

An Improved Method for Synthesizing Cyclic Bis(3′–5′)diguanylic Acid (*c*-di-GMP)

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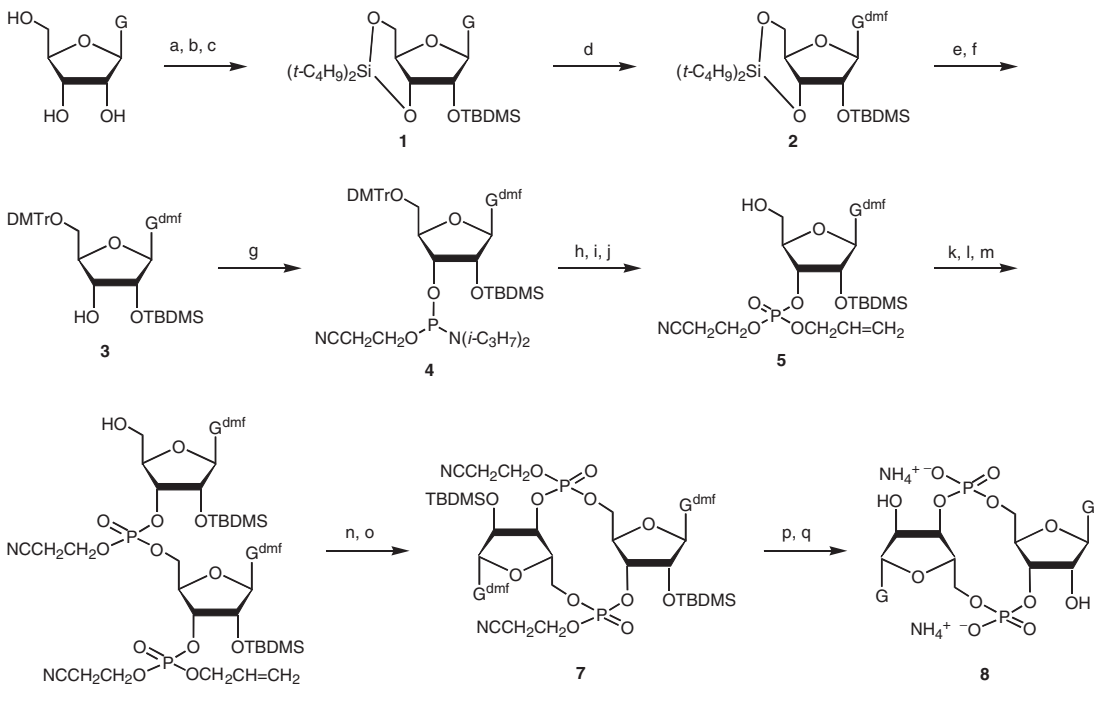
This paper describes a new method for synthesizing biologically important cyclic bis(3′–5′)diguanylic acid (*c*-di-GMP) in a higher yield than that previously reported to be available by our synthetic method. In the new synthesis, the following two means, in place of those used in the previously reported synthesis, are employed as main strategies to obtain an increase in product yield. One is the use of di-*tert*-butylsilanediyl protection for 3′- and 5′-hydroxy groups of guanosine; this method allows regioselective production of a 2′-*O*-(*tert*-butyldimethylsilyl)guanosine derivative that is a key intermediate for the synthesis. The other is the use of a dimethylformamidine group as a protector for the 2-NH_2 function of the guanine base, which can be easily introduced and results in an excellent yield.

Cyclic bis(3′–5′)diguanylic acid (*c*-di-GMP) (**8** in Scheme 1) is a naturally-occurring nucleotide that is now attracting great attention in many research fields. This substance demonstrates various important properties, such as regulation of cellulose synthesis in the bacterium *Acetobacter xylinum*,[1,2] acceleration of DNA synthesis and retarding of cell division in Molt4 cells,[3] and elevation of an expression of the CD4 receptor and cell cycle arrest in Jurkat cells.[4] The cell-growth retarding activities of *c*-di-GMP have suggested that this compound may have anti-cancer activity. Further, on the basis of studies on activity



Scheme 1. (a) $(t\text{-}C_4H_9)_2Si(OTf)_2$, 0 °C, 45 min; (b) imidazole, 25 °C, 30 min; (c) TBDMSCl, 60 °C, 2 h; (d) $(CH_3)_2NCH(OCH_3)_2$, 50 °C, 5 h; (e) HF·pyridine, pyridine, CH_2Cl_2, 0 °C, 2 h; (f) DMTrCl, pyridine, 25 °C, 12 h; (g) $NCCH_2CH_2OP[N(i\text{-}C_3H_7)_2]Cl$, 2,4,6-collidine, *N*-methylimidazole, THF, 25 °C, 1 h; (h) allyl alcohol, IMP, MS 3A, CH_3CN, 25 °C, 30 min; (i) a 6.7% BPO/toluene solution, 25 °C, 5 min; (j) a 20% $Cl_2CHCOOH/CH_2Cl_2$ solution, 25 °C, 10 min; (k) the phosphoroamidite **4**, IMP, MS 3A, CH_3CN, 25 °C, 30 min; (l) a 6.7% BPO/toluene solution, 25 °C, 5 min; (m) a 20% $Cl_2CHCOOH/CH_2Cl_2$ solution, 25 °C, 10 min; (n) NaI, acetone, reflux, 2 h; (o) TPSCl, *N*-methylimidazole, 25 °C, 36 h; (p) conc. aq. NH_3–CH_3OH (1:1 v/v), 50 °C, 12 h; (q) $(C_2H_5)_3N\cdot 3HF$, 25 °C, 12 h.

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of a regulatory protein, termed the regulation of cell signaling (RocS) protein, in the switch between the smooth and rugose phenotypes of *V. cholerae*, it was proposed that *c*-di-GMP may have an important function in regulating exopolysaccharide production, biofilm formation, and other phenotypes.[5] Thus, we were stimulated to carry out extensive investigations of the biological properties of *c*-di-GMP. Such investigations required a sufficient amount of the substance. It was necessary to develop an efficient chemical method for synthesizing *c*-di-GMP in order to meet this requirement. Thus far, there have been two methods developed, one by van Boom et al.,[2] the other by us.[6] Based on a comparison between these two methods, our method appears more advantageous because it affords the target compound in fewer steps and in a higher yield. Our method still includes at least two unfavorable processes, which do not provide satisfactory yields. One process is the *tert*-butyldimethylsilyl (TBDMS) protection of the 2′-hydroxy of guanosine. This protection was carried out using the 2′-*O*- and 3′-*O*-free material, and took place in a nonregioselective manner to give nearly equal amounts of 2′-*O*-TBDMS, 3′-*O*-TBDMS, and 2′,3′-bis-*O*-TBDMS products.[7] Therefore, isolation of the desired 2′-*O*-TBDMS compound requires tedious chromatographic purification, causing a loss of the product. In this approach, the desired 2′-*O*-TBDMS product was isolated in a yield of only ca. 30%. Another process is the introduction of allyloxycarbonyl and allyl protecting groups to N^2- and O^6-functions of the guanine base, respectively.[8] This double protection requires multiple steps, and the desired compound was provided in an overall yield of only ca. 50%.[7] Accordingly, we investigated development of an improved method that would eliminate these drawbacks. This objective was achieved by means of the following two new strategies. The first problem was resolved by use of the di-*tert*-butylsilanediyl ribonucleoside-3′,5′-di-*O*-protection method (Furusawa method)[9] and selective deblocking of this protector from the 3′,5′-*O*-di-*tert*-butylsilanediyl-2′-*O*-TBDMS guanosine intermediate.[10] The second drawback was removed by use of an N^2-dimethylformamidine (dmf)-protected/O^6-unprotected guanosine derivative[11] as a building block.

Results and Discussion

Scheme 1 illustrates a newly developed synthetic pathway to *c*-di-GMP. Successive single-flask treatment of guanosine in DMF with di-*tert*-butylsilanediyl di(triflate) at 0 °C for 45 min, with imidazole at 25 °C for 30 min, and with TBDMS chloride at 60 °C for 2 h gave **1** in a 70% isolated yield.[12] Subsequently, the 2-amino function of the guanine base was protected by the reaction of **1** and $(CH_3)_2NCH(OCH_3)_2$ in methanol (50 °C, 5 h) to provide **2** in a 98% isolated yield. The product **2** was converted to the 3′-*O*-free guanosine **3** in an 86% overall yield through removal of the di-*tert*-butylsilanediyl protector by exposure to a mixture of HF·pyridine complex and pyridine (4.0:7.4 molar ratio) in dichloromethane (0 °C, 2 h), and subsequent dimethoxytritylation using *p*,*p*′-dimethoxytrityl chloride (DMTrCl) in pyridine (25 °C, 12 h). Condensation of **3** with $NCCH_2CH_2OP[N(i\text{-}C_3H_7)_2]Cl$ in the presence of 2,4,6-collidine and *N*-methylimidazole in THF (25 °C, 1 h) afforded the phosphoramidite **4** with >90% purity in a 78% yield. The ^{31}P NMR signals observed at δ 148.9 and 150.0 supported the structure of **4** (a 68:32 mixture of two diastereomers). The compound **4** was treated with allyl alcohol (1.2 molar amount) in the presence of imidazolium perchlorate (IMP)[13] (2.0 mol. amt.) and molecular sieves 3A (MS 3A)[14] in acetonitrile (25 °C, 30 min), followed treatment with 2-butanone peroxide (BPO) (2.0 mol. amt.) in toluene (25 °C, 5 min),[15] and then, from the resulting product, the DMTr protecting group was removed by exposure to a 20% solution of dichloroacetic acid in dichloromethane to furnish the phosphotriester **5** (an 86% overall yield from **3**). The structure of **5** was confirmed by the ^{31}P NMR spectrum that showed signals at δ −2.3, and by the ESI-TOF mass spectrum that indicated a molecular peak at m/z 626.2628 [calcd for $(C_{25}H_{41}N_7O_8Si)^+$: 626.2518]. The ^{1}H NMR data were also consistent with **5** as a mixture of diastereomers. The compound **5** was reacted with the phosphoramidite **4** by the aid of IMP in acetonitrile containing MS 3A (25 °C, 30 min), and then oxidized by BPO in toluene (25 °C, 5 min) to give the diguanylic acid ester, which led to **6** (an 82% overall yield from **5**) by detritylation using a 20% dichloroacetic acid/dichloromethane solution (25 °C, 10 min). The ^{31}P NMR signals that appear at δ −1.78, −1.72, −1.45, −1.32, −1.05, and −0.83 and an ESI-TOF-mass peak that appears at m/z 1193.5944 [calcd for $(C_{47}H_{75}N_{14}O_{15}P_2Si_2)^+$: 1193.4545] supported the structure of the product. From the 3′-terminal phosphotriester moiety of **6**, the allyl group was removed by the action of sodium iodide in refluxing acetone (2 h), and the resulting product was converted to the fully protected cyclic dinucleotide **7** by treatment with 2,4,6-triisopropylbenzenesulfonyl chloride (TPSCl) in the presence of *N*-methylimidazole in THF (25 °C, 36 h).[16] In this process, **7** was obtained in an 85% yield. This product showed a molecular peak at m/z 1135.4455 [calcd for $(C_{44}H_{69}N_{14}O_{14}P_2Si_2)^+$: 1135.4126] in the ESI-TOF mass spectrum and a signal at δ 0.75 in the ^{31}P NMR spectrum. These data were consistent with **7**. Finally, **7** was exposed to a 1:1 mixture of concentrated aqueous ammonia and methanol (50 °C, 12 h)[17] to remove the dmf and cyanoethyl protecting groups and then treated with $(C_2H_5)_3N\cdot 3HF$[18] (25 °C, 12 h) to deblock the TBDMS protector. The resulting crude product was purified by reverse-phase column chromatography to give *c*-di-GMP (**8**) as the diammonium salt in an 89% isolated yield. The ^{1}H NMR, ^{31}P NMR, UV, and ESI-TOF mass spectral data of this product were identical to those of the authentic sample of **8**.[6]

We have thus developed a novel synthesis of *c*-di-GMP, which has eliminated two major drawbacks of our previous method. One improvement is perfectly regioselective production of a 2′-*O*-TBDMS-protected guanosine derivative obtained using the Furusawa method for the 3′,5′-di-*O*-protection of guanosine. The other improvement is an easy and high-yielding preparation of the N^2-protected guanosine achieved using the dmf group as the protector. These modifications greatly improved the yield of the target product. Actually, when the synthesis using the same amount of the starting guanosine is carried out by the present new method and by our previously reported method, the former method gives the target product in an amount approximately ten-fold larger than does the latter method.

Experimental

General Procedures, Materials, and Solvents. Each UV spectrum was measured on a JASCO V-500 spectrometer. NMR spectra were taken on a JEOL JNM-α400 or ECA-500 instrument. The ^{1}H, ^{13}C, and ^{31}P NMR chemical shifts are described as δ values in ppm relative to $(CH_3)_4Si$ (for ^{1}H and ^{13}C) and 85% H_3PO_4, respectively. ESI-TOF high resolution mass (HRMS) spectra were obtained on Applied Biosystems Voyager MDE and Mariner spectrometers, respectively. 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 washing with anhydrous dichloromethane, and then heating at 100 °C. Imidazolium perchlorate,[13] a 6.7% 2-butanone peroxide/dimethyl phthalate–toluene solution,[6,15] and $NCCH_2CH_2OP[N(i\text{-}C_3H_7)_2]Cl$[19] were prepared by the reported methods. Diethyl ether, THF, and toluene were used after drying by reflux over sodium–diphenyl ketyl. Acetonitrile, DMF, 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 over P_2O_5 at 50–60 °C for 8–12 h under 133–400 Pa. Powdery molecular sieves (MS) 3A were employed after drying the commercially supplied product (Nacalai tesque) at 200 °C for 12 h under 133–400 Pa.

2′-*O*-(*tert*-Butyldimethylsilyl)-3′,5′-*O*-(di-*tert*-butylsilanediyl)guanosine (1). To a stirred suspension of guanosine (11.3 g, 40 mmol) in DMF (80 mL) was added di-*tert*-butylsilanediyl di(triflate) (19.4 g, 16.0 mL, 44 mmol) at 0 °C over 15 min. After stirring for 30 min at the same temperature, imidazole (13.6 g, 200 mmol) was added. The resulting mixture was stirred at 0 °C for 5 min and then at room temperature for 25 min. To this was added *tert*-butyldimethylchlorosilane (7.2 g, 48 mmol) and the reaction mixture was heated at 60 °C for 2 h. The occurring precipitate was collected by filtration, washed with cold methanol, and dried under reduced pressure to give **1** (15.0 g, 70% yield) as a colorless powder: ^{1}H NMR (DMSO-d_6) δ 0.07 (s, 3H), 0.09 (s, 3H), 0.86 (s, 9H), 1.01 (s, 9H), 1.06 (s, 9H), 3.93–4.00 (m, 2H), 4.27–4.35 (m, 2H), 4.57 (d, J = 5.6 Hz, 1H), 5.72 (s, 1H), 6.34 (br, 2H), 7.90 (s, 1H), 10.64 (s, 1H).

N^2-(Dimethylaminomethylene)-2′-*O*-(*tert*-butyldimethylsilyl)-3′,5′-*O*-(di-*tert*-butylsilanediyl)guanosine (2). *N*,*N*-Dimethylformamide dimethyl acetal (11.9 g, 13.3 mL, 100 mmol) was added with stirring to a suspension of **1** (14.3 g, 25 mmol) in methanol (150 mL). The reaction was heated at 50 °C for 5 h. Cooling the reaction mixture afforded a colorless precipitate, which was collected by filtration, washed with cold methanol, and dried under reduced pressure to give **2** (14.5 g, 98% yield) as a colorless powder: ^{1}H NMR ($CDCl_3$) δ 0.14 (s, 6H), 0.93 (s, 9H), 1.05 (s, 9H), 1.07 (s, 9H), 3.13 (s, 3H), 3.34 (s, 3H), 4.00–4.06 (m, 1H), 4.18–4.21 (m, 2H), 4.42 (d, J = 3.6 Hz, 1H), 4.49–4.52 (m, 1H), 5.93 (s, 1H), 7.60 (s, 1H), 8.59 (s, 1H); HRMS (ESI^+) calcd for $C_{27}H_{49}N_6O_5Si_2^+$ ($M + H^+$) m/z 593.3298, found m/z 593.3330.

N^2-(Dimethylaminomethylene)-2′-*O*-(*tert*-butyldimethylsilyl)-5′-*O*-(*p*,*p*′-dimethoxytrityl)guanosine (3). A chilled solution of hydrogen fluoride–pyridine (2.0 mL, 100 mmol) in pyridine (12 mL) was added to a solution of **2** (14.8 g, 25 mmol) in dichloromethane (100 mL) at 0 °C. The reaction mixture was stirred for 2 h. The reaction mixture was washed with water and with an aqueous sodium hydrogencarbonate-saturated solution. The organic layer was evaporated under reduced pressure to give a residual material. This resulting product was dissolved in pyridine (50 mL) and to this was added dimethoxytrityl chloride (9.3 g, 27.5 mmol). The mixture was stirred for 12 h, then the reaction was quenched by adding methanol (5 mL). Concentration of the resulting mixture gave an oily product. This material was dissolved in ethyl acetate and washed with water, a sodium hydrogencarbonate solution, and brine. The organic layer was dried and subjected to silica gel (400 g) column chromatography to give **3** (16 g, 86% yield) as an amorphous solid: ^{1}H NMR ($CDCl_3$) δ −0.13 (s, 3H), 0.01 (s, 3H), 0.83 (s, 9H), 2.80 (d, J = 3.7 Hz, 2H), 3.02 (s, 3H), 3.07 (s, 3H), 3.35 (dd, J = 4.0, 10.5 Hz, 2H), 3.77 (s, 6H), 4.20 (q, J = 3.5 Hz, 1H), 4.30 (q, J = 3.5 Hz, 1H), 4.69 (t, J = 5.5 Hz, 1H), 5.97 (d, J = 6.0 Hz, 1H), 6.80–6.82 (m, 4H), 7.18–7.32 (m, 13H), 7.41–7.43 (m, 2H), 7.80 (s, 1H), 8.51 (s, 1H), 9.46 (s, 1H); HRMS (ESI^+) calcd for $C_{40}H_{50}N_6O_7Si^+$ ($M + H^+$) m/z 755.3583, found m/z 755.3579.

N^2-(Dimethylaminomethylene)-2′-*O*-(*tert*-butyldimethylsilyl)-5′-*O*-(*p*,*p*′-dimethoxytrityl)guanosine 3′-[(2-Cyanoethyl) *N*,*N*-Diisopropylaminophosphoramidite] (4). To a solution of **3** (3.8 g, 5 mmol), 2,4,6-collidine (4.4 g, 4.8 mL, 36 mmol), and *N*-methylimidazole (205 mg, 0.2 mL, 2.5 mmol) in THF (25 mL) was added $NCCH_2CH_2OP[N(i\text{-}C_3H_7)_2]Cl$ (3.0 g, 12.5 mmol) at 0 °C. The mixture was stirred at 25 °C for 1 h and then diluted with ethyl acetate. The resulting mixture was washed with an aqueous sodium hydrogencarbonate solution, then with brine, and the organic layer was concentrated to give an oily material. This crude product was dissolved in dichloromethane (20 mL) and the resulting solution was added dropwise to hexane (1 L). The resulting precipitate was collected by filtration and dried under reduced pressure to give **5** in ca. 90% purity (3.7 g, 78% yield) as a colorless powder: ^{1}H NMR ($CDCl_3$) δ −0.13, −0.10, 0.02, 0.03 (4 s, 6H), 0.81, 0.82 (2 s, 9H), 1.16–1.30 (m, 12H), 2.24–2.39 (m, 2H), 2.95, 3.07, 3.08, 3.09 (4 s, 6H), 3.24–3.28 (m, 1H), 3.53–3.64 (m, 4H), 3.78, 3.79, 3.80, 3.81 (4 s, 6H), 4.27–4.39 (m, 2H), 4.67–4.74 (m, 1H), 5.97, 6.04 (2 d, J = 6.4 Hz, 1H), 6.71–6.85 (m, 4H), 7.21–7.48 (m, 13H), 7.85, 7.88 (2 s, 1H), 8.50, 8.58 (2 s, 1H), 8.67, 8.69 (2 s, 1H); ^{31}P NMR ($CDCl_3$) δ 148.88, 150.02; HRMS (ESI^+) calcd for $C_{49}H_{68}N_8O_8Si^+$ ($M + H^+$) m/z 955.4662, found m/z 955.4639.

N^2-(Dimethylaminomethylene)-2′-*O*-(*tert*-butyldimethylsilyl)guanosine 3′-(Allyl 2-Cyanoethyl Phosphate) (5). A heterogeneous mixture of the phosphoramidite **4** (3.82 g, 4.0 mmol), allyl alcohol (0.33 mL, 279 mg, 4.8 mmol), and powdery MS 3A (200 mg) in acetonitrile (16 mL) was stirred at 25 °C for 30 min. To this was added imidazolium perchlorate (1.35 mg, 8.0 mmol) and stirring was continued for an additional 30 min. To the resulting mixture was added a 6.7% solution of 2-butanone peroxide/dimethyl phthalate in toluene (8.0 mL). The mixture was stirred for 5 min and then MS 3A were removed by passage through a Celite 545 pad. The filtrate was diluted with ethyl acetate (100 mL) and then washed with an aqueous sodium hydrogen carbonate-saturated solution, followed by washing with brine. The organic layer was concentrated to give a residue material. This

product was dissolved in dichloromethane (30 mL) and then cooled at 0 °C. To the resulting solution was slowly added dichloroacetic acid (6.6 mL, 10.4 g, 80 mmol) at 0 °C, and then the mixture was stirred at the same temperature for 5 min. The reaction mixture was poured into an aqueous sodium hydrogen carbonate-saturated solution (100 mL), and the organic layer was separated. The aqueous layer was extracted with dichloromethane (100 mL, 50 mL × 2). The combined organic extracts were dried and concentrated to give a residual product. This crude material was subjected to column chromatography on silica gel (100 g) using a 1:20 mixture of methanol and dichloromethane, followed by a 1:10 mixture of methanol and dichloromethane as eluents, to afford **5** (2.15 g, 86% yield; a mixture of two diastereomers) as a colorless amorphous solid: ^{1}H NMR ($CDCl_3$) δ −0.274, −0.23 (2 s, 3H), −0.09 (s, 3H), 0.78, 0.79 (2 s, 9H), 2.80 (t, $J = 6.0$ Hz, 2H), 3.12 (s, 3H), 3.19 (s, 3H), 3.76 (q, $J = 11.6$ Hz, 1H), 3.88–3.91 (m, 1H), 4.27–4.35 (m, 2H), 4.46–4.48 (m, 1H), 4.63–4.67 (m, 2H), 4.97 (m, 1H), 5.08–5.17 (m, 1H), 5.31–5.37 (m, 1H), 5.41–5.45 (m, 1H), 5.69–5.72 (m, 1H), 5.95–6.03 (m, 1H), 7.63 (s, 1H), 8.40 (s, 1H), 9.37 (br, 1H); ^{31}P NMR ($CDCl_3$) δ −2.34; HRMS (ESI^+) calcd for $C_{25}H_{40}N_7O_8PSi^+$ ($M + H^+$) m/z 626.2518, found m/z 626.2628.

The Guanylyl(3′–5′)guanosine 3′-Phosphate 6. A mixture of the phosphoramidite **4** (1.7 g, 1.6 mmol) and the 5′-*O*-free nucleoside phosphate **5** (980 mg, 1.6 mmol) in the presence of powdery MS 3A (200 mg) in acetonitrile (10 mL) was stirred at 25 °C for 30 min. The mixture was treated with imidazolium perchlorate (540 mg, 3.2 mmol) and stirred for an additional 30 min. To this mixture was added a 6.7% 2-butanone peroxide/dimethyl phthalate–toluene solution (3.2 mL). After 5 min, the reaction mixture was passed through a Celite 545 pad to remove MS 3A. The filtrate was then concentrated to afford a viscous oil. This material was dissolved in dichloromethane (20 mL). To this solution was added dichloroacetic acid (3.3 mL, 5.2 g, 40 mmol) at 0 °C. After stirring for 5 min, 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 using a 1:20 methanol–dichloromethane mixture and then a 1:10 methanol–dichloromethane mixture as an eluent to afford **6** (1.53 g, 82% yield; a mixture of four diastereomers) as a colorless amorphous solid: ^{1}H NMR ($CDCl_3$) δ −0.26 to 0.03 (m, 12H), 0.74–0.96 (m, 18H), 2.72–2.80 (m, 4H), 3.09, 3.11, 3.18, 3.22 (4 s, 12H), 3.70–3.81 (m, 2H), 4.17–4.64 (m, 11H), 4.88–5.25 (m, 4H), 5.31–5.44 (m, 2H), 5.73–6.00 (m, 3H), 7.51–7.82 (m, 4H), 8.37 (s, 1H), 8.61 (s, 1H), 9.24 (br, 2H); ^{31}P NMR ($CDCl_3$) δ −1.78, −1.72, −1.45, −1.32, −1.05, −0.83; HRMS (ESI^+) calcd for $C_{47}H_{74}N_{14}O_{15}P_2Si_2^+$ ($M + H^+$) m/z 1193.4545, found m/z 1193.5944.

Conversion of 6 to the Protected Cyclic Bis(3′–5′)diguanylic Acid 7. To a solution of **6** (848 mg, 0.71 mmol) in acetone (10 mL) was added sodium iodide (1.06 g, 7.1 mmol), and the resulting solution was stirred under reflux for 2 h. The resulting colorless precipitate was collected by filtration, washed with chilled acetone (50 mL), and dried. This solid was suspended in THF (120 mL) and to this mixture were successively added *N*-methylimidazole (0.11 mL, 115 mg, 1.4 mmol) and 2,4,6-triisopropylbenzenesulfonyl chloride (424 mg, 1.0 mmol). The resulting solution was stirred for 36 h. To the reaction mixture was added water (50 mL); stirring was then continued for an additional 1 h. The reaction mixture was extracted with ethyl acetate (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 mixture of methanol and dichloromethane and then a 1:10 mixture of methanol and dichloromethane afforded **7** (676 mg, 85% yield) as an amorphous solid: ^{1}H NMR (CD_3OD) δ −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 (2 d, $J = 5.0$ 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) δ 0.75; HRMS (ESI^+) calcd for $C_{44}H_{69}N_{14}O_{14}P_2Si_2^+$ ($M + H^+$) m/z 1135.4126, found m/z 1135.4455.

Preparation of *c*-di-GMP (8) by Full Deprotection of 7. To a suspension of **7** (116 mg, 0.1 mmol) in methanol (8 mL) was added a concentrated aqueous ammonia solution (8 mL), and the resulting mixture was stirred at 50 °C for 12 h. The reaction mixture was concentrated under reduced pressure, and the residue was dried in vacuo. The resulting product was mixed with $(C_2H_5)_3N \cdot 3HF$ (1.0 mL) and this mixture was stirred for 12 h. To the reaction mixture was added a 1 M (= 1 mol dm^{-3}) ammonium acetate buffer solution (10 mL). The mixture was vigorously stirred at 30–40 °C to precipitate a pale yellow solid. After removal of the resulting precipitate, the aqueous solution was subjected to preparative HPLC using a COSMOSIL 5C_{18}-AR-300 column [25 (diameter) × 200 (height) mm]. Elution was carried out under these 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 give the diammonium salt of **8** (67 mg, 89% yield): UV (a 50 mM solution of NH_4OAc in H_2O) λ_{max} 254 nm (ε 23700); ^{1}H NMR (D_2O) δ 4.04–4.06 (m, 2H), 4.38–4.44 (m, 4H), 5.08 (s, 2H), 5.33 (s, 2H), 6.12 (s, 2H), 8.25 (s, 2H); ^{13}C NMR (D_2O) δ 62.8, 70.9, 73.7, 80.8, 90.6, 116.4, 136.9 (weak), 150.4 (weak), 154.1, 157.8; ^{31}P NMR (D_2O) δ −0.61; HRMS (ESI^-) calcd for $C_{20}H_{23}N_{10}O_{18}P_2^-$ ($M - H^-$) m/z 689.0876, found m/z 689.0853. These spectral data were identical to those of the authentic sample.[6]

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17 In Ref. 6, we reported that, on the basis of only HPLC analysis, *c*-di-GMP is decomposed extensively by treatment with hot concentrated aqueous ammonia to give undesired products which might be those resulting from cleavage of the internucleotide linkage. However, our recent studies indicated that this matter is a misunderstanding by us. Treatment of *c*-di-GMP with hot concentrated aqueous ammonia (50 °C, 12 h) actually changed *c*-di-GMP to give some other products, but they were not products undergoing undesired internucleotide-bond cleavage. The products were several kinds of aggregates of *c*-di-GMP without skeletal change, which all revert to the monomer under certain conditions such as in a 0.9% aqueous sodium chloride solution or in a >100 mM ammonium acetate buffer solution. Further, it has been disclosed that *c*-di-GMP easily aggregates under various conditions such as dissolving in water and in a low concentration of aqueous sodium acetate and ammonium acetate solution. We are carrying out investigations on the aggregation of *c*-di-GMP, including structure determination of the aggregates, and will report the results of the investigation in separated papers in the future. Consequently, hot ammonia treatment does not cleave internucletide bonds of *c*-di-GMP and thus the dmf group was used as an effective protector for the guanine base, causing no decomposition of the target compound by the deprotection.

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