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Synthesis of Biotinylated c-di-GMP and c-di-AMP using Click Conjugation

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Abstract

The biotinylated c-di-GMP and c-di-AMP conjugates **10a/b** were synthesized by a straightforward set of procedures from standard, commercially available phosphoramidites. Their availability should allow isolation and characterization of new protein and RNA receptors for these key bacterial signaling molecules.

INTRODUCTION

The bacterial signaling molecules cyclic diguanosine monophosphate (c-di-GMP, **6a**) and cyclic diadenosine monophosphate (c-di-AMP, **6b**) are both key second messengers.^{1, 2} c-di-GMP has received significant attention as recent research has begun to elucidate its complex, multi-faceted role in facilitating a rapid response to environmental cues by regulating biofilm formation as well as expression of virulence factors.^{3–8} Of several identified protein receptors for c-di-GMP, the best studied is the PilZ domain,^{3, 4, 9} to which it can bind as either a monomer or a self-intercalated dimer.¹⁰ In addition, c-di-GMP can bind to two different classes of riboswitch, which are noncoding regulatory mRNA domains.^{11–17} c-di-AMP has only recently been discovered in bacteria, where it serves as a signal to help regulate cell wall synthesis and cell division, including sporulation.^{18–21} In addition, although neither c-di-GMP nor c-di-AMP functions as a second messenger in eukaryotes, they both are known to trigger an innate immune response in infected hosts.^{22, 23} A mouse transmembrane protein named STING in the innate immune sensing pathway has recently been identified as a specific receptor for cyclic dinucleotides.²⁴ Because of their critical functions in essential bacterial pathways, c-di-GMP and c-di-AMP clearly have major implications for human health. However, in spite of advances in the identification of proteins and RNA to which c-di-GMP and c-di-AMP bind, a great many additional receptors remain unknown.²⁵ Biotinylated analogs of c-di-GMP and c-di-AMP should prove to be valuable tools for detection of crucial new receptors.

We recently reported a one-flask, gram-scale synthesis for c-di-GMP and its hydrolysis-resistant [R_p, R_p] and [R_p, S_p] dithiophosphate analogs.²⁶ In addition, we have described their concentration- and metal-dependent polymorphism, demonstrating that they can associate to form not only self-intercalated dimers, but also higher order guanine quartet assemblies.^{27–29}

We now present a straightforward approach for attaching hexynyl phosphoramidite to the 2'-OH of an intermediate readily available from our method. The resulting alkyne, once fully

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deprotected, can then be conjugated to a biotinylated azide using click chemistry^{30–32} to give 2'-biotinylated c-di-GMP, **10a**. We also describe the preparation of c-di-AMP, **6b**, as well as its biotinylated conjugate, **10b**, by the same procedures used for c-di-GMP. Beaucage has recently reported a method to synthesize a different 2'-biotinylated c-di-GMP, starting with 2'-propargylated protected guanosine.³³ Their conjugate differs in having a single methylene between the 2'-oxygen and the triazole ring formed by a click reaction. In contrast, **10a/10b** has a charged phosphate and a (CH₂)₄ tether to the triazole. In addition, Sintin has very recently described the reduced binding to several protein and RNA receptors of yet a third 2'-biotinylated c-di-GMP, which has a carbamate connection between the 2'-oxygen and a biotinylated linker.³⁴ The net favorabilities of the many interactions involved in the binding of these biotinylated conjugates to a wide range of receptors are difficult to predict. Therefore, the availability of a variety of different biotinylated c-di-GMP and c-di-AMP conjugates should prove useful for isolation and identification of new receptors.

EXPERIMENTAL PROCEDURES

General Methods

5'-Hexyn-1-yl-(2-cyanoethyl)-(N,N-diisopropyl)-phosphoramidite and N-biotin-2-aminoethyl-diethyleneglycolyl-ethyl azide were purchased from Glen Research. CuBr and tris((1-benzyl-1H-1,2,3-triazol-4-yl)methyl)amine (TBTA) were purchased from Sigma-Aldrich. The DMSO and *tert*-BuOH used in the click reactions were both anhydrous. Benzoyl adenosine phosphoramidite was purchased from Thermo Fisher Scientific.

Analytical RP HPLC was performed on a Waters 2960 system with an Atlantis C18 column, 100 Å, 4.6 mm × 50 mm, 3 μm, using gradients of CH₃CN and 0.1M aq Et₃NHOAc (TEAA) (pH 6.8) at a flow rate of 1.0 mL/min. ESI-MS was acquired in negative mode using a Waters Micromass single quadrupole LCZ system. Normal phase purifications were done on disposable Varian SuperFlash SF25 cartridges at 10 mL/min. Semi-preparative RP HPLC purifications were performed on a Waters Novapak C18 19 × 300 mm column, using gradients of CH₃CN and 0.1 M aq NH₄HCO₃ (pH 6.8) at 6 mL/min. Li⁺ salts were obtained by passing samples through a small column (1.5 cm diameter) containing ~1 mL of AG 50W-X2 sulfonic acid resin, which had been converted to its Li⁺ form using LiOH.

Maximum UV absorbances were determined at 25 °C in the solvent indicated on an Aviv 14DS UV/VIS spectrophotometer. Molar extinction coefficients, ϵ , were determined by dissolving a known mass of a lyophilized or precipitated sample in water in a volumetric flask, and measuring the absorbances of four diluted portions at 25 °C using different path length cells. The slope of a plot of absorbance/path length vs concentration gave the value of ϵ . Masses for these molar extinction coefficient determinations, as well as for the final yields of **9a/b** and **10a/b**, were determined in Corning centrifuge tubes exposed to an anti-static U-electrode before weighing.

All NMR spectra were acquired on a Varian VNMRS 500 MHz spectrometer at 25 °C in the solvents specified. Samples were heated to 50 °C for ten min prior to acquisition. The ¹H and ¹³C spectra in *d*₆-DMSO were referenced to that solvent, while those in D₂O were referenced indirectly to 3-(trimethylsilyl)-1-propane-sulfonic acid, sodium salt. The ¹H spectra in D₂O were acquired with frequency presaturation for water suppression, and therefore the resonances adjacent to 4.8 ppm are diminished. The ³¹P spectra were referenced indirectly to neat phosphoric acid.

One-flask synthesis of **4a**

A portion of **4a** was prepared following our previously published procedure,²⁶ starting with 3.00 mmol of **1a**. Both diastereomers of **4a** were purified together on 80 g of silica gel, using a gradient of 0 to 25% CH₃OH in CH₂Cl₂, to give 1.615 g of **4a** (1.36 mmol, 45% from **1a**), which was characterized as follows: *m/z* (**M-H**) 1110 (calculated for C₄₃H₆₆N₁₁O₁₆P₂Si₂⁻: 1110); UV (CH₃OH) λ_{max} 255 nm; ¹H NMR (*d*₆-DMSO): δ (extra resonances indicate aggregation) 12.22 – 11.61 (8s, 8H), 8.39 – 8.17 (4s, 4H), 7.79 (br, 3H), 6.03 – 5.85 (4d, 4H), 5.32 – 4.02 (m, 20H), 3.03 – 2.67 (m, 8H), 1.22 – 1.06 (m, 37H), 0.87 – 0.65 (4s, 36H), 0.10 – -0.15 (8s, 24H); ¹³C NMR (*d*₆-DMSO) 25 °C: δ 180.2, 180.0, 154.8, 154.7, 149.2, 148.8, 148.4, 148.2, 120.7, 119.8, 119.7, 118.0, 117.9, 86.4, 86.3, 85.1, 84.8, 83.9, 80.4, 72.4, 62.9, 62.8, 62.7, 56.0, 54.8, 51.0, 46.2, 34.9, 34.8, 34.7, 34.6, 27.1, 25.8, 25.4, 25.3, 18.9, 18.8, 18.7, 17.8, 17.7, 17.5, -3.2, -4.9, -5.1, -5.2, -5.5, -5.6, -5.8; ³¹P NMR (*d*₆-DMSO) 25°C: δ (extra resonances indicate aggregation) 1.40, 1.26, 0.91, -2.34.

Monodesilylation to give **7a**

To a portion of **4a** (0.795 g, 0.671 mmol) in a 50 mL plastic centrifuge tube with a short stir bar was added 18 mL of dry CH₃CN. To the stirring suspension was added pyridine•HF (0.128 mL, 4.93 mmol, 7.3 equiv). The mixture was stirred for 18 h. The stir bar was removed, silica gel (100 mg) was added, and the tube was shaken for 30 min to consume excess F⁻. The mixture was filtered through a sintered glass funnel, and the silica gel washed 4× with 6 mL portions of 20% CH₃OH in CH₂Cl₂. The filtrate was concentrated to about 1 mL (not dryness), and 3 mL of CH₂Cl₂ was added. This mixture was purified on 60 g of silica gel, using a gradient of 2 to 30% CH₃OH in CH₂Cl₂, to give 0.211 g of nearly pure **7a** as a mixture of four isomers, *m/z* (**M-H**) 996 (calculated for C₃₇H₅₂N₁₁O₁₆P₂Si⁻: 996), and 0.313 g of recovered **4a**. This **7a** was used as follows without further purification.

Coupling to give protected dimer alkyne, **8a**

To **7a** (0.211 g) from above in a 100 mL RBF with a stir bar was added pyridinium trifluoroacetate (0.485 g, 2.51 mmol, 3 equiv rel to amidite). The mixture was dried 3× by evaporation of CH₃CN, never letting it go dry, and ending with ~3 mL. The flask was sealed with a septum, then evacuated and refilled 10× with dry N₂. 5'-Hexyn-1-yl-(2-cyanoethyl)-(N,N-diisopropyl)-phosphoramidite (0.25 g, 0.838 mmol, ~4.6 equiv) in a sealed bottle was dissolved by addition of 1.5 mL of dry CH₃CN through the septum, and the contents were then transferred to the flask. The solution was stirred for 30 min, and 5.5 M *tert*-butyl hydroperoxide (0.46 mL, 2.51 mmol, 3 equiv rel to amidite) was added. The solution was stirred for 30 min, then concentrated and purified on 40 g of silica gel, using a gradient of 0 to 20% CH₃OH in CH₂Cl₂, to give 0.170 g of nearly pure **8a** as a mixture of eight isomers. This **8a** was used as follows without further purification.

Deprotection to give dimer alkyne, **9a**

To **8a** (0.170 g) from above in a 100 mL RBF with a stir bar was added 10 mL (80 mmol, ~300 equiv rel to each ib) of CH₃NH₂ in anhydrous EtOH (33% by weight). After 3 h, the mixture was concentrated to dryness. Pyridine (3 mL) and Et₃N (2 mL) were added and the mixture was concentrated to an oil. This process was repeated two more times to convert the *tert*-BuNH₃⁺ to the Et₃NH⁺ salt. Pyridine (2 mL) was added and the flask with a vent needle was placed in an oil bath at 55 °C, and the contents stirred for 5 min. Et₃N (1.4 mL) and Et₃N•3HF (0.8 mL, 14.7 mmol F⁻, ~110 equiv) were added simultaneously through separate syringes. *Caution, HF: rinse all needles, syringes, septa, etc with aq K₂CO₃ before discarding.* The solution was stirred for 1h, with occasional swirling. The flask was then removed from the oil bath and placed on a different stir plate. HPLC grade acetone (25 mL)

was added in a slow stream over 1 min, and the resulting suspension of white solid was stirred for 15 min. The product was collected by filtration in a sintered glass funnel, washed 3× with 5 mL of acetone, and dried in a desiccator overnight over KOH. The product was then dissolved in 2 mL of 0.1 M NH_4HCO_3 and purified by semi-preparative RP chromatography, using a gradient of 2 to 40% CH_3CN in 0.1 M NH_4HCO_3 , to give pure **9a** (0.0445 g) as primarily the Et_3NH^+ salt.

Another portion of **9a** was prepared from **4a** by the same method and was then converted to the Li^+ salt. This second portion, **9a**, Li^+ (0.0344 g, 0.0396 mmol), was used to determine a molar extinction coefficient of 22,700 $\text{OD M}^{-1} \text{cm}^{-1}$. Assuming that **9a**, Et_3NH^+ and **9a**, Li^+ have the same extinction coefficient, the original preparation of **9a**, Et_3NH^+ was determined by UV to be 0.0500 mmol, 12% from **4a**, taking into account the recovered **4a** from the monodesilylation step. The Li^+ salt of **9a** was characterized as follows: *m/z* (**M-H**) 849 (calculated for $\text{C}_{26}\text{H}_{32}\text{N}_{10}\text{O}_{17}\text{P}_3^-$: 849); **UV** (H_2O) λ_{max} 253 nm, $\epsilon = 22,700 \text{ OD M}^{-1} \text{cm}^{-1}$; **^1H NMR** (D_2O): δ 7.99 (s, 1H), 7.98 (s, 1H), 5.93 (s, 1H), 5.70 (s, 1H), 5.33 – 5.27 (m, 1H), 5.12 – 5.00 (m, 2H), 4.41 – 4.25 (m, 4H), 3.99 – 3.84 (m, 4H), 2.17 – 2.06 (m, 3H), 1.66 – 1.56 (m, 2H), 1.52 – 1.41 (m, 2H); **^{13}C NMR** (D_2O): δ 158.8, 158.6, 156.5, 156.4, 152.2, 151.5, 139.3, 138.7, 116.6, 116.3, 93.2, 91.4, 88.6, 83.2, 82.9, 78.4, 75.7, 72.6, 71.9, 71.7, 68.7, 64.8, 64.6, 31.6, 31.5, 26.8; **^{31}P NMR** (D_2O): δ 0.14, –0.47, –1.17.

Click conjugation to give biotinylated c-di-GMP, **10a**

A portion of **9a**, Et_3NH^+ (0.0160 mmol determined by UV) was lyophilized in a 15 mL centrifuge tube. The tube was evacuated and refilled 10× with Ar in a small desiccator. A 3:1 mixture of DMSO:*tert*-BuOH (2.0 mL) was added by syringe to a 0.025 mmol sealed bottle of *N*-biotin-2-aminoethyl-diethyleneglycolyl-ethyl azide to make a 0.0125 M solution. This entire solution was transferred by syringe to the lyophilized **9a**, and the mixture was vortexed until it all dissolved. CuBr (0.0092 g, 0.064 mmol, 4 equiv rel to **9a**) was weighed into a 15 mL centrifuge tube pre-filled with Ar. A 3:1 mixture of DMSO:*tert*-BuOH (0.94 mL) was added to a 50 mg bottle of tris((1-benzyl-1H-1,2,3-triazol-4-yl)methyl)amine (TBTA, 0.0942 mmol, 1.47 equiv rel to CuBr) to make a 0.10 M solution. The bottle was evacuated and refilled 10× with Ar, and the solution was then transferred by syringe to the tube of CuBr. This tube was vortexed, then evacuated and refilled 10× with Ar. The CuBr/TBTA solution was transferred using a glass pipet under a flow of Ar to the tube containing **9a**/azide. This final tube was vortexed and left at room temperature.

After 20 h, a thick suspension of Amberlyte CG50 anion exchange resin was prepared in 3:1 DMSO:*tert*-BuOH, and ~2 mL (3.0 meq) was added to the tube containing crude **10a**, which was shaken for 1h. Another 2 mL portion of the resin suspension was placed in a small column (1.5 cm diameter) and allowed to drain. The reaction mixture was then applied to this column and collected in a 50 mL centrifuge tube. The column was washed 15× with 1mL portions of H_2O . The collected solution was divided among four 50 mL centrifuge tubes, diluted further with water, and lyophilized. Water (1 mL) was added to one of the tubes, the solid was scraped from the walls of the tube with a spatula, and the mixture was transferred in turn to each of the other tubes, with scraping. The final mixture was filtered through a 25 mm × 0.45 μm filter into a new 50 mL centrifuge tube. The original tubes were rinsed and scraped sequentially 10× with 1 mL portions of H_2O , with each rinse filtered. The solution of crude **10a** in the final tube was lyophilized.

Crude **10a** was purified by semi-preparative RP chromatography to give pure **10a**, primarily as the Et_3NH^+ salt. This product was converted to the Li^+ salt, to give **10a**, Li^+ (0.00895 g, 0.00682 mmol, 43% from **9a**), which was characterized as follows: *m/z* (**M-H**) 1293 (calculated for $\text{C}_{44}\text{H}_{64}\text{N}_{16}\text{O}_{22}\text{P}_3\text{S}^-$: 1293); **UV** (H_2O) λ_{max} 253 nm, $\epsilon = 18,100 \text{ OD M}^{-1}$

cm⁻¹; ¹H NMR (D₂O): δ 7.96 (s, 1H), 7.93 (s, 1H), 7.63 (s, 1H), 6.06 (s, 1H), 5.87 (s, 1H), 5.12 – 5.05 (m, 1H), 4.95 – 4.87 (m, 1H), 4.84 – 4.75 (s, 1H), 4.65 – 4.57 (m, 2H), 4.49 – 4.38 (m, 3H), 4.32 – 4.15 (m, 4H), 4.02 – 3.93 (m, 2H), 3.86 – 3.75 (m, 4H), 3.54 – 3.38 (m, 10H), 3.23 (t, J = 5 Hz, 2H), 3.15 – 3.07 (m, 2H), 2.82 (dd, J = 13 Hz, 5 Hz, 1H), 2.62 (d, J = 13 Hz, 1 H), 2.54 (t, J = 7 Hz, 1H), 2.10 (t, J = 7 Hz, 2H), 1.61 – 1.33 (m, 8H), 1.27 – 1.15 (m, 2H); ¹³C NMR (D₂O): δ 177.0, 165.5, 159.0, 154.1, 153.9, 148.5, 137.5, 123.5, 116.7, 116.3, 89.2, 87.7, 80.0, 76.1, 73.6, 71.0, 70.0, 69.9, 69.8, 69.7, 69.6, 69.0, 66.2, 62.2, 60.4, 55.5, 50.1, 39.9, 39.1, 39.0, 35.6, 29.4, 28.0, 27.9, 25.3, 24.3; ³¹P NMR (D₂O): δ 0.29, -0.43, -0.92.

One-flask synthesis of **4b**

A portion of adenosine phosphoramidite, **1b** (2.57 g, 2.60 mmol, 1.3 equiv), was dried 3× by evaporation of 16 mL of dry CH₃CN, the last time leaving 8 mL. Four 3 Å molecular sieves were added. To a 2nd portion of **1b** (1.98 g, 2.00 mmol) dissolved in 10 mL of CH₃CN and water (0.072 mL, 4 mmol, 2 equiv) was added pyridinium trifluoroacetate (0.464 g, 2.4 mmol, 1.2 equiv). After 1 min, 10 mL of *tert*-BuNH₂ was added. After another 10 min, the mixture was concentrated to a foam, the residue was dissolved in 20 mL of CH₃CN, and concentrated again to a foam. This addition and concentration were repeated one more time. To the residue dissolved in 24 mL of CH₂Cl₂ was added H₂O (0.360 mL, 20 mmol, 10 equiv), followed by 24 mL of 6% dichloroacetic acid (DCA, 17.6 mmol) in CH₂Cl₂. After 10 min, the reaction was quenched by addition of pyridine (2.8 mL, 35 mmol, 2 equiv rel to DCA). The mixture was then concentrated, and the residue was dissolved in 16 mL of CH₃CN and concentrated again. This process was repeated two more times, the last time leaving **2b** in 5 mL.

To the above solution of **2b** was added the dried solution of **1b** using a syringe with a bent needle (to access as much of the solution as possible). After 2 min, anhydrous *tert*-butyl hydroperoxide 5.5 M in decane (1.1 mL, 6 mmol, 3 equiv) was added. After 30 min, the solution was cooled in an ice bath, and 0.50 g of NaHSO₃ dissolved in 1 mL H₂O was added. The ice bath was immediately removed, the mixture was stirred for 5 min, and then concentrated to a small volume. The residual oil was dissolved in 32 mL of CH₂Cl₂, followed by addition of H₂O (0.36 mL, 20 mmol, 10 equiv) and then 32 mL of 6% DCA (23 mmol) in CH₂Cl₂. After 10 min, the reaction was quenched with 20 mL of pyridine. The mixture was concentrated to a small volume, 60 mL more pyridine was added, and the solution was concentrated again, leaving **3b** in 40 mL.

To the above solution of **3b** was added 5,5-dimethyl-2-oxo-2-chloro-1,3,2-dioxaphosphinane (DMOCP, 1.36 g of 95% reagent, 7 mmol, 3.5 equiv). After 10 min, the reaction was quenched by addition of H₂O (1.28 mL, 70 mmol, 10 equiv rel to DMOCP), and I₂ (0.66 g, 2.6 mmol, 1.3 equiv) was added immediately. After 5 min, the mixture was poured into 280 mL of H₂O containing 0.4 g NaHSO₃. After 5 min of stirring, 8 g of NaHCO₃ was slowly added. After 5 min more of stirring, the aqueous solution was partitioned with 320 mL 1:1 EtOAc:Et₂O. The separated aqueous layer was then partitioned with an additional 80 mL of 1:1 EtOAc:Et₂O. The organic layers were combined and concentrated to an oil, with excess pyridine removed by evaporation of three 15 mL portions of EtOAc.

The above oil was dissolved in CH₂Cl₂ and purified on 80 g of silica gel, using a gradient of 0 to 20% CH₃OH in CH₂Cl₂. Pure fractions of both diastereomers were combined together, concentrated to a foam, and dried in a desiccator over KOH overnight, giving **4b** (1.079 g, 0.884 mmol, 44% from **1b**), which was characterized as follows: *m/z* (M-H) 1146 (calculated for C₄₉H₆₂N₁₁O₁₄P₂Si₂⁻: 1146; UV (CH₃OH) λ_{max} 280 nm; ¹H NMR (*d*₆-

DMSO): δ (extra resonances indicate aggregation) 11.26 – 11.04 (4s, 4H), 8.85 – 8.54 (8s, 8H), 8.08 – 7.92 (m, 8H), 7.67 – 7.50 (m, 12H), 6.18 – 6.03 (4d, 4H), 5.37 – 4.08 (m, 20H), 3.04 – 2.82 (m, 4H), 1.16 – 0.98 (m, 13H), 0.86 – 0.71 (4s, 36H), 0.16 – –0.12 (8s, 24H); ^{13}C NMR (d_6 -DMSO): δ 165.6, 151.8, 151.7, 151.3, 150.5, 150.4, 150.2, 142.7, 142.2, 133.3, 132.5, 129.1, 128.8, 128.5, 125.8, 125.5, 118.1, 118.0, 117.9, 88.3, 80.3, 74.9, 73.9, 73.2, 71.9, 71.4, 63.2, 63.1, 63.0, 62.9, 62.8, 62.7, 50.8, 46.0, 25.8, 25.7, 25.6, 25.5, 25.4, 19.1, 18.5, 18.4, 17.8, 17.6, 17.5, –3.2, –4.4, –5.0, –5.1, –5.2, –5.4, –5.5, –5.6; ^{31}P NMR (d_6 -DMSO): δ (extra resonances indicate aggregation) 0.20, –0.08, –1.09, –2.81.

One-flask synthesis of **5b**

Another portion of **4b** was prepared from **1b** as above on a 2.65 mmol scale. Instead of chromatography, one-half of the organic solution containing **4b** was dissolved in 10 mL CH_3CN and converted to **5b** by addition of 10 mL of *tert*- BuNH_2 . After 10 min, the mixture was concentrated to a foam, the residue was dissolved in 10 mL of CH_3CN , and concentrated again. This addition and concentration were repeated one more time. The residue was then dissolved in 10 mL of CH_3OH , filtered, and concentrated to a foam. The product was crystallized by addition of 10 mL of CH_2Cl_2 , collected by filtration, washed with minimal CH_2Cl_2 , and dried in a desiccator over KOH overnight, giving pure **5b** (0.467 g, 0.376 mmol, 28% from 1.32 mmol of **1b**), which was characterized as follows: m/z (**M-H**) 1093 (calculated for $\text{C}_{46}\text{H}_{59}\text{N}_{10}\text{O}_{14}\text{P}_2\text{Si}_2^-$: 1093); UV (CH_3OH) λ_{max} 280 nm; ^1H NMR (d_6 -DMSO): δ 11.31 (br, 1H), 8.89 (s, 1H), 8.73 (s, 1H), 8.02 (d, $J = 8$ Hz, 2H), 7.64 – 7.58 (m, 1H), 7.49 (t, $J = 8$ Hz, 2H), 6.05 (s, 1H), 4.66 – 4.57 (m, 1H), 4.46 – 4.40 (m, 1H), 4.21 – 4.14 (m, 1H), 4.14 – 4.05 (m, 1H), 3.82 – 3.74 (m, 1H), 0.98 (s, 9H), 0.97 (s, 9H), 0.26 (s, 3H), 0.22 (s, 3H); ^{13}C NMR (d_6 -DMSO): δ 165.5, 151.7, 151.3, 149.9, 141.5, 133.2, 132.4, 128.5, 128.3, 124.9, 89.7, 76.0, 74.5, 69.4, 61.5, 50.6, 26.8, 25.9, 17.9, –4.2, –5.1; ^{31}P NMR (d_6 -DMSO): δ –3.08.

c-di-AMP, **6b**

To a portion of **5b** (0.443 g, 0.357 mmol) in a 250 mL RBF was added 25 mL of CH_3NH_2 in anhydrous EtOH (33% by weight, 200 mmol, 280 equiv rel to each benzoyl). After 1 h at room temperature, the mixture was concentrated to an oil, to which 3 mL of pyridine and 1 mL of Et_3N were added. The mixture was concentrated to an oil, and this process was repeated two more times to convert the *tert*- BuNH_3^+ to the Et_3NH^+ salt. To the oil was added 10 mL of pyridine, and the flask with a vent needle was placed in an oil bath at 55 °C. Et_3N (4.4 mL) and $\text{Et}_3\text{N}\cdot 3\text{HF}$ (2.6 mL, 48 mmol F^- , 67 eq rel to each TBS) were added simultaneously through two syringes. Caution, HF: rinse all needles, syringes, septa, etc with aq K_2CO_3 before discarding. The mixture was swirled and then stirred at 55 °C. After 20 h, the flask was removed from the oil bath and placed on a different stir plate. HPLC grade acetone (200 mL) was immediately added in a slow stream over 1 min to the stirring mixture. After 10 min, the solid was collected by filtration, washed 5 $\times$ with 5 mL portions of acetone, and dried in a desiccator over KOH overnight, giving pure **6b** (0.247 g, 0.288 mmol, 81% from **5b**, 23% from **1b**), which was characterized as follows: mp 196–198 °C dec; m/z (**M-H**) 657 (calculated for $\text{C}_{20}\text{H}_{23}\text{N}_{10}\text{O}_{12}\text{P}_2^-$: 657); UV (CH_3OH) λ_{max} 258 nm, $\epsilon = 18,600 \text{ OD M}^{-1} \text{ cm}^{-1}$; ^1H NMR (d_6 -DMSO): δ 8.22 (s, 1H), 7.82 (s, 1H), 5.99 (s, 1H), 4.83 – 4.72 (m), 4.67 – 4.65 (s), 4.43 – 4.34 (m, 2H), 4.07 – 3.97 (m, 1H), 3.05 (q, $J = 7$ Hz, 12H), 1.13 (t, $J = 7$ Hz, 18H); ^{13}C NMR (d_6 -DMSO): δ 157.2, 154.7, 149.3, 141.0, 120.8, 92.7, 82.4, 76.4, 72.7, 64.9, 49.3, 10.8; ^{31}P NMR (d_6 -DMSO): δ –0.83.

Monodesilylation to give **7b**

To a portion of **4b** (0.488 g, 0.400 mmol) in a 50 mL plastic centrifuge tube with a short stir bar was added 15 mL of dry CH_3CN . To the stirring suspension was added pyridine $\cdot$ HF

(0.160 mL, 6.16 mmol, 15.4 equiv). The mixture was stirred for 18 h. The stir bar was removed, silica gel (50 mg) was then added, and the tube was shaken for 30 min to consume excess F^- . The mixture was filtered through a sintered glass funnel, and the silica gel washed 4 $\times$ with 6 mL portions of 20% CH_3OH in CH_2Cl_2 . The filtrate was concentrated to about 10 mL, 5 mL of pyridine was added, and the mixture was concentrated to an oil. Additional CH_2Cl_2 was added (10 mL) and the mixture concentrated once more. Then 3 mL of CH_2Cl_2 was added and the mixture was purified on 60 g of silica gel, using a gradient of 2 to 30% CH_3OH in CH_2Cl_2 , to give 0.137 g of nearly pure **7b** as a mixture of four isomers, m/z (**M-H**) 1032 (calculated for $C_{43}H_{48}N_{11}O_{14}P_2Si^-$: 1032), and 0.086 g of recovered **4b**. This **7b** was used as follows without further purification.

Coupling to give protected dimer alkyne, **8b**

To **7b** (0.137 g) from above in a 100 mL RBF with a stir bar was added pyridinium trifluoroacetate (0.970 g, 5.03 mmol, 3 equiv rel to amidite). The mixture was dried 3 $\times$ by evaporation of CH_3CN , never letting it go dry, and ending with ~ 3 mL. The flask was sealed with a septum, then evacuated and refilled 10 $\times$ with dry N_2 . 5'-Hexyn-1-yl-(2-cyanoethyl)-(N,N-diisopropyl)-phosphoramidite (0.50 g, 1.68 mmol, ~ 13.5 equiv) in two sealed bottles was dissolved by addition of 1.5 mL of dry CH_3CN through the septum of the first bottle, the contents transferred to the second bottle, and then to the flask. The solution was stirred for 45 min, and 5.5 M *tert*-butyl hydroperoxide (1.22 mL, 6.72 mmol, 4 equiv rel to amidite) was added. The solution was stirred for 40 min, then concentrated, and purified on 40 g of silica gel, using a gradient of 0 to 25% CH_3OH in CH_2Cl_2 , to give **8b** as a mixture of eight isomers. This **8b** was used as follows without further purification.

Deprotection to give dimer alkyne, **9b**

To **8b** from above in a 100 mL RBF with a stir bar was added 18 mL (144 mmol, ~ 180 equiv rel to each bz) of CH_3NH_2 in anhydrous EtOH (33% by weight). After 3 h, the mixture was concentrated to dryness. Pyridine (3 mL) and Et_3N (2 mL) were added and the mixture was concentrated to an oil. This process was repeated two more times to convert the *tert*- $BuNH_3^+$ to the Et_3NH^+ salt. Pyridine (2 mL) was added and the flask with a vent needle was placed in an oil bath at 55 $^\circ C$, and stirred for 5 min. Et_3N (3.0 mL) and $Et_3N \cdot 3HF$ (1.9 mL, 35 mmol F^- , ~ 85 equiv) were added simultaneously through separate syringes. Caution, HF: rinse all needles, syringes, septa, etc with aq K_2CO_3 before discarding. The solution was stirred overnight. The flask was then removed from the oil bath and placed on a different stir plate. HPLC grade acetone (95 mL) was added over 1 min, and the resulting suspension of white solid was stirred for 15 min. The product was collected by filtration in a sintered glass funnel, washed 3 $\times$ with 5 mL of acetone, and dried in a desiccator overnight over KOH. The product was then dissolved in 2 mL of 0.1 M NH_4HCO_3 and purified by semi-preparative RP chromatography, using a gradient of 2 to 40% CH_3CN in 0.1 M NH_4HCO_3 , to give pure **9b** (9.51 mg) as primarily the Et_3NH^+ salt.

Another portion of **9b** was prepared from **4b** by the same method and was then converted to the Li^+ salt. This second portion, **9b**, Li^+ (0.0755 g, 0.00903 mmol), was used to determine an extinction coefficient of 25,000 OD $M^{-1} cm^{-1}$. Assuming that **9b**, Et_3NH^+ and **9b**, Li^+ have the same extinction coefficient, the original preparation of **9b**, Et_3NH^+ was determined by UV to be 0.0102 mmol, 3% from **4b**, taking into account the recovered **4b** from the monodesilylation. The Li^+ salt of **9b** was characterized as follows: m/z (**M-H**) 817 (calculated for $C_{26}H_{32}N_{10}O_{15}P_3^-$: 817); UV (H_2O) λ_{max} 258 nm, $\epsilon = 25,000$ OD $M^{-1} cm^{-1}$; 1H NMR (D_2O): δ 8.44_s, 1H), 8.43 (s, 1H), 8.21 (s, 1H), 8.20 (s, 1H), 6.43 (s, 1H), 6.19 (s, 1H), 5.22 – 5.16 (m, 1H), 4.59 – 4.41 (m, 4H), 4.18 – 4.01 (m, 4H), 2.37 – 2.22 (m, 3H), 1.82 – 1.72 (m, 2H), 1.67 – 1.57 (m, 2H); ^{13}C NMR (D_2O): δ 153.2, 152.6, 149.9,

148.9, 147.7, 147.4, 140.6, 139.9, 118.5, 118.4, 89.9, 88.1, 86.2, 79.8, 79.7, 76.9, 74.0, 70.3, 69.4, 66.4, 62.3, 62.6, 29.3, 29.2, 24.4, 17.5; ^{31}P NMR (D_2O): δ 0.37, -0.68, -1.30.

Click conjugation to give biotinylated c-di-AMP, 10b

A portion of **9b**, Et_3NH^+ (0.00500 mmol determined by UV) was lyophilized in a 15 mL centrifuge tube. The tube was evacuated and refilled 10 $\times$ with Ar in a small desiccator. A 3:1 mixture of DMSO:*tert*-BuOH (2.5 mL) was added by syringe to a 0.025 mmol sealed bottle of *N*-biotin-2-aminoethyl-diethyleneglycolyl-ethyl azide to make a 0.0100 M solution. A portion of this solution (1.0 mL, 0.010 mmol, 2.0 equiv) was transferred by syringe to the lyophilized **9b**, and the mixture was vortexed until it all dissolved. CuBr (0.0072 g, 0.050 mmol, 10 equiv rel to **9b**) was weighed into a 15 mL centrifuge tube pre-filled with Ar. A 3:1 mixture of DMSO:*tert*-BuOH (0.94 mL) was added to a 50 mg bottle of TBTA (0.0942 mmol) to make a 0.10 M solution. The bottle was evacuated and refilled 10 $\times$ with Ar. A portion of this solution (0.75 mL, 0.075 mmol, 1.5 equiv rel to CuBr) was then transferred by syringe to the tube of CuBr. This tube was vortexed, then evacuated and refilled 10 $\times$ with Ar. The CuBr/TBTA solution was transferred using a glass pipet under a flow of Ar to the tube containing **9b**/azide. This final tube was vortexed and left at room temperature.

After 20 h, a thick suspension of Amberlyte CG50 anion exchange resin was prepared in 3:1 DMSO: *tert*-BuOH, and ~2 mL (3.0 meq) was added to the tube containing **10b**, which was shaken for 1h. Another 2 mL portion of the resin suspension was placed in a small column (1.5 cm diameter) and allowed to drain. The reaction mixture was then applied to this column and collected in a 50 mL centrifuge tube. The column was washed 15 $\times$ with 1 mL portions of H_2O . The collected solution was divided among four 50 mL centrifuge tubes, diluted further with water, and lyophilized. Water (1 mL) was added to one of the tubes, the solid was scraped from the walls of the tube with a spatula, and the mixture was transferred in turn to each of the other tubes, with scraping. The final mixture was filtered through a 25 mm $\times$ 0.45 μm filter into a new 50 mL centrifuge tube. The original tubes were washed and scraped sequentially 10 $\times$ with 1 mL of H_2O , with each rinse filtered. The solution of crude **10b** in the final tube was lyophilized.

Three other portions of **9b** (5.00, 7.45, and 9.00 μmol) were used to prepare additional crude **10b** by the same procedure, which was combined with the first. This combined crude **10b** was purified by semi-preparative RP chromatography to give pure **10b**, primarily as the Et_3NH^+ salt. This product was converted to the Li^+ salt, to give **10b**, Li^+ (0.01020 g, 0.0080 mmol, 30% from 0.0265 mmol of **9b**), which was characterized as follows: *m/z* (**M-H**) 1261 (calculated for $\text{C}_{44}\text{H}_{64}\text{N}_{16}\text{O}_{20}\text{P}_3\text{S}^-$: 1261); UV (H_2O) λ_{max} 259 nm, ϵ = 13,200; ^1H NMR (D_2O): δ 8.33 – 8.29 (2s, 2H), 8.02 – 7.95 (2s, 2H), 7.58 (s, 1H), 6.22 (s, 1H), 6.03 (s, 1H), 5.15 – 5.10 (m, 1H), 4.97 – 4.90 (m, 1H), 4.86 – 4.80 (m, 1H), 4.65 – 4.61 (m, 1H), 4.44 – 4.17 (m, 8H), 4.06 – 3.97 (m, 2H), 3.92 – 3.81 (m, 2H), 3.77 (t, J = 5 Hz, 2H), 3.48 – 3.37 (m, 10 H), 3.21 (t, J = 5 Hz, 2H), 3.06 (p, J = 4 Hz, 1H), 2.78 (dd, J = 13 Hz, J = 5 Hz, 1H), 2.62 – 2.45 (m, 3H), 2.07 (t, J = 7 Hz, 2H), 1.55 – 1.31 (m, 8H), 1.22 – 1.12 (m, 2H); ^{13}C NMR (D_2O): δ 177.0, 167.8, 155.5, 155.4, 152.9, 152.8, 148.4, 148.3, 147.8, 139.9, 139.3, 123.4, 118.7, 118.6, 89.7, 87.5, 79.9, 79.7, 76.6, 73.9, 70.6, 70.5, 69.8, 69.7, 69.6, 69.0, 66.3, 66.2, 62.2, 60.4, 55.5, 50.0, 39.9, 39.1, 35.6, 29.4, 28.0, 27.8, 25.3, 25.0, 24.3; ^{31}P NMR (D_2O): δ 0.29, -0.43, -0.92.

RESULTS AND DISCUSSION

Our previously described one-flask route to c-di-GMP²⁶ started with standard commercial *N*-ib-2'-*O*-TBS-5'-*O*-DMT guanosine phosphoramidite, **1a**. In brief, as shown in Scheme 1, one portion was converted to the H-phosphonate and detritylated with trichloroacetic acid to give *N*-ib-2'-*O*-TBS-guanosine 3'-H-phosphonate, **2a**. Quenching of the acid with pyridine

generated pyridinium trichloroacetate which, along with the pyridinium trifluoroacetate from the previous step, catalyzed the subsequent amidite coupling. After that amidite coupling of **2a** to a second portion of **1a**, followed by oxidation and detritylation, the resulting linear dimer, **3a**, was cyclized by an H-phosphonate coupling and oxidized to give **4a**. To prepare **6a**, first the single cyanoethyl group was removed, and the resulting symmetric product, **5a**, was readily crystallized. This pure intermediate was then fully deprotected and precipitated/crystallized to give **6a**.

Our strategy for the new work reported here on the synthesis of biotinylated c-di-GMP, as shown in Scheme 2, was to perform a monodesilylation of **4a**, while retaining the cyanoethyl group for good chromatographic behavior and reasonable solubility in organic solvents. The resulting intermediate, **7a**, could then be reacted with hexynyl phosphoramidite with minimal side reactions. We therefore prepared a portion of **4a** from **1a** according to Scheme 1, and then purified it by silica chromatography. We had previously carried out similar chromatography to separate dithiophosphate diastereomers,²⁶ but here we combined the two diastereomers to give **4a** in 45% yield from **1a**. We then carried out the monodesilylation of **4a**, using only enough pyridine•HF to give about 50% of **7a** and 10% full desilylation, leaving about 40% of starting **4a**. Silica chromatography of the mixture gave reasonably pure **7a** as four isomers, and the recovered **4a**, which could later be reused.

Commercial hexynyl phosphoramidite (4.5 equiv) was reacted with **7a** to give **8a**. Silica chromatography gave a reasonably pure mixture of eight isomers, which was then fully deprotected with CH₃NH₂ followed by Et₃N•3HF to give the dimer alkyne, **9a**, as a single compound. Although the product was pure after semi-preparative RP chromatography, it was not completely the Et₃NH⁺ salt, as found by NMR. Therefore, a new portion of **9a** was prepared from **4a** the same way, and then converted to the Li⁺ salt, a non-volatile cation that does not favor guanine quartet assemblies. This **9a**, Li⁺ salt was used to determine a molar extinction coefficient of 22,700 OD M⁻¹ cm⁻¹. Assuming **9a**, Li⁺ and **9a**, Et₃NH⁺ salt have the same extinction coefficient, the yield for the original synthesis of **9a**, Et₃NH⁺ salt from **4a** was determined to be 12%, taking into account the recovered **4a** from the monodesilylation. The Et₃NH⁺ salt of **9a** was used in the subsequent click reaction, rather than the Li⁺ salt, because of its better solubility in organic solvents.

Conditions were then optimized for the click reaction with commercial biotinylated azide. We found that 1.5 equiv of the azide in the presence of 4 equiv of CuBr with 1.5 equiv (rel to CuBr) of the stabilizing ligand tris((1-benzyl-1H-1,2,3-triazol-4-yl)methyl)amine (TBTA) in a solvent mixture of 3:1 DMSO: *tert*-BuOH gave complete conversion by HPLC of **9a**, Et₃NH⁺ salt to the biotinylated c-di-GMP, **10a**, after 20 h at room temperature. However, we discovered that a significant amount of **10a** was lost by associating with the TBTA that precipitated upon addition of H₂O. We therefore shook the completed reaction mixture with a suspension of Amberlyte CG50 weak anion exchange resin in the same solvent for an hour to release the product, and then applied it to a small column containing additional resin. The resulting solution was diluted with H₂O and lyophilized. Purification by semi-preparative RP chromatography gave pure **10a**, but again not completely the Et₃NH⁺ salt. It was therefore converted to the Li⁺ salt and lyophilized to give **10a**, Li⁺ salt in 43% isolated yield from **9a**.

We also report here that c-di-AMP, **6b**, can be prepared by the same one-flask procedure we used for c-di-GMP. The symmetric intermediate **5b** was obtained in 28% yield from **1b**, and c-di-AMP, **6b**, was isolated in 81% yield from **5b**, 23% yield from **1b**. The only difference in the procedure was a longer time for the final removal of the TBS groups. In both cases, the use of an amidite coupling to prepare the linear dimer, followed by the extremely fast and specific H-phosphonate cyclization, is an ideal combination that gives a high-yield,

scalable, and efficient set of reactions that do not require chromatography. In recent years, others have prepared c-di-AMP on fairly small scales using an amidite coupling with a phosphotriester cyclization,^{15, 35} as well as phosphotriester reactions for both steps.³⁶

In addition to the facile preparation of c-di-AMP, we demonstrate here that its biotinylated conjugate, **10b**, can be synthesized by the same method used for biotinylated c-di-GMP, **10a**. The intermediate dimer alkynes described in this work, **9a/b**, are versatile synthons and could readily be conjugated with longer biotinylated azides or even with azides linked to other functionalities.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS

bz	benzoyl
c-di-AMP	cyclic diadenosine monophosphate
c-di-GMP	cyclic diguanosine monophosphate
DCA	dichloroacetic acid
DMOCP	5,5-dimethyl-2-oxo-2-chloro-1,3,2-dioxaphosphinane
DMSO	dimethylsulfoxide
ib	isobutyl
RBF	round bottom flask
RP HPLC	reverse phase high pressure liquid chromatography
TBS	<i>tert</i> -butyl-dimethylsilyl
TBTA	tris((1-benzyl-1H-1,2,3-triazol-4-yl)methyl)amine
TEAA	triethylammonium acetate

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Scheme 1.

Syntheses of c-di-GMP and c-di-AMP from commercially available phosphoramidites **1a** and **1b**.

Scheme 2.
Syntheses of biotinylated c-di-GMP and c-di-AMP.