1H), 11.78 (s, 1H), 10.69 (bs, 1H), 8.29 (s, 1H), 6.55 (d, *J* = 595.7 Hz, 1H), 5.88 (d, *J* = 7.4 Hz, 1H), 5.30 (s, 1H), 5.12-5.02 (m, 1H), 4.36 (dd, *J* = 4.8 Hz, *J* = 1.6 Hz, 1H), 3.97-3.88 (m, 1H), 3.55 (ddt, 1H), 2.91 (q, *J* = 7.3 Hz, 6H), 2.76 (hept, *J* = 6.9 Hz, 1H), 1.11 (dd, *J* = 7.3 Hz), 1.07 (t, *J* = 7.3 Hz, 9H), 0.89 (s, 9H), 0.13 and 0.13 (s, 3H).

¹³C NMR (101 MHz, DMSO) δ 180.37, 155.00, 149.39, 148.41, 138.06, 120.16, 87.26, 84.72 (d, *J* = 5.1 Hz), 74.61 (d, *J* = 4.1 Hz), 72.63, 61.25, 45.36, 34.92, 26.00, 19.08, 18.18, 8.50, -4.26, -4.88.

³¹P NMR (202.4 MHz, DMSO-*d*₆) δ 2.17 (s).

(1R,6R,8R,9R,10S,15R,17R,18R)-17-(2-amino-6-oxo-6,9-dihydro-1H-purin-9-yl)-8-(6-amino-9H-purin-9-yl)-9,18-dihydroxy-3,12-disulfanyl-2,4,7,11,13,16-hexaoxa-3λ⁵,12λ⁵-diphosphatricyclo[13.2.1.0^{6,10}]octadecane-3,12-dione (**CDN-35**, **-36**, **-37**)

A mixture of **S6** (55 mg, 0.1 mmol) and pyridinium trifluoroacetate (29 mg, 0.15 mmol) was codistilled with dry MeCN (3x 3 mL), suspended in dry MeCN (1 mL) and stirred overnight in a sealed vessel over activated molecular sieves. In a separate flask, *N*-benzoyl-5'-*O*-[bis(4-methoxyphenyl)phenylmethyl]-2'-*O*-[(1,1-dimethylethyl)dimethylsilyl]-adenosine (123 mg, 0.125 mmol, CAS # 136834-22-5, purchased from Sigma-Aldrich) was codistilled with dry MeCN (3x 3 mL), dissolved in dry MeCN (1 mL) and stirred overnight in a sealed vessel over activated molecular sieves. A solution of the commercial phosphoramidite was transferred *via syringe* to the flask with the suspension of **S6** with py-TFA and the resulting solution was stirred for 1 hour at ambient temperature. 3-((*N,N*-dimethylaminomethylidene)amino)-3H-1,2,4-dithiazole-5-thione (23 mg, 0.11 mmol) was added and the reaction mixture was stirred for further 30 min. Volatiles were evaporated, residue was dissolved in DCM (3 mL) and water (18 mL, 1 mmol) was added followed by a solution of DCA (74 mL, 0.9 mmol) in DCM (3 mL). After stirring the reaction mixture for 30 min, triethylsilane (1.5 mL) was added and the reaction mixture was stirred for further 90 min, after which it was quenched by the addition of pyridine (1.5 mL). Volatiles were evaporated and crude **S7** was codistilled with dry pyridine (3x 3 mL) and used in the next reaction without further purification.

To a solution of crude **S7** in pyridine (2 mL) was added DMOCP (65 mg, 0.35 mmol) and reaction mixture was stirred at ambient temperature for 1 hour. Water (18 mL, 1 mmol) was added followed by 3*H*-1,2-benzodithiol-3-one (25 mg, 0.15 mmol) and reaction mixture was stirred for 10 min. Volatiles were evaporated and product was isolated on reverse phase FCC (MeCN in 50 mM aqueous NH₄HCO₃ 0-70%) to afford **S8** as a mixture of diastereomers.

A solution of **S8** (23 mg) in CH₃NH₂ (33% in ethanol, 1 mL) was stirred at ambient temperature for 3 hours. Volatiles were evaporated and the residue was codistilled with pyridine (3x 2 mL), dissolved in pyridine-TEA (1:1, v/v, 1 mL) and HF-TEA (160 μL, 1 mmol) was added dropwise. Reaction mixture was stirred at 50 °C for 1 hour, quenched with 1M ammonium acetate (2 mL) and purified on preparative HPLC (C18, ACN in 0.1M TEAB, 0-30%). Appropriate fractions were pooled, evaporated, codistilled with water (3x 5 mL) and methanol (3x 5 mL) to afford diastereoisomers **CDN-35**, **-36** and **-37** in approx. 2:3:3:1 ratio, in order of their elution from HPLC, as triethylammonium salts. NMR and HRMS data can be found in Table S4 and Table S5.

Molecular docking.

We used the DOCK6 algorithm to perform computational docking for selected set of 17 CDN ligands to the wt-STING target protein (4KSY.pdb code). To establish the accuracy of the DOCK algorithm for this system, the native ligand in 4KSY.pdb X-ray structure was re-docked to crystal structures with RMSD = 1.1 Å for docked ligand conformation/native conformation. The DOCK 6.6 calculations² were set up within CHIMERA graphical interface³ according to protocol recommended in official documents of DOCK (including protonation states, molecular surface generation, charge calculation at AM1-BCC level). Rigid ligand docking with optimization and flexible ligand “anchor and grow” was tested with grid scoring of 50 conformers in each run. The ten best scored poses for each complex were further rescored using the amber score.

Details of QM/MM Calculations.

In our QM/MM approach, the protein and solvent are split into three subsystems: The QM region (system 1) contains most of the atoms relevant for the chemical process under consideration (i.e., the active site and its nearest vicinity) and is relaxed by QM/MM forces. System 2 consists of all residues within 2.5 Å of any atom in system 1 and is relaxed by full MM minimization in each step of the QM/MM geometry optimization. Finally, system 3 contains the remaining part of the protein and surrounding solvent molecules and is kept fixed at the original (crystallographic) coordinates. When there is a bond spanning the boundary of systems 1 and 2 (a junction), the quantum region is reduced to hydrogen atoms (the hydrogen link approach). The total energy is calculated as

$$E_{\text{tot}} = E_{\text{QM}} + E_{\text{MM},123} - E_{\text{MM},1}$$

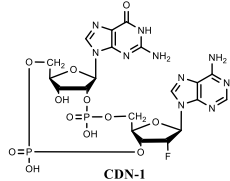
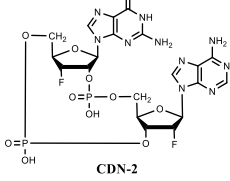
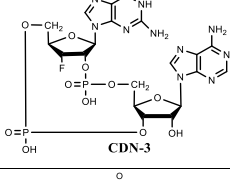
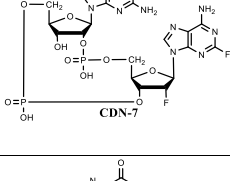
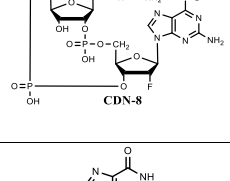
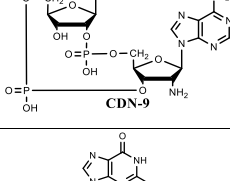
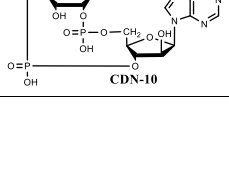
E_{QM} is the QM energy of the quantum system truncated by hydrogen atoms in the field of the surrounding point charges, but excluding the self-energy of the point charges. $E_{\text{MM},1}$ is the MM energy of the quantum system, still truncated by hydrogen atoms but without any electrostatic interactions. Finally, $E_{\text{MM},123}$ is the MM energy of all atoms in the system with original atoms at the junctions and with the charges of the quantum system set to zero (to avoid double counting of the electrostatic interactions). The actual charges used for all atoms can be found in the sample PDB file in the Supporting Information (last column).

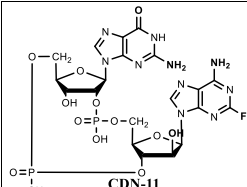
In the quantum chemical calculations, the QM system is represented by a wave function, whereas all of the other atoms are represented by an array of partial point charges, one per atom, taken from Amber libraries (see SI for the actual charges used). Thereby, the polarization of the quantum

chemical system by the surroundings is included in a self-consistent manner. In the MM calculations for the QM/MM forces and energies, all atoms are represented by the Amber force field. When there is a bond spanning the boundary of systems 1 and 2 (a junction), the quantum region is reduced to hydrogen atoms, the positions of which are linearly related to the corresponding carbon atoms in the full system (the hydrogen link approach). In order to avoid over- polarization of the quantum system, point charges on the atoms in the MM region bound to the junction atoms are omitted, and the remaining charges on the truncated amino acid are adjusted to keep the fragment neutral.

3. SUPPLEMENTAL DATA

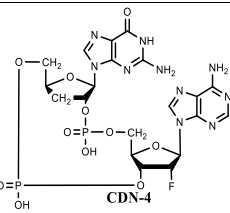
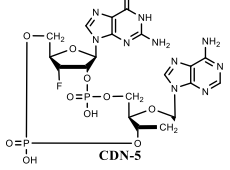
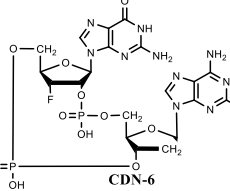
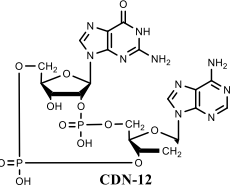
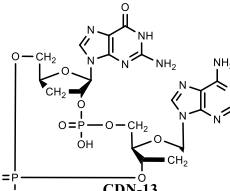
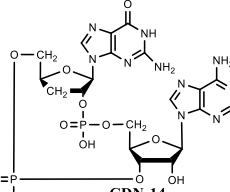
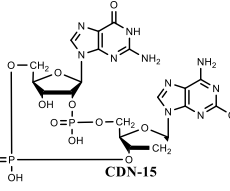
Table S1a. The ^1H and ^{31}P NMR data of 2',3'-CDNs **1 – 3** and **7 – 11** in D_2O .*

Structure	Res.	H-1'	H-2'	H-3'	H-4'	H-5'a	H-5'b	Base	^{31}P	^{19}F
 CDN-1	G	5.98 d 1,2 = 8.3	5.58 td 2,1 = 8.3 2,3 = 4.3 2,P = 8.4	4.60 d 3,2 = 4.3 3,4 = 0	4.395 q 4,3 = 0 4,5a = 2.6 4,5b = 2.8 4,P = 3.0	4.27 ddd 5a,4 = 2.6 5a,5b = 12.0 5a,P = 4.5	4.25 ddd 5b,4 = 2.8 5b,5a = 12.0 5b,P = 6.3	H-8: 7.86 s	1.18 0.40	--
	A	6.47 d 1,2 = 0 1,F = 15.1	5.60 dd 2,1 = 0 2,3 = 3.7 2,F = 51.5	5.16 dddd 3,2 = 3.7 3,4 = 9.2 3,F = 23.2 3,P = 5.6	4.55 dm 4,3 = 9.2	4.45 ddd 5a,4 = 2.5 5a,5b = 12.1 5a,P = 1.0	4.155 ddd 5b,4 = 1.3 5b,5a = 12.1 5b,P = 3.7	H-2: 8.26 s H-8: 8.24 s		-199.06
 CDN-2	G	6.04 d 1,2 = 8.6	5.73 dddd 2,1 = 8.6 2,3 = 3.4 2,P = 7.2 2,F = 26.7	5.44 dd 3,2 = 3.4 3,4 = 0 3,F = 53.4	4.70 overlap	4.33 m	4.17 dtd 5b,4 = 2.5 5b,5a = 11.8 5b,P = 2.7 5b,F = 1.6	H-8: 7.86 s	-0.57 -1.61	-196.28 -199.80
	A	6.46 d 1,2 = 0 1,F = 13.7	5.55 dd 2,1 = 0 2,3 = 3.5 2,F = 51.5	5.11 dddd 3,2 = 3.5 3,4 = 9.5 3,F = 24.0 3,P = 6.5	4.57 dm 4,3 = 9.5	4.52 ddd 5a,4 = 2.3 5a,5b = 12.2 5a,P = 1	4.21 ddd 5b,4 = 1.2 5b,5a = 12.2 5b,P = 3.0	H-2: 8.28 s H-8: 8.25 s		
 CDN-3	G	6.03 d 1,2 = 8.8	5.73 dddd 2,1 = 8.8 2,3 = 3.4 2,P = 6.8 2,F = 37.0	5.45 dd 3,2 = 3.4 3,4 = 0 3,F = 53.3	4.70 dtd 4,3 = 0 4,5a = 3.5 4,5b = 1.5 4,P = 3.4	4.31 ddd 5a,4 = 3.5 5a,5b = 11.7 5a,P = 3.9	4.13 ddd 5b,4 = 1.5 5b,5a = 11.7 5b,P = 2.4 5b,F = 2.3	H-8: 7.86 s	-0.42 -1.63	--
	A	6.18 s 1,2 = 0	4.79 d 2,1 = 0 2,3 = 4.1	5.04 ddd 3,2 = 4.1 3,4 = 9.3 3,P = 7.1	4.50 m	4.515 m	4.20 m	H-2: 8.28 s H-8: 8.29 s		--
 CDN-7	G	5.96 d 1,2 = 8.4	5.66 td 2,1 = 8.4 2,3 = 4.3 2,P = 8.2	4.585 d 3,2 = 4.3 3,4 = 0	4.40 q 4,3 = 0 4,5a = 2.8 4,5b = 2.3 4,P = 3.1	4.26 ddd 5a,4 = 2.8 5a,5b = 11.8 5a,P = 5.7	4.23 ddd 5b,4 = 2.3 5b,5a = 11.8 5b,P = 3.7	H-8: 7.86 s	-0.34 -1.13	-- 2F: -49.22 2'F: -199.52
	2-F-A	6.375 d 1,2 = 0 1,F = 14.0	5.52 ddd 2,1 = 0 2,3 = 3.4 2,F = 51.1	5.13 dddd 3,2 = 3.4 3,4 = 9.6 3,F = 23.9 3,P = 5.8	4.55 dm 4,3 = 9.6	4.47 bdd 5a,4 = 2.3 5a,5b = 12.1 5a,P < 1	4.14 ddd 5b,4 = 1.1 5b,5a = 12.1 5b,P = 3.4	H-8: 8.21 s		
 CDN-8	G	5.96 d 1,2 = 8.4	5.66 td 2,1 = 8.6 2,3 = 4.3 2,P = 8.0	4.59 d 3,2 = 4.3 3,4 = 0	4.40 q 4,3 = 0 4,5a = 2.8 4,5b = 2.8 4,P = 2.8	4.32 m	4.28 m	H-8: 7.88 s	-1.13 -0.40	-- -199.15
	DAP	6.29 d 1,2 = 0 1,F = 14.5	5.51 dd 2,1 = 0 2,3 = 3.5 2,F = 51.3	5.14 dddd 3,2 = 3.5 3,4 = 9.5 3,F = 24.0 3,P = 5.7	4.51 dm 4,3 = 9.5	4.45 bdd 5a,4 = 2.3 5a,5b = 11.7 5a,P ~ 1.0	4.13 ddd 5b,4 = 1.2 5b,5a = 11.7 5b,P = 3.5	H-8: 7.96 s		
 CDN-9	G	5.96 d 1,2 = 8.5	5.60 ddd 2,1 = 8.5 2,3 = 4.1 2,P = 8.0	4.60 d 3,2 = 4.1 3,4 = 0	4.415 ddd 4,3 = 0 4,5a = 2.8 4,5b = 1.8 4,P = 3.4	4.26 dd 5a,4 = 2.8 5a,5b = 11.8 5a,P = 5.1	4.165 ddd 5b,4 = 1.8 5b,5a = 11.8 5b,P = 2.4	H-8: 7.91 s	-0.18 -1.00	-- --
	A	6.11 d 1,2 = 1.6	4.16 dd 2,1 = 1.6 2,3 = 5.2	5.11 ddd 3,2 = 5.2 3,4 = 8.2 3,P = 7.0	4.51 dddd 4,3 = 8.2 4,5a = 3.0 4,5b = 1.5 4,P = 3.4	4.43 dt 5a,4 = 3.0 5a,5b = 12.0 5a,P = 3.2	4.13 ddd 5b,4 = 1.5 5b,5a = 12.0 5b,P = 3.5	H-2: 8.28 s H-8: 8.36 s		
 CDN-10	G	6.04 d 1,2 = 8.5	5.465 td 2,1 = 8.5 2,3 = 4.4 2,P = 8.5	4.97 d 3,2 = 4.4 3,4 = 0	4.41 q 4,3 = 0 4,5a = 2.2 4,5b = 2.1 4,P = 2.7	4.305 ddd 5a,5b = 12.2 5a,4 = 2.2 5a,P = 7.8	4.20 ddd 5b,5a = 12.2 5b,4 = 2.1 5b,P = 3.7	H-8: 8.03 s	0.72 0.00	-- --
	A	6.50 d 1,2 = 5.0	4.78 overlap	4.97 dt 3,2 = 6.1 3,4 = 4.8 3,P = 5.0	4.39 ddd 4,3 = 4.8 4,5a = 6.0 4,5b = 3.0	4.275 dt 5a,5b = 11.3 5a,4 = 6.0 5a,P = 6.0	4.15 ddd 5b,5a = 11.3 5b,4 = 3.0 5b,P = 5.0	H-2: 8.24 s H-8: 8.37 s		

	G	6.03 d <i>1,2 = 8.5</i>	5.47 td <i>2,1 = 8.5</i> <i>2,3 = 4.4</i> <i>2,P = 9.0</i>	4.56 d <i>3,2 = 4.4</i> <i>3,4 = 0</i>	4.38 q <i>4,3 = 0</i> <i>4,5a = 2.2</i> <i>4,5b = 2.2</i> <i>4,P = 2.8</i>	4.30 ddd <i>5a,4 = 2.2</i> <i>5a,5b = 12.1</i> <i>5a,P = 7.7</i>	4.20 ddd <i>5b,4 = 2.2</i> <i>5b,5a = 12.1</i> <i>5b,P = 3.9</i>	H-8: 8.02 s	-0.10 0.79	---
	2-F-A	6.37 d <i>1,2 = 5.1</i>	4.78 overlap	4.96 dt <i>3,2 = 5.0</i> <i>3,4 = 5.4</i> <i>3,P = 6.2</i>	4.37 tdd <i>4,3 = 5.4</i> <i>4,5a = 5.6</i> <i>4,5b = 2.8</i> <i>4,P = 1.5</i>	4.27 dt <i>5a,4 = 5.4</i> <i>5a,5b = 11.3</i> <i>5a,P = 5.8</i>	4.14 ddd <i>5b,4 = 2.8</i> <i>5b,5a = 11.3</i> <i>5b,P = 4.8</i>	H-8: 8.29 s		-49..79

* Coupling constants are written in italics in a shortened form (e.g. instead $J(I',2') = 8.6 \text{ Hz}$ we type simply $1,2 = 8.6$).

Table S1b. The ^1H and ^{31}P NMR data of 3'- or 2'-deoxy- 2',3'-CDNs **4 – 6** and **12 – 15** in D_2O .*

Structure	Res.	H-1'	H-2'a	H-2'b	H-3a'	H-3b'	H-4'	H-5'a	H-5'b	Base	^{31}P	^{19}F
 CDN-4	G	5.83 d <i>1,2 = 6.4</i>	--	5.56 dq <i>2,1 = 6.4</i> <i>2,3a = 9.0</i> <i>2,3b = 6.5</i> <i>2,P = 5.2</i>	2.62 dt <i>3a,2 = 9.0</i> <i>3a,3b = 13.0</i> <i>3a,4 = 8.6</i>	2.49 ddd <i>3b,2 = 6.5</i> <i>3b,3a = 13.0</i> <i>3b,4 = 3.4</i>	4.60 m	4.35 m	4.16 m	H-8: 7.84 s	-0.32 -1.14	--
	A	6.47 d <i>1,2 = 0</i> <i>1,F = 14.4</i>	--	5.61 dd <i>2,1 = 0</i> <i>2,3 = 3.5</i> <i>2,F = 51.0</i>	--	5.105 dddd <i>3,2 = 3.5</i> <i>3,4 = 9.5</i> <i>3,F = 23.8</i> <i>3,P = 5.2</i>	4.56 dm <i>4,3 = 9.5</i>	4.49 dd <i>5a,4 = 2.3</i> <i>5a,5b = 12.2</i> <i>5a,P < 1</i>	4.22 ddd <i>5b,4 = 1.1</i> <i>5b,5a = 12.2</i> <i>5b,P = 3.4</i>	H-2: 8.28 s H-8: 8.32 s	--	-199.33
 CDN-5	G	6.05 d <i>1,2 = 8.6</i>	--	5.69 dddd <i>2,1 = 8.6</i> <i>2,3 = 3.4</i> <i>2,P = 7.7</i> <i>2,F = 26.8</i>	--	5.44 dd <i>3,2 = 3.4</i> <i>3,4 = 0</i> <i>3,F = 53.3</i>	4.70 overlap	4.34 dt <i>5a,4 = 3.3</i> <i>5a,5b = 11.5</i> <i>5a,P = 3.3</i>	4.16 ddd <i>5b,4 = 1.3</i> <i>5b,5a = 11.5</i> <i>5b,P = 3.5</i>	H-8: 7.91 s	-0.10 -1.10	-196.33
	A	6.45 dd <i>1,2a = 6.6</i> <i>1,2b = 2.2</i>	2.80 ddd <i>2a,1 = 6.6</i> <i>2a,2b = 13.5</i> <i>2a,3 = 9.0</i>	2.99 ddd <i>2b,1 = 2.2</i> <i>2b,2a = 13.5</i> <i>2b,3 = 6.4</i>	--	5.17 dq <i>3,2a = 9.0</i> <i>3,2b = 6.4</i> <i>3,4 = 7.0</i> <i>3,P = 6.7</i>	4.31 m	4.30 m	4.17 m	H-8: 8.28 s	--	--
 CDN-6	G	6.05 d <i>1,2 = 8.6</i>	--	5.69 dddd <i>2,1 = 8.6</i> <i>2,3 = 3.4</i> <i>2,P = 7.8</i> <i>2,F = 26.8</i>	--	5.43 dd <i>3,2 = 3.4</i> <i>3,4 = 0</i> <i>3,F = 53.5</i>	4.71 overlap	4.30 ddd <i>5a,4 = 3.2</i> <i>5a,5b = 11.6</i> <i>5a,P = 4.9</i>	4.18 dtd <i>5b,4 = 2.5</i> <i>5b,5a = 11.6</i> <i>5b,P = 2.5</i> <i>5b,F = 1.7</i>	H-8: 7.91 s	0.10 -1.08	-196.28
	2F-A	6.34 dd <i>1,2a = 6.6</i> <i>1,2b = 2.2</i>	2.775 ddd <i>2a,1 = 6.6</i> <i>2a,2b = 13.5</i> <i>2a,3 = 8.9</i>	2.96 ddd <i>2b,1 = 2.2</i> <i>2b,2a = 13.5</i> <i>2b,3 = 6.4</i>	--	5.17 dq <i>3,2a = 8.9</i> <i>3,2b = 6.4</i> <i>3,4 = 6.7</i> <i>3,P = 6.7</i>	4.30 m	4.32 dt <i>5a,4 = 3.4</i> <i>5a,5b = 11.3</i> <i>5a,P = 3.4</i>	4.15 ddd <i>5b,4 = 1.1</i> <i>5b,5a = 11.3</i> <i>5b,P = 3.6</i>	H-8: 8.20 s	--	-49.68
 CDN-12	G	5.98 d <i>1,2 = 8.4</i>	--	5.59 td <i>2,1 = 8.4</i> <i>2,3 = 4.2</i> <i>2,P = 8.2</i>	--	4.595 d <i>3,2 = 4.2</i> <i>3,4 = 0</i>	4.42 ddd <i>4,3 = 0</i>	4.235 ddd <i>5a,4 = 2.8</i> <i>5a,5b = 11.8</i> <i>5a,P = 5.3</i>	4.21 ddd <i>5b,4 = 2.2</i> <i>5b,5a = 11.8</i> <i>5b,P = 3.5</i>	H-8: 7.93 s	0.28 -0.59	--
	A	6.465 dd <i>1,2a = 6.6</i> <i>1,2b = 2.7</i>	2.81 ddd <i>2a,1 = 6.6</i> <i>2a,2b = 13.6</i> <i>2a,3 = 8.6</i>	3.00 ddd <i>2b,1 = 2.7</i> <i>2b,2a = 13.6</i> <i>2b,3 = 6.3</i>	--	5.19 dq <i>3,2a = 8.6</i> <i>3,2b = 6.3</i> <i>3,4 = 6.5</i> <i>3,P = 6.5</i>	4.32 dddd <i>4,3 = 6.5</i> <i>4,5a = 4.0</i> <i>4,5b = 1.5</i> <i>4,P = 2.5</i>	4.295 ddd <i>5a,4 = 4.0</i> <i>5a,5b = 11.5</i> <i>5a,P = 3.2</i>	4.105 ddd <i>5b,4 = 1.5</i> <i>5b,5a = 11.5</i> <i>5b,P = 4.0</i>	H-2: 8.25 s H-8: 8.30 s	--	--
 CDN-13	G	5.875 d <i>1,2 = 5.8</i>	--	5.46 dq <i>2,1 = 5.8</i> <i>2,3a = 8.1</i> <i>2,3b = 6.2</i> <i>2,P = 5.6</i>	2.65 dt <i>3a,2 = 8.1</i> <i>3a,3b = 13.1</i> <i>3a,4 = 8.2</i>	2.48 ddd <i>3b,2 = 6.2</i> <i>3b,3a = 13.1</i> <i>3b,4 = 4.0</i>	4.61 m	4.19 ddd <i>5a,4 = 3.2</i> <i>5a,5b = 11.6</i> <i>5a,P = 7.7</i>	4.11 ddd <i>5b,4 = 4.2</i> <i>5b,5a = 11.6</i> <i>5b,P = 7.8</i>	H-8: 7.90 s	0.29 -0.75	--
	A	6.475 dd <i>1,2a = 6.7</i> <i>1,2b = 3.0</i>	2.83 ddd <i>2a,1 = 6.7</i> <i>2a,2b = 13.8</i> <i>2a,3 = 8.1</i>	3.04 ddd <i>2b,1 = 3.0</i> <i>2b,2a = 13.8</i> <i>2b,3 = 6.4</i>	--	5.14 dq <i>3,2a = 8.1</i> <i>3,2b = 6.4</i> <i>3,4 = 6.0</i> <i>3,P = 6.0</i>	4.33 m	4.27 dt <i>5a,4 = 3.9</i> <i>5a,5b = 11.8</i> <i>5a,P = 3.9</i>	4.18 ddd <i>5b,4 = 1.9</i> <i>5b,5a = 11.8</i> <i>5b,P = 4.4</i>	H-2: 8.25 s H-8: 8.34 s	--	--
 CDN-14	G	5.80 d <i>1,2 = 6.8</i>	--	5.58 dtd <i>2,1 = 6.8</i> <i>2,3a = 9.7</i> <i>2,3b = 6.6</i> <i>2,P = 5.9</i>	2.59 ddd <i>3a,2 = 9.7</i> <i>3a,3b = 12.7</i> <i>3a,4 = 8.5</i>	2.51 ddd <i>3b,2 = 6.6</i> <i>3b,3a = 12.7</i> <i>3b,4 = 2.9</i>	4.60 dq <i>4,3a = 8.5</i> <i>4,3b = 2.9</i> <i>4,5a = 3.0</i> <i>4,5b = 2.9</i>	4.14 ddd <i>5a,4 = 3.0</i> <i>5a,5b = 11.6</i> <i>5a,P = 6.3</i>	4.10 ddd <i>5b,4 = 2.9</i> <i>5b,5a = 11.6</i> <i>5b,P = 6.5</i>	H-8: 7.84 s	-0.07 -1.08	--
	A	6.19 s <i>1,2 = 0</i>	--	4.84 d <i>2,1 = 0</i> <i>2,3 = 4.1</i>	--	5.04 ddd <i>3,2 = 4.1</i> <i>3,4 = 9.0</i> <i>3,P = 6.0</i>	4.50 m	4.48 m	4.215 m	H2: 8.28 s H-8: 8.36 s	--	--
 CDN-15	G	5.985 d <i>1,2 = 8.4</i>	--	5.595 td <i>2,1 = 8.4</i> <i>2,3 = 4.2</i> <i>2,P = 8.4</i>	--	4.59 d <i>3,2 = 4.2</i> <i>3,4 = 0</i>	4.42 ddd <i>4,3 = 0</i> <i>4,5a = 2.7</i> <i>4,5b = 2.2</i> <i>4,P = 3.3</i>	4.24 ddd <i>5a,4 = 2.7</i> <i>5a,5b = 11.8</i> <i>5a,P = 5.3</i>	4.21 ddd <i>5b,4 = 2.2</i> <i>5b,5a = 11.8</i> <i>5b,P = 3.5</i>	H-8: 7.94 s	0.29 -0.65	--
	2Cl-A	6.39 dd <i>1,2a = 6.6</i> <i>1,2b = 2.7</i>	2.80 ddd <i>2a,1 = 6.6</i> <i>2a,2b = 13.7</i> <i>2a,3 = 8.5</i>	2.96 ddd <i>2b,1 = 2.7</i> <i>2b,2a = 13.7</i> <i>2b,3 = 6.4</i>	--	5.19 dq <i>3,2a = 8.5</i> <i>3,2b = 6.4</i> <i>3,4 = 6.5</i> <i>3,P = 6.5</i>	4.31 m	4.29 m	4.10 m	H-8: 8.26 s	--	--

* Coupling constants are written in italics in a shortened form (e.g. instead $J(1',2') = 8.6 \text{ Hz}$ we type simply $1,2 = 8.6$).

Table S2. The ^1H and ^{31}P NMR data of 2',3'-CDNs **16 - 26** in D_2O .*

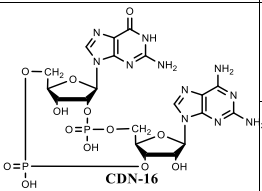
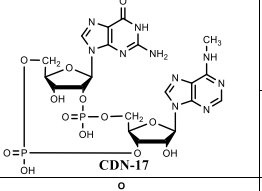
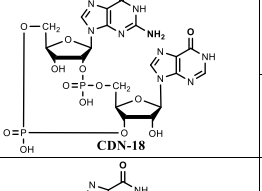
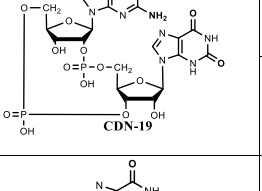
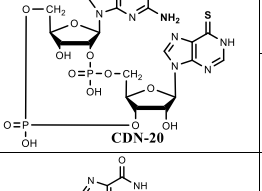
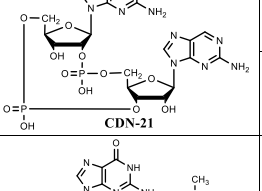
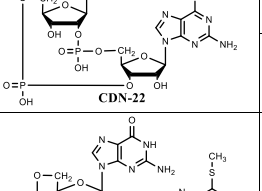
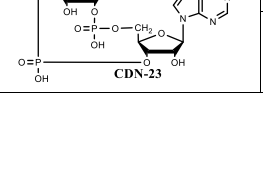
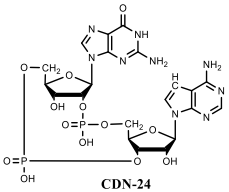
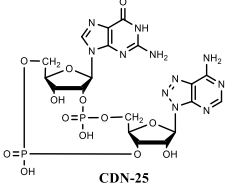
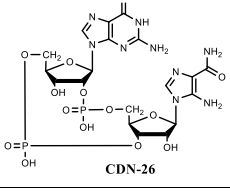
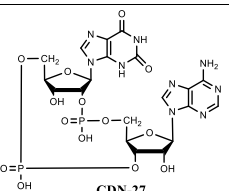
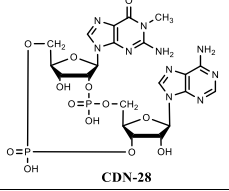
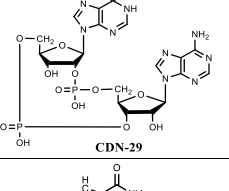
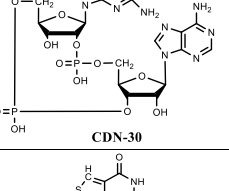
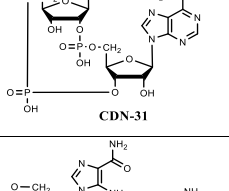
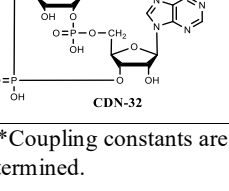
Structure	Residue	H-1'	H-2'	H-3'	H-4'	H-5'a	H-5'b	Base	^{31}P
 CDN-16	G	5.95 d $1,2 = 8.5$	5.65 ddd $2,1 = 8.5$ $2,3 = 4.2$ $2,P = 7.6$	4.60 d $3,2 = 4.2$ $3,4 = 0$	4.41 ddd $4,3 = 0$ $4,5a = 3.0$ $4,5b = 1.9$ $4,P = 3.6$	4.25 ddd $5a,5b = 11.7$ $5a,4 = 3.0$ $5a,P = 5.2$	4.18 ddd $5b,5a = 11.7$ $5b,4 = 1.9$ $5b,P = 2.5$	H-8: 7.87 s	-0.21 -1.11
	DAP	6.01 s $1,2 = 0$	4.74 d $2,1 = 0$ $2,3 = 4.1$	5.055 ddd $3,2 = 4.1$ $3,4 = 9.0$ $3,P = 6.5$	4.44 m	4.44 m	4.13 m	H-8: 7.99 s	
 CDN-17	G	5.935 d $1,2 = 8.5$	5.63 ddd $2,1 = 8.5$ $2,3 = 4.1$ $2,P = 7.5$	4.61 d $3,2 = 4.1$ $3,4 = 0$	4.41 ddd $4,3 = 0$ $4,5a = 3.2$ $4,5b = 1.7$ $4,P = 3.6$	4.24 ddd $5a,5b = 11.7$ $5a,4 = 3.2$ $5a,P = 4.8$	4.15 ddd $5b,5a = 11.7$ $5b,4 = 1.7$ $5b,P = 2.3$	H-8: 7.86 s	-0.26 -1.26
	6-Me-A	6.16 s $1,2 = 0$	4.775 overlap	5.055 ddd $3,2 = 4.1$ $3,4 = 8.9$ $3,P = 6.8$	4.49 m	4.49 m	4.16 m	H-2: 8.28 bs H-8: 8.28 bs	
 CDN-18	G	5.97 d $1,2 = 8.4$	5.66 td $2,1 = 8.4$ $2,3 = 4.2$ $2,P = 8.4$	4.59 d $3,2 = 4.2$ $3,4 = 0$	4.395 ddd $4,3 = 0$ $4,5a = 2.6$ $4,5b = 2.0$ $4,P = 3.2$	4.25 ddd $5a,4 = 2.6$ $5a,5b = 11.8$ $5a,P = 6.0$	4.18 ddd $5b,4 = 2.0$ $5b,5a = 11.8$ $5b,P = 3.2$	H-8: 7.91 s	-0.81 -0.15
	Hpx	6.195 d $1,2 = 1.0$	4.80 overlap	5.06 ddd $3,2 = 4.3$ $3,4 = 8.6$ $3,P = 6.8$	4.49 dm $4,3 = 8.6$	4.42 dt $5a,4 = 2.4$ $5a,5b = 11.8$ $5a,P = 2.0$	4.11 ddd $5b,4 = 1.3$ $5b,5a = 11.8$ $5b,P = 3.5$	H-2: 8.21 s H-8: 8.27 s	
 CDN-19	G	5.95 d $1,2 = 8.4$	5.72 td $2,1 = 8.4$ $2,3 = 4.3$ $2,P = 8.4$	4.58 d $3,2 = 4.3$ $3,4 = 0$	4.39 ddd $4,3 = 0$ $4,5a = 2.7$ $4,5b = 2.2$ $4,P = 3.2$	4.25 ddd $5a,4 = 2.7$ $5a,5b = 11.8$ $5a,P = 6.1$	4.21 ddd $5b,4 = 2.2$ $5b,5a = 11.8$ $5b,P = 3.5$	H-8: 7.87 s	-0.77 -0.15
	Xan	5.95 d $1,2 = 1.1$	4.70 dd $2,1 = 1.1$ $2,3 = 4.3$	5.04 ddd $3,2 = 4.3$ $3,4 = 8.8$ $3,P = 6.1$	4.44 dtd $4,3 = 8.6$ $4,5a = 2.7$ $4,5b = 1.3$ $4,P = 2.7$	4.38 dt $5a,4 = 2.7$ $5a,5b = 12.0$ $5a,P = 1.4$	4.08 ddd $5b,4 = 1.3$ $5b,5a = 12.0$ $5b,P = 3.6$	H-8: 7.855 s	
 CDN-20	G	5.97 d $1,2 = 8.4$	5.70 td $2,1 = 8.4$ $2,3 = 4.2$ $2,P = 8.1$	4.575 d $3,2 = 4.2$ $3,4 = 0$	4.38 ddd $4,3 = 0$ $4,5a = 2.6$ $4,5b = 2.2$ $4,P = 3.0$	4.25 ddd $5a,4 = 2.6$ $5a,5b = 12.0$ $5a,P = 6.7$	4.18 ddd $5b,4 = 2.2$ $5b,5a = 12.0$ $5b,P = 3.6$	H-8: 7.91 s	-0.61 -0.08
	6-S-Hpx	6.20 s $1,2 = 0$	4.81 overlap	5.04 ddd $3,2 = 4.3$ $3,4 = 8.5$ $3,P = 6.1$	4.50 dtd $4,3 = 8.5$ $4,5a = 2.5$ $4,5b = 1.6$ $4,P = 2.5$	4.42 dt $5a,4 = 2.5$ $5a,5b = 11.7$ $5a,P = 2.5$	4.105 ddd $5b,4 = 1.6$ $5b,5a = 11.7$ $5b,P = 3.8$	H-2: 8.34 s H-8: 8.40 s	
 CDN-21	G	5.955 d $1,2 = 8.7$	5.59 ddd $2,1 = 8.7$ $2,3 = 4.1$ $2,P = 7.5$	4.62 d $3,2 = 4.1$ $3,4 = 0$	4.42 td $4,3 = 0$ $4,5a = 3.0$ $4,5b = 1.7$ $4,P = 3.6$	4.25 ddd $5a,5b = 11.8$ $5a,4 = 3.0$ $5a,P = 5.0$	4.17 ddd $5b,5a = 11.8$ $5b,4 = 1.7$ $5b,P = 2.8$	H-8: 7.89 s	-0.18 -1.00
	2-NH2-Pur	6.11 d $1,2 = 1.0$	4.82 overlap	5.05 ddd $3,2 = 4.1$ $3,4 = 8.6$ $3,P = 6.7$	4.47 m	4.46 m	4.15 m $5b,5a = 12.2$ $5b,4 = 1.3$ $5b,P = 3.4$	H-8: 8.24 s H-6: 8.63 s	
 CDN-22	G	5.94 d $1,2 = 8.5$	5.64 ddd $2,1 = 8.5$ $2,3 = 4.1$ $2,P = 7.0$	4.60 d $3,2 = 4.1$ $3,4 = 0$	4.40 td $4,3 = 0$ $4,5a = 3.0$ $4,5b = 2.0$ $4,P = 3.3$	4.24 ddd $5a,5b = 11.8$ $5a,4 = 3.0$ $5a,P = 5.3$	4.16 ddd $5b,5a = 11.8$ $5b,4 = 2.0$ $5b,P = 2.6$	H-8: 7.87 s	-0.22 -1.10
	6-S-Me-2-NH2-Pur	6.05 s $1,2 = 0$	4.83 d $2,3 = 4.2$	5.035 ddd $3,2 = 4.2$ $3,4 = 9.1$ $3,P = 6.5$	4.47 m	4.47 m	4.16 m SM: 2.67 s	H-8: 8.18 s	
 CDN-23	G	5.94 d $1,2 = 8.6$	5.63 ddd $2,1 = 8.6$ $2,3 = 4.1$ $2,P = 6.8$	4.61 d $3,2 = 4.1$ $3,4 = 0$	4.40 ddd $4,3 = 0$ $4,5a = 3.0$ $4,5b = 1.8$ $4,P = 3.5$	4.235 ddd $5a,5b = 11.8$ $5a,4 = 3.0$ $5a,P = 5.0$	4.14 ddd $5b,5a = 11.8$ $5b,4 = 1.8$ $5b,P = 2.5$	H-8: 7.87 s	-0.24 -1.19
	6-S-Me-Pur	6.25 s $1,2 = 0$	4.89 d $2,1 = 0$ $2,3 = 4.1$	5.04 ddd $3,2 = 4.1$ $3,4 = 9.2$ $3,P = 6.6$	4.53 m	4.515 dt $5a,4 = 2.9$ $5a,5b = 10.8$ $5a,P = 2.9$	4.19 ddd $5b,4 = 1.3$ $5b,5a = 10.8$ $5b,P = 3.0$	H-2: 8.71 s H-8: 8.58 s SM: 2.76 s	

Table S2 - continued. The ^1H and ^{31}P NMR data of 2',3'-CDNs **16 - 26** in D_2O .*

Structure	Res.	H-1'	H-2'	H-3'	H-4'	H-5'a	H-5'b	Base	^{31}P
 CDN-24	G	5.97 d <i>1,2 = 8.4</i>	5.61 ddd <i>2,1 = 8.4</i> <i>2,3 = 4.1</i> <i>2,P = 7.4</i>	4.62 d <i>3,2 = 4.1</i> <i>3,4 = 0</i>	4.44 ddd <i>4,3 = 0</i> <i>4,5a = 3.1</i> <i>4,5b = 1.6</i> <i>4,P = 3.5</i>	4.27 ddd <i>5a,4 = 3.1</i> <i>5a,5b = 11.7</i> <i>5a,P = 5.0</i>	4.21 ddd <i>5b,4 = 1.6</i> <i>5b,5a = 11.7</i> <i>5b,P = 2.5</i>	H-8: 7.91 s	-0.24 -0.92
	7-deaza-A	6.29 d <i>1,2 = 1.0</i>	4.64 ddd <i>2,1 = 1.0</i> <i>2,3 = 4.2</i>	5.08 ddd <i>3,2 = 4.2</i> <i>3,4 = 8.6</i> <i>3,P = 6.6</i>	4.42 ddd <i>4,3 = 8.6</i> <i>4,5a = 2.2</i> <i>4,5b = 1.3</i>	4.40 dt <i>5a,4 = 2.2</i> <i>5a,5b = 11.6</i> <i>5a,P = 2.2</i>	4.13 ddd <i>5b,4 = 1.3</i> <i>5b,5a = 11.6</i> <i>5b,P = 3.2</i>	H-2: 8.17 s H-7: 6.24 d H-8: 7.37 d <i>8,7 = 3.7</i>	
 CDN-25	G	6.03 d <i>1,2 = 8.4</i>	5.44 ddd <i>2,1 = 8.4</i> <i>2,3 = 4.3</i> <i>2,P = 8.2</i>	4.62 d <i>3,2 = 4.3</i> <i>3,4 = 0</i>	4.445 ddd <i>4,3 = 0</i> <i>4,5a = 2.4</i> <i>4,5b = 2.0</i> <i>4,P = 3.1</i>	4.32 ddd <i>5a,4 = 2.4</i> <i>5a,5b = 12.0</i> <i>5a,P = 6.7</i>	4.255 ddd <i>5b,4 = 2.0</i> <i>5b,5a = 12.0</i> <i>5b,P = 3.6</i>	H-8: 8.06 s	-0.11 -0.40
	8-aza-A	6.42 d <i>1,2 = 1.9</i>	5.21 dd <i>2,1 = 1.9</i> <i>2,3 = 4.7</i>	5.46 ddd <i>3,2 = 4.7</i> <i>3,4 = 7.4</i> <i>3,P = 6.4</i>	4.50 dddd <i>4,3 = 7.4</i> <i>4,5a = 3.7</i> <i>4,5b = 2.3</i> <i>4,P = 2.8</i>	4.15 dt <i>5a,4 = 3.7</i> <i>5a,5b = 12.4</i> <i>5a,P = 3.8</i>	4.03 ddd <i>5b,4 = 2.3</i> <i>5b,5a = 12.4</i> <i>5b,P = 4.2</i>	H-2: 8.33 s	
 CDN-26	G	5.955 d <i>1,2 = 8.4</i>	5.65 td <i>2,1 = 8.4</i> <i>2,3 = 4.2</i> <i>2,P = 8.1</i>	4.585 d <i>3,2 = 4.2</i> <i>3,4 = 0</i>	4.42 td <i>4,3 = 0</i> <i>4,5a = 3.0</i> <i>4,5b = 1.7</i> <i>4,P = 3.3</i>	4.265 ddd <i>5a,4 = 3.0</i> <i>5a,5b = 11.7</i> <i>5a,P = 5.3</i>	4.21 ddd <i>5b,4 = 1.7</i> <i>5b,5a = 11.7</i> <i>5b,P = 2.7</i>	H-8: 7.87 s	-0.18 -1.12
	AlCA	5.77 d <i>1,2 = 0.9</i>	4.67 bd <i>2,1 = 0.9</i> <i>2,3 = 4.4</i>	5.05 ddd <i>3,2 = 4.4</i> <i>3,4 = 9.0</i> <i>3,P = 6.5</i>	4.39 m	4.38 m	4.09 m	H-8: 7.485 s	

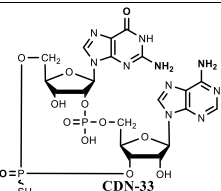
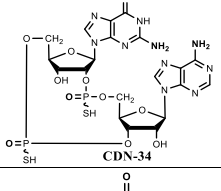
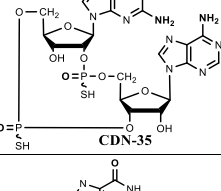
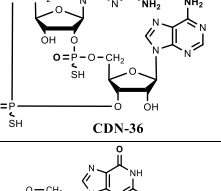
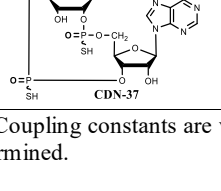
*Coupling constants are written in italics in a shortened form (e.g. instead $J(1',2') = 8.6 \text{ Hz}$ we type simply $1,2 = 8.6$).

Table S3. The ^1H and ^{31}P NMR data of 2',3'-CDNs **27** - **32** in D_2O .*

Structure	Residue	H-1'	H-2'	H-3'	H-4'	H-5'a	H-5'b	Base	^{31}P
 CDN-27	Xan	6.075 d <i>1,2 = 8.4</i>	5.08 td <i>2,1 = 8.4</i> <i>2,3 = 4.2</i> <i>2,P = 8.7</i>	4.63 d <i>3,2 = 4.2</i> <i>3,4 = 0</i>	4.42 dt <i>4,3 = 0</i> <i>4,5a = 1.8</i> <i>4,5b = 1.7</i> <i>4,P = 3.2</i>	4.28 ddd <i>5a,4 = 1.8</i> <i>5a,5b = 12.0</i> <i>5a,P = 6.4</i>	4.12 ddd <i>5b,4 = 1.7</i> <i>5b,5a = 12.0</i> <i>5b,P = 3.6</i>	H-8: 8.06 s	0.16 -0.26
	A	6.155 d <i>1,2 = 3.1</i>	4.87 dd <i>2,1 = 3.1</i> <i>2,3 = 4.7</i>	4.925 td <i>3,2 = 4.7</i> <i>3,4 = 6.8</i> <i>3,P = 6.8</i>	4.50 ddt <i>4,3 = 6.8</i> <i>4,5a = 4.5</i> <i>4,5b = 2.5</i> <i>4,P = 2.1</i>	4.34 dt <i>5a,4 = 4.5</i> <i>5a,5b = 11.7</i> <i>5a,P = 4.5</i>	4.165 ddd <i>5b,4 = 2.5</i> <i>5b,5a = 11.7</i> <i>5b,P = 4.4</i>	H-2: 8.25 s H-8: 8.25 s	
 CDN-28	N1-Me-G	5.95 d <i>1,2 = 8.5</i>	5.705 ddd <i>2,1 = 8.5</i> <i>2,3 = 4.2</i> <i>2,P = 7.6</i>	4.61 d <i>3,2 = 4.2</i> <i>3,4 = 0</i>	4.41 td <i>4,3 = 0</i> <i>4,5a = 3.0</i> <i>4,5b = 1.8</i> <i>4,P = 3.6</i>	4.26 ddd <i>5a,4 = 3.0</i> <i>5a,5b = 11.7</i> <i>5a,P = 5.2</i>	4.19 ddd <i>5b,4 = 1.8</i> <i>5b,5a = 11.7</i> <i>5b,P = 2.7</i>	H-8: 7.85 s N-Me: 2.89 s	-1.18 -0.11
	A	6.21 s <i>1,2 = 0</i>	4.77 overlap	5.12 ddd <i>3,2 = 4.1</i> <i>3,4 = 9.2</i> <i>3,P = 6.5</i>	4.495 tdd <i>4,3 = 5.4</i> <i>4,5a = 5.6</i> <i>4,5b = 2.8</i> <i>4,P = 1.5</i>	4.47 m	4.15 m	H-2: 8.27s H-8: 8.29 s	
 CDN-29	Hpx	6.225 d <i>1,2 = 8.2</i>	5.305 td <i>2,1 = 8.2</i> <i>2,3 = 4.2</i> <i>2,P = 8.4</i>	4.695 d <i>3,2 = 4.2</i> <i>3,4 = 0</i>	4.50 q <i>4,3 = 0</i> <i>4,5a = 2.0</i> <i>4,5b = 1.9</i> <i>4,P = 3.0</i>	4.35 ddd <i>5a,4 = 2.0</i> <i>5a,5b = 12.0</i> <i>5a,P = 6.6</i>	4.22 ddd <i>5b,4 = 1.9</i> <i>5b,5a = 12.0</i> <i>5b,P = 3.7</i>	H-2: 7.96 s H-8: 8.40 s	1.43 0.97
	A	6.02 d <i>1,2 = 2.2</i>	4.79 dd <i>2,1 = 2.2</i> <i>2,3 = 4.7</i>	4.985 td <i>3,2 = 4.7</i> <i>3,4 ~ 7.0</i> <i>3,P ~ 7.0</i>	4.50 m	4.41 dt <i>5a,4 = 3.5</i> <i>5a,5b = 12.0</i> <i>5a,P = 3.5</i>	4.19 ddd <i>5b,4 = 2.0</i> <i>5b,5a = 12.0</i> <i>5b,P = 4.2</i>	H-2: 8.01 s H-8: 8.10 s	
 CDN-30	7-deaza-G	5.96 d <i>1,2 = 8.6</i>	5.48 ddd <i>2,1 = 8.6</i> <i>2,3 = 4.2</i> <i>2,P = 7.2</i>	4.60 d <i>3,2 = 4.2</i> <i>3,4 ~ 0</i>	4.375 ddd <i>4,3 ~ 0</i> <i>4,5a = 2.7</i> <i>4,5b = 3.1</i> <i>4,P = 2.2</i>	4.24 ddd <i>5a,4 = 2.7</i> <i>5a,5b = 11.8</i> <i>5a,P = 5.0</i>	4.16 ddd <i>5b,4 = 3.1</i> <i>5b,5a = 11.8</i> <i>5b,P = 1.8</i>	H-7: 6.27 d <i>7,8 = 3.7</i> H-8: 6.98 d <i>8,7 = 3.7</i>	-0.16 -1.01
	A	6.17 d <i>1,2 = 1.2</i>	4.83 dd <i>2,1 = 1.2</i> <i>2,3 = 4.2</i>	5.00 ddd <i>3,2 = 4.2</i> <i>3,4 = 8.6</i> <i>3,P = 6.6</i>	4.495 dtd <i>4,3 = 8.6</i> <i>4,5a = 3.0</i> <i>4,5b = 1.5</i> <i>4,P = 3.0</i>	4.445 ddd <i>5a,4 = 3.0</i> <i>5a,5b = 11.8</i> <i>5a,P = 2.0</i>	4.185 ddd <i>5b,4 = 1.5</i> <i>5a,5b = 11.8</i> <i>5a,P = 3.5</i>	H-2: 8.275 s H-8: 8.26 s	
 CDN-31	8-5-7,9-deaza-G	nd	nd	4.575 d <i>3,2 = 4.2</i> <i>3,4 = 0</i>	4.34 um	4.275 br	4.125 vbd	--	-0.32 -1.14
	A	6.14 d <i>1,2 = 1.0</i>	4.845 overlap	nd	4.495 dddd <i>4,3 = 8.5</i> <i>4,5a = 3.0</i> <i>4,5b = 1.5</i> <i>4,P = 3.0</i>	4.44 ddd <i>5a,4 = 3.0</i> <i>5a,5b = 12.0</i> <i>5a,P = 1.7</i>	4.215 ddd <i>5b,4 = 1.5</i> <i>5b,5a = 12.0</i> <i>5b,P = 3.5</i>	H-2: 8.25 s H-8: 8.20 s	
 CDN-32	AICA	5.84 d <i>1,2 = 8.6</i>	5.095 ddd <i>2,1 = 8.6</i> <i>2,3 = 4.3</i> <i>2,P = 7.8</i>	4.61 d <i>3,2 = 4.3</i> <i>3,4 = 0</i>	4.42 dt <i>4,3 = 0</i> <i>4,5a = 1.7</i> <i>4,5b = 1.6</i>	4.30 ddd <i>5a,4 = 1.7</i> <i>5a,5b = 12.0</i> <i>5a,P = 5.6</i>	4.14 ddd <i>5b,4 = 1.6</i> <i>5b,5a = 12.0</i> <i>5b,P = 3.4</i>	H-2: 7.51 s	-0.60 -0.80
	A	6.16 d <i>1,2 = 2.0</i>	4.78 overlap	5.015 ddd <i>3,2 = 4.7</i> <i>3,4 = 7.7</i> <i>3,P = 6.7</i>	4.48 dtd <i>4,3 = 7.7</i> <i>4,5a = 3.0</i> <i>4,5b = 1.9</i> <i>4,P = 3.6</i>	4.40 ddd <i>5a,4 = 3.0</i> <i>5a,5b = 11.9</i> <i>5a,P = 3.2</i>	4.20 ddd <i>5b,4 = 4.0</i> <i>5b,5a = 11.9</i> <i>5b,P = 4.0</i>	H-2: 8.25 s H-8: 8.26 s	

*Coupling constants are written in italics in a shortened form (e.g. instead $J(1',2') = 8.6 \text{ Hz}$ we type simply *1,2 = 8.6*); nd, not determined.

Table S4. The ^1H and ^{31}P NMR data of 2',3'-CDNs with thiophosphate groups **33** - **37** in D_2O .*

Structure	Res.	H-1'	H-2'	H-3'	H-4'	H-5'a	H-5'b	Base	^{31}P
 CDN-33	G	5.97 d <i>1,2 = 8.6</i>	5.44 ddd <i>2,1 = 8.6</i> <i>2,3 = 4.0</i> <i>2,P = 7.6</i>	4.62 d <i>3,2 = 4.0</i> <i>3,4 = 0</i>	4.46 dd <i>4,3 = 0</i> <i>4,5a = 3.3</i> <i>4,5b = 1.3</i>	4.25 ddd <i>5a,4 = 3.3</i> <i>5a,5b = 11.7</i> <i>5a,P = 4.8</i>	4.17 ddd <i>5b,4 = 1.3</i> <i>5b,5a = 11.7</i> <i>5b,P = 3.2</i>	H-8: 8.03 s	-1.22 53.59
	A	6.20 s <i>1,2 = 0</i>	5.09 d <i>2,1 = 0</i> <i>2,3 = 4.3</i>	5.04 td <i>3,2 = 4.3</i> <i>3,4 = 8.9</i> <i>3,P = 8.9</i>	4.49 m	4.47 m	4.16 bdd <i>5b,4 < 1</i> <i>5b,5a = 11.0</i> <i>5b,P = 3.0</i>	H-2: 8.27 s H-8: 8.24 s	
 CDN-34	G	5.96 d <i>1,2 = 8.6</i>	5.51 td <i>2,1 = 8.5</i> <i>2,3 = 3.9</i> <i>2,P = 8.5</i>	4.78 overlap <i>3,2 = 4.0</i> <i>3,4 = 0</i>	4.47 dd <i>4,3 = 0</i> <i>4,5a = 3.3</i> <i>4,5b = 1.3</i> <i>4,P = 3.4</i>	4.25 ddd <i>5a,4 = 3.3</i> <i>5a,5b = 11.8</i> <i>5a,P = 5.1</i>	4.17 ddd <i>5b,4 = 1.3</i> <i>5b,5a = 11.8</i> <i>5b,P = 3.1</i>	H-8: 8.025 s	52.88 53.56
	A	6.20 d <i>1,2 = 0.8</i>	5.095 dd <i>2,1 = 0.8</i> <i>2,3 = 4.3</i>	5.05 td <i>3,2 = 4.3</i> <i>3,4 = 8.9</i> <i>3,P = 8.9</i>	4.53 m	4.52 m	4.19 m	H-2: 8.27 s H-8: 8.26 s	
 CDN-35	G	6.04 d <i>1,2 = 8.5</i>	5.46 ddd <i>2,1 = 8.5</i> <i>2,3 = 4.0</i> <i>2,P = 12.4</i>	4.59 d <i>3,2 = 4.0</i> <i>3,4 = 0</i>	4.44 td <i>4,3 = 0</i> <i>4,5a = 2.7</i> <i>4,5b = 1.8</i> <i>4,P = 2.8</i>	4.26 ddd <i>5a,4 = 2.7</i> <i>5a,5b = 11.8</i> <i>5a,P = 5.7</i>	4.18 ddd <i>5b,4 = 1.8</i> <i>5b,5a = 11.8</i> <i>5b,P = 4.0</i>	H-8: 8.29 s	55.36 56.08
	A	6.18 d <i>1,2 = 3.7</i>	5.10 dd <i>2,1 = 3.7</i> <i>2,3 = 4.4</i>	5.29 ddd <i>3,2 = 4.4</i> <i>3,4 = 5.8</i> <i>3,P = 10.2</i>	4.54 tdd <i>4,3 = 5.8</i> <i>4,5a = 6.0</i> <i>4,5b = 2.2</i> <i>4,P ~ 1.0</i>	4.49 ddd <i>5a,4 = 6.0</i> <i>5a,5b = 11.2</i> <i>5a,P = 7.2</i>	4.06 ddd <i>5b,4 = 2.2</i> <i>5b,5a = 11.2</i> <i>5b,P = 4.1</i>	H-2: 8.26 s H-8: 8.44 s	
 CDN-36	G	6.00 d <i>1,2 = 8.4</i>	5.665 ddd <i>2,1 = 8.4</i> <i>2,3 = 4.2</i> <i>2,P = 11.7</i>	4.555 d <i>3,2 = 4.2</i> <i>3,4 = 0</i>	4.44 t <i>4,3 = 0</i> <i>4,5a = 2.7</i> <i>4,5b = 2.8</i> <i>4,P = 1.7</i>	4.42 ddd <i>5a,4 = 2.7</i> <i>5a,5b = 11.7</i> <i>5a,P = 8.0</i>	4.11 ddd <i>5b,4 = 2.8</i> <i>5b,5a = 11.7</i> <i>5b,P = 1.3</i>	H-8: 7.97 s	56.67 56.98
	A	6.20 d <i>1,2 = 2.4</i>	4.86 dd <i>2,1 = 2.4</i> <i>2,3 = 4.2</i>	5.30 ddd <i>3,2 = 4.2</i> <i>3,4 = 7.0</i> <i>3,P = 8.3</i>	4.53 ddt <i>4,3 = 7.0</i> <i>4,5a = 5.0</i> <i>4,5b = 1.7</i> <i>4,P = 1.7</i>	4.47 ddd <i>5a,4 = 5.0</i> <i>5a,5b = 11.4</i> <i>5a,P = 5.4</i>	4.06 ddd <i>5b,4 = 1.7</i> <i>5b,5a = 11.4</i> <i>5b,P = 3.8</i>	H-2: 8.27 s H-8: 8.51 s	
 CDN-37	G	6.01 d <i>1,2 = 8.5</i>	5.39 td <i>2,1 = 8.5</i> <i>2,3 = 3.9</i> <i>2,P = 8.8</i>	4.81 d <i>3,2 = 3.9</i> <i>3,4 = 0</i>	4.46 td <i>4,3 = 0</i> <i>4,5a = 2.8</i> <i>4,5b = 1.5</i> <i>4,P = 3.4</i>	4.235 ddd <i>5a,4 = 2.8</i> <i>5a,5b = 11.6</i> <i>5a,P = 4.7</i>	4.18 ddd <i>5b,4 = 1.5</i> <i>5b,5a = 11.6</i> <i>5b,P = 4.2</i>	H-8: 8.16 s	53.38 55.12
	A	6.18 d <i>1,2 = 2.0</i>	5.11 dd <i>2,1 = 2.0</i> <i>2,3 = 4.4</i>	5.08 ddd <i>3,2 = 4.4</i> <i>3,4 = 9.6</i> <i>3,P = 7.4</i>	4.56 ddd <i>4,3 = 9.6</i> <i>4,5a = 4.3</i> <i>4,5b = 2.0</i>	4.43 dt <i>5a,4 = 4.3</i> <i>5a,5b = 11.8</i> <i>5a,P = 4.9</i>	4.22 ddd <i>5b,4 = 2.0</i> <i>5b,5a = 11.8</i> <i>5b,P = 4.9</i>	H-2: 8.25 s H-8: 8.22 s	

*Coupling constants are written in italics in a shortened form (e.g. instead $J(1',2') = 8.6 \text{ Hz}$ we type simply *1,2 = 8.6*); nd, not determined.

Table S5. Yields of CDNs from enzymatic preparation and their HRMS data for prepared CDNs analysis.

Compound	Enzyme used for CDN preparation	Relative conversion of NTP to CDN [%]	Calculated Mass [M-H] ⁻	Measured Mass [M-H] ⁻
CDN-1	mouse cGAS-TR	45	675.08834	675.08820
CDN-2	mouse cGAS-TR	59,5	677.08400	677.08278
CDN-3	mouse cGAS-FL	76	675.08834	675.08734
CDN-4	mouse cGAS-TR	55	659.09343	659.09297
CDN-5	mouse cGAS-TR	28	659.09343	659.09259
CDN-6	mouse cGAS-TR	44	677.08400	677.08372
CDN-7	mouse cGAS-TR	74	693.07892	693.07934
CDN-8	mouse cGAS-TR	53	690.09924	690.09967
CDN-9	mouse cGAS-FL	74	672.10866	672.10803
CDN-10	mouse cGAS-TR	43	673.09268	673.09142
CDN-11	mouse cGAS-FL	86	691.08326	691.08289
CDN-12	mouse cGAS-FL	50	657.09776	657.09652
CDN-13	mouse cGAS-FL	43	642.4185	nd
CDN-14	mouse cGAS-FL	85	657.09776	657.09747
CDN-15	mouse cGAS-FL	38	691.05879	691.05764
CDN-16	mouse cGAS-FL	65	688.10358	688.10255
CDN-17	mouse cGAS-TR	45	687.10833	687.10823
CDN-18	mouse cGAS-TR	53	674.07669	674.07623
CDN-19	mouse cGAS-FL	94	690.07161	690.07072
CDN-20	mouse cGAS-TR	70	690.05385	690.05287
CDN-21	human cGAS-TR	77	673.09268	673.09210
CDN-22	human cGAS-TR	56	719.08040	719.07920
CDN-23	mouse cGAS-TR	94	704.06950	704.06919
CDN-24	mouse cGAS-TR	93	672.09743	672.09589
CDN-25	mouse cGAS-TR	58	674.08793	674.08681
CDN-26	mouse cGAS-FL	54	664.09234	664.09209
CDN-27	mouse cGAS-FL	87	674.07669	674.07557
CDN-28	mouse cGAS-TR	73	687.10833	687.10727
CDN-29	*	*	658.08178	658.08061
CDN-30	mouse cGAS-FL	96	672.09743	672.09596
CDN-31	mouse cGAS-FL	78	689.05860	689.05747
CDN-32	mouse cGAS-FL	59	648.09743	648.09582
CDN-33	mouse cGAS-TR	64,5	689.06983	689.06894
CDN-34	mouse cGAS-TR	58	705.04699	705.04568
CDN-35	*	*	705.04699	705.04688
CDN-36	*	*	705.04699	705.04644
CDN-37	*	*	705.04699	705.04674

cGAS-TR, truncated version of cGAS, cGAS-FL, full length version of cGAS *CDNs prepared by organic synthesis; nd, not determined. Reaction conversions were determined by HPLC using UV detection at 260 nm. Conversions are defined as follows; a ratio of AUC of a CDN over the sum of AUCs of the CDN, NTPs and NDPs.

Table S6. Crystallography data collection and processing

Crystal	<i>wt</i> STING_CDN-1	<i>wt</i> STING_CDN-24
Diffraction source	Rotating Anode, Rigaku Micromax-007 HF	BESSY ID 14-2
Detector	Pixel, Dectris Pilatus 200K	Pixel, Dectris Pilatus 2M
Space group	P4 ₁ 2 ₁ 2	P 2 ₁ 2 ₁ 2
Resolution (Å)	2.8	2.05
R_{sym} (deg)*	0.1567	0.1350
Completeness (%)	99.62	99.17
<i>a</i> (Å)	111.441	93.776
<i>b</i> (Å)	111.441	116.478
<i>c</i> (Å)	35.24	35.845

*R_{sym} = $\frac{\sum_{hkl} \sum_j |I_{hkl,j} - \langle I_{hkl} \rangle|}{\sum_{hkl} \sum_j I_{hkl,j}}$ where $\langle I_{hkl} \rangle$ is the average symmetry-related observations of a unique reflection.

Table S7. Crystallography structure solution and refinement

Crystal	wtSTING_CDN-1	wtSTING_CDN-24
Resolution	35.24 - 2.802*	33.48 - 2.05*
R_{cryst}[#]	0.2037	0.1958
R_{free}	0.2761	0.2431
RMS deviation bond length (Å)	0.012	0.014
RMS deviation bond angle (deg)	1.54	1.51
PDB code	6S27	6S26

* It has been proven that including data with mean $I/\sigma(I) < 2.0$ leads to improved electron density maps.⁴ We have decided to cut the data at 2.8 Å leading to $I/\sigma(I) = 1.89$ in the highest resolution shell for **wtSTING_CDN-1** and 2.05 Å leading to $I/\sigma(I) = 1.21$ in the highest resolution shell for **wtSTING_CDN-24**. $I/\sigma(I) = 2$ in case of **wtSTING_CDN-1** corresponds to 3 Å resolution and in case of for **wtSTING_CDN-24** corresponds to 2.2 Å.

[#] $R_{\text{cryst}} = \frac{\sum_{\text{hkl}} |F_{\text{hkl}}^{\text{obs}} - F_{\text{hkl}}^{\text{calc}}|}{\sum_{\text{hkl}} F_{\text{hkl}}^{\text{obs}}}$ where F is the structure factor. R_{free} differs from R_{cryst} in the set of reflections, which were used for its calculation (for R_{free} usually 10% of working set).

Table S8. Effect of CDNs on CD14+ monocyte and CD3+ T-cell populations and cytokine induction in PBMC Assay (Donor 2).

Compound	Monocyte viability ^a	T-lymphocyte viability ^b	Cytokines (fold of 2'3'cGAMP induced cytokines) ^c		
			INF γ	TNF α	IFN α
CDN-1	2.1%	101.3%	1.30	1.75	2.48
CDN-2	1.5%	106.0%	0.74	1.37	1.81
CDN-3	1.0%	108.7%	1.05	1.50	1.70
CDN-4	1.95%	97.0%	0.93	1.38	1.97
CDN-5	5.5%	103.3%	1.28	2.48	0.23
CDN-6	0.3%	106.7%	0.11	0.67	0.08
CDN-7	0.5%	109.2%	1.35	1.73	0.71
CDN-8	2.3%	102.9%	1.10	1.37	1.45
CDN-9	21.3%	109.2%	1.14	1.91	1.25
CDN-10	3.9%	100.4%	1.71	2.60	2.10
CDN-11	7.8%	107.3%	2.01	2.33	1.79
CDN-12	6.1%	113.7%	1.82	2.84	1.23
CDN-13	nd	nd	n.d.	n.d.	n.d.
CDN-14	3.0%	112.0%	1.14	1.34	1.16
CDN-15	0.4%	97.0%	1.13	2.41	0.34
CDN-16	3.2%	105.7%	1.84	3.80	0.88
CDN-17	3.6%	106.0%	1.55	1.84	1.05
CDN-18	nd	nd	n.d.	n.d.	n.d.
CDN-19	85.8%	102.2%	0.05	0.08	0.03
CDN-20	6.4%	106.5%	1.59	1.53	0.69
CDN-21	2.5%	108.4%	1.25	1.76	0.46
CDN-22	28.2%	112.6%	1.08	2.00	0.05
CDN-23	nd	nd	n.d.	n.d.	n.d.
CDN-24	4.0%	100.2%	0.94	2.01	0.45
CDN-25	nd	nd	n.d.	n.d.	n.d.
CDN-26	29.1%	106.4%	0.49	0.27	0.36
CDN-27	35.0%	110.2%	0.33	0.20	0.13
CDN-28	2.7%	98.5%	0.91	1.05	0.61
CDN-29	4.7%	109.5%	1.10	1.12	1.28
CDN-30	1.3%	110.0%	1.13	1.63	0.56
CDN-31	1.0%	100.8%	1.83	1.60	1.58
CDN-32	39.8%	110.6%	0.67	0.12	0.70
CDN-33	7.6%	103.6%	1.48	1.42	1.19
CDN-34	1.5%	98.9%	1.44	1.80	3.80
CDN-35	4.7%	107.0%	1.12	0.94	0.64
CDN-36	0.7%	99.8%	0.31	1.08	0.34
CDN-37	1.0%	95.8%	1.06	0.81	1.61

2'2'cGAMP	1.6%	105.3%	1.21	1.40	1.42
3'3'c-diGMP	106.5%	104.1%	0.00	0.02	0.02
3'3'cGAMP	3.4%	109.5%	1.05	1.03	0.78
2'3'cGAMP	5.6%	110.5%	1.00	1.00	1.00

^a Viability of monocytes in PBMC culture treated with 12.5μM CDN for 16 h. Values are the mean of three independent determinations from one PBMC donor. Viability of monocytes in untreated control equals 100%.

^b Viability of CD3 T-lymphocytes in PBMC culture treated with 12.5μM CDN for 16 h. Values are the mean of three independent determinations from one PBMC donor. Viability of T-lymphocytes in untreated control equals 100%.

^c Levels of INF α, INF γ and TNFα secreted by PBMC treated with 12.5μM CDN for 16 h relative to levels secreted by PBMC treated with 12.5μM 2'3'cGAMP. Values are the mean of three independent determinations from the same PBMC donor as in monocyte/CD3 T-lymphocyte cytotoxicity assay; Amount of cytokines induced by 2'3'cGAMP treatment: Interferon γ: 28579 pg/ml, TNFα: 5340 pg/ml, Interferon α: 295 pg/ml. nd, not determined

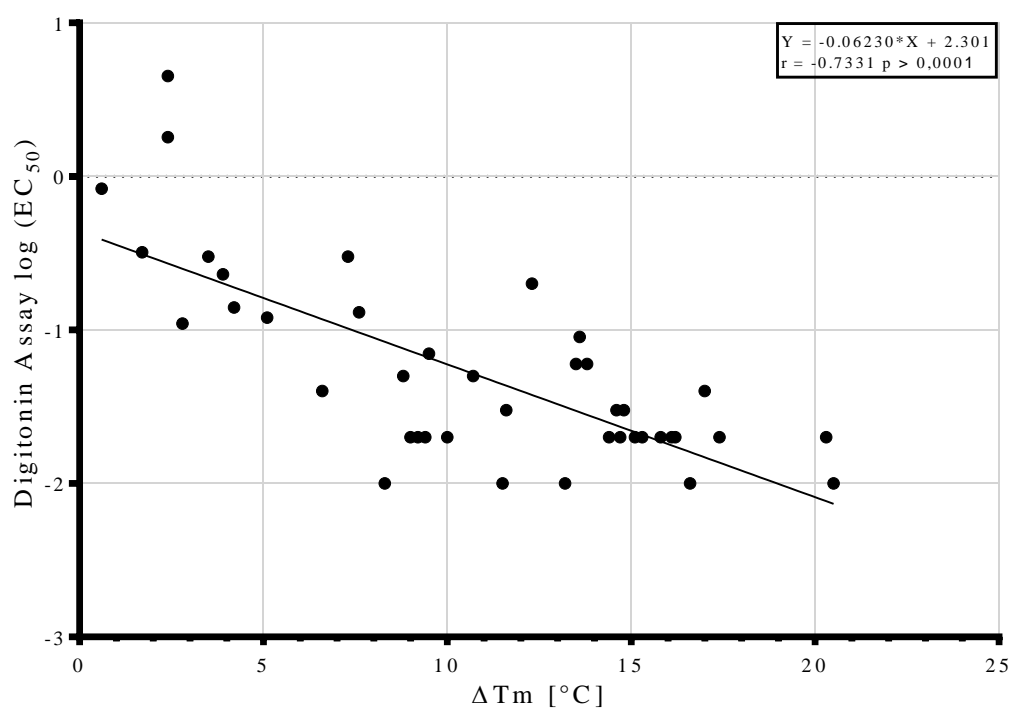
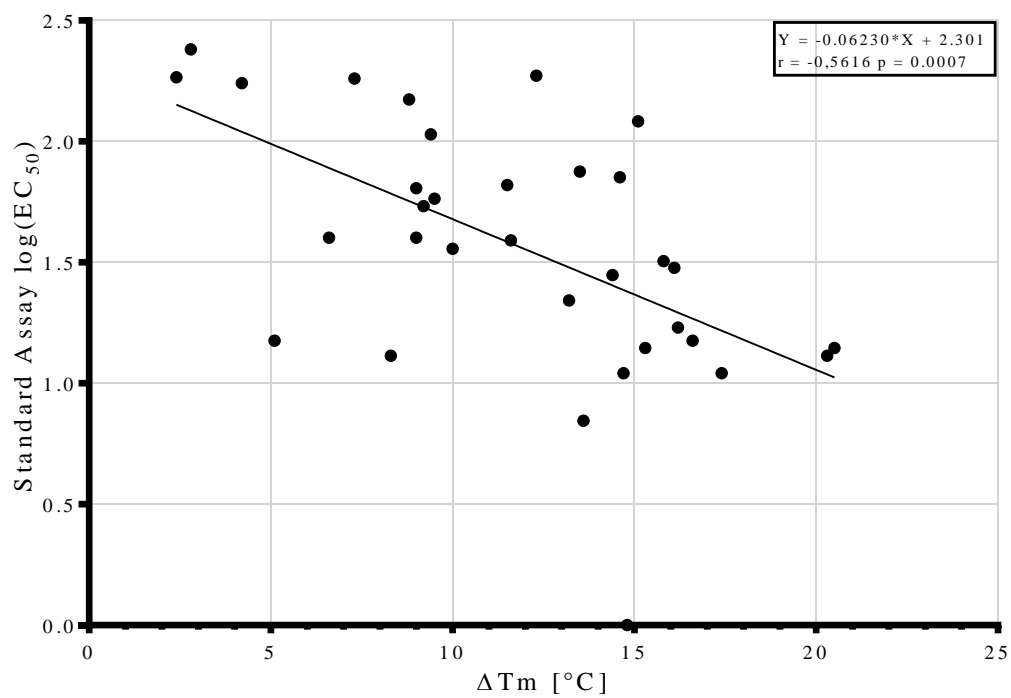


Figure S1. Correlation of ΔT_m values from DSF Assay and log EC_{50} values from Standard or Digitonin Assay of prepared CDNs.

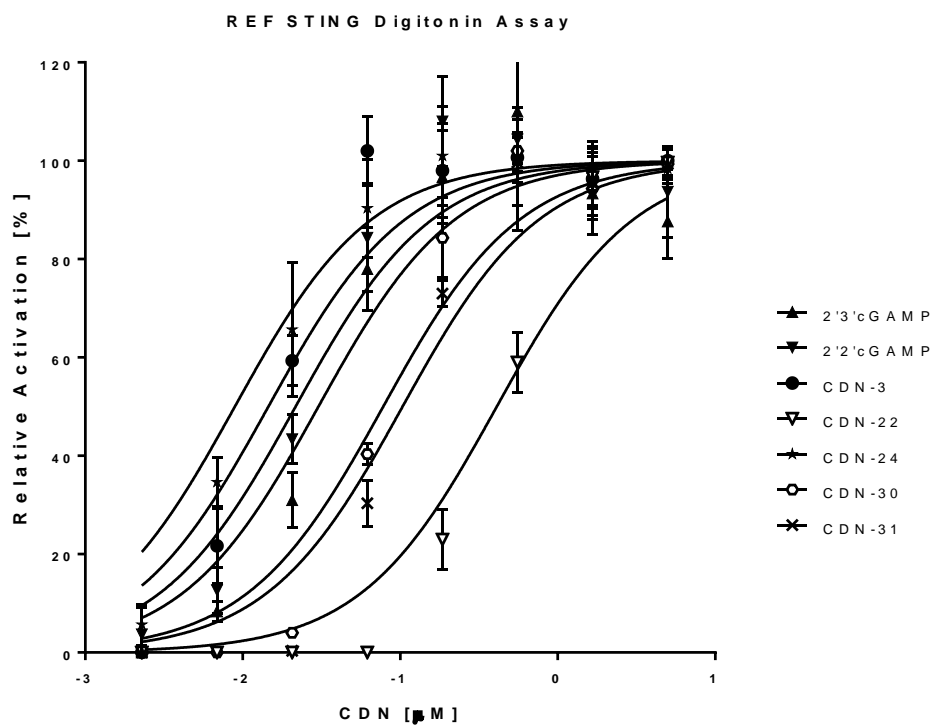
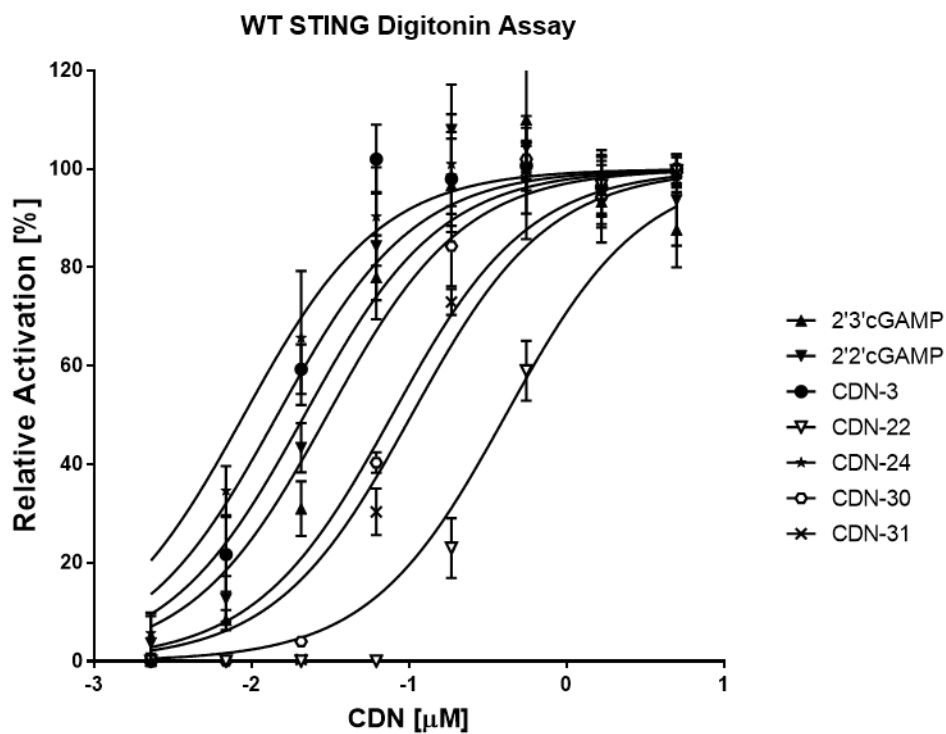


Figure S2. Representative dose response curves of 5 CDNs from Digitonin Assay on WT or REF STING haplotypes.

Supplementary files

Structures obtained from computational modeling are provided in file jm9b01062_si_002.zip, which contains three folders:

confsampl: structures of ligands identified by conformational sampling as global minima and those obtained from QM/MM calculation

QMMM: QM/MM structures for a series of ligands. Full system with 2'3'cGAMP is provided. Only the QM region is provided for the other ligands (non-QM region was kept identical). These include CDN-1, CDN-10, and four diastereoisomers of CDN-34.

docking: selected CDNs docked into 4KSY pdb structure

Molecular formula strings can be found in SI file jm9b01062_si_003.csv

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