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Synthesis of cyclic bis(3′-5′)diguanylic acid (*c*-di-GMP) analogs

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Abstract—This paper reports the synthesis of cyclic bis(3′-5′)diguanylic acid (*c*-di-GMP) analogs, including the monophosphorothioic acid of *c*-di-GMP (*c*-GpGps), cyclic bis(3′-5′)guanylic/adenylic acid (*c*-GpAp), and cyclic bis(3′-5′)guanylic/inosinic acid (*c*-GpIp). These compounds are expected to be important, both in elucidating the mechanism of bioactive *c*-di-GMP and in designing and creating new bioactive *c*-di-GMP-related artificial derivatives.
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1. Introduction

Cyclic bis(3′-5′)diguanylic acid (*c*-di-GMP) has attracted great interest due to its various biological activities, including regulation of cellulose synthesis in the bacterium *Acetobacter xylinum*,[1,2] acceleration of DNA synthesis and retarding of cell division in Molt 4 cells,[3] elevation of CD4 receptor expression and cell cycle arrest in Jurkat cells,[4] inhibition of basal and growth factor-stimulated human colon cancer cell proliferation,[5] inhibition of *Staphylococcus aureus* cell–cell interactions and biofilm formation,[6] and reduction of the virulence of biofilm-forming *S. aureus* strains in a mouse model of mastitis infection.[7] Further, *c*-di-GMP is conceived to play an important role in regulating exopolysaccharide production, biofilm formation, and other phenotypes.[8] These attractive biological properties of *c*-di-GMP have prompted us to carry out a systematical investigation on the bioactivity of *c*-di-GMP related compounds, including derivatives with modified nucleoside bases, carbohydrates, or internucleotide bonds. This investigation may lead not only to the discovery of new bioactive compounds, but also to a determination of which function of *c*-di-GMP is involved in its various biological activities: that is, how *c*-di-GMP works on receptors in cells. Thus, we attempted the synthesis of various *c*-di-GMP analogs.[9] This paper describes the synthesis of the monophosphorothioic acid of *c*-di-GMP (*c*-GpGps) (**5**), cyclic bis(3′-5′)guanylic/adenylic acid (*c*-GpAp) (**9**), and cyclic bis(3′-5′)guanylic/inosinic acid (*c*-GpIp) (**13**).

2. Results and discussion

c-GpGps **5** was prepared via the procedure shown in Scheme 1. The nucleoside phosphoramidite **1**[10] was condensed with the 5′-*O*-free nucleoside 3′-phosphate **2**[10] using imidazolium perchlorate (IMP)[11] as a promoter in acetonitrile containing 3 Å molecular sieves (MS 3 Å)[12] (30 min). The resulting phosphite product was sulfurized with bis[3-(triethoxysilyl)propyl] tetrasulfide (TEST)[13] (30 min); then, the 5′-*O*-*p*,*p*′-dimethoxytrityl (DMTr) group was removed by treatment with a 20% dichloroacetic acid/dichloromethane solution (30 min) to give the nucleoside monophosphorothioate **3** in a 78% overall yield. Subsequently, the allyl group on the 3′-terminal phosphotriester moiety of **3** was removed by exposure to sodium iodide in refluxing acetone[14] (2 h), and the resulting linear dinucelotide 3′-phosphodiester was treated with a high-dilution mixture of 2,4,6-trisipropylbenzenesulfonyl chloride (TPSCl) (5 equiv) and *N*-methylimidazole (5 equiv) (36 h) in THF[15] to provide fully protected *c*-GpGps **4** in a 76% overall yield. Finally, deprotection was carried out using a 1:1 (v/v) mixture of concd aqueous ammonia and methanol at 50 °C (12 h) for dimethylformamidine (dmf) and cyanoethyl protecting groups, followed by $(C_2H_5)_3N\cdot 3HF$[16] for *tert*-butyldimethylsilyl (TBDMS) protecting groups (12 h) to afford the target compound **5**. The overall yield of this deprotection procedure was 64%.

Synthesis of *c*-GpAp (**9**) was achieved according to the strategy shown in Scheme 2, which is fundamentally identical to that for the synthesis of *c*-GpGps. Thus, condensation of the phosphoramidite **6**[17] and the 5′-*O*-free nucleoside 3′-phosphate **2** using IMP as a promoter in acetonitrile containing MS 3 Å (30 min) and subsequent oxidation with *tert*-butyl hydroperoxide (TBHP)[18] (30 min)

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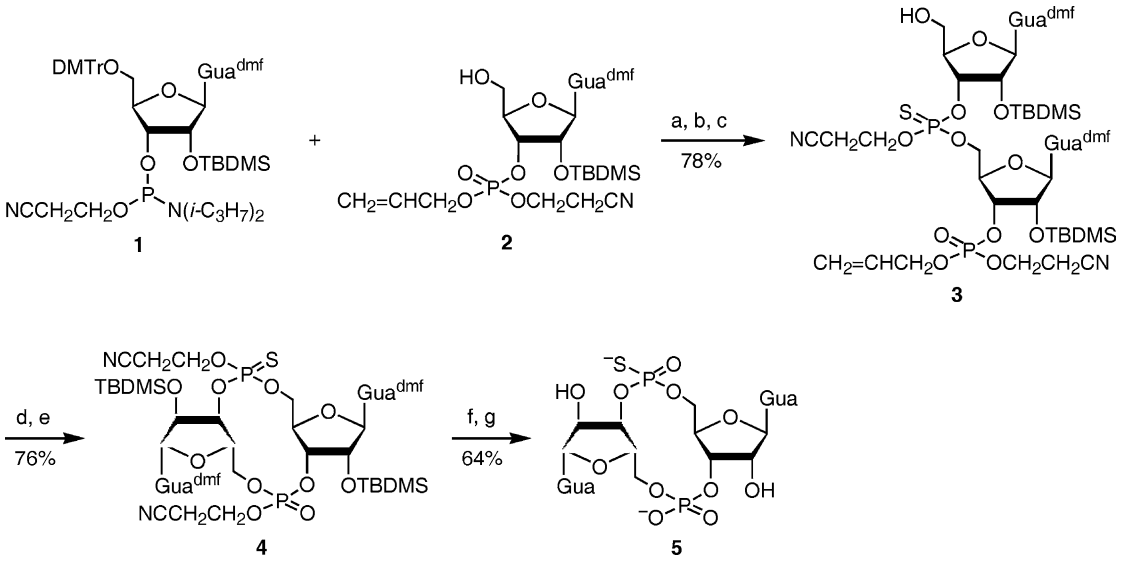
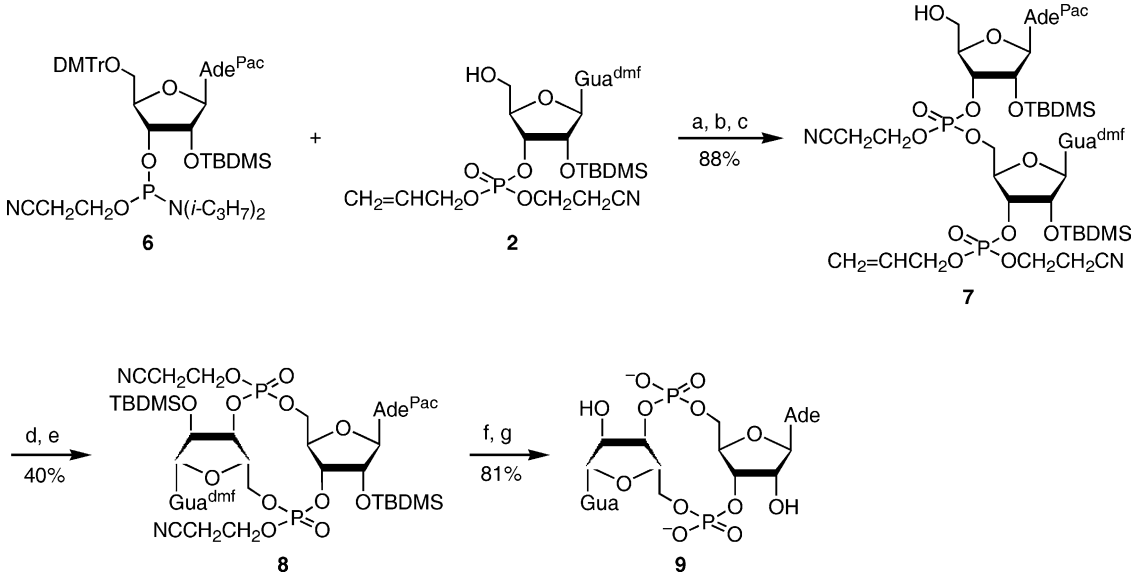


Scheme 1. Synthesis of *c*-GpGps (**5**): (a) IMP, MS 3 Å, CH_3CN, rt, 30 min; (b) TEST, rt, 30 min; (c) a 20% $Cl_2CHCOOH/CH_2Cl_2$ solution, rt, 30 min; (d) NaI, acetone, reflux, 2 h; (e) TPSCI, *N*-methylimidazole, THF, rt, 36 h; (f) concd aqueous NH_3–CH_3OH (1/1 v/v), 50 °C, 12 h; (g) $(C_2H_5)_3N\cdot 3HF$, rt, 12 h.

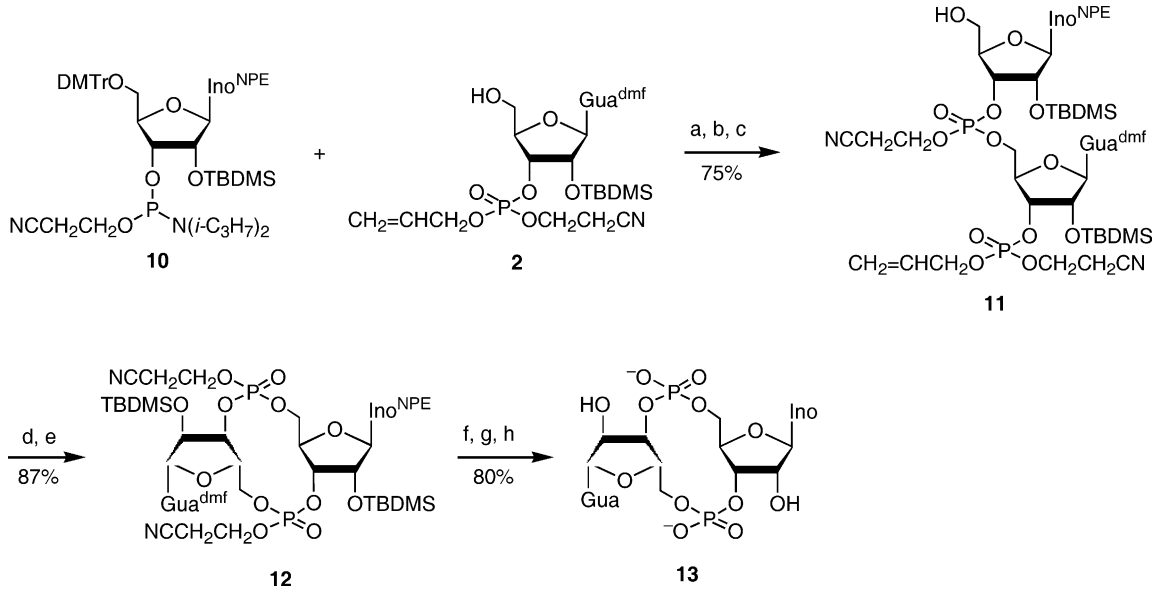
and detritylation using a 20% dichloroacetic acid solution in dichloromethane (20 min) furnished the dinucleotide **7** in an 88% overall yield. Then, **7** was converted to **8** in a 40% overall yield by removal of the allyl protector using sodium iodide in refluxing acetone followed by cyclization of the resulting product by treatment with TPSCl (5 equiv) in the presence of *N*-methylimidazole (5 equiv) in THF under high-dilution conditions. Finally, all protecting groups of **8** were removed by successive treatment with a 1:1 (v/v) mixture of concd aqueous ammonia and methanol (14 h) and with $(C_2H_5)_3N\cdot 3HF$ to produce the target compound **9** in an 81% overall yield.

c-GpIp (**13**) could be also prepared using **10**[19] as a starting material by a method essentially identical to that for the synthesis of the above-mentioned two analogs **5** and **9**. The synthetic route is shown in Scheme 3. In this process, 2-butanone peroxide (BPO)[20] was employed for oxidation of the dinucleoside phosphite intermediate obtained by condensation of **2** and **10** in the first step. Further, use of DBU (10 equiv) in pyridine (3 h)[21] was most effective for removal of cyanoethyl and *p*-nitrophenylethyl (NPE) protectors from the intermediate **12**. Yields of products were 75% for **11** from **2** and **10**, 87% for **11** from **12**, and 80% in for **13** from **12**, respectively.

The present synthesis of *c*-di-GMP analogs has a remarkable advantage over previously reported syntheses of related cyclic bis(3′-5′)dinucleic acids;[1,2,9f] that is, the present synthesis is capable of yielding a large amount of the desired compound. This advantage mainly results from the elimination of two major drawbacks of the previous syntheses. The first one concerns the regioselective production of a 2′-*O*-protected ribonucleoside, which is



Scheme 2. Synthesis of *c*-GpAp (**9**): (a) IMP, MS 3 Å, CH_3CN, rt, 30 min; (b) a 3.3 M TBHP/toluene solution, rt, 30 min; (c) a 20% $Cl_2CHCOOH/CH_2Cl_2$ solution, rt, 20 min; (d) NaI, acetone, reflux, 2 h; (e) TPSCI, *N*-methylimidazole, THF, rt, 30 h; (f) concd aqueous NH_3–CH_3OH (1/1 v/v), 50 °C, 14 h; (g) $(C_2H_5)_3N\cdot 3HF$, rt, 14 h.



Scheme 3. Synthesis of *c*-Gplp (**13**): (a) IMP, MS 3 Å, CH_3CN, rt, 30 min; (b) a 6.7% BPO/CH_2Cl_2 solution, rt, 30 min; (c) a 20% $Cl_2CHCOOH/CH_2Cl_2$ solution, rt, 30 min; (d) NaI, acetone, reflux, 2 h; (e) TPSCI, *N*-methylimidazole, THF, rt, 55 h; (f) DBU, pyridine, rt, 3 h; (g) concd aqueous NH_3–CH_3OH (1/1 v/v), 50 °C, 12 h; (h) $(C_2H_5)_3N\cdot 3HF$, rt, 12 h.

required as a precursor of a starting synthetic unit, namely, a ribonucleoside 3′-phosphoramidite[9f] or 3′-phosphotriester.[1,2] In previous syntheses, introduction of a protecting group was carried out using 2′-*O*- and 3′-*O*-free material, and took place in a nonregioselective manner to give nearly equal amounts of 2′-*O*- and 3′-*O*-protected products; in addition, a considerable amount of the 2′,3′-di-*O*-protected compound was produced. Consequently, yield of the desired 2′-*O*-protected compound was very low. The present synthesis eliminated this drawback by achieving perfectly regioselective, higher-yielding production of the 2′-*O*-protected material by means of a previously reported manner[10] that employs 3′,5′-di-*O*-protection of the 2′,3′,5′-tri-*O*-free ribonucleoside by the di-*tert*-butylsilanediyl group using Furusawa method,[22] followed by introduction of the TBDMS protecting group to the 2′-hydroxy group, and then removal of the 3′,5′-di-*O*-silanediyl protector. The other improvement in the present synthesis is the use of our original phosphoramidite strategy with imidazolium perchlorate as a promoter in the presence of MS 3 Å,[11,12] in place of the phosphoramidite method with 1*H*-tetrazole as a promoter without MS[9f] or the phosphotriester method[1,2] employed in previous syntheses, for production of a linear dinucleotide intermediate, such as **3**, **7**, or **11**. As reported before, the phosphoramidite method employed in the present synthesis is particularly effective for the internucleotide linkage formation using a ribonucleoside phosphoramidite with a bulky protecting group on the 2′-hydroxy function, such as **1**, **6**, or **10**. Thus, this modification also increased the yield of the product.

3. Conclusion

We developed a facile synthesis of three analogs of *c*-di-GMP: namely, *c*-GpGps (**5**), *c*-GpAp (**9**), and *c*-GpIp (**13**) using a fairly common procedure. These analogs are expected to have bioactivity similar to that of *c*-di-GMP. We are investigating the biological activities of these analogs and will report the results in the near future.

4. Experimental

4.1. General

A UV spectrum was measured on a JASCO V-500 spectrometer. NMR spectra were taken on a JEOL JNM-α400 or ECA-500 instrument. The ^1H, ^{13}C, and ^{31}P NMR chemical shifts are described as δ values in ppm relative to $(CH_3)_4Si$ (for ^1H and ^{13}C NMR) and 85% H_3PO_4 (for ^{31}P NMR), respectively. ESI-TOF high resolution mass (HRMS) spectra were obtained on Applied Biosystems Voyager MDE and Mariner spectrometers. HPLC analysis was carried out using a COSMOSIL $5C_{18}$-MS column (Nacalai Tesque, ODS-5 mm, 4.6×250 mm) on a Waters 2695 Separations Module chromatograph with a Waters 2996 Photodiode Array detector. Preparative HPLC was achieved using a COSMOSIL $5C_{18}$-AR-300 column (Nacalai Tesque, 25×200 mm) on an ÄKTA explorer (Amersham Biosciences). Column chromatography was performed using Nacalai Tesque silica gel 60 (neutrality, 75 mm). Unless otherwise noted, synthetic reactions were carried out at ambient temperature. The reactions requiring anhydrous conditions were achieved under an argon atmosphere in flasks dried by heating at 400 °C under 133–400 Pa, or by washing with a 5% solution of dichlorodimethylsilane in dichloromethane followed by anhydrous dichloromethane, and then heating at 100 °C.

4.2. Material and solvents

The nucleoside 3′-phosphoramidites **1**,[9] **6**,[16] **10**,[19] the 5′-*O*-free nucleoside 3′-phosphate **2**,[9] imidazolium perchlorate (IMP),[10] a 3.30 M solution of TBHP in toluene solution,[18] and a 6.7% 2-butanone peroxide/dimethyl phthalate–toluene

solution[17] were prepared by the reported methods. Bis[3′-(triethoxysilyl)propyl] tetrasulfide (TEST)[12] was supplied from Shin-etsu, Tokyo, Japan. THF was used after drying by reflux over sodium-diphenyl ketyl. Acetone, acetonitrile, and dichloromethane were distilled from calcium hydride. Other organic reagents were used as commercially supplied without any purification. Solid and amorphous organic substances were used after drying at 50–60 °C for 8–12 h under 133–400 Pa. Powdery molecular sieves (MS) 3 Å were employed after drying the commercially supplied product (Nacalai tesque) at 200 °C for 12 h under 133–400 Pa.

4.2.1. Preparation of the guanylyl(3′-5′)guanosine 3′-phosphate monophosphorothioate 3. A mixture of the phosphoramidite **1** (1.02 g, 1.07 mmol) and the 5′-*O*-free nucleoside phosphate **2** (674 mg, 1.07 mmol) in the presence of powdery MS 3 Å (200 mg) in acetonitrile (10 mL) was stirred at 25 °C for 30 min. To this mixture was added IMP (370 mg, 2.2 mmol) and stirring was continued for an additional 30 min. TEST (1.10 mL, 1.20 g, 2.20 mmol) was added to the resulting mixture. After stirring for 30 min, the reaction mixture was passed through a Celite 545 pad to remove MS 3 Å. The filtrate was concentrated to afford a viscous oil. This material was dissolved in dichloromethane (20 mL). To this solution was added dichloroacetic acid (1.50 mL, 2.30 g, 18.0 mmol) at 0 °C. After stirring for 30 min at 25 °C, the reaction mixture was poured into an aqueous sodium hydrogen carbonate solution (100 mL) and the organic layer was separated. The aqueous layer was extracted with dichloromethane (100 mL, 50 mL×2). The organic solutions were combined and concentrated. The resulting material was chromatographed on a silica gel (50 g) column successively using a 1:30 methanol/dichloromethane mixture, a 1:20 methanol/dichloromethane mixture, and a 1:10 methanol/dichloromethane mixture as eluents to afford **3** (923 mg, a 78% overall yield; a mixture of four diastereomers) as a colorless amorphous solid: ^{1}H NMR ($CDCl_3$) −0.28–0.01 (m, 12H), 0.73–0.97 (m, 18H), 2.72–2.79 (m, 4H), 3.08–3.22 (m, 12H), 3.48 (d, J=4.8 Hz, 6H), 3.70–3.83 (m, 2H), 4.23–4.63 (m, 10H), 5.02–5.15 (m, 3H), 5.30–5.44 (m, 3H), 5.67–6.00 (m, 3H), 7.59–7.86 (m, 2H), 8.38, (s, 1H), 8.57–8.61 (m, 1H), 8.79–8.90 (br, 2H); ^{31}P NMR ($CDCl_3$) −0.92, −0.86, 67.07, 67.15, 68.20, 68.25; HRMS (ESI^+) calcd for $C_{47}H_{74}N_{14}O_{14}P_2SSi_2Na^+$ ($M+Na^+$) *m/z* 1231.4136, found *m/z* 1231.4430.

4.2.2. Conversion of 3 to the protected cyclic bis(3′-5′)diguanylic acid monophosphorothioate 4. To a solution of **3** (876 mg, 0.72 mmol) in acetone (15 mL) was added sodium iodide (1.10 g, 7.20 mmol) and the resulting solution was stirred under reflux for 2 h. The resulting colorless precipitate was collected by filtration through a filter paper and washed with chilled acetone (50 mL). This precipitate is highly hygroscopic and thus was immediately subjected to the next procedure. The collected precipitate was dissolved in methanol (50 mL) and the solution was concentrated. The resulting oily material was co-evaporated with toluene (50 mL×3). The residue was suspended in THF (200 mL) and to this mixture were successively added *N*-methylimidazole (0.30 mL, 287 mg, 3.50 mmol) and 2,4,6-triisopropylbenzenesulfonyl chloride (1.06 g, 3.50 mmol). The resulting solution was stirred at 25 °C for 36 h. To the reaction mixture was added water (50 mL) and stirring was continued for an additional 1 h. The reaction mixture was extracted with dichloromethane (50 mL×3) and the organic layer was concentrated. The resulting residual material was subjected to column chromatography on silica gel (50 g). Elution with a 1:20 methanol/dichloromethane mixture and then a 1:10 methanol/dichloromethane mixture afforded **4** (836 mg, a 76% overall yield from **3**) as an amorphous solid: ^{1}H NMR (CD_3OD) −0.14 (s, 6H), 0.09 (s, 6H), 0.76 (s, 18H), 2.95 (t, J=6.0 Hz, 4H), 3.14 (s, 6H), 3.31 (s, 6H), 4.13–4.21 (m, 2H), 4.35–4.68 (m, 10H), 5.36, 5.38 (2d, J=5.0 Hz, 2H), 5.31–5.44 (m, 2H), 5.91–5.96 (m, 4H), 7.08 (s, 2H), 7.94 (s, 2H), 8.69 (s, 2H); ^{31}P NMR (CD_3OD) −0.97, −0.75, 66.91, 67.75; HRMS (ESI^+) calcd for $C_{44}H_{69}N_{14}O_{13}P_2SSi_2Na^+$ ($M+Na^+$) *m/z* 1173.3717, found *m/z* 1173.4057.

4.2.3. Preparation of *c*-GpGps (5) by deprotection of 4. To a suspension of **4** (155 mg, 0.13 mmol) in methanol (10 mL) was added a concd aqueous ammonia solution (10 mL), and the resulting mixture was stirred at 50 °C for 12 h. The reaction mixture was concentrated and the residue was dried under reduced pressure using a vacuum oil pump. The resulting product was mixed with $(C_2H_5)_3N\cdot 3HF$ (6.0 mL, 5.93 g, 37 mmol) and the mixture was stirred for 12 h. To the reaction mixture was added a 1 M (1 mol dm^{-3}) ammonium acetate buffer solution (30 mL) and the mixture was vigorously stirred at 30–40 °C for 10 min. After removing the resulting pale yellow precipitate by filtration, the aqueous filtrate was subjected to preparative HPLC using a COSMOSIL 5C_{18}-AR-300 column [25 (diameter)×200 (height) mm]. Elution carried out under the following conditions [A=a 1.0 mM ammonium acetate buffer solution, B=a 0.2 mM ammonium acetate solution in a 20:80 mixture of H_2O and acetonitrile; gradient: 0–8 min A 100%, 8–55 min linear gradient A 100% to A 40%/B 60%, 55–63 min B 100%; detection 254 nm; flow rate 10 mL/min] gave rise to the diammonium salt of **5** (64 mg, a 64% overall yield from **4**, a mixture of two diastereomers): UV (a 50 mM solution of ammonium acetate in water) λ_{max} 254 nm (ε 23,700); ^{1}H NMR (DMSO-d_6) 3.76–4.15 (m, 6H), 4.35–4.92 (m, 4H), 5.67–5.72 (m, 2H), 6.54 (br, 2H), 7.92, 7.94 (2s, 2H); ^{31}P NMR (DMSO-d_6) 1.27, 54.21; HRMS (ESI^-) calcd for $C_{20}H_{23}N_{10}O_{17}P_2S^-$ ($M-H^-$) *m/z* 705.0648, found *m/z* 705.0804.

4.2.4. Preparation of the adenylyl(3′-5′)guanosine 3′-phosphate 7. The phosphoramidite **6** (916 mg, 0.90 mmol) and the 5′-*O*-free nucleoside phosphate **2** (378 mg, 0.60 mmol) were mixed in acetonitrile (3 mL) containing powdery MS 3 Å (200 mg) and stirred at 25 °C for 30 min. The mixture was added IMP (207 mg, 1.20 mmol) and stirred for additional 30 min. To the resulting mixture was added a 3.30 M solution of TBHP in toluene (0.30 mL, 0.90 mmol of the peroxide). The mixture was left at 25 °C with stirring for 30 min and then passed through a Celite 545 pad for removing MS 3 Å. The filtrate was concentrated to afford oily material, which was dissolved in dichloromethane (20 mL). To this solution was added dichloroacetic acid (1.10 mL, 1.70 g, 15.6 mmol) at 0 °C. After stirring for 20 min at 25 °C, the reaction mixture was poured into an aqueous sodium hydrogen carbonate solution (100 mL).

The organic layer was separated and the aqueous layer was extracted with dichloromethane (100 mL, 50 mL×2). The combined organic layers were dried and concentrated. The resulting material was subjected to silica gel (50 g) column chromatography using a 1:30 methanol/dichloromethane mixture, a 1:20 methanol/dichloromethane mixture and then a 1:10 methanol/dichloromethane mixture as an eluent to afford **7** (660 mg, an 88% overall yield; a mixture of four diastereomers) as a colorless amorphous solid: ^{1}H NMR ($CDCl_3$) −0.37–0.09 (m, 12H), 0.70–0.87 (m, 18H), 2.77–2.82 (m, 4H), 3.11 (s, 3H), 3.21, 3.22 (2s, 3H), 3.63–3.90 (m, 3H), 4.32–5.46 (m, 16H), 5.93–6.24 (m, 3H), 7.06–7.08 (m, 3H), 7.36 (t, J=7.4 Hz, 2H), 7.85–7.92 (m, 1H), 8.55–8.66, (m, 1H), 8.77 (br, 2H), 9.72 (br, 1H); ^{31}P NMR ($CDCl_3$) −3.08, −2.86, −2.71, −2.64, −2.52, −2.46, −2.34, −2.27; HRMS (ESI^+) calcd for $C_{52}H_{76}N_{13}O_{16}P_2Si_2^+$ ($M+H^+$) m/z 1256.4541, found m/z 1256.4742.

4.2.5. Transformation of 7 to the protected cyclic (3′-5′)adenylic-guanylic acid 8. A mixture of **7** (660 mg, 0.53 mmol) and sodium iodide (566 mg, 5.30 mmol) in acetone (30 mL) was refluxed with stirring for 2 h. The occurring yellow precipitate was collected by filtration and washed with chilled acetone (50 mL). This solid was suspended in THF (120 mL) and to this heterogeneous mixture were successively added *N*-methylimidazole (0.211 mL, 218 mg, 2.65 mmol) and 2,4,6-triisopropylbenzenesulfonyl chloride (803 mg, 2.65 mmol). The resulting mixture was stirred at 25 °C for 30 h. The reaction was quenched by addition of water (50 mL) at 25 °C and the resulting aqueous mixture was stirred at the same temperature for additional 1 h. The reaction mixture was extracted with dichloromethane (50 mL×3) and the organic layer was separated and concentrated. The residue was subjected to column chromatography on silica gel (50 g) eluted with a 1:20 mixture of methanol and dichloromethane and then a 1:10 mixture of methanol and dichloromethane to provide **8** (180 mg, a 40% overall yield from **7**) as an amorphous solid: ^{1}H NMR (CD_3OD) −0.23 (s, 3H), −0.09 (s, 3H), 0.10 (s, 6H), 0.74, 0.79 (2s, 18H), 2.46–2.48 (m, 2H), 2.65, 2.67 (2s, 6H), 3.12–3.22 (m, 4H), 3.47–3.48 (m, 2H), 3.76 (s, 2H), 4.05–5.00 (m, 4H), 5.34–5.43 (m, 2H), 5.91–5.99 (m, 3H), 6.13–6.19 (m, 1H), 6.96–7.16 (m, 3H), 7.33–7.45 (m, 2H), 7.95–7.99 (m, 1H), 8.27–8.32 (m, 1H), 8.54–8.61 (m, 1H), 8.67–8.78 (m, 2H); NMR (CD_3OD) −0.66, 0.00; HRMS (ESI^+) calcd for $C_{49}H_{70}N_{13}O_{15}P_2Si_2^+$ ($M+H^+$) m/z 1198.4123, found m/z 1198.4466.

4.2.6. Preparation of *c*-GpAp (9) from 8. A suspension of **8** (180 mg, 0.21 mmol) in methanol (5 mL) was mixed with a concd aqueous ammonia solution (10 mL) and the resulting mixture was stirred at 50 °C for 14 h. The reaction mixture was concentrated and the resulting residue was dried in vacuo. The dried material was mixed with $(C_2H_5)_3N\cdot 3HF$ (4.0 mL, 3.96 g, 25 mmol) and stirred at 25 °C for 14 h. To this mixture was added a 1 M (1 mol dm^{-3}) ammonium acetate buffer solution (30 mL) and the mixture was vigorously stirred at 30–40 °C for 10 min. The resulting precipitate was removed by passage of the reaction mixture through a column packed with Celite 545 and the filtrate was subjected to preparative HPLC using a COSMOSIL 5C_{18}-AR-300 column [25 (diameter)×200 (height) mm] eluted under the following conditions [A=a 1.0 mM ammonium acetate buffer solution, B=a 0.2 mM ammonium acetate solution in a 20:80 mixture of H_2O and acetonitrile; gradient: 0–8 min A 100%, 8–55 min linear gradient A 100% to A 40%/B 60%, 55–63 min B 100%; detection 254 nm; flow rate 10 mL/min] to afford the diammonium salt of **9** (80 mg, an 81% overall yield from **8**): ^{1}H NMR (DMSO-d_6) 3.77–3.96 (m, 4H), 4.12–4.19 (m, 2H), 4.58–4.63 (m, 3H), 4.89–4.91 (m, 1H), 5.73 (d, J=8.1 Hz, 1H), 5.84 (d, J=8.0 Hz, 1H), 6.57 (br, 2H), 7.23 (br, 2H), 7.92 (s, 1H), 8.12 (s, 1H), 8.38 (s, 1H); ^{31}P NMR (DMSO-d_6) 1.16; HRMS (ESI^-) calcd for $C_{20}H_{23}N_{10}O_{13}P_2^-$ ($M-H^-$) m/z 673.0927, found m/z 673.1104.

4.2.7. Preparation of the inosinylyl(3′-5′)guanosine 3′-phosphate 11. A mixture of the phosphoramidite **10** (620 mg, 0.60 mmol) and the 5′-*O*-free nucleoside phosphate **2** (313 mg, 0.50 mmol) in acetonitrile (1 mL) containing powdery MS 3 Å (200 mg) was stirred at 25 °C for 30 min. To this mixture was added IMP (168 mg, 1.00 mmol). After stirring at the same temperature for 30 min, to the reaction mixture was added a 6.7% solution of 2-butanone peroxide/dimethyl phthalate in toluene (1.00 mL, 1.0 mmol of the peroxide) and the mixture was stirred for additional 30 min. The reaction mixture was passed through a column packed with Celite 545 pad to remove MS 3 Å. Concentration of the filtrate afforded a viscous oil, which was dissolved in dichloromethane (20 mL). To this solution was added dichloroacetic acid (0.50 mL, 0.77 g, 6.00 mmol) at 0 °C. After stirring for 30 min at 25 °C, the reaction mixture was poured into an aqueous sodium hydrogen carbonate solution (100 mL) and the organic layer was separated. The aqueous layer was extracted with dichloromethane (100 mL, 50 mL×2). The organic solutions were collected and concentrated to give an oily material. This crude product was chromatographed on a silica gel (50 g) column using a 1:30 methanol/dichloromethane mixture, a 1:20 methanol/dichloromethane mixture, and then a 1:10 methanol/dichloromethane mixture as eluents to afford **11** (579 mg, a 75% overall yield from **10**; a mixture of four diastereomers) as a colorless amorphous solid: ^{1}H NMR ($CDCl_3$) −0.39–0.22 (m, 12H), 0.68–0.91 (m, 18H), 2.73–2.82 (m, 4H), 3.12–3.23 (m, 6H), 3.33 (t, J=6.8 Hz, 2H), 3.75–3.90 (m, 2H), 4.24–4.62 (m, 10H), 4.82–5.45 (m, 8H), 5.68–6.17 (m, 4H), 7.48–7.51 (m, 2H), 7.75–7.86 (m, 1H), 8.15 (d, J=8.8 Hz, 2H), 8.27–8.33 (m, 1H), 8.47–8.48 (m, 1H), 8.53–8.62 (m, 1H) 9.38–9.46 (m, 1H); ^{31}P NMR ($CDCl_3$) −2.93, −2.82, −2.64, −2.53, −2.38, −2.27; HRMS (ESI^+) calcd for $C_{52}H_{75}N_{13}O_{17}P_2Si_2Na^+$ ($M+Na^+$) m/z 1294.4310, found m/z 1294.4623.

4.2.8. Conversion of 11 to the fully protected cyclic (3′-5′)guanylic/inosinic acid 12. To a solution of **11** (398 mg, 0.30 mmol) in acetone (10 mL) was added sodium iodide (420 mg, 3.00 mmol) and the resulting solution was stirred at the reflux temperature for 2 h. The reaction mixture was poured into a 10 mM of triethylammonium carbonate solution in water (100 mL) and extracted with dichloromethane (100 mL, 50 mL×2). The collected organic solutions were concentrated. The resulting residue was mixed with THF (100 mL) and to this mixture were successively added *N*-methylimidazole (0.11 mL, 123 mg,

1.50 mmol) and 2,4,6-triisopropylbenzenesulfonyl chloride (460 mg, 1.50 mmol). The resulting solution was stirred at 25 °C for 55 h. To the reaction mixture was added water (50 mL) at 25 °C and stirring was continued at the same temperature for an additional 1 h. The reaction mixture was extracted with dichloromethane (50 mL×3) and the organic layer was concentrated. The resulting residual material was subjected to column chromatography on silica gel (50 g) and eluted with a 1:20 mixture of methanol and dichloromethane and then a 1:10 mixture of methanol and dichloromethane. The desired product **12** was obtained as an amorphous solid (324 mg, an 87% overall yield from **11**): ^{1}H NMR (CD_3OD) −0.13–0.20 (6s, 12H), 0.80–0.97 (3s, 18H), 2.89–3.06 (m, 2H), 3.17–3.50 (m, 10H), 3.90 (d, J= 1.5 Hz, 2H), 4.21–5.02 (m, 12H), 5.42–5.66 (m, 2H), 5.95 (s, 1H), 6.11–6.12 (m, 1H), 6.93–6.95 (m, 2H), 7.66–7.69 (m, 2H), 7.87–7.99 (m, 1H), 8.28–8.32 (m, 2H), 8.64–8.78 (m, 2H); ^{31}P NMR (CD_3OD) 4.47, 5.33; HRMS (ESI^+) calcd for $C_{49}H_{70}N_{13}O_{16}P_2Si_2^+$ ($M+H^+$) m/z 1214.4072, found m/z 1214.5455.

4.2.9. Deprotection of 12 giving *c*-GpIp (13). To a solution of **12** (296 mg, 0.23 mmol) in pyridine (10 mL) was added DBU (0.4 mL, 407 mg, 2.7 mmol) and the mixture was stirred at 25 °C for 3 h. To this mixture was added an aqueous solution concentrated with ammonia (10 mL) and the mixture was stirred at 50 °C for 12 h. The reaction mixture was concentrated under reduced pressure and the obtained residue was dried in vacuo. The resulting product was mixed with $(C_2H_5)_3N\cdot 3HF$ (4.4 mL, 4.35 g, 27 mmol) and the mixture was stirred at 25 °C for 12 h. The reaction mixture was added a 1 M (1 mol dm^{-3}) ammonium acetate buffer solution (30 mL) and vigorously stirred at 30–40 °C for 10 min to precipitate a pale yellow solid. After removal of the resulting precipitate by filtration by passing through a Celite 545 pad, the aqueous filtrate was subjected to preparative HPLC using a COSMOSIL 5C_{18}-AR-300 column [25 (diameter)×200 (height) mm]. Elution was performed under the following conditions [A=a 1.0 mM ammonium acetate buffer solution, B=a 0.2 mM ammonium acetate solution in a 20:80 mixture of water and acetonitrile; gradient: 0–8 min A 100%, 8–55 min linear gradient A 100% to A 40%/B 60%, 55–63 min B 100%; detection 254 nm; flow rate 10 mL/min] to give the diammonium salt of **13** (125 mg, an 80% overall yield from **12**, a mixture of two diastereomers): ^{1}H NMR (DMSO-d_6) 3.73–3.99 (m, 4H), 4.11–4.21 (m, 2H), 4.57–4.87 (m, 4H), 5.72 (d, J=8.0 Hz, 1H), 5.82 (d, J=8.0 Hz, 1H), 6.75 (br, 2H), 7.91 (s, 1H), 8.03 (br, 1H), 8.32 (s, 1H); ^{31}P NMR (DMSO-d_6) 1.02, 1.16; HRMS (ESI^-) calcd for $C_{20}H_{23}N_9O_{14}P_2^-$ ($M-H^-$) m/z 674.0767, found m/z 674.1057.

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