New Approach for the Synthesis of c-di-GMP and Its Analogues

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Abstract: The synthesis of cyclic bis(3′-5′)diguanylic acid (c-di-GMP) by using a new flexible approach is reported. The flexibility of the method is exemplified by the synthesis of base-modified analogues that will find applications in the elucidation of c-di-GMP biological modes of action.

Key words: c-di-GMP, nucleotide, cyclization, nucleobases, phosphotriester methodology

Cyclic bis(3′-5′)diguanylic acid (c-di-GMP) **1** (Figure 1) has been recently identified as a universal bacterial secondary messenger.[1–5] It was first related to the regulation of the cellulose synthesis in the bacterium *Acetobacter xylinum*[6] but it became clear later that c-di-GMP is involved in several biological events including regulation of biofilm formation in *Vibrio cholerae*[7] and *Yersinia pestis*,[8] and the transition from motility to sessility of *Escherichia coli* and *Salmonella typhimurium*.[9] It is involved in the inhibition of *Staphylococcus aureus* cell–cell interactions and biofilm formation, as well as in the reduction of the virulence of the biofilm-forming strains of the same bacterium in a mouse model of mastitis infection.[10] The biological activity might be even wider since reports have pointed out that this compound may have anticancer activity.[11]

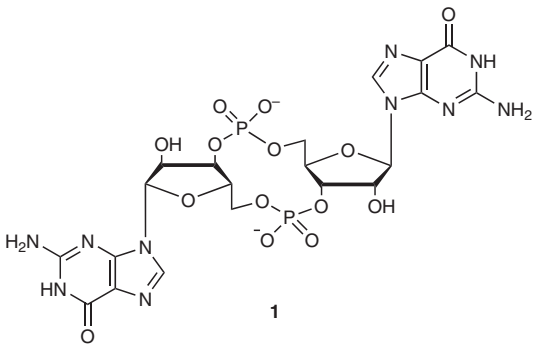

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Figure 1 c-di-GMP

Thus, c-di-GMP represents an excellent platform for drug design in medicinal chemistry and especially in the field of antibiotics where compounds with new modes of action are required. However, the mechanisms of c-di-GMP dependent signaling remain unknown, mainly because very little data is available on c-di-GMP targets.[1,4] In order to study the biochemistry of c-di-GMP in more detail we have started a research program dedicated to the synthesis of c-di-GMP and its analogues. Because the literature procedures start from protected guanosines and are therefore limited to the synthesis of c-di-GMP,[6,12–15] we have designed a new and flexible approach towards c-di-GMP and its base derivatives.

We have decided to develop a new approach where a cyclic sugar backbone **7** was synthesized first and the base was introduced at the latest stage. The synthesis started from readily available furanose building blocks **2** and **3**[16,17] (Scheme 1). The phosphotriester methodology[6,18] was used to produce dimer **5**, which was deprotected with ceric ammonium nitrate (CAN) in methanol[19] (**5** → **6**). In the next step, cyclization was achieved using 2-chlorophenyl phosphorodichloridate in dilute pyridine solution. Two fractions with notably different R_f values on TLC, which contain, due to the chiral phosphates, three stereoisomers of **7**, were isolated. The highest yields were obtained after an hour of reaction time, any prolonged reaction resulted in cleavage of phosphate linkages. The cyclic sugar building block **7** was produced in yields of 33% over three steps, and the synthesis could be reproduced in a multi-gram scale.

In this paper we describe the synthesis of c-di-GMP using the higher-R_f fraction. However, the lower-R_f fraction gives equally good results and leads in the same way to c-di-GMP. In the next step, the cyclic acetals were cleaved and replaced by acetates[20] that are required for the base introduction reactions (Scheme 2). Modified Vorbrüggen reaction conditions[21,22] were used to obtain fully protected c-di-GMP **9**. In the first step, the guanine building block **8**[21] was silylated. In the second step, the acetylated cyclic precursor reacted with this activated nucleophile in presence of large excess of TMSOTf. The highest yields were obtained if 7.5 equivalents of the Lewis acid were added and the reaction time did not exceed 30 minutes. Any longer reaction time resulted in degradation of the compound, most probably due to nucleophilic attack on the aryl protected phosphate linkage that results in phosphate bond cleavage. The problem of the **9** versus **7** selectivity encountered in the guanine base introduction was addressed by using the bulky *p*-nitrophenylethyl (Npe) protecting group at the O-6 position and high reaction temperature as previously reported.[21,23] The solvent of the reaction was very important and toluene gave the highest yields of the protected c-di-GMP **9**. The *o*-chlorophenyl as well as the Npe groups were removed using *syn*-pyri-

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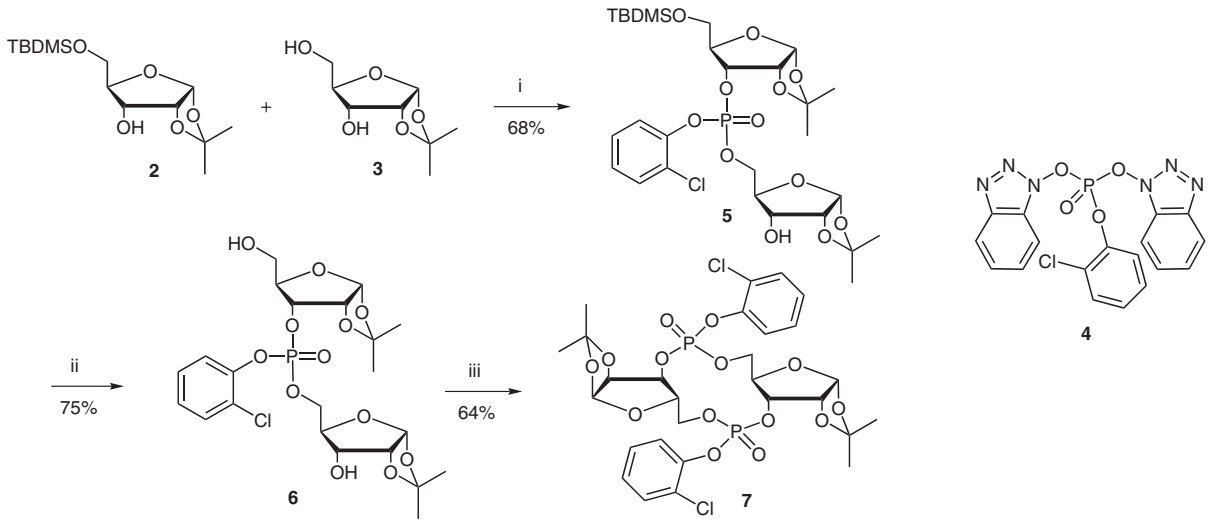


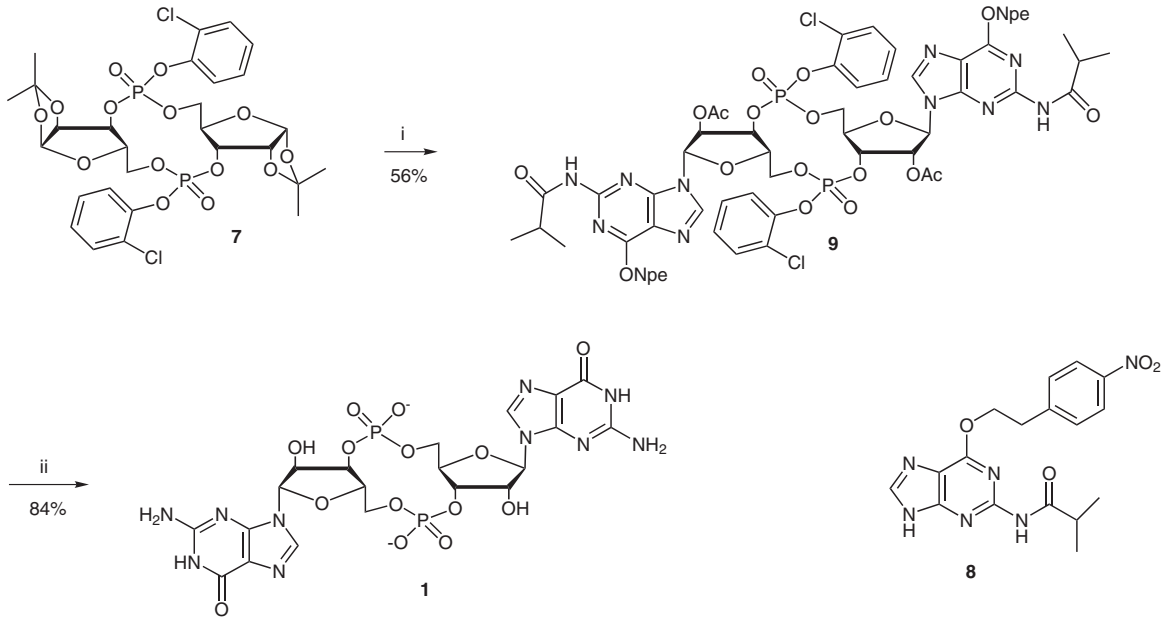
Scheme 1 Synthesis of cyclic sugar backbone **5**. *Reaction conditions*: (i) **4**, THF, 4 Å molecular sieves; (ii) CAN, MeOH; (iii) 2-chlorophenyl phosphorodichloridate, pyridine.

dine-2-carbaldoxime in the presence of *N*,*N*,*N*,*N*-tetramethylguanidine.[6] Therefore the use of DBU[23] for the deprotection of the Npe group was not necessary.

Finally, the acetyl and isobutyryl protecting groups were removed by treatment with aqueous ammonium hydroxide for two days at 50 °C.[6] Pure c-di-GMP **1** was obtained after purification using gel filtration chromatography and HPLC.

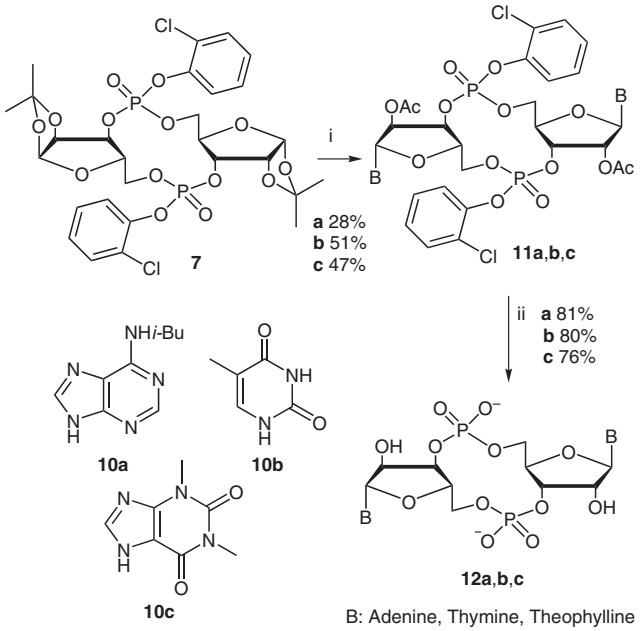
Using this completely new approach, the bacterial secondary messenger was synthesized in an efficient and up-scalable manner. One batch of c-di-GMP was used to study the activity of PleD, a response regulator that plays an important role in the *Caulobacter crescentus* cell cycle, and that has been shown to be part of the biofilm formation regulation system. It was shown that the GGDEF domain, also called domain of unknown function 1 (DUF1), is in fact a diguanylate cyclase domain (DGC).[2] The GGDEF domain catalyzes the biosynthesis of c-di-GMP starting from two GTP molecules. This synthetic compound was also used to crystallize PleD and to get the crystal structure of a c-di-GMP binding protein.[24]

Although these results are of prime importance, the elucidation of the biochemistry of c-di-GMP remains to be achieved. We believe that base-modified analogues can be useful in addressing this problem. Following our new approach, we have synthesized three new derivatives of c-di-GMP and proved that our synthesis can be employed to synthesize this new class of compounds.



Scheme 2 Synthesis of c-di-GMP. *Reaction conditions*: (i) AcOH, Ac_2O, H_2SO_4; **8**, *N*,*O*-Bis(trimethylsilyl)acetamide (BSA), $ClCH_2CH_2Cl$ (DCE), 80 °C; TMSOTf, toluene, 80 °C; (ii) *syn*-pyridine-2-carbaldoxime, *N*,*N*,*N*,*N*-tetramethylguanidine; NH_4OH, 50 °C.

The base-modified c-di-GMP **11a–c** could be synthesized from building block **7** under similar conditions (Scheme 3) as for the synthesis of **1**. Base introduction works well for thymidine and theophylline with yields of 51% and 47% to provide **11b** and **11c**, respectively. However, care had to be taken during the introduction of the adenine since degradation products were observed.



Scheme 3 Synthesis of analogues of c-di-GMP. *Reaction conditions*: (i) AcOH, Ac_2O, H_2SO_4; **10a**, **10b** or **10c**, BSA, DCE, 80 °C, respectively; TMSOTf, toluene, 80 °C; (ii) *syn*-pyridine-2-carbaldoxime, *N*,*N*,*N*,*N*-tetramethylguanidine; NH_4OH, 50 °C.

The highest yields were obtained with an equimolar ratio of adenine building block **10a**.[25] Moreover, the purification by silica gel chromatography had to be carried out quickly after the work-up, otherwise the base would cleave the chlorophenyl protected phosphate group. Final deprotection was achieved following the same reaction sequence used in the synthesis of c-di-GMP and gave **12a**, **12 b** and **12c** with yields of about 80%.

In summary, we have successfully designed a new synthetic pathway towards c-di-GMP. The synthesis of different based modified analogues has also shown that this procedure is flexible and that starting from compound **7**, new molecules can be created. These compounds are expected to play an important role in the elucidation of the biological mode of action of c-di-GMP and experiments to address this question are currently under way.

Reactions were monitored by TLC on silica gel 60 F_{254} (Merck) with detection under UV and subsequent charring with 15% H_2SO_4 in MeOH. Flash column chromatography was performed on silica gel 60 (0.040–0.063 mm, Fluka). NMR spectra were recorded on Bruker VRX 500 instrument. Chemical shits are relative to TMS (0.00). When necessary, assignments were based on DEPT, COSY, HMQC, HMBC, NOESY and TOCSY. MALDI-ToF spectra were measured with an Applied Biosystems Voyager DE Pro using *p*-nitroaniline as matrix. Chemicals were purchased from Fluka AG and Sigma-Aldrich Chemical Company Inc. TEAC buffer was prepared by bubbling CO_2 through a 0.25 M Et_3N solution in H_2O until pH 7.0.

Phosphorylating Agent 4 (1 M in THF)

To a solution of HOBt (13.78 g, 0.1 mol, 2.03 equiv) and pyridine (8.33 mL, 0.1 mol, 2.07 equiv) in THF (25 mL) was added a solution of 2-chlorophenyl phosphorodichloridate (8.07 mL, 0.05 mol, 1 equiv) and THF (7 mL) under N_2 at r.t. After a white suspension had crashed out from the clear solution, the stirring was continued for 18 h. The solution was filtered under N_2 providing a 1 M stock solution of phosphorylating agent that can be kept for several weeks in the freezer.

(5′-*O*-*tert*-Butyldimethylsilyl-1′,2′-bis-*O*-isopropylidene-D-ribofuranosyl)-(3′-5′)-(1′,2′-bis-*O*-isopropylidene-D-ribofuranosyl)-2-chlorophenyl Phosphate (5)

5′-*O*-*tert*-Butyldimethylsilyl-1′,2′-bis(*O*-isopropylidene)-D-ribofuranoside (**2**; 2.0 g, 6.6 mmol, 1.0 equiv) was dissolved in THF (60 mL) and 4 Å molecular sieves were added. The solution was then stirred for 3 h at r.t. under N_2. Phosphorylating agent **4** (7.9 mL of 1 M stock solution, 7.9 mol, 1.2 equiv) was then added at r.t. under N_2. The mixture was stirred for 15 min after which time only zero-mobility product was detected on TLC. 1′,2′-Bis(*O*-isopropylidene)-D-ribofuranoside (**3**; 1.88 g, 9.9 mmol, 1.5 equiv) in THF (30 mL) and 4 Å molecular sieves were added quickly and the mixture was stirred for 1 h. The mixture was then filtered through Celite and washed with CH_2Cl_2 (200 mL). The organic phase was washed with 0.1 M TEAC buffer (200 mL) and then brine (200 mL). The organic phase was then dried ($MgSO_4$), filtered and the solvent was removed under reduced pressure. Flash chromatography using a gradient from hexane (100%) to hexane–EtOAc (50:50) provided **5** (2.98 g, 68%, mixture of 2 diastereoisomers) as a colorless oil; R_f 0.61 (hexane–EtOAc, 50:50).

Diastereoisomer 1

^{1}H NMR (500.0 MHz, DMSO-d_6): δ = 7.58 (d, *J* = 7.9 Hz, 1 H, H_{arom}), 7.44 (d, *J* = 11.7 Hz, 1 H, H_{arom}), 7.38 (m, 1 H, H_{arom}), 7.26 (m, 1 H, H_{arom}), 5.75 (d, *J* = 0.3 Hz, 1 H, H-1′a), 5.67 (d, *J* = 3.6 Hz, 1 H, H-1′b), 5.32 (d, *J* = 6.8 Hz, 1 H, H-3′b), 4.72 (t, *J* = 4.3 Hz, 1 H, H-2′a), 4.63 (m, 1 H, H-3′a), 4.47 (m, 1 H, H-2′b), 4.43 (m, 1 H, H-5′b), 4.16 (sext, *J* = 5.8 Hz, 1 H, H-5′b), 4.01 (m, 1 H, H-4′a), 3.95 (m, 1 H, H-4′b), 3.83 (m, 1 H, H-5′a), 3.75 (m, 1 H, H-3′b), 3.71 (m, 1 H, H-5′a), 1.43 (s, 6 H, CH_3a, CH_3b), 1.26 (s, 6 H, CH_3a, CH_3b), 0.82 (s, 9 H, CH_3), 0.015 (s, 3 H, CH_3), 0.008 (s, 3 H, CH_3).

^{13}C NMR (125.8 MHz, DMSO-d_6): δ = 145.9 (C_{arom}), 130.5, 128.5, 126.6, 124.4, 121.3 (CH_{arom}), 112.3 (Cq), 111.6 (Cq), 103.6 (C-1′a), 103.4 (C-1′b), 78.9 (C-2′b), 78.3 (C-4′a), 77.6 (C-4′b), 77.3 (C-2′a), 74.2 (C-3′a), 70.4 (C-3′b), 67.7 (C-5′b), 60.7 (C-5′a), 26.6 (CH_3a), 26.6 (CH_3b), 26.4 (CH_3a), 26.4 (CH_3b), 25.8 (CH_3), 18.0 (Cq), –5.4 (CH_3), –5.4 (CH_3).

Diastereoisomer 2

^{1}H NMR (500.0 MHz, DMSO-d_6): δ = 7.58 (d, *J* = 7.9 Hz, 1 H, H_{arom}), 7.44 (d, *J* = 11.7 Hz, 1 H, H_{arom}), 7.38 (m, 1 H, H_{arom}), 7.26 (m, 1 H, H_{arom}), 5.75 (d, *J* = 0.3 Hz, 1 H, H-1′a), 5.62 (d, *J* = 3.6 Hz, 1 H, H-1′b), 5.32 (d, *J* = 6.8 Hz, 1 H, OH-3′b), 4.76 (t, *J* = 4.3 Hz, 1 H, H-2′a), 4.59 (m, 1 H, H-3′a), 4.46 (m, 1 H, H-2′b), 4.43 (m, 1 H, H-5′b), 4.16 (sext, *J* = 5.8 Hz, 1 H, H-5′b,), 4.01 (m, 1 H, H-4′a), 3.95 (m, 1 H, H-4′b), 3.79 (m, 1 H, H-5′a), 3.74 (m, 1 H, H-3′b), 3.60 (m, 1 H, H-5′a), 1.46 (s, 6 H, CH_3a, CH_3b), 1.29 (s, 6 H, CH_3a, CH_3b), 0.81 (s, 9 H, CH_3), –0.011 (s, 3 H, CH_3), –0.015 (s, 3 H, CH_3).

^{13}C NMR (125.8 MHz, DMSO-d_6): δ = 146.5, 146.0 (C_{arom}), 130.6, 128.5, 126.7, 124.4, 121.35 (CH_{arom}), 112.4 (Cq), 111.6 (Cq), 103.7 (C-1′a), 103.6 (C-1′b), 78.9 (C-2′b), 78.4 (C-4′a), 77.6 (C-4′b), 77.3 (C-2′a), 74.3 (C-3′a), 70.5 (C-3′b), 68.1 (C-5′b), 60.7 (C-5′a), 26.6

(CH_3a, CH_3b), 26.5 (CH_3a), 26.4 (CH_3b), 25.8 (CH_3), 18.0 (C_q), –5.5 (CH_3), –5.4 (CH_3).

Anal. Calcd for $C_{24}H_{44}ClO_{12}PSi$ (667.17): C, 50.41; H, 6.65; O, 28.78. Found: C, 50.45; H, 6.62; O, 28.77.

(1′,2′-Bis-*O*-isopropylidene-D-ribofuranosyl)-(3′-5′)-(1′,2′-bis-*O*-isopropylidene-D-ribofuranosyl)-2-chlorophenyl Phosphate (6)

Compound **5** (2.97 g, 4.45 mol, 1 equiv) was dissolved in MeOH (60 mL) and CAN (2.45 g, 4.47 mol, 1 equiv) was added and the mixture was stirred for 18 h under N_2. The mixture was diluted with EtOAc (100 mL) and 0.25 M TEAC buffer (200 mL) was added. The aqueous phase was then extracted with EtOAc (4 × 50 mL). The combined organic phases were dried ($MgSO_4$) and the solvent was removed under reduced pressure. Purification was achieved by flash chromatography over silica gel using a mixture of CH_2Cl_2–MeOH (90:10) as eluent to afford **6** (1.87 g, 76%, mixture of 2 diastereoisomers) as a colorless oil; R_f 0.23 (CH_2Cl_2–MeOH, 95:5).

Diastereoisomer 1

^{1}H NMR (500.0 MHz, DMSO-d_6): δ = 7.59 (m, 4 H, H_{arom}), 5.77 (d, *J* = 3.7 Hz, 1 H, H-1′a), 5.68 (d, *J* = 3.5 Hz, 1 H, H-1′b,), 5.32 (dd, *J* = 2.6, 4.2 Hz, 1 H, OH-3′b), 4.89 (m, 1 H, OH-5′a), 4.71 (m, 1 H, H-2′a), 4.61 (m, 1 H, H-3′a), 4.48 (br t, 1 H, H-2′b), 4.44 (m, 1 H, H-5′b), 4.18 (m, 1 H, H-5′b), 3.99 (m, 1 H, H-4′a), 3.96 (m, 1 H, H-4′b), 3.79 (m, 1 H, H-3′b), 3.69 (m, 1 H, H-5′ a), 3.47 (m, 1 H, H-5′a), 1.44 (s, 6 H, CH_3a, CH_3b), 1.27 (s, 6 H, CH_3a, CH_3b).

Diastereoisomer 2

^{1}H NMR (500.0 MHz, DMSO-d_6): δ = 7.59 (m, 4 H, H_{arom}), 5.77 (d, *J* = 3.7 Hz, 1 H, H-1′a), 5.63 (d, *J* = 3.6 Hz, 1 H, H-1′b), 5.32 (dd, *J* = 2.6, 4.2 Hz, 1 H, OH-3′b), 4.86 (m, 1 H, OH-5′a), 4.75 (m, 1 H, H-2′a), 4.61 (m, 1 H, H-3′a), 4.47 (br t, 1 H, H-2′b), 4.44 (m, 1 H, H-5′b), 4.18 (m, 1 H, H-5′b), 3.99 (m, 1 H, H-4′a), 3.96 (m, 1 H, H-4′b), 3.77 (m, 1 H, H-3′ b), 3.63 (m, 1 H, H-5′a), 3.47 (m, 1 H, H-5′a), 1.44 (s, 6 H, CH_3a, CH_3b), 1.29 (s, 3 H, CH_3a), 1.27 (s, 3 H, CH_3b).

^{13}C NMR (125.8 MHz, DMSO-d_6): δ = 146.5, 146.0 (C_{arom}), 130.6, 128.6, 126.61, 121.41 (CH_{arom}), 112.2 (Cq), 111.6 (Cq), 103.6 (C-1′a), 103.4 (C-1′b), 79.0 (C-2′b), 78.6 (C-4′a), 77.6 (C-4′b), 77.4 (C-2′a), 74.6 (C-3′a), 70.4 (C-3′b), 67.9 (C-5′b), 59.1 (C-5′a), 26.6 (CH_3a, CH_3b), 26.4 (CH_3a, CH_3b).

Anal. Calcd for $C_{22}H_{30}Cl_2O_{12}P$ (552.90): C, 47.79; H, 5.47; O, 34.72. Found: C, 47.62; H, 5.67; O, 34.70.

Cyclic Bis(3′-5′)-(1′,2′-bis-*O*-isopropylidene-D-ribofuranosyl)-2-chlorophenyl Phosphate (7)

Dimer **6** (1.80 g, 3.26 mmol, 1 equiv) was co-evaporated with pyridine (2 × 10 mL) and dissolved in pyridine (900 mL). 2-Chlorophenyl phosphorodichloridate (0.79 mL, 4.89 mmol, 1.5 equiv) was added and the mixture was stirred at r.t. under N_2 for 1 h. The solvent was evaporated, the residue taken up in CH_2Cl_2 (200 mL) and washed with 0.25 M TEAC buffer (200 mL). The organic phase was dried ($MgSO_4$) and the solvent was removed under reduced pressure. Flash chromatography over silica gel was used for the purification with a gradient from hexane (100%) to hexane–EtOAc (50:50). Two fractions containing the different possible stereoisomers of **7** were isolated. The first fraction of higher R_f on silica gel TLC plate was isolated as a white oily residue (683 mg, 29%) whereas the lower-R_f fraction was isolated as a colorless oil (826 mg, 35%).

High-R_f Stereoisomer 7

R_f 0.71 (hexane–EtOAc, 30:70).

^{1}H NMR (500 MHz, DMSO-d_6): δ = 7.73 (m, 8 H, H_{arom}), 5.88 (d, *J* = 3.5 Hz, 1 H, H-1′a), 5.75 (d, *J* = 3.6 Hz, 1 H, H-1′b), 4.93–4.87 (m, 2 H, H-3′a, H-2′b), 4.85–4.81 (m, 1 H, H-3′b), 4.74 (t, *J* = 4.2 Hz, 1 H, H-2′b), 4.52–4.45 (m, 2 H, H-5′a, H-5′b), 4.34–4.24 (m, 4 H, H-5′a, H-5′b, H-4′a, H-4′b), 1.52, 1.43, 1.32, 1.27 (4 s, 12 H, CH_3a, CH_3b, CH_3a, CH_3b).

^{13}C NMR (125.8 MHz, DMSO-d_6): δ = 145.7, 145.6 (C_{arom}), 130.8, 130.6, 128.8, 128.5, 127.0, 126.8, 121.4, 121.0 (CH_{arom}), 112.8, 112.6 (Cq), 103.6 (C-1′a), 103.5 (C-1′b), 77.4 (C-2′a), 77.3 (C-2′b), 74.4 (C-4′a), 74.3 (C-4′b), 74.1(C-3′a), 74.1 (C-3′b), 65.2, 65.1 (C-5′a, C-5′b), 26.6, 26.4, 26.3 (CH_3).

^{31}P NMR (202 MHz, DMSO-d_6): δ = –8.35, –8.52.

Anal. Calcd for $C_{28}H_{32}Cl_2O_{14}P_2$ (724.06): C, 46.36; H, 4.45; O, 30.88. Found: C, 46.29; H, 4.51; O, 30.98.

Low-R_f Stereoisomer 7

R_f 0.49 (hexane–EtOAc, 30:70).

^{1}H NMR (500 MHz, DMSO-d_6): δ = 7.60–7.28 (m, 8 H, H_{arom}), 5.85 (d, *J* = 3.4 Hz, 2 H, H-1′a, H-1′b), 4.82–4.78 (m, 4 H, H-2′a, H-2′b, H-3′a, H-3′b), 4.46–4.45 (m, 4 H, H-5′a, H-5′b), 4.25–4.22 (m, 2 H, H-4′a, H-4′b), 1.42, 1.28 (2 s, 12 H, CH_3).

^{13}C NMR (125.8 MHz, DMSO-d_6): δ = 145.6 (C_{arom}), 130.6, 128.9, 128.4, 121.3 (CH_{arom}), 112.5, 112.8 (Cq), 103.7 (C-1′), 77.3 (C-2′), 74.6 (C-4′), 73.5 (C-3′), 65.7 (C-5′), 26.5, 26.3 (CH_3).

^{31}P NMR (202.5 MHz, DMSO-d_6): δ = –8.08.

Anal. Calcd for $C_{28}H_{32}Cl_2O_{14}P_2$ (724.06): C, 46.36; H, 4.45; O, 30.88. Found: C, 46.27; H, 4.49; O, 30.95.

Cyclic Bis(3′-5′)-(2′-*O*-acetyl-2-*N*-isobutyryl-6-*O*-*p*-nitrophenylethylguanosine)-2-chlorophenyl Phosphate (9)

Cyclic compound **7** (670 mg, 0.93 mmol, 1 equiv) was dissolved in glacial AcOH (10 mL). Ac_2O (1 mL, 10.6 mmol, 11 equiv) was added as well as H_2SO_4 (0.4 mL) and the mixture was stirred for 18 h at r.t. under N_2. The mixture was then poured into ice water (50 mL) and the aqueous phase was extracted with CH_2Cl_2 (4 × 50 mL). The combined organic phases were washed with aq sat. $NaHCO_3$ (100 mL), dried ($MgSO_4$) and the solvent was removed under reduced pressure. The residue was dissolved in CH_2Cl_2 and the solution was filtered through a short pad of silica gel. The acetylated product was used in the next step without further purification. Guanosine building block **8** (820 mg, 2.22 mmol, 3 equiv) was suspended into DCE (20 mL). BSA (1.08 mL, 4.48 mmol, 6 equiv) was added and the mixture was heated at 80 °C for 16 h in a sealed flask. The excess of BSA and DCE were removed by evaporation under reduced pressure. The resulting residue was dissolved in toluene (20 mL). TMSOTf (0.70 mL, 3.87 mmol, 5 equiv) was added together with the previously obtained cyclic acetylated precursor (600 mg, 0.74 mmol, 1 equiv) dissolved in toluene (10 mL). The mixture was stirred for 30 min at 80 °C in a sealed flask. The mixture was diluted with EtOAc (60 mL) and the solution was washed with 0.25 M TEAC buffer (100 mL). The aqueous layer was then extracted with EtOAc (2 × 100 mL). The combined organic phases were dried ($MgSO_4$), filtered and the solvent was evaporated under reduced pressure. The cyclic diguanosine product **9** was purified by flash chromatography over silica gel column using a gradient from pure CH_2Cl_2 to CH_2Cl_2–MeOH (95:5). Fully protected c-di-GMP **9** was obtained as a white oily residue (779 mg, 66%); R_f 0.53 (CH_2Cl_2–MeOH, 95:5).

^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.78, 8.70 (2 s, 2 H, NH), 8.14 (m, 4 H, H_{arom}, Npe), 7.85, 7.76 (2 s, 2 H, H-8a, H-8b), 7.52 (m, 4 H, H_{arom}, Npe), 7.50–7.00 (m, 8 H, H_{arom}, chlorophenyl), 6.33 (m, 1 H, H-3′a), 6.19 (dd, *J* = 4.6, 5.6 Hz, 1 H, H-2′a), 6.15 (dd, *J* = 4.0, 5.2 Hz, 1 H, H-2′b), 6.02 (d, *J* = 4.4 Hz, 1 H, H-1′b,), 5.99 (m, 1 H, H-3′b), 5.86 (d, *J* = 3.6 Hz, 1 H, H-1′a,), 4.98 (ddd, *J* = 6.5, 7.8, 10.7 Hz, 1 H, H-5′b), 4.91–4.80 (m, 4 H, OCH_2, Npe), 4.70 (m, 1 H, H-4′b), 4.65–4.49 (m, 3 H, H-5′a, H-4′a, H-5′a), 4.45 (ddd, *J* = 3.6,

5.0, 10.7 Hz, 1 H, H-5′b), 3.34–3.30 (m, 4 H, CH_2C, NPe), 2.92 (br d, 2 H, CH, isobutyryl), 1.99, 1.94 (2 s, 6 H, CH_3, acetyl), 1.21–1.15 (m, 12 H, CH_3, isobutyryl).

^{13}C NMR (125.8 MHz, $CDCl_3$): δ = 175.9 (C=O, isobutyryl), 169.4, 169.2 (C=O, acetyl), 160.8 (C=O-4), 152.4, 152.3, 152.1, 151.9 (C-2, C-6), 146.9, 146.8, 146.0, 145.9, 145.8, 145.7 (C_{arom}), 141.1 (C-8), 130.9, 130.7, 130.1, 130.0, 128.1, 127.9, 126.9, 126.6, 125.5, 125.0, 123.8, 123.7, 121.4, 120.9 (CH_{arom}), 118.9, 118.6 (C-5), 88.0, 87.0 (C-1′), 81.2, 79.0 (C-4′), 75.6, 74.6 (C-3′), 72.4, 72.2 (C-2′), 66.1, 65.9 (C-5′), 35.8, 35.0 (CH, isobutyryl), 20.4, 20.3, 19.4, 19.3 (CH_3, acetyl, isobutyryl).

^{31}P NMR (202.5 MHz, $CDCl_3$): δ = –5.8, –9.5.

Anal. Calcd for $C_{60}H_{60}Cl_2N_{12}O_{22}P_2$ (1432.28): C, 50.25; H, 4.22; O, 24.55. Found: C, 50.14; H, 4.29; O, 24.64.

c-di-GMP 1

Fully protected c-di-GMP **9** (430 mg, 0.30 mmol) in pyridine (5 mL) and *syn*-pyridine-2-carbaldoxime (1.5 g, 12.3 mmol, 40 equiv) were mixed with *N*,*N*,*N*,*N*-tetramethylguanidine (1.35 mL, 10.7 mmol, 35 equiv). The mixture was stirred for 16 h at r.t. Aq 14 M NH_4OH (100 mL) was then added and the mixture was stirred for 2 days at 50 °C in a sealed flask. The solution was evaporated to 1/10 of its volume and then washed with CH_2Cl_2 (2 × 20 mL). A first purification step was achieved using size-exclusion chromatography with Sephadex G15 and nanopure H_2O as eluent. A final purification by reverse-phase HPLC chromatography yielded pure c-di-GMP **1** (173 mg, 84%, 99.99% pure).

^{1}H NMR (500 MHz, D_2O): δ = 7.90 (s, 2 H, H-8), 5.84 (d, *J* = 1.3 Hz, 2 H, H-1′), 4.74 (dd, *J* = 8.5, 5.0 Hz, 2 H, H-3′), 4.59 (dd, *J* = 5.0 Hz, 2 H, H-2′), 4.27 (dd, *J* = 8.5 Hz, 2 H, H-4′), 4.21, 3.96 (2 m, 4 H, H-5′).

^{13}C NMR (125.8 MHz, D_2O): δ = 158.8, 153.8 (C-4, C-6), 150.8 (C-2), 137.1 (C-8), 116.3 (C-5), 89.2 (C-1′), 79.8 (C-4′), 73.3 (C-2′), 70.5 (C-3′), 62.2 (C-5′).

HRMS-ESI: *m*/*z* [M – H]$^-$ calcd for $C_{20}H_{23}N_{10}O_{14}P_2$: 689.0870; found: 689.0871.

Cyclic Bis(3′-5′)-(2′-*O*-acetyl-6-*N*-isobutyryladenosine)-2-chlorophenyl Phosphate (11a)

Cylic compound **7** (70 mg, 0.10 mmol, 1 equiv) was dissolved in glacial AcOH (2 mL). Ac_2O (0.2 mL, 2.13 mmol, 21 equiv) was added as well as H_2SO_4 (42 μL) and the mixture was stirred for 18 h at r.t. under N_2. The mixture was then poured into ice water (10 mL) and the aqueous phase was extracted with CH_2Cl_2 (4 × 10 mL). The organic phase was washed with aq sat. $NaHCO_3$ (20 mL), dried ($MgSO_4$) and the solvent was removed under reduced pressure. The residue was dissolved in CH_2Cl_2 and the solution was filtered through a short pad of silica gel. The acetylated residue was used in the next step without further purification. Adenine building block **10a** (35 mg, 0.19 mmol, 2 equiv) was suspended in DCE (2.5 mL). BSA (83 μL, 0.39 mmol, 4 equiv) was added and the mixture was heated at 80 °C for 16 h in a sealed flask. The excess of BSA and DCE were removed by evaporation under reduced pressure. The resulting residue was dissolved in toluene (2 mL). TMSOTf (78 μL, 0.43 mmol, 5 equiv) was added together with the previously obtained cyclic acetylated precursor dissolved in toluene (2 mL). The mixture was stirred for 30 min at 80 °C in a sealed flask. The mixture was diluted with EtOAc (20 mL) and the solution was washed with 0.25 M TEAC buffer (20 mL). The aqueous layer was then extracted with EtOAc (2 × 10 mL). The combined organic phases were dried ($MgSO_4$), filtered and the solvent was evaporated under reduced pressure. The cyclic diadenosine product **11a** was purified by preparative TLC using a mixture of CH_2Cl_2–MeOH (95:5) as eluent shortly after work-up. Fully protected c-di-AMP **11a** was obtained as a colorless oily residue (29 mg, 28%); R_f 0.46 (CH_2Cl_2–MeOH, 95:5).

^{1}H NMR (500 MHz, DMSO-d_6): δ = 10.74, (s, 1 H, NHa), 10.70 (s, 1 H, NHb), 8.74 (s, 1 H, H-8a), 8.68 (s, 1 H, H-2a), 8.66 (s, 1 H, H-8b), 8.60 (s, 1 H, H-2b), 7.64 (m, 1 H, H_{arom}b), 7.49 (m, 1 H, H_{arom}a), 7.45 (m, 1 H, H_{arom}b), 7.38 (m, 1 H, H_{arom}a), 7.32 (m, 1 H, H_{arom}b), 7.31 (m, 1 H, H_{arom}b), 7.20 (m, 1 H, H_{arom}a), 7.15 (m, 1 H, H_{arom}a), 6.44 (d, *J* = 6.4 Hz, 1 H, H-1′a,), 6.38–6.44 (m, 1 H, H-1′b), 6.36 (m, 1 H, H-2′a), 6.17–6.44 (d, *J* = 5.2 Hz, 1 H, H-2′b), 5.86 (m, 1 H, H-3′b), 5.74 (m, 1 H, H-3′a), 4.87 (m, 1 H, H-4′b), 4.80 (m, 1 H, H-4′a), 4.60 (m, 2 H, H-5′a, H-5′b), 4.50 (m, 2 H, H-5′a, H-5′b), 2.94 (q, *J* = 6.9 Hz, 2 H, isobutyryl), 1.95 (s, 3 H, CH_3, acetyl a), 1.94 (s, 3 H, CH_3, acetyl b), 1.13 (d, *J* = 6.9 Hz, 12 H, CH_3, isobutyryl).

^{13}C NMR (125.8 MHz, DMSO-d_6): δ = 175.3 (C=O, isobutyryl), 169.2 (C=O, acetyl a), 169.1 (C=O, acetyl b), 151.9 (C-6a), 151.8 (C-6b), 151.6 (C-2a), 151.3 (C-2b), 150.2 (2 s, C-5), 145.6 (2 s, C-4), 145.5 (2 s, C_{arom}), 143.3 (C-8a), 143.2 (C-8b), 130.8, 130.6, 128.8, 128.2, 127.3, 126.8 (CH_{arom}), 124.3 (2 s, C_{arom}), 121.7, 120.8 (CH_{arom}), 85.7 (C-1′a), 85.1 (C-1′b), 80.5 (2 s, C-4′), 78.2 (2 s, C-3′), 71.4 (C-2′a), 70.8 (C-2′b), 65.5 (C-5′a), 65.4 (C-5′b), 39.0 (CH, isobutyryl), 20.2 (CH_3, acetyl), 19.3 (CH_3, isobutyryl).

MS (MALDI-ToF): *m*/*z* calcd for $C_{44}H_{46}Cl_2N_{10}O_{18}P_2$ (1102.13); found: 1125.13 [M + Na]$^+$.

c-di-AMP 12a

Fully protected c-di-AMP **11a** (29 mg, 0.03 mmol, 1 equiv) in pyridine (2 mL) and *syn*-pyridine-2-carbaldoxime (118 mg, 0.97 mmol, 40 equiv) was mixed with *N*,*N*,*N*,*N*-tetramethylguanidine (110 μL, 0.87 mmol, 35 equiv). The mixture was stirred for 16 h at r.t. Aq 14 M NH_4OH (30 mL) was then added and the mixture was stirred for 2 days at 50 °C in a sealed flask. The solution was evaporated to 1/10 of its volume and then washed with CH_2Cl_2 (2 × 10 mL). A first purification step was achieved using gel filtration chromatography with Sephadex G10 and a mixture of MeOH–nanopure H_2O (1:1) as eluent. A final purification by reverse-phase HPLC chromatography yielded pure c-di-AMP **12a** (14 mg, 81%).

^{1}H NMR (500 MHz, DMSO-d_6): δ = 8.45 (s, 2 H, H-8), 8.14 (s, 2 H, H-2), 7.32 (s, 4 H, NH_2), 5.88 (d, *J* = 4.4 Hz, 2 H, H-1′), 4.93 (m, 2 H, H-2′), 4.68 (t, *J* = 5.3 Hz, 2 H, H-3′), 4.22 (dd, *J* = 5.3, 9.7 Hz, 2 H, H-4′), 4.00–3.98 (m, 4 H, H-5′).

^{13}C NMR (125.8 MHz, DMSO-d_6): δ = 156.1 (C-4), 152.7 (C-6), 149.6 (C-5), 139.7 (C-8), 119.2 (C-2), 87.0 (C-1′), 81.1 (C-2′), 73.5 (C-3′), 71.3 (C-4′), 63.9 (C-5′).

HRMS-ESI: *m*/*z* [M – H]$^-$ calcd for $C_{20}H_{23}N_{10}O_{12}P_2$: 657.0972; found: 657.0967.

Cyclic Bis(3′-5′)-(2′-*O*-acetylthymidine)-2-chlorophenyl Phosphate (11b)

Compound **7** (62 mg, 0.09 mmol, 1 equiv) was dissolved in glacial AcOH (3 mL). Ac_2O (0.3 mL, 3.2 mmol, 35 equiv) was added as well as H_2SO_4 (14 μL) and the mixture was stirred for 18 h at r.t. under N_2. The mixture was then poured into ice water and the aqueous phase was extracted with CH_2Cl_2 (4 × 10 mL). The organic phase was washed with 0.1 M TEAC buffer (10 mL), dried ($MgSO_4$) and the solvent was removed under reduced pressure. The residue was dissolved in CH_2Cl_2 and the solution was filtered through a short pad of silica gel. The acetylated residue was used in the next step without further purification. Thymine **10b** (22.8 mg, 0.18 mmol, 2.1 equiv) was suspended into DCE (3 mL) and BSA (088 μL, 0.36 mmol, 4.2 equiv) was added. The mixture was heated at 80 °C for 16 h in a sealed flask. The excess of BSA and DCE were removed by evaporation under reduced pressure. The resulting residue was dissolved in toluene (3 mL). TMSOTf (117 μL, 0.68 mmol, 7.5 equiv) was added to the mixture together with the previously obtained cyclic sugar dissolved in toluene (1.5 mL). The mixture was

stirred for 30 min at 80 °C in a sealed flask. The mixture was diluted with EtOAc (10 mL) and the solution was washed with 0.25 M TEAC buffer (10 mL). The aqueous layer was then extracted with EtOAc (2 × 10 mL). The combined organic phases were dried ($MgSO_4$), filtered and the solvent was evaporated under reduced pressure. Flash chromatography (gradient of CH_2Cl_2 to CH_2Cl_2–MeOH, 95:5) afforded a white oily residue (41.4 mg, 51%); R_f 0.41 (CH_2Cl_2–MeOH, 9:1).

^{1}H NMR (500.0 MHz, DMSO-d_6): δ = 7.73 (m, 2 H, H-6), 7.63 (m, 2 H, H_{arom}), 7.46 (m, 2 H, H_{arom}), 7.43 (m, 2 H, H_{arom}), 7.31 (m, 2 H, H_{arom}), 5.92 (d, *J* = 5.9 Hz, 2 H, H-1′), 5.63 (t, *J* = 5.9 Hz, 2 H, H-2′), 5.36 (m, 2 H, H-3′a), 4.70 (m, 2 H, H-5′), 4.38 (m, 4 H, H-4′, H-5′), 1.89 (s, 6 H, CH_3), 1.76 (s, 6 H, CH_3, acetyl).

^{13}C NMR (125.8 MHz, DMSO-d_6): δ = 169.1 (C=O, acetyl), 163.6 (C=O-4), 150.4 (C=O-2), 145.6 (C_{arom}), 136.9 (C-6), 130.8, 128.8, 127.2 (CH_{arom}), 124.4 (C_{arom}), 121.6 (CH_{arom}), 110.1 (C-5), 86.7 (C-1′), 79.1 (C-4′), 73.5 (C-3′), 70.6 (C-2′), 65.5 (C-5′), 20.2 (CH_3, acetyl), 12.0 (CH_3).

MS (MALDI-ToF): *m*/*z* calcd for $C_{36}H_{36}Cl_2N_4O_{18}P_2$ (944.09); found: 967.76 $[M + Na]^+$.

c-di-TMP 12b

Compound **11b** (29 mg, 0.03 mmol, 1 equiv) was dissolved in pyridine (2 mL) and treated with *syn*-pyridine-2-carbaldoxime (150 mg, 1.23 mmol, 40 equiv) as well as *N*,*N*,*N*,*N*-tetramethylguanidine (134 μL, 1.08 mmol, 35 equiv). The mixture was stirred for 16 h at r.t.. The solvents were evaporated and the residue was taken up in H_2O (10 mL) and washed with CH_2Cl_2 (2 × 10 mL). The aqueous phase was then evaporated under reduced pressure. A first purification step was achieved using size-exclusion chromatography with Sephadex G10 and a mixture of nanopure H_2O–MeOH (1:1) as eluent. A final purification by reverse-phase HPLC chromatography yielded pure c-di-TMP **12b** (23.2 mg, 80%).

^{1}H NMR (500.0 MHz, DMSO-d_6): δ = 11.32 (s, 2 H, NH), 9.33 (s, 1 H, OH), 8.38 (s, 1 H, OH), 7.73 (s, 2 H, H-6), 5.75 (d, *J* = 5.1 Hz, 2 H, H-1′), 4.47 (m, 2 H, H-3′), 4.19 (t, *J* = 4.9 Hz, 2 H, H-2′), 4.07 (m, 2 H, H-4′), 3.95 (m, 2 H, H-5′), 3.87 (m, 2 H, H-5′), 1.77 (s, 6 H, CH_3).

^{13}C NMR (125.8 MHz, DMSO-d_6): δ = 163.6 (C=O-4), 150.4 (C=O-2), 135.6 (C-6), 109.7 (C-5), 87.6 (C-1′), 80.0 (C-4′), 72.0 (C-3′), 71.7 (C-2′), 62.9 (C-5′), 11.9 (CH_3).

HRMS-ESI: *m*/*z* $[M - H]^-$ calcd for $C_{20}H_{25}N_4O_{16}P_2$: 639.0741; found: 639.0735.

Cyclic Bis(3′-5′)-(2′-*O*-acetyltheophylline)-2-chlorophenyl Phosphate (11c)

Compound **7** (62 mg, 0.09 mmol, 1 equiv) was dissolved in glacial AcOH (3 mL). Ac_2O (0.3 mL, 3.2 mmol, 35 equiv) was added as well as H_2SO_4 (14 μL) and the mixture was stirred for 18 h at r.t. under N_2. The mixture was then poured into ice water and the aqueous phase was extracted with CH_2Cl_2 (4 × 10 mL). The combined organic phases were washed with 0.1 M TEAC buffer (10 mL), dried ($MgSO_4$) and the solvent was removed under reduced pressure. The residue was dissolved in CH_2Cl_2 and the solution was filtered through a short pad of silica gel. The acetylated residue was used in the next step without further purification. Theophylline **10c** (32.5 mg, 0.18 mmol, 2.1 equiv) was suspended into DCE (3 mL) and BSA (88 μL, 0.36 mmol, 4.2 equiv) was added. The mixture was heated at 80 °C for 16 h in a sealed flask. The excess of BSA and DCE were removed by evaporation under reduced pressure. The resulting residue was dissolved in toluene (3 mL). TMSOTf (117 μL, 0.64 mmol, 7.5 equiv) was added to the mixture together with the previously obtained cyclic acetylated sugar dissolved in toluene (1.5 mL). The mixture was stirred for 30 min at 80 °C in a sealed flask. The mixture was diluted with EtOAc (10 mL) and the solution was washed with 0.25 M TEAC buffer (10 mL). The aqueous layer was then extracted with EtOAc (2 × 10 mL). The combined organic phases were dried ($MgSO_4$), filtered and the solvent was evaporated under reduced pressure. Flash chromatography (gradient of CH_2Cl_2 to CH_2Cl_2–MeOH, 95:5) afforded **11c** as a white residue (42 mg, 47%); R_f 0.32 (CH_2Cl_2–MeOH, 9:1).

^{1}H NMR (500 MHz, DMSO-d_6): δ = 8.49 (s, 1 H, H-8a), 8.44 (s, 1 H, H-8b), 7.63 (d, *J* = 7.6 Hz, 1 H, H_{arom}a), 7.56 (d, *J* = 7.9 Hz, 1 H, H_{arom}b), 7.47 (m, 1 H, H_{arom}b), 7.45 (m, 1 H, H_{arom}b), 7.38 (m, 1 H, H_{arom}a), 7.32 (m, 1 H, H_{arom}b), 7.26 (m, 1 H, H_{arom}a), 7.24 (m, 1 H, H_{arom}a), 6.39 (d, *J* = 6.7 Hz, 2 H, H-1′a, H-1′b), 6.08 (m, 1 H, H-2′b), 6.04 (t, *J* = 6.3 Hz, 1 H, H-2′a,), 5.62 (m, 1 H, H-3′b), 5.58 (m, 1 H, H-3′a), 4.79 (m, 1 H, H-5′a), 4.76 (m, 1 H, H-5′b), 4.51 (m, 1 H, H-4′b), 4.50 (m, 1 H, H-4′a), 4.33 (m, 2 H, H-5′a, H-5′b), 3.43 (s, 3 H, H-4a), 3.41 (s, 3 H, H-4b), 3.24 (s, 3 H, H-2a), 3.23 (s, 3 H, H-2b), 1.95 (s, 3 H, CH_3, acetyl b), 1.93 (s, 3 H, CH_3, acetyl a).

^{13}C NMR (125.8 MHz, DMSO-d_6): δ = 169.1 (C=O, acetyl b), 168.8 (C=O, acetyl a), 154.1 (C=O-3a), 153.9 (C=O-3b), 150.7 (C=O-1a), 150.6 (C=O-1a), 149.7 (C-5b), 149.6 (C-5a), 145.7 (C_{arom}b), 145.6 (C_{arom}a), 143.6 (C-8b), 142.9 (C-8a), 130.8 (CH_{arom}a), 130.7 (CH_{arom}b), 128.8 (CH_{arom}b), 128.0 (CH_{arom}a), 127.3 (CH_{arom}b), 126.7 (CH_{arom}a), 124.6 (C_{arom}a), 124.2 (C_{arom}b), 121.7 (CH_{arom}a), 120.7 (CH_{arom}b), 105.6 (C-6b), 105.5 (C-6a), 86.8 (C-1′b), 86.5 (C-1′a), 80.5 (C-4′a), 80.0 (C-4′b), 75.4 (C-3′b), 74.4 (C-3′a), 71.7 (C-2′a), 71.6 (C-2′b), 65.5 (C-5′a), 64.8 (C-5′b), 29.7 (C-4), 27.9 (C-2), 20.2 (CH_3, acetyl b), 20.1 (CH_3, acetyl a).

MS (MALDI-ToF): *m*/*z* calcd for $C_{40}H_{40}Cl_2N_8O_{18}P_2$ (1052.13); found: 1075.17 $[M + Na]^+$.

Cyclic Bis(3′-5′)-theophylline Monophosphate 12c

Compound **11c** (22 mg, 0.02 mmol, 1 equiv) was dissolved in pyridine (2 mL) and treated with *syn*-pyridine-2-carbaldoxime (150 mg, 0.84 mmol, 40 equiv) and *N*,*N*,*N*,*N*-tetramethylguanidine (92 μL, 0.73 mmol, 35 equiv). The mixture was stirred for 16 h at r.t. The solvents were evaporated and the residue was taken up in H_2O (10 mL) and washed with CH_2Cl_2 (2 × 10 mL). The aqueous phase was then evaporated under reduced pressure. A first purification step was achieved using size-exclusion chromatography with Sephadex G10 and a mixture MeOH–nanopure H_2O (1:1) as eluent. A final purification by reverse-phase HPLC chromatography yielded pure **12c** (11.8 mg, 76%).

^{1}H NMR (500 MHz, DMSO-d_6): δ = 8.51 (s, 1 H, H-8), 7.17 (s, 1 H, OH-2′), 6.12 (d, *J* = 5.8 Hz, 1 H, H-1′), 4.57 (m, 2 H, H-2′, H-3′), 4.18 (m, 1 H, H-4′), 3.91 (m, 2 H, H-5′), 3.43 (s, 3 H, H-4), 3.23 (s, 3 H, H-2).

^{13}C NMR (125.8 MHz, DMSO-d_6): δ = 154.1 (C=O-1), 150.9 (C=O-3), 148.9 (C-5), 141.0 (C-8), 105.9 (C-6), 89.1 (C-1′), 81.2 (C-4′), 73.0 (C-3′), 72.4 (C-2′), 63.3 (C-5′), 29.5 (C-4), 27.7 (C-2).

HRMS-ESI: *m*/*z* $[M - H]^-$ calcd for $C_{24}H_{29}N_8O_{16}P_2$: 747.1177; found: 747.1171.

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