Chemical Synthesis of the Fluorescent, Cyclic Dinucleotides $c^\text{th}$GAMP

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1. **EXPERIMENTAL SECTION**

1.1 Chemicals

Reagents were purchased from Sigma-Aldrich, Acros Organics, TCI Europe, Link Technologies and Alfa Aesar, were stored under nitrogen and were used without further purification unless otherwise specified. Dry solvents were purchased from Acros Organics, were stored under argon over molecular sieves and used as received applying standard Schlenk techniques. HPLC grade solvents were purchased from VWR. Water was purified by a Milli-Q Plus system from Merck Millipore.

Chemicals used include acetic acid (HOAc), allyl alcohol, BTT activator, 2-cyanoethyl N,N,N',N'-tetraisopropylphosphorodiamidite, dichloroacetic acid (DCA), 2-[4-(2-hydroxyethyl)piperazine-1-yl]ethanesulfonic acid (HEPES; Sigma Aldrich), imidazole, 1-methylimidazole, methoxytrimethylsilane, pyridinium trifluoroacetate, silica gel, sodium bisulfite, sodium hydrogen carbonate, sodium iodide, sodium sulfate, triethylamine, triethanolamine trihydrofluoride (NEt₃·3HF), 2,4,6-triisopropylbenzolsulfonyl chloride, and tert-Butyldimethylsilyl chloride (TBSCI), tert-Butyl hydroperoxide (t-BuOOH).

Dry solvents comprise acetone, acetonitrile (MeCN), deuterated chloroform-d (CDCl₃), dichloromethane (DCM), ethylacetate (EtOAc), iso-hexane (iHex), methanol (MeOH), pyridine, tetrahydrofuran (THF), toluene, were stored (if necessary) under septum and used without further purification.

For cell culture, we used Roswell Park Memorial 1640 medium (RPMI 1640; ThermoFisher Scientific), L-Alanyl-L-Glutamine (Sigma Aldrich), Pen-Strep (Sigma Aldrich), HEPES (1M, Sigma Aldrich), Lipofectamine™ RNAiMAX (Thermo Fischer), and Poly-D-Lysine (PDL, Sigma Aldrich), which were stored at 4°C and used only under sterile conditions. Antibiotics, including blasticidin, Normocin™, and Zeocin™ were purchased from Invivogen and kept at -20°C. Fetal Bovine Serum (FBS, Pan Biotech), Quanti-Luc™ (Invivogen) were aliquoted and stored at -20°C as well. Phosphate buffered saline (PBS, Sigma Aldrich) was kept at room temperature.

1.2 Methods and Characterization

**Photophysical characterization by UV-Vis and Fluorescence spectroscopy:** UV-Vis spectra of compounds were recorded using a spectrophotometer (Implen Nanophotometer N60). The weight purity of CDNs was measured by UV spectroscopy using the estimated extinction coefficient at maximum absorbance, i.e. 25050 M⁻¹ cm⁻¹ at 256 nm for 2'3'- or 3'3'-cGAMP and 4150 M⁻¹ cm⁻¹ at 321 nm for 2'3'- or 3'3'-cGAMP. Fluorescence based measurements were performed on a FLS 1000 Fluorimeter (Edinburgh Instruments). Emission and excitation spectra were recorded on a photon multiplier tube (PMT 900 detector, Hamamatsu) by exciting the sample with a 450 W Xenon lamp, an excitation/emission slit width of 1 nm and 0.5 nm steps with 1 s of dwell time.

**Infrared (IR) spectroscopy:** IR spectroscopy was performed on an FT-IR spectrometer (PerkinElmer Spectrum BX FT-IR) with a diamond-ATR (ATTenuated Total Reflection) mode. Samples were applied directly on the ATR unit. The range of analysis was 4500 to 600 cm⁻¹.

**TLC:** All reactions were monitored with analytical TLC (Merck silica gel 60 F254).
**RP-HPLC:** Separations and purifications by reversed-phase HPLC were carried out with an Agilent Technologies 1260 Infinity II machine consisting of 1260 Quat Pump VL, 1260 man. Inj. and 1260 MWD using a VP 250/10 NUCLEODUR 100-5 C18ec column (Macherey-Nagel). The flow rate applied was 0.5 mL/min.

**RP-LCMS:** LC-MS and low resolution ESI-MS were measured on a Dionex micro UHPLC-System (mobile phase: water and acetonitrile with 0.01 % formic acid) using a Hypersil Gold C18 selectivity column (100 × 2.1 mm) coupled to a MSQ Plus single-quadrupole mass spectrometer.

**NMR spectroscopy:** NMR spectra were measured on a Bruker Ascend 400 or Bruker ARX 600 at room temperature operating at 400 MHz or 600 MHz for 1H-nuclei and at 101 or 151 MHz for 13C-nuclei. For 29Si-NMR and 31P-NMR measurements, 80 MHz or 162 MHz measurements respectively, were performed. The chemical shift (δ) in the NMR spectra is reported in parts per million (ppm) and referenced to the residual solvent signal. Measurements were performed in CDCl₃ or D₂O. The spectra were referenced to the residual protons and carbons of the solvent (CDCl₃: δ(1H) = 7.26 ppm; δ(13C) = 39.52 ppm; D₂O δ(1H) = 4.79 ppm). All spectra were analyzed with the software MestReNova from Mestrelab Research.

**Mass spectroscopy:** High resolution mass spectra (HRMS) were recorded on a Thermo Finnigan MAT 95 (EI) and a Thermo Finnigan LTQ FTCR (ESI).

**Isothermal titration calorimetry:** ITC measurements were carried out on a Malvern PEAQ-ITC system with 52 μM (mSTING) or 251 μM (hSTING) protein in ITC-buffer (20 mM HEPES pH 7.5, 150 mM NaCl) in the cell. Compound 2 was titrated in a concentration of 200 μM (mSTING) into the cell by 19 injections of 2 μL or in a concentration of 600 μM (hSTING) into the cell by 13 injections of 3 μL, spaced 150 seconds apart, at 25 °C. The results were analyzed using the MicroCal PEAQ-ITC analysis software provided with the instrument. All titrations were repeated to confirm robustness of the assay.

**Nano differential scanning fluorimetry:** Thermal melting experiments of STING constructs were performed using a Tycho NT.6 instrument (NanoTemper Technologies). In brief, the samples were heated up in a glass capillary and while heating, the internal fluorescence at 330 nm and 350 nm was recorded. Data analysis, data smoothing, and calculation of derivatives was done using the internal evaluation features of the NT.6 instrument. All measurements were repeated to confirm robustness of the assay.
2. SYNTHESIS STEPS AND CHARACTERIZATION OF C-THGAMP

Unless otherwise specified, all reactions were magnetically stirred under an N₂ atmosphere. Reactions vessels were dried under high vacuum at 550 °C prior to use. Synthesis of C-thGAMPs was verified by a set of different techniques, including RP-HPLC, 1H-NMR, 13C-NMR, 29Si-NMR, Infrared spectroscopy, ESI-HRMS and ESI-LRMS.

2.1. Preparation of 5'-O-DMTr-NA-DMF-2'-O-TBS-thguanosine (6) and 5'-O-DMTr-NA-DMF-3'-O-TBS-thguanosine (5)

To a stirred solution of 5'-O-DMTr-NA-DMF-thguanosine (4, 4.60 g, 7.00 mmol, 1.0 eq.) in dry pyridine (125 mL), imidazole (0.95 g, 14.1 mmol, 1.0 eq.) and TBSCI (1.59 g, 10.5 mmol, 1.5 eq.) were added and the reaction mixture was stirred under nitrogen atmosphere for 16 hours at rt. The reaction was quenched by the addition of MeOH (15 mL), all volatile components were removed in vacuo and the residue was co-evaporated with toluene (2 x 100 mL). The crude product was purified by column chromatography (silica gel, Hex/EtOAc, 4:1 → 2:1 → 1:1 → 1:3 → 1:4 → EtOAc) to yield the title compounds (3.84 g, 4.98 mmol, 71%) as a colorless foam. The product was a mixture of the 2'-O-TBS regiosomer 6 and 3'-O-TBS regioisomer 5 inseparable by flash column chromatography and was used without further purification.

For analysis of 5 and 6 a small sample was purified by preparative RP-HPLC (isocratic elution, 80% MeCN in H₂O for 30 min, R₆) = 11.4 – 14.2 min, R₅ = 16.0 – 19.4 min).

2.1.1. Compound 6 (2'-O-TBS)

R₆ = 0.58 (DCM/MeOH, 10:1).

1H-NMR (400 MHz, CDCl₃): δ/ppm = 8.68 (s, 1H, H-dmf), 8.66 (s, 1H, NH), 8.16 (s, 1H, H-5), 7.55 – 7.51 (m, 2H, Ph-ο-CH), 7.44 – 7.38 (m, 4H, MeO-Ph-ο-CH), 7.30 – 7.25 (m, 2H, Ph-m-CH₂), 7.23 – 7.17 (m, 1H, Ph-p-CH), 6.84 – 6.80 (m, 4H, MeO-Ph-m-CH₂), 5.63 (d, 3J = 6.9 Hz, 1H, H-1'), 4.45 (dd, 3J = 6.9 Hz, 3J = 4.9 Hz, 1H, H-2'), 4.18 – 4.15 (m, 1H, H-4'), 4.16 – 4.14 (m, 1H, H-3'), 3.784 (s, 3H, OCH₃), 3.780 (s, 3H, OCH₃), 3.50 (dd, 3J = 10.2 Hz, 3J = 2.5 Hz, 1H, H-5), 3.19 (dd, 3J = 10.2 Hz, 3J = 3.5 Hz, 1H, H-5'), 3.13 (s, 3H, CH₃-dmf), 3.08 (s, 3H, CH₃-dmf), 2.88 (d, 3J = 3.1 Hz, 1H, OH-3'), 0.83 (s, 9H, Si(CH₃)₃), -0.03 (s, 3H, Si(CH₃)₂), -0.16 (s, 3H, Si(CH₃)₂).

13C-NMR (100.6 MHz, CDCl₃): δ/ppm = 159.7 (C-4), 158.54 (MeO-Ph), 158.52 (MeO-Ph), 157.9 (CH-dmf), 153.8 (C-2), 147.9 (C-7a), 145.1 (Ph-CC), 136.31 (MeO-Ph-CC), 136.11 (MeO-Ph-CC), 130.30 (2x MeO-Ph-ο-CH), 130.26 (2x MeO-Ph-ο-CH), 128.9 (C-7), 128.4 (2x
Ph-o-CH), 127.9 (2x Ph-m-CH), 126.8 (Ph-p-CH), 125.9 (C-5), 125.4 (C-4a), 113.23 (2x MeO-Ph-m-CH), 113.21 (2x MeO-Ph-sm-CH), 86.2 (CPh(Ph-OME)_2), 84.0 (C-4'), 79.6 (C-2'), 77.0 (C-1'), 73.0 (C-3'), 64.3 (C-5'), 55.3 (2x OCH₃), 41.3 (CH₃-dmf), 35.1 (CH₃-dmf), 25.8 (SiC(CH₃)₃), 18.1 (SiC(CH₃)₃), -4.8 (Si(CH₃)₂), -5.1 (Si(CH₃)₂).

^{29}Si-NMR (80 MHz, CDCl₃): δ/ppm = 23.5.

IR (ATR): ν/cm⁻¹ = 2929, 2248, 1679, 1629, 1572, 1508, 1250, 1112, 835, 729.

ESI-HRMS calculated for [C₄₁H₅₀N₄O₂SSi + H]⁺: 771.3242, found: 771.3243.

ESI-HRMS calculated for [C₄₁H₅₀N₄O₂SSi - H]⁻: 769.3096, found: 769.3105.

2.1.2. Compound 5 (3'-O-TBS)

R_f = 0.55 (DCM/MeOH, 10:1).

^1H-NMR (400 MHz, CDCl₃): δ/ppm = 9.03 (s, 1H, CH), 8.61 (s, 1H, H-dmf), 8.12 (s, 1H, H-5), 7.52 – 7.47 (m, 2H, Ph-o-CH), 7.41 – 7.35 (m, 4H, MeO-Ph-o-CH), 7.29 – 7.24 (m, 2H, Ph-m-CH), 7.22 – 7.17 (m, 1H, Ph-p-CH), 6.83 – 6.79 (m, 4H, MeO-Ph-m-CH), 5.53 (d, 3J = 7.0 Hz, 1H, H-1'), 4.31 (dd, 3J = 5.3 Hz, 3J = 3.3 Hz, 1H, H-3'), 4.24 (dd, 3J = 7.0 Hz, 3J = 5.3 Hz, 1H, H-2'), 4.11 (dd, 3J = 6.9 Hz, 3J = 3.3 Hz, 1H, H-4'), 3.775 (s, 3H, OCH₃), 3.773 (s, 3H, OCH₃), 3.42 (dd, 3J = 10.3 Hz, 3J = 3.4 Hz, 1H, H-5'), 3.14 (dd, 3J = 10.3 Hz, 3J = 3.9 Hz, 1H, H-5'), 3.12 (s, 3H, CH₃-dmf), 3.05 (s, 3H, CH₃-dmf), 0.88 (s, 9H, SiC(CH₃)₃), 0.06 (s, 3H, Si(CH₃)₂), -0.02 (s, 3H, Si(CH₃)₂).

^13C-NMR (100.6 MHz, CDCl₃): δ/ppm = 159.7 (C-4), 158.50 (MeO-C-Ph), 158.49 (MeO-C-Ph), 157.8 (CH-dmf), 154.1 (C-2), 146.3 (C-7a), 144.9 (Ph-CC), 136.2 (MeO-Ph-CC), 136.0 (MeO-Ph-CC), 130.2 (4x MeO-Ph-o-CH), 129.5 (C-7), 128.3 (2x Ph-o-CH), 127.9 (2x Ph-m-CH), 126.8 (Ph-p-CH), 125.4 (C-4a), 125.1 (C-5), 113.19 (2x MeO-Ph-m-CH), 113.18 (2x MeO-Ph-m-CH), 86.2 (CPh(Ph-OME)_2), 84.0 (C-4'), 78.3 (C-1'), 77.8 (C-2'), 73.7 (C-3'), 63.5 (C-5'), 55.3 (2x OCH₃), 41.3 (CH₃-dmf), 35.1 (CH₃-dmf), 25.9 (SiC(CH₃)₃), 18.3 (SiC(CH₃)₃), -4.5 (Si(CH₃)₂), -4.6 (Si(CH₃)₂).

^{29}Si-NMR (80 MHz, CDCl₃): δ/ppm = 22.3.

IR (ATR): ν/cm⁻¹ = 2930, 2247, 1680, 1629, 1572, 1508, 1258, 1111, 832, 725.

ESI-HRMS calculated for [C₄₁H₅₀N₄O₂SSi + H]⁺: 771.3242, found: 771.3239.

ESI-HRMS calculated for [C₄₁H₅₀N₄O₂SSi - H]⁻: 769.3096, found: 769.3107.
2.2. Preparation of \(N^2\)-DMF-3'-O-PO(Oallyl, OCE)-2'-O-TBS-th guanosine (8) and \(N^2\)-DMF-2'-O-PO(Oallyl, OCE)-3'-O-TBS-th guanosine (7)

To a stirred solution of 5 and 6 (2.30 g, 2.98 mmol, 1.0 eq.) in dry DCM (23.1 mL), 2-cyanoethyl \(N,N,N,N\)-tetraisopropylphosphorodiamidite (1.14 mL, 3.58 mmol, 1.2 eq.) was added under argon atmosphere at rt. After stirring for 10 min pyridinium trifluoroacetate (1.14 mL, 3.58 mmol, 1.2 eq.) was added and the reaction mixture was stirred for additional 3 h. The reaction progress was monitored by TLC. After full conversion allyl alcohol (1.01 mL, 14.9 mmol, 5.0 eq.) and BTT activator (0.3 M in MeCN, 19.9 mL, 5.96 mmol, 2.0 eq.) were added and the reaction mixture was stirred for 90 min. The intermediate products were oxidized by addition of t-BuOOH (5.5 M in decane, 1.60 mL, 8.94 mmol, 3.0 eq.) and further stirring for 30 min. The reaction mixture was cooled to 0 °C and the reaction was quenched by the addition of an aqueous solution of NaHSO₃ (500 mg/mL, 3.74 mL, 17.8 mmol, 6.0 eq.). The resulting yellow suspension was diluted with EtOAc (100 mL) and washed with brine (100 mL). The aqueous layer was extracted with EtOAc (3 x 50 mL) and the combined organic layers were dried over Na₂SO₄, filtered and concentrated in vacuo.

The residue was dissolved in DCM (48.0 mL) and H₂O (0.54 mL) and a solution of DCA (6% v/v in DCM, 48.0 mL) was added. The resulting red solution was stirred for 15 min at rt and quenched by the addition of sat. aq. NaHCO₃ solution (200 mL). The resulting yellow suspension was diluted with EtOAc (500 mL) and H₂O (200 mL), the layers were separated and the aqueous layer was extracted with EtOAc (2 x 300 mL). The combined organic layers were dried over Na₂SO₄, filtered and concentrated under reduced pressure. The crude product was purified by column chromatography (silica gel, DCM/MeOH, 49:1 → 19:1) to yield the title compounds (1.32 g, 2.06 mmol, 69%) as a yellow foam. The product was a mixture of 4 isomers: 2 diastereomers of 8 (a/b) and 2 diastereomers of 7 (a/b), inseparable by flash column chromatography and was used without further purification.

For analysis of 8a/8b and 7a/7b a small sample was purified by preparative RP-HPLC (gradient elution, 30% to 60% MeCN in H₂O over 45 min, \(R(8a) = 24.5 – 26.0\) min, \(R(8b) = 26.4 – 28.8\) min, \(R(7a) = 31.2 – 32.7\) min, \(R(7b) = 33.3 – 35.8\) min).
2.2.1. Diastereomer 8a (2’-O-TBS)

$R_f = 0.41$ (DCM/MeOH, 10:1).

$R_f$ (RP-LCMS) = 5.91 min (gradient elution, 5 % to 80 % MeCN in H$_2$O over 7 min).

$^1$H-NMR (400 MHz, CDCl$_3$): $\delta$/ppm = 8.99 (s, 1H, NH), 8.39 (s, 1H, H-dmf), 8.09 (s, 1H, H-5), 6.03 – 5.91 (m, 1H, OCH$_2$CH=CH$_2$), 5.56 (bs, 1H, OH-5’), 5.44 – 5.37 (m, 1H, OCH$_2$CH=CH$_2$), 5.32 – 5.28 (m, 1H, OCH$_2$CH=CH$_2$), 5.09 – 5.03 (m, 1H, H-2’), 4.95 – 4.91 (m, 1H, H-3’), 4.93 (d, $^3$J = 8.8 Hz, 1H, H-1’), 4.65 – 4.58 (m, 2H, OCH$_2$CH=CH$_2$), 4.40 – 4.36 (m, 1H, H-4’), 4.34 – 4.26 (m, 2H, OCH$_2$CH=CH$_2$), 3.84 (dd, $^2$J = 12.5 Hz, $^3$J = 2.3 Hz, 1H, H-5’), 3.71 (d, $^2$J = 12.5 Hz, 1H, H-5’), 3.15 (s, 3H, CH$_3$-dmf), 3.07 (s, 3H, CH$_3$-dmf), 2.81 – 2.76 (m, 2H, OCH$_2$CH=CH$_2$), 0.75 (s, 9H, Si(CH$_3$)$_3$), -0.16 (s, 3H, Si(CH$_3$)$_2$), -0.28 (s, 3H, Si(CH$_3$)$_2$).

$^{13}$C-NMR (100.6 MHz, CDCl$_3$): $\delta$/ppm = 159.4 (C-4), 158.1 (CH-dmf), 155.3 (C-2), 146.4 (C-7a), 132.1 (d, OCH$_2$CH=CH$_2$), 126.3 (C-4a), 125.4 (C-5), 124.5 (C-7), 119.1 (OCH$_2$CH=CH$_2$), 116.4 (OCH$_2$CH=CH$_2$), 85.0 (d, C-4’), 80.7 (d, C-3’), 79.1 (C-1’), 75.1 (d, C-2’), 69.0 (d, OCH$_2$CH=CH$_2$), 63.0 (C-5’), 62.1 (d, OCH$_2$CH=CH$_2$), 41.2 (CH$_3$-dmf), 35.1 (CH$_3$-dmf), 25.7 (Si(CH$_3$)$_3$), 19.7 (d, OCH$_2$CH=CH$_2$), 18.2 (Si(CH$_3$)$_3$), -5.0 (Si(CH$_3$)$_2$), -5.3 (Si(CH$_3$)$_2$).

$^{29}$Si-NMR (80 MHz, CDCl$_3$): $\delta$/ppm = 23.0.

$^{31}$P-NMR (162 MHz, CDCl$_3$): $\delta$/ppm = -1.5 - -1.8 (m).

IR (ATR): $\tilde{\nu}$/cm$^{-1}$ = 3191, 2929, 1681, 1630, 1565, 1463, 1421, 1257, 1022, 726.

ESI-LRMS calculated for [C$_2$H$_4$NO$_3$PSSi + H]$^+$: 642.2, found: 642.4.

ESI-HRMS calculated for [C$_2$H$_4$NO$_3$PSSi + H]$^+$: 642.2177, found: 642.2180.

ESI-HRMS calculated for [C$_2$H$_4$NO$_3$PSSi + Na]$^+$: 664.1996, found: 664.2003.

2.2.2. Diastereomer 8b (2’-O-TBS)

$R_f = 0.41$ (DCM/MeOH, 10:1).

$R_f$ (RP-LCMS) = 6.08 min (gradient elution, 5 % to 80 % MeCN in H$_2$O over 7 min).

$^1$H-NMR (400 MHz, CDCl$_3$): $\delta$/ppm = 9.01 (s, 1H, NH), 8.36 (s, 1H, H-dmf), 8.06 (s, 1H, H-5), 6.01 – 5.88 (m, 1H, OCH$_2$CH=CH$_2$), 5.57 (bs, 1H, OH-5’), 5.41 – 5.35 (m, 1H, OCH$_2$CH=CH$_2$), 5.30 – 5.25 (m, 1H, OCH$_2$CH=CH$_2$), 5.04 – 4.98 (m, 1H, H-2’), 4.93 – 4.88 (m, 1H, H-3’), 4.92 (d, $^3$J = 8.8 Hz, 1H, H-1’), 4.64 – 4.58 (m, 2H, OCH$_2$CH=CH$_2$), 4.39 – 4.36 (m, 1H, H-4’), 4.28 – 4.21 (m, 2H, OCH$_2$CH=CH$_2$), 3.83 (dd, $^2$J = 12.5 Hz, $^3$J = 2.3 Hz, 1H, H-5’), 3.71 (d, $^2$J = 12.5 Hz, 1H, H-5’), 3.12 (s, 3H, CH$_3$-dmf), 3.04 (s, 3H, CH$_3$-dmf), 2.79 – 2.74 (m, 2H, OCH$_2$CH=CH$_2$), 0.73 (s, 9H, Si(CH$_3$)$_3$), -0.18 (s, 3H, Si(CH$_3$)$_2$), -0.31 (s, 3H, Si(CH$_3$)$_2$).

$^{13}$C-NMR (100.6 MHz, CDCl$_3$): $\delta$/ppm = 159.3 (C-4), 158.0 (CH-dmf), 155.3 (C-2), 146.4 (C-7a), 132.1 (d, OCH$_2$CH=CH$_2$), 126.3 (C-4a), 125.2 (C-5), 124.5 (C-7), 119.2 (OCH$_2$CH=CH$_2$), 116.4 (OCH$_2$CH=CH$_2$), 85.0 (d, C-4’), 80.7 (d, C-3’), 79.1 (C-1’), 75.1 (d, C-2’), 69.0 (d, OCH$_2$CH=CH$_2$), 63.0 (C-5’), 62.0 (d, OCH$_2$CH=CH$_2$), 41.1 (CH$_3$-dmf), 35.0 (CH$_3$-dmf), 25.7 (Si(CH$_3$)$_3$), 19.8 (d, OCH$_2$CH=CH$_2$), 18.1 (Si(CH$_3$)$_3$), -5.1 (Si(CH$_3$)$_2$), -5.4 (Si(CH$_3$)$_2$).

$^{29}$Si-NMR (80 MHz, CDCl$_3$): $\delta$/ppm = 22.9.

$^{31}$P-NMR (162 MHz, CDCl$_3$): $\delta$/ppm = -1.6 - -1.9 (m).

IR (ATR): $\tilde{\nu}$/cm$^{-1}$ = 3397, 2929, 1681, 1631, 1566, 1422, 1280, 1259, 1023, 726.
2.2.3. Diastereomer 7a (3'-O-TBS)

$R_f = 0.41$ (DCM/MeOH, 10:1).
$R_f$(RP-LCMS) = 6.30 min (gradient elution, 5 % to 80 % MeCN in H2O over 7 min).

$^1$H-NMR (400 MHz, CDCl3): $\delta$/ppm = 8.81 (s, 1H, NH$_2$), 8.57 (s, 1H, H-dmf), 8.09 (s, 1H, H-5), 5.76 – 5.65 (m, 1H, OCH$_2$CH=CH$_2$), 5.56 – 5.49 (m, 1H, H-2'), 5.27 (d, $^3$J = 6.9 Hz, 1H, H-1'), 5.22 – 5.16 (m, 1H, OCH$_2$CH=CH$_2$), 5.15 – 5.11 (m, 1H, OCH$_2$CH=CH$_2$), 4.48 (dd, $^3$J = 4.6 Hz, $^3$J = 3.0 Hz, 1H, H-3'), 4.32 – 4.27 (m, 2H, OCH$_2$CH=CH$_2$), 4.18 – 4.08 (m, 1H, OCH$_2$CH=CN), 4.11 – 4.07 (m, 1H, H-4'), 4.02 – 3.94 (m, 1H, OCH$_2$CH=CN), 3.87 (dd, $^2$J = 12.4 Hz, $^2$J = 2.3 Hz, 1H, H$_5$-5'), 3.64 (dd, $^2$J = 12.4 Hz, $^2$J = 1.8 Hz, 1H, H$_5$-5'), 3.18 (s, 3H, CH$_3$-dmf), 3.05 (s, 3H, CH$_3$-dmf), 2.70 – 2.54 (m, 2H, OCH$_2$CH=CN), 0.93 (s, 9H, SiC(CH$_3$)$_3$), 0.14 (s, 3H, Si(CH$_2$)$_3$), 0.12 (s, 3H, Si(CH$_3$)$_3$).

$^{13}$C-NMR (100.6 MHz, CDCl3): $\delta$/ppm = 159.2 (C-4), 158.6 (CH-dmf), 155.1 (C-2), 146.5 (C-7a), 131.8 (d, OCH$_2$CH=CH$_2$), 126.3 (C-4a), 125.5 (C-5), 124.3 (C-7), 118.7 (OCH$_2$CH=CH$_2$), 116.3 (OCH$_2$CH=CN), 86.3 (C-4'), 79.4 (d, C-2'), 77.6 (d, C-1'), 72.9 (d, C-3'), 68.7 (d, OCH$_2$CH=CH$_2$), 62.5 (C-5'), 61.9 (d, OCH$_2$CH=CN), 41.3 (CH$_3$-dmf), 35.1 (CH$_3$-dmf), 25.9 (SiC(CH$_3$)$_3$), 19.6 (d, OCH$_2$CH=CN), 18.3 (SiC(CH$_3$)$_3$), 4.5 (Si(CH$_3$)$_3$), -4.7 (Si(CH$_3$)$_3$).

$^{29}$Si-NMR (80 MHz, CDCl3): $\delta$/ppm = 22.1.

IR (ATR): $\tilde{\nu}$/cm$^{-1}$ = 3438, 2929, 1682, 1630, 1568, 1423, 1352, 1260, 1032, 838.

ESI-LRMS calculated for [C$_{26}$H$_{46}$N$_3$O$_3$P$_2$Si$^+$ + H$^+$]: 642.2, found: 642.4.

ESI-HRMS calculated for [C$_{26}$H$_{46}$N$_3$O$_3$P$_2$Si$^+$ + Na$^+$]: 664.1996, found: 664.2003.

2.2.4. Diastereomer 7b (3'-O-TBS)

$R_f = 0.41$ (DCM/MeOH, 10:1).
$R_f$(RP-LCMS) = 6.43 min (gradient elution, 5 % to 80 % MeCN in H2O over 7 min).

$^1$H-NMR (400 MHz, CDCl3): $\delta$/ppm = 8.87 (s, 1H, NH$_2$), 8.59 (s, 1H, H-dmf), 8.09 (d, $^4$J = 1.1 Hz, 1H, H-5), 5.86 – 5.75 (m, 1H, OCH$_2$CH=CH$_2$), 5.55 – 5.48 (m, 1H, H-2'), 5.30 – 5.24 (m, 1H, OCH$_2$CH=CH$_2$), 5.29 (d, $^3$J = 6.9 Hz, 1H, H-1'), 5.22 – 5.18 (m, 1H, OCH$_2$CH=CH$_2$), 4.53 – 4.45 (m, 1H, OCH$_2$CH=CH$_2$), 4.48 – 4.45 (m, 1H, H-3'), 4.37 – 4.29 (m, 1H, OCH$_2$CH=CH$_2$), 4.11 – 4.08 (m, 1H, H-4'), 4.00 – 3.85 (m, 2H, OCH$_2$CH=CN), 3.87 (dd, $^2$J = 12.4 Hz, $^2$J = 2.0 Hz, 1H, H$_5$-5'), 3.65 (dd, $^2$J = 12.4 Hz, $^2$J = 1.7 Hz, 1H, H$_5$-5'), 3.19 (s, 3H, CH$_3$-dmf), 3.06 (s, 3H, CH$_3$-dmf), 2.59 – 2.42 (m, 2H, OCH$_2$CH=CN), 0.92 (s, 9H, SiC(CH$_3$)$_3$), 0.14 (s, 3H, Si(CH$_2$)$_3$), 0.12 (s, 3H, Si(CH$_3$)$_3$).

$^{13}$C-NMR (100.6 MHz, CDCl3): $\delta$/ppm = 159.2 (C-4), 158.7 (CH-dmf), 155.2 (C-2), 146.5 (C-7a), 131.9 (d, OCH$_2$CH=CH$_2$), 126.3 (C-4a), 125.5 (C-5), 124.5 (C-7), 118.8 (OCH$_2$CH=CH$_2$), 116.3 (OCH$_2$CH=CN), 86.3 (C-4'), 79.5 (d, C-2'), 77.7 (d, C-1'), 72.9 (d, C-3'), 68.8 (d,
OCH₂CH=CH₂), 62.5 (C-5'), 61.9 (d, OCH₂CH₂CN), 41.4 (CH₃-dmf), 35.2 (CH₂-dmf), 25.9 (SiC(CH₃)₃), 19.5 (d, OCH₂CH₂CN), 18.3 (SiC(CH₃)₃), -4.5 (Si(CH₃)₂), -4.8 (Si(CH₃)₂).

29Si-NMR (80 MHz, CDCl₃): δ/ppm = 22.0.

IR (ATR): ν/cm⁻¹ = 3410, 2928, 1685, 1631, 1571, 1424, 1352, 1261, 1116, 1034.

ESI-LRMS calculated for [C₂₆H₄₀N₅O₈PSSi + H]⁺: 642.2, found: 642.4.

ESI-HRMS calculated for [C₂₆H₄₀N₅O₈PSSi + H]⁺: 642.2177, found: 642.2176.

calculated for [C₂₆H₄₀N₅O₈PSSi + Na]⁺: 664.1996, found: 664.1999.
2.3. \( N^6''\)-Bz-2''\-'O-TBS-adenosin-3''\-'5'-\( O\)-PO(OCE)-\( N^2\)-DMF-3'-'O-PO(Oallyl, OCE)-2'-'O-TBS-thguanosine (10) and \( N^6''\)-Bz-2''\-'O-TBS-adenosin-3''\-'5'-\( O\)-PO(OCE)-\( N^2\)-DMF-3'-'O-PO(Oallyl, OCE)-2'-'O-TBS-thguanosine (9)

To a stirred solution of the diastereomeric mixture of 7 and 8 (1.32 g, 2.06 mmol, 1.0 eq.) in dry MeCN (12.7 mL), a solution of DMT-2'-O-TBS-rA(Bz) phosphoramidite (2.24 g, 2.27 mmol, 1.10 eq.) in dry MeCN (1.00 mL) was added under argon atmosphere at rt. After stirring for 10 min BTT activator (0.3 M in MeCN, 13.7 mL, 4.12 mmol, 2.0 eq.) was and the reaction mixture was stirred for additional 90 min at rt. The intermediate products were oxidized by addition of \( t\) -BuOOH (5.5 M in decane, 1.12 mL, 6.18 mmol, 3.0 eq.) and further stirring for 30 min. The reaction mixture was cooled to 0 °C and the reaction was quenched by the addition of an aqueous solution of NaHSO\(_3\) (500 mg/mL, 2.57 mL, 12.4 mmol, 6.0 eq.). The resulting yellow suspension was diluted with EtOAc (50 mL) and washed with brine (50 mL). The aqueous layer was extracted with EtOAc (3 x 25 mL) and the combined organic layers were dried over Na\(_2\)SO\(_4\), filtered and concentrated under reduced pressure. The residue was dissolved in DCM (18.2 mL) and H\(_2\)O (0.21 mL) and a solution of DCA (6% v/v in DCM, 18.2 mL) was added. The resulting red solution was stirred for 15 min at rt and quenched by the addition of sat. aq. NaHCO\(_3\) solution (100 mL). The resulting yellow suspension was diluted with EtOAc (300 mL) and H\(_2\)O (100 mL), the layers were separated and the aqueous layer was extracted with EtOAc (2 x 300 mL). The combined organic layers were dried over Na\(_2\)SO\(_4\), filtered and concentrated in vacuo. The crude product was purified by column chromatography (silica gel, DCM/MeOH, 49:1 \(\to\) 19:1) to yield the title compounds (1.61 g, 1.29 mmol, 63%) as a yellow foam. The product was a mixture of 8 isomers: 4 diastereomers of 10 and 4 diastereomers of 9, inseparable by flash column chromatography and was used without further purification.

For analysis of 10 and 9 a small sample was purified by preparative RP-HPLC (gradient elution, 30% to 60% MeCN in H\(_2\)O over 45 min, \( R_t(10) = 21.9 - 22.9\) min, \( R_t(9) = 36.4 - 37.3\) min). For compounds 10 and 9 a single diastereomer was isolated for analysis.
2.3.1. Compound 10 (3',3'-Dimer)

\( R_f = 0.27 \) (DCM/MeOH, 10:1).
\( R_t (\text{RP-LCMS}) = 7.31, 7.45 \text{ min (gradient elution, 5 \% to 80 \% MeCN in H}_2\text{O over 7 min).} \)

\(^1\text{H}-\text{NMR} \) (400 MHz, CDCl\(_3\)): \( \delta/\text{ppm} = 9.25 \) (bs, 1H, A-NH), 8.80 (s, 1H, H-2'''), 8.65 (s, 1H, H-dmf), 8.57 (bs, 1H, G-NH), 8.133 (s, 1H, H-5), 8.128 (s, 1H, H-8'''), 8.06 – 8.01 (m, 2H, Bz-o-CH), 7.64 – 7.58 (m, 1H, Bz-p-CH), 7.56 – 7.50 (m, 2H, Bz-m-CH), 6.01 – 5.89 (m, 1H, OCH\(_2\)CH=CH\(_2\)), 5.94 (d, \(^3\)J = 7.8 Hz, 1H, H-1'''), 5.50 (d, \(^3\)J = 4.9 Hz, 1H, H-1'), 5.42 – 5.36 (m, 1H, OCH\(_2\)CH=CH\(_2\)), 5.30 – 5.26 (m, 1H, OCH\(_2\)CH=CH\(_2\)), 5.25 – 5.18 (m, 1H, H-2''), 5.09 – 5.03 (m, 1H, H-3''), 4.89 – 4.83 (m, 1H, H-3'), 4.66 – 4.60 (m, 2H, OCH\(_2\)CH=CH\(_2\)), 4.59 – 4.52 (m, 1H, H-2'), 4.57 – 4.50 (m, 1H, H-4'''), 4.51 – 4.45 (m, 1H, H-5'), 4.46 – 4.40 (m, 1H, H-4'), 4.37 – 4.27 (m, 2H, A-OCH\(_2\)CH\(_2\)CN), 4.35 – 4.25 (m, 2H, G-OCH\(_2\)CH\(_2\)CN), 3.96 – 3.87 (m, 1H, H-2'''), 3.85 – 3.76 (m, 1H, H-5'''), 3.18 (s, 3H, CH\(_3\)-dmf), 3.08 (s, 3H, CH\(_3\)-dmf), 2.83 – 2.76 (m, 2H, G-OCH\(_2\)CH\(_2\)CN), 2.81 – 2.69 (m, 2H, A-OCH\(_2\)CH\(_2\)CN), 0.84 (s, 9H, G-SiC(CH\(_3\))\(_3\)), 0.73 (s, 9H, A-SiC(CH\(_3\))\(_3\)), 0.03 (s, 3H, G-SiC(CH\(_3\))\(_2\)), -0.05 (s, 3H, G-SiC(CH\(_3\))\(_2\)), -0.11 (s, 3H, A-SiC(CH\(_3\))\(_2\)), -0.33 (s, 3H, A-SiC(CH\(_3\))\(_2\)).

\(^{13}\text{C}-\text{NMR} \) (100.6 MHz, CDCl\(_3\)): \( \delta/\text{ppm} = 164.5 \) (aryl-CCONH), 159.4 (C-4), 157.8 (CH-dmf), 154.2 (C-2), 152.4 (C-2''), 150.5 (C-6''), 150.4 (C-4''), 147.4 (C-7a), 143.4 (C-8''), 133.6 (aryl-CCONH), 133.1 (Bz-p-CH), 131.9 (d, OCH\(_2\)CH=CH\(_2\)), 129.0 (2x Bz-o-CH), 128.0 (2x Bz-m-CH), 127.0 (C-4a), 126.0 (C-7), 125.9 (C-5), 124.4 (C-5'''), 119.6 (OCH\(_2\)CH=CH\(_2\)), 116.6 (G-OCH\(_2\)CH\(_2\)CN), 116.5 (A-OCH\(_2\)CH\(_2\)CN), 90.4 (C-1'''), 86.1 (C-4''), 79.7 (C-4'), 79.4 (C-1'), 79.3 (C-3''), 77.1 (d, C-3'), 77.0 (d, C-2'), 73.5 (d, C-2''), 69.3 (d, OCH\(_2\)CH=CH\(_2\)), 68.1 (C-5'), 62.6 (C-5''), 62.4 (d, G-OCH\(_2\)CH\(_2\)CN), 62.3 (d, A-OCH\(_2\)CH\(_2\)CN), 41.4 (CH\(_3\)-dmf), 35.3 (CH\(_3\)-dmf), 25.8 (SiC(CH\(_3\))\(_3\)), 25.7 (SiC(CH\(_3\))\(_3\)), 19.9 (d, G-OCH\(_2\)CH\(_2\)CN), 19.8 (d, A-OCH\(_2\)CH\(_2\)CN), 18.2 (G-SiC(CH\(_3\))\(_3\)), 18.1 (A-SiC(CH\(_3\))\(_3\)), -4.7 (G-SiC(CH\(_3\))\(_2\)), -4.8 (G-SiC(CH\(_3\))\(_2\)), -4.9 (A-SiC(CH\(_3\))\(_2\)), -5.5 (A-SiC(CH\(_3\))\(_2\)).

\(^{29}\text{Si}-\text{NMR} \) (80 MHz, CDCl\(_3\)): \( \delta/\text{ppm} = 22.5 \) (G-TBS), 25.1 (A-TBS).

\(^{31}\text{P}-\text{NMR} \) (162 MHz, CDCl\(_3\)): \( \delta/\text{ppm} = -1.5 - -1.7 \) (m, 1P, G-P), -2.0 - -2.3 (m, 1P, G-P).

IR (ATR): \( \tilde{\nu} / \text{cm}^{-1} = 3150, 2931, 1684, 1630, 1572, 1458, 1347, 1255, 1031, 838. \)

ESI-LRMS calculated for [C\(_{52}\)H\(_{78}\)N\(_{11}\)O\(_{15}\)P\(_2\)SSi\(_2\) + H]\(^+\): 1242.4, found: 1242.7.

ESI-HRMS calculated for [C\(_{52}\)H\(_{78}\)N\(_{11}\)O\(_{15}\)P\(_2\)SSi\(_2\) + H]\(^+\): 1242.4095, found: 1242.4120.

calculated for [C\(_{52}\)H\(_{78}\)N\(_{11}\)O\(_{15}\)P\(_2\)SSi\(_2\) - H]\(^-\): 1240.3949, found: 1240.3960.
2.3.2. Compound 9 (2',3'-Dimer)

$R_f = 0.27$ (DCM/MeOH, 10:1).

$R_f$ (RP-LC-MS) = 7.58, 7.63 min (gradient elution, 5 % to 80 % MeCN in H$_2$O over 7 min).

$^1$H-NMR (400 MHz, CDCl$_3$): $\delta$/ppm = 9.25 (d, $^3$J = 2.2 Hz, 1H, A-NH$_2$), 8.94 (s, 1H, H-dmf), 8.80 (s, 1H, H-2'''), 8.70 (bs, 1H, G-NH$_2$), 8.11 (s, 1H, H-8'''), 8.08 (s, 1H, H-5), 8.06 – 8.02 (m, 2H, Bz-o-CH$_3$), 7.64 – 7.59 (m, 1H, Bz-p-CH$_3$), 7.55 – 7.50 (m, 2H, Bz-m-CH$_3$), 5.99 – 5.84 (m, 1H, OCH$_2$CH$_3$), 5.92 (d, $^3$J = 7.8 Hz, 1H, H-1'''), 5.68 (d, $^3$J = 2.6 Hz, 1H, H-1'), 5.49 – 5.43 (m, 1H, H-2'), 5.39 – 5.36 (m, 1H, OCH$_2$CH$_3$), 5.30 – 5.26 (m, 1H, OCH$_2$CH$_3$), 5.24 – 5.19 (m, 1H, H-2'''), 5.10 – 5.05 (m, 1H, H-3'''), 4.66 – 4.55 (m, 2H, OCH$_2$CH$_3$), 4.59 – 4.53 (m, 1H, H-4'''), 4.46 – 4.40 (m, 1H, H$_3$-5''), 4.34 – 4.27 (m, 1H, H$_3$-5'), 4.36 – 4.27 (m, 2H, A-OCH$_2$CH$_2$CN), 4.27 – 4.21 (m, 1H, H-4'), 4.25 – 4.19 (m, 1H, H-3'), 4.19 – 4.11 (m, 2H, G-OCH$_2$CH$_2$CN), 3.99 – 3.91 (m, 1H, H$_3$-5''), 3.87 – 3.87 (m, 1H, H$_3$-5''), 3.24 (s, 3H, CH$_3$-dmf), 3.08 (s, 3H, CH$_3$-dmf), 2.81 – 2.71 (m, 2H, A-OCH$_2$CH$_2$CN), 2.71 – 2.61 (m, 2H, G-OCH$_2$CH$_2$CN), 0.93 (s, 9H, G-SiC(CH$_3$)$_3$), 0.74 (s, 9H, G-SiC(CH$_3$)$_3$), 0.15 (s, 3H, G-SiC(CH$_3$)$_2$), 0.12 (s, 3H, G-SiC(CH$_3$)$_2$), -0.08 (s, 3H, A-Si(CH$_3$)$_3$), -0.33 (s, 3H, A-Si(CH$_3$)$_3$).

$^{13}$C-NMR (100.6 MHz, CDCl$_3$): $\delta$/ppm = 164.5 (aryl-CCONH), 159.3 (C-4), 159.1 (CH-dmf), 154.6 (C-2), 152.5 (C-2''), 150.7 (C-6''), 150.6 (C-4''), 146.1 (C-7a), 143.2 (C-8''), 133.6 (aryl-CONH), 133.1 (Bz-p-CH$_3$), 132.0 (d, OCH$_2$CH$_3$), 129.1 (2x Bz-o-CH$_3$), 128.1 (2x Bz-m-CH$_3$), 126.0 (C-4a), 125.9 (C-7), 125.8 (C-5), 124.5 (C-5''), 119.2 (OCH$_2$CH$_3$), 116.5 (G-OCH$_2$CH$_2$CN), 116.4 (A-OCH$_2$CH$_2$CN), 90.7 (C-1'''), 86.3 (C-4'''), 81.1 (d, C-2'), 80.4 (C-4'), 79.13 (C-1'), 79.08 (C-3''), 73.4 (d, C-2''), 71.6 (d, C-3'), 69.1 (d, OCH$_2$CH$_3$), 68.6 (C-5'), 62.6 (C-5''), 62.5 (d, A-OCH$_2$CH$_2$CN), 62.2 (d, G-OCH$_2$CH$_2$CN), 41.4 (CH$_3$-dmf), 35.2 (CH$_3$-dmf), 25.9 (G-SiC(CH$_3$)$_3$), 19.9 (d, G-OCH$_2$CH$_2$CN), 19.7 (d, A-OCH$_2$CH$_2$CN), 18.2 (G-SiC(CH$_3$)$_3$), 18.0 (A-SiC(CH$_3$)$_3$), -4.4 (G-Si(CH$_3$)$_2$), -4.88 (G-Si(CH$_3$)$_2$), -4.93 (A-Si(CH$_3$)$_3$), -5.5 (A-Si(CH$_3$)$_3$).

$^{28}$Si-NMR (80 MHz, CDCl$_3$): $\delta$/ppm = 23.4 (G-TBS), 25.1 (A-TBS).

$^{31}$P-NMR (162 MHz, CDCl$_3$): $\delta$/ppm = -2.2 (A-P), -2.5 (G-P).

IR (ATR): $\tilde{\nu}$/cm$^{-1}$ = 3145, 2931, 1684, 1630, 1573, 1458, 1349, 1255, 1034, 839.

ESI-LRMS calculated for [C$_{52}$H$_{73}$N$_{11}$O$_{15}$P$_2$SSi$_2$ + H]$^+$: 1242.4, found: 1242.7.

ESI-HRMS calculated for [C$_{52}$H$_{73}$N$_{11}$O$_{15}$P$_2$SSi$_2$ + H]$^+$: 1242.4095, found: 1242.4122.

calculated for [C$_{52}$H$_{73}$N$_{11}$O$_{15}$P$_2$SSi$_2$ - H]$^-$: 1240.3949, found: 1240.3964.
2.4. Preparation of 3',3'-c<sup>th</sup>GAMP (3) and 2',3'-c<sup>th</sup>GAMP (2)

The cyclization and deprotection protocol starting from a mixture of 9 and 10 required three major steps: 1) allyl deprotection, 2) cyclization and 3) final deprotection. The characterization of intermediate products was limited to RP-LCMS und ESI-LRMS mostly.

1) Allyl deprotection
A stirred suspension of a mixture of 9 and 10 (538 mg, 0.433 mmol, 1.0 eq.) and sodium iodide (649 mg, 4.33 mmol, 10 eq.) in dry acetone (20 mL) under argon atmosphere was heated to 56 °C in the dark for 3 h. The reaction mixture was allowed to cool to rt and all volatile components were removed under reduced pressure. The residue was purified by column chromatography (silica gel, DCM/MeOH, 19:1 → 9:1 → 4:1) to yield the allyl-deprotected dinucleotides (367 mg, 0.305 mmol, 70%) as a yellow solid. The isolated product was a mixture of 4 isomers.

\[ R_f = 0.32 \ (DCM/MeOH, \ 4:1). \]
\[ R_f (RP-LCMS) = 6.52, 6.58, 6.72, 6.77 \text{ min} \ (\text{gradient elution, 5 \% to 80 \% MeCN in H}_2\text{O over 7 min}). \]
\[ \text{ESI-LRMS calculated for [C}_{49}\text{H}_{69}\text{N}_{11}\text{O}_{15}\text{P}_{2}\text{Si}_{2}^{+} + \text{H}]^{+} : 1202.4, \text{found: 1202.7.} \]
\[ \text{ESI-HRMS calculated for [C}_{49}\text{H}_{69}\text{N}_{11}\text{O}_{15}\text{P}_{2}\text{Si}_{2}^{-} - \text{H}]^{-} : 1200.3636, \text{found: 1200.3645.} \]

2) Cyclization
The allyl-deprotected dinucleotides (367 mg, 305 μmol, 1.0 eq.) were dissolved in dry THF (72 mL) and 4 Å MS, 1-methylimidazole (1.22 mL, 15.3 mmol, 50 eq.) and 2,4,6-triisopropyl-benzolsulfonylchloride (4.62 g, 15.3 mmol, 50 eq.) were added. The reaction mixture was stirred under argon atmosphere for 25 h at rt. The reaction progress was monitored by RP-LCMS. After full conversion, the reaction was quenched by the addition of H<sub>2</sub>O (23.1 mL). After stirring for an additional hour, the solution was concentrated in vacuo, diluted with brine (100 mL) and extracted with EtOAc (200 mL) and DCM (3 × 200 mL). The combined organic layers were dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under reduced pressure. The crude product was purified by column chromatography (silica gel, DCM/MeOH, 49:1 → 97:3 → 19:1) to yield the protected cyclic dinucleotides 11 and 12 (177 mg, 149 μmol, 49%) as a yellow solid. The isolated product was a mixture of 8 isomers.

\[ R_f = 0.28 \ (DCM/MeOH, \ 19:1). \]
3) Final deprotection

The protected cyclic dinucleotides 11 and 12 (33.0 mg, 27.9 μmol) were dissolved in MeOH (5 mL) and ammonium hydroxide solution (28-30% NH₃, 5 mL) was added. The reaction mixture was stirred for 16 h at room temperature. The reaction progress was monitored by RP-LCMS. After full conversion, all volatile components were removed under reduced pressure and the residue was co-evaporated with pyridine (3 x 20 mL). The crude product was used in the next step without further purification.

$R_{t}$ (RP-LCMS) = 4.87, 5.40 min (gradient elution, 5 % to 80 % MeCN in H₂O over 7 min).

ESI-LRMS calculated for [C₄₉H₇₁N₁₁O₁₄P₂SSi₂ + H]$^+$: 1184.4, found: 1184.4.

The crude product was dissolved in dry pyridine (1 mL) and dry THF (2 mL). After dropwise addition of NEt₃ (0.56 mL) and NEt₃-3HF (0.56 mL, 3.41 mmol, 50 eq.) the reaction mixture was stirred for 48 h at room temperature. The reaction progress was monitored by RP-LCMS. After full conversion, the mixture was quenched by the addition of methoxytrimethylsilane (1.4 mL) and all volatile components were removed in vacuo. The solid residue was dissolved in MeOH (0.5 mL), the crude product was precipitated by the addition of cold acetone (15 mL) and collected by centrifugation. The products 3',3'-c⁷thGAMP (3, 2.5 mg, 3.62 μmol, 13%) and 2',3'-c⁷thGAMP (2, 2.5 mg, 3.62 μmol, 13%) were separated and purified by two RP-HPLCs as follows:

First preparative RP-HPLC: Isocratic and gradient elution, 0% buffer B for 5 min, then 0% to 40% buffer B in buffer A over 25 min; buffer A: 0.1 M NEt₃/HOAc in H₂O, buffer B: 0.1 M NEt₃/HOAc in MeCN/H₂O (4:1); $R_{t}(3)$ = 13.6 – 14.1 min and $R_{t}(2)$ = 15.5 – 16.5 min.

Second preparative RP-HPLC: Isocratic and gradient elution, 0% MeCN+0.1% TFA for 5 min, then 0% to 40% MeCN+0.1% TFA in H₂O+0.1% TFA over 18 min: $R_{t}(3)$ = 12.5 – 13.1 min and $R_{t}(2)$ = 13.6 – 14.6 min.

2.4.1. 3',3'-c⁷thGAMP (3)

$^1$H-NMR (800 MHz, D₂O): $\delta$/ppm = 8.65 (s, 1H, A_H-2), 8.51 (s, 1H, G_H-5), 8.46 (s, 1H, A_H-8), 6.28 (s, 1H, A_H-1'), 5.58 (d, $^3J$ = 2.6 Hz, 1H, G_H-1'), 4.97 – 4.91 (m, 1H, G_H-3'), 4.91 – 4.85 (m, 1H, A_H-3'), 4.83 – 4.78 (m, 1H, A_H-4'), 4.61 – 4.58 (m, 1H, G_H-2'), 4.54 – 4.49 (m, 1H, A_H-2'), 4.46 – 4.43 (m, 1H, G_H-4'), 4.44 – 4.40 (m, 1H, G_H-5'), 4.27 – 4.22 (m, 1H, A_H-5'), 4.14 – 4.08 (m, 1H, G_H-5'), 4.07 – 4.01 (m, 1H, A_H-5').

$^{13}$C-NMR (100.6 MHz, D₂O): $\delta$/ppm = 157.7 (G_C-4), 151.2 (G_C-2), 149.9 (A_C-6), 147.6 (A_C-4), 144.8 (A_C-8), 142.0 (A_C-2), 132.6 (G_C-5), 132.3 (G_C-7a), 120.3 (G_C-4a), 119.4 (G_C-7), 118.8 (A_C-5), 90.0 (A_C-1'), 79.9 (A_C-2'), 78.1 (G_C-4'), 76.9 (G_C-1'), 73.8 (A_C-4'), 73.4 (G_C-3'), 72.6 (G_C-2'), 70.3 (A_C-3'), 63.3 (A_C-5'), 62.2 (G_C-5').

$^{31}$P-NMR (162 MHz, D₂O): $\delta$/ppm = -1.1 - -1.3 (m, 1P, A_C-5'-P), -1.5 - -1.8 (m, 1P, G_C-5'-P).
ESI-HRMS calculated for $[C_{21}H_{24}N_8O_{13}P_2S + H]^+$: 691.0732, found: 691.0740.
calculated for $[C_{21}H_{24}N_8O_{13}P_2S - H]^-$: 689.0586, found: 689.0585.

The connectivity of the phosphodiesters was verified by $^1$H-$^{31}$P-HMBC measurements:

**2.4.2. 2',3'-cAMP (2)**

$^1$H-NMR (800 MHz, D$_2$O): $\delta$/ppm = 8.44 (s, 1H, A_H-2), 8.31 (s, 1H, A_H-8), 7.77 (s, 1H, G_H-5), 6.11 (s, 1H, A_H-1'), 5.40 (s, 1H, G_H-1'), 4.89 – 4.83 (m, 1H, A_H-3'), 4.85 – 4.79 (m, 1H, A_H-2'), 4.72 – 4.64 (m, 1H, G_H-2'), 4.52 – 4.46 (m, 1H, A_H-4'), 4.48 – 4.42 (m, 1H, G_H-4'), 4.40 – 4.37 (m, 1H, A_Ha-5'), 4.38 – 4.32 (m, 1H, G_Ha-5'), 4.30 – 4.24 (m, 1H, G_H-3'), 4.08 (dd, $^2$J = 11.8 Hz, $^3$J = 4.4 Hz, 1H, G_Hb-5'), 4.03 (dd, $^2$J = 11.7 Hz, $^3$J = 4.1 Hz, 1H, A_Hb-5').

$^{13}$C-NMR (100.6 MHz, D$_2$O): $\delta$/ppm = 156.0 (G_C-4), 153.1 (G_C-2), 149.8 (A_C-6), 147.0 (A_C-4), 145.0 (A_C-8), 142.0 (A_C-2), 130.7 (G_C-5), 129.9 (G_C-7a), 123.6 (G_C-7), 120.0 (G_C-4a), 118.5 (A_C-5), 90.9 (A_C-1'), 81.7 (G_C1'), 80.4 (A_C-4'), 78.5 (G_C-3'), 75.8 (G_C-4'), 73.6 (A_C-2'), 71.2 (G_C-2'), 70.0 (A_C-3'), 62.2 (A_C-5'), 61.9 (G_C-5').

$^{31}$P-NMR (162 MHz, D$_2$O): $\delta$/ppm = -1.6 - -1.9 (2P).

ESI-HRMS calculated for $[C_{21}H_{24}N_8O_{13}P_2S + H]^+$: 691.0732, found: 691.0743.
calculated for $[C_{21}H_{24}N_8O_{13}P_2S - H]^-$: 689.0586, found: 689.0585.
The connectivity of the phosphodiesters was verified by $^1$H-$^{31}$P-HMBC measurements:
3. **CELL CULTURE**

3.1. Culturing of THP-1 wt and THP-1 STING-KO cell lines

THP-1-Dual™ wt cells and THP-1-Dual™ STING-KO cells were purchased from Invivogen. Both cell lines were cultured in initial growth medium containing RPMI 1640 (Sigma Aldrich), 20% FBS (for ESC, Pan Biotech), 25 mM HEPES (1M, Sigma Aldrich), 2 mM L-Alanyl-L-Glutamine (Sigma Aldrich), 100 µg/ml Normocin™ (Invivogen), Pen-Strep (100 U/ml-100 µg/ml, Sigma Aldrich) upon manufacturer's instructions. After the cells were passaged twice, they were cultured in maintaining medium (same as growth medium but with 10% FBS) with selective antibiotics blasticidin and Zeocin™ (Invivogen). The cells were cultured between 4x10^5 and 1.5x10^6 cells/mL. For experiments a test medium was used which contained no Normocin™, blasticidin, or Zeocin™. The cells were cultured in humidity saturated 37°C, 5% CO2 containing incubators (Heracell 150 CO2 incubator) and handled in sterile conditions.

3.2. Preparation of Poly-D-Lysin - coated Slides

18 mm diameter glass cover slides (Carl Roth) were placed in 12-well plates (VWR) in sterile conditions. Poly-D-Lysin (PDL; Sigma Aldrich) was prepared in sterile ddH2O to an end concentration of 0.1 mg/mL and applied on the cover slides with the adjusted amount of 40 L/cm². The incubation last for 30 minutes under saturated humidity at 37°C and 5% CO2 within the incubator. The slides were washed twice with ddH2O, incubated for 5 minutes during each washing step in the incubator. Before use, the slides were dried at least for two hours in a laminal hood. All steps were performed in sterile conditions.

3.3. Feeding compounds to THP-1 cells

For a feeding of 24 hours, approximately 100.000 cells per well/condition in a 96-well plate were used. The compounds were added according to the desired concentration in test medium - reaching an end volume of 100 µL of test medium, i.e. medium without Normocin™. The concentration of the compounds was measured prior to each feeding via the UV-Vis spectrophotometer (Implen Nanophotometer N60) applying the extinction coefficient on Beer-Lambert law.

3.4. Immobilization of THP-1 cells for TPE imaging

A volume of 100 µL medium containing fed or unfed cells, respectively, was transferred on the dried cover slides and incubated for 20 minutes under saturated humidity at 37°C in the incubator containing 5% CO2. After the cells were observed immobile on the slides under the microscope, the medium was washed away with sterile PBS for 5 minutes in the incubator. The samples were fixed with 4% paraformaldehyde in water, for 15-20 minutes on the bench. The slides were washed three times with PBS for 5 minutes and mounted on a microscope slide wit Fluoroshield™ mounting medium without DAPI (Sigma Aldrich).

3.5. Interferon activation in THP-1 wt cells

Cells described above have a luciferase reporter for interferon production, which is measured by Quanti-Luc™, following supplier’s recommendations. The cells were transfected with Lipofectamine™ RNAiMAX according to supplier’s protocol. 100.000 cells per well were used. The amount of compound transfected was calculated to give 200 nM 2’3’-cGAMP (1) and 1,1 µM 2’3’-cGAMP (2) end concentrations in the well. An empty transfection with no compound served as an indicator of the effect of transfection on interferon production. 48 hours after transfection, 20 µl of the cell medium was transferred to an opaque white 96-well plate in
technical duplicates and 50 μl Quanti-Luc was injected to then measure the luminescence of the well via a Tecan Microplate Reader as relative light units (RLUs).

3.6. Cloning, expression and purification

Human STING AA139-379 and mouse STING AA138-378 constructs employed for ITC studies were cloned, expressed and purified according to previous studies.[1] The plasmids were used to transform E. coli Rosetta (DE3) protein expression strain cells (Novagen). The cells were grown in 1 L of Turbo BrothQ media (Molecular Dimensions) supplemented with Kanamycin (50 mg/mL) and Chloramphenicol (34 mg/mL) at 37°C to an OD600 = 1.3 and expression was induced by adding IPTG to a final concentration of 0.2 mM.

4. TWO-PHOTON EXCITATION IMAGING AND LIFETIME ANALYSIS OF cGAMP IN THP-1 CELLS

Fluorescence imaging was carried out with two-photon excitation (TPE) imaging at constant laser power to first compare the mean brightness per cell area of THP-1 cells (wild type and STING knock-out) in the presence and absence of CDNs. To prove the presence of CDNs in cells, we additionally performed lifetime analysis.

4.2. Brightfield and Confocal laser scanning microscopy

Imaging was carried out on a home-built, multimodal confocal scanning microscope with bright-field illumination and camera.[2] Briefly, a two-photon excitation source was a fiber-based, frequency-double erbium laser (FemtoFiber dichro bioMP; Toptica Photonics) running at 774 nm served as two-photon excitation. The laser light was coupled into the microscope via a low pass dichroic mirror (HC BS 749 SP; AHF Analysetechnik) that separates laser excitation and fluorescence emission. Scanning of the sample was achieved by using a xyz piezo stage (BIO3.200; PiezoConcept). The laser excitation was focused onto the sample with a 60x (water) 1.20-NA plan apochromat objective (Plan APO VC 60x 1.2 NA, Nikon). The emission was collected by the same objective and passed afterwards through a dichroic filter (HC BS 614 SPUV; AHF Analysetechnik). After spectrally dividing the emission signal, the green channel (< 610 nm) was filtered with a 447/60 bandpass (447/60 BrightLine HC; AHF Analysetechnik) for CDN detection and the red channel > 610 nm with a 620/60 bandpass filter (620/60 ET Bandpass; AHF Analysetechnik) for cellular auto-fluorescence. To ensure blocking of the excitation laser wavelength, an additional 780 notch filter (BNF-785-OD4-11M; Opti-Grate) was mounted in the red channel (> 610 nm). The emission was recorded on two APD detectors (< 610 nm: Count Blue; > 610 nm: Count Red; Laser Components) and its photon streams registered using a TCSPC card (TH260 pico dual; PicoQuant GmbH). The experiment was controlled using a home-written program written in C#. The confocal data was extracted and evaluated afterwards by PAM[3] and Fiji[4]. Lifetime data was analyzed using the Phasor approach.[5]

Brightfield pictures of the scanned area were taken before the experiment followed by a confocal scan. Each 2D image had a range of 100 μm, an acquisition time of 180 s and a step size of 200 nm. The laser power was 2.28 mW. For brightness comparison, the sum over triplets was compared to avoid scanning artefacts and increase statistics.
4.3. Brightness analysis

For comparing the brightness quantitatively, it was necessary to determine the mean brightness of all measured cells. The data analysis process carried out in Fiji\cite{Fiji} is exemplary shown for one data set in Supplementary Fig. S4.1. After cumulating over three scans (step 1), the resulting images were converted into binary images (step 2), with a threshold of 7 kHz. The binary image enables the automatic cell detection and analysis using the ‘Analyze Particles’ plugin (step 3), in order to distinguish between inner and outer cell area in the scan image and define the region of interests (ROI). The overlay between the map of ROIs and the averaged scan image is depicted after step 4. The average brightness, volume, size and shape per cell is extracted afterwards within the ROIs (step 5). Since only cells contribute to the average brightness, the results can be compared between the different conditions (see Fig. 4c).

Figure S4.1: Brightness analysis of fluorescence imaging data. Step 1: Accumulate scan images. Step 2: Conversion into binary image. Step 3: Identification of cells including their size and shape. Step 4: Application of ROI on cumulated image. Step 5: Determination of average fluorescence per cell area.

4.4. Phasor analysis of fluorescence lifetime

Fluorescence lifetime imaging exploits the time a fluorophore spends in an excited state before emitting a photon in order to distinguish and characterize the local micro-environment. After a molecule is electronically excited to a higher energy level $S_n$ by absorbing a photon, it will return to the electronic ground state $S_0$ via different decay (radiative and/or non-radiative) pathways. In the ensemble, the molecule relaxes to the ground state via different decay paths whose likelihood is statistically given by their decay rate $k_i$. Fluorescence refers to the radiative process occurring from the lowest excited electronic state $S_1$. The observed time the molecule needs for its emissive return to the electronic ground state is called lifetime $\tau$. It is equal to the temporal decay of the observed fluorescence $I(t)$ to which it is linked via

\[
I(t) = I_0 \cdot e^{-t/\tau} \quad \text{and} \quad \frac{1}{\tau} = \sum k_i \quad \text{Eq. (1)}
\]

The precise likelihood for the accessible pathways, and hence detected fluorescence lifetime depends on multiple factors, not only the molecule but also its local environment. The fluorescent signal will hence show differences in the exponential decay (Figure S4.2a).
Figure S4.2: Phasor analysis of fluorescence Lifetime imaging data. a) Fluorescence lifetime decays with different durations. b) Phasor-Transformation of lifetimes. c) FLIM image (color-coded according to the lifetime extracted from the phasor plot). d) Conversion of lifetime information derived from the phasor analysis in e) into pixel ‘brightness’ within the FLIM image. e) Phasor plot of the pixel wise lifetimes measured in a confocal fluorescence microscope image with TCSPC. f) Theoretical phasor plot for a two-component mix, e.g., the cellular autofluorescence. The mixture (purple dot) is located on the weighted vector connecting the pure species. g) Theoretical phasor plot of a three-component mix upon addition of a third fluorescent species with long lifetime. The purple population would be observed as mixed population between all components, i.e., as mixed population connecting the signatures of the THP-1 autofluorescence and the the added compound 2’3’-cGAMP.

Fluorescence lifetimes can be determined in the temporal domain by exponential fitting. For samples with complex photo-chemistry or mixtures of fluorescent species with different lifetimes, a phasor transformation[6] is employed to unravel the underlying lifetime components. Based on time-correlated single-photon counting (TCSPC), the histogram of the photon arrival times is transformed into Fourier space. The phasor coordinates $g$ and $s$ of the phasor plot (Figure S4.2b) equals the sine and cosine Fourier components of the transformation and are derived using the following relations
\[
g(\omega) = \int_0^T I(t) \cdot \cos(n \omega t) \, dt / \left( \int_0^T I(t) \, dt \right) \quad \text{Eq. (2a)}
\]
\[
s(\omega) = \int_0^T I(t) \cdot \sin(n \omega t) \, dt / \left( \int_0^T I(t) \, dt \right) \quad \text{Eq. (2b)}
\]

in which \( T \) is repetition frequency of the data acquisition and \( n \) and \( \omega \) the harmonic and angular frequency of excitation. The values of \( g \) range between 0 and 1, while \( s \) scales 0 and 0.5. The coordinates \((g, s)\) per decay are shown in polar coordinates, where \( m \) is the modulus of the phasor and \( \phi \) the phase. In the phasor transformation, mono-exponential fluorescent decays appear on the universal semicircle with decreasing lifetime from left to right (as shown in Figure S4.2a-b qualitatively for the decays and data points depicted in blue, red and green).

In Fluorescence Lifetime Imaging (FLIM), the lifetime for each individual pixel of an image is analyzed (Figure S4.2c-d). Using the phasor approach on FLIM measurements allows for a graphical visualization of the detected lifetimes per pixel and prevents the need of a complex fit-based analysis.\(^{[5b, 7]}\) In this case, the sine and cosine Fourier components of the lifetime decay for every pixel of the image are calculated, resulting in the \( i \) times \( j \) pairs of phasor coordinates \( g_{i,j} \) and \( s_{i,j} \)

\[
g_{i,j}(\omega) = \int_0^T I_{i,j}(t) \cdot \cos(\omega t - \phi_{\text{inst}}) \, dt / \left( m_{\text{inst}} \cdot \int_0^T I_{i,j}(t) \, dt \right) \quad \text{Eq. (3a)}
\]
\[
s_{i,j}(\omega) = \int_0^T I_{i,j}(t) \cdot \sin(\omega t - \phi_{\text{inst}}) \, dt / \left( m_{\text{inst}} \cdot \int_0^T I_{i,j}(t) \, dt \right) \quad \text{Eq. (3b)}
\]

\( I_{i,j}(t) \) refers to the number of photon counts in the photon arrival histogram at time bin \( t \) at the corresponding pixel \((i,j)\). For FLIM, the frequency \( \omega \) corresponds to \( 2m/T \), with \( T \) being the repetition timescale of the lifetime decay histogram (here 12.4 ns). The phase shift and demodulation due to the instrument response function are corrected by \( \phi_{\text{inst}} \) and \( m_{\text{inst}} \). They are determined from a calibration measurement of samples with known lifetime (such as a solution of Atto532 in PBS (Atto-Tec) with a lifetime of 3.8 ns).

The phasor coordinates \( g \) and \( s \) of a population allow for determining the phase \((\tau_\phi)\) and modulation \((\tau_m)\) lifetime according to

\[
\tau_\phi(\omega) = \frac{1}{\omega} \cdot \frac{s}{g} \quad \text{Eq. (4a)}
\]
\[
\tau_m(\omega) = \frac{1}{\omega} \cdot \sqrt{\frac{1}{g^2 + s^2} - 1} \quad \text{Eq. (4b)}
\]

As a result, samples with a pure mono-exponential decay are located directly on the semicircle of the phasor plot (Figure S4.2e or S4.2b). For this case, the phase and modulation state the same value and show the correct lifetime. In mixed populations, lifetime times add up according to the weighted abundance of each compound. The mixture of two species lies on a straight line connecting the phasors of the pure species (Figure S4.2f), as observed in the case of the autofluorescence of THP-1 cells that is dominated by two lifetime species. In the case of three or more components \( k \) (Figure S4.2g), the mix is located inside the \( k \)-sided polygon where the corners are given by the pure lifetime species. This holds in particular true for multi-exponential species. In order to derive a single apparent lifetime, the mean phase and
modulation were calculated for all pixels above a threshold of ~300 photons. The lifetime of the sample then consists of the arithmetic average of the different lifetimes.

**Lifetime analysis via the software PAM – PIE analysis in MATLAB:**

The lifetime data is analyzed by a self-written program PAM – PIE analysis in MATLAB[3]. At first the calibration sample Atto532 is loaded into the program and set as reference (known lifetime of 3.8ns). Following, the measurements of the different cells are evaluated. For the phasor plots, an averaging of the photons by 3x3 matrix was calculated.
5. SUPPLEMENTAL FIGURES

Figure S5.1

![Graph showing binding affinity of CDNs to the STING receptor in humans and mice.](image)

**Figure S5.1:** Binding affinity of CDNs to the STING receptor in humans and mice. For monitoring the binding of CDNs to a) murine and b) human STING, differential scanning fluorimetry was performed using 10 μM mSTING/hSTING with 500 μM of ligand. The physiological compounds 2′3′-cGAMP and 3′3′-cGAMP were used as control.

Figure S5.2

![Graph showing interferon activation by 2′3′-cGAMP and 2′3′-cthGAMP.](image)

**Figure S5.2:** Interferon activation by 2′3′-cGAMP and 2′3′-cthGAMP. Both CDN are biologically active and lead to interferon production. By quantifying the expressed luciferase-based reporter for interferon activation, a 4-fold lower activation was found for 2′3′-cthGAMP and compared to 2′3′-cGAMP. The mean values and standard deviations represent the average of biological repeats with n = 4. The interferon induction is significant at a level of 0.05 according to a two-sample t-test.
Figure S5.3: Two-photon microscopy. a) Experimental setup with two-photon excitation at 774 nm for confocal fluorescence microscopy including lifetime information. b) Energy diagram of fluorescence with direct and two-photon excitation. c) Fluorescence emission of 2 mM of 2'3'-cGAMP in water between 400 and 650 nm as function of laser power. The quadratic dependence confirms a two-photon dependence of the excitation for the molecule.
Figure S5.4

**a)** Calibration curves for FLIM measurements. The fluorescence decay of 200 nM 2’3’-cGAMP in water, PBS, and cell medium. Increasing salinity leads to fluorescence quenching.

**b)** Phasor analysis for determining the fluorescence lifetime of 200 nM 2’3’-cGAMP in PBS (top) and cell medium (middle) and 1 μM Atto532 (bottom).

**c)** Phasor analysis of the fluorescence lifetime distribution of THP-1 STING KO cells in medium only (top), in presence of 2’3’-cGAMP (middle) and in presence of 2’3’-cGAMP (bottom). Upon addition of fluorescent 2’3’-cGAMP, the average detected lifetime shifts towards the population of the pure 2’3’-cGAMP in solution (visualized by the black bar connecting the pure 2’3’-cGAMP and the autofluorescence). For 2’3’-cGAMP, neither a shift in the phasor plot nor change in lifetime is observed.

Color bar encodes from blue to red (i.e. from lowest to highest occurrence).
6. **Supplemental References**

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