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Synthesis of 2′-Modified Cyclic Bis(3′–5′)diadenylic Acids (*c*-di-AMPs) and Their Promotion of Cell Division in a Freshwater Green Alga

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Three 2′-modified cyclic bis(3′–5′)diadenylic acids (*c*-di-AMPs) were synthesized from commercially available adenosine phosphoramidites and the effects of *c*-di-AMP derivatives on the cell division of *Chlamydomonas reinhardtii*, a kind of freshwater green algae, were investigated. This structure–activity relationship study suggests that the di(2′-*O*-methyl)-*c*-di-AMP showed the highest activity and that 2′-substituents influenced the cell division of *C. reinhardtii*.

c-di-AMP (Chart 1)[1,2] is a recently identified second-messenger molecule in bacteria, and it has great potential in many research fields. This molecule performs numerous important functions in bacteria, such as monitoring DNA integrity during sporulation and playing an essential role in cell wall homeostasis in *Bacillus subtilis*,[3,4] and controlling cell size and envelope stress in *Staphylococcus aureus*.[5] Although the existence of endogenous *c*-di-AMP in animals has not been disclosed, *c*-di-AMP secreted by intracellular *Listeria monocytogenes* has been shown to activate a host type I interferon response in cultured cells.[6] Moreover, mucosally administered *c*-di-AMP exerted strong adjuvant activities in mice.[7]

To date, the investigations of the physiological action of *c*-di-AMP have been performed using bacteria[1–6] and animals.[6,7] However, as far as we know, there has been no report of an investigation in planta. Therefore, we examined the potential physiological actions by *c*-di-AMP in *Chlamydomonas reinhardtii*, a kind of freshwater green algae frequently used as a model alga,[8] and found that *c*-di-AMP promotes the cell division of *C. reinhardtii*. Moreover, we synthesized various *c*-di-AMP analogs to investigate the structure–activity relationship related to this phenomenon.

Among various analogs, 2′-modified *c*-di-AMPs are interesting based on reports that the substituents at the 2′ positions of nucleotides and nucleic acids are correlated with phosphodiesterase stability,[9,10] cell permeability,[11,12] and nucleic acid duplex stability,[13] which in turn influence biological activities. However, there are only a few reports on the synthesis of *c*-di-AMPs with 2′ modification.[14–21] For example, cyclic deoxydiadenylic acid (**1a**) (Chart 1) was synthesized by phosphotriester[14–17] and *H*-phosphonate[18] methods. In addition, previously published methods for synthesis of *c*-di-AMP such as homopolymerization of adenosine 3′-monophosphate,[22] construction of a cyclic sugar backbone followed by introduction of the protected adenine,[23] and the phosphotriester method[24,25] cannot be applied for synthesis of various 2′-modified analogs due to the low yield of the final product and/or multistep reactions. Thus, we synthesized the three *c*-di-AMP analogs shown in Chart 1 by a combination of the phosphoramidite and phosphotriester approaches based on methods previously published by us[20] and by Hayakawa.[26]

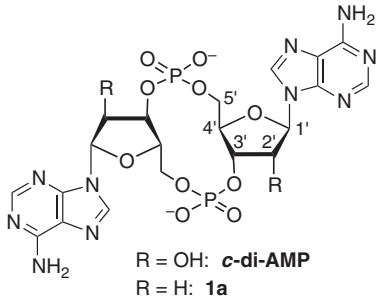
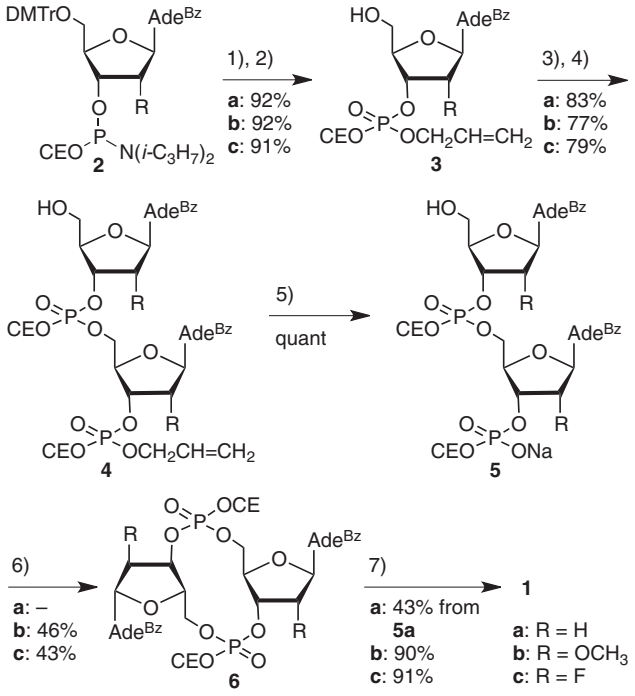


Chart 1.

The synthesis starts from the commercially available adenosine cyanoethyl (CE) phosphoramidites **2** with the substituent R (H, OCH_3, and F) at the 2′-position as shown in Scheme 1. First, the amidites **2** were converted into the 3′-phosphotriesters **3** by a three-step procedure consisting of condensation with allyl alcohol in the presence of imidazolium perchlorate (IMP),[27] oxidation of the resulting phosphite triester by *tert*-butyl hydroperoxide,[28,29] and removal of the 5′-*O*-*p*,*p*′-dimethoxytrityl (DMTr) group under acidic conditions. The 5′-hydroxy-free nucleotides **3** were then elongated with the amidites **2** by the above three-step procedure to afford the protected linear dimers **4**. The overall yields of **4** (71–76%) from the beginning of synthesis were almost the same as in the synthesis of *c*-di-AMP [R = *tert*-butyldimethylsilyloxy (OTBDMS), 79%].[20]

Subsequently, the allyl groups on the 3′-terminal phosphates of **4** were deprotected by sodium iodide. However, the aqueous workup using a triethylammonium hydrogen carbonate solution, followed by extraction with dichloromethane, was unable to collect the linear dinucleotide 3′-phosphodiesters **5** as triethylammonium salts due to their hydrophilic properties. Fortunately, the sodium salts **5** were precipitated quantitatively as white powder from the reaction mixture and were characterized by FAB-MS and NMR measurements. This is quite in contrast with the more hydrophobic 2′-*O*-TBDMS analog (R = OTBDMS), which can be isolated only by aqueous workup.[20]

The sodium salts **5** thus obtained were cyclized by a mixture of 2,4,6-triisopropylbenzenesulfonyl chloride and *N*-methylimidazole in THF under high-dilution conditions (substrate concentration: ca. 6 mM) to give the fully protected *c*-di-AMP analogs **6**.[20,26,30] Yields of this cyclization were 46% and 43% for **6b** and **6c**, respectively. The precise yield of **6a** could not be



Scheme 1. *Reagents and conditions*: 1) (i) allyl alcohol, imidazolium perchlorate (IMP), molecular sieves 3 Å (MS 3 Å), CH_3CN, rt, (ii) *t*-C_4H_9OOH/toluene, rt; 2) $CHCl_2COOH$, CH_2Cl_2, 0 °C; 3) (i) **2**, IMP, MS 3 Å, CH_3CN, rt, (ii) *t*-C_4H_9OOH/toluene, rt; 4) $CHCl_2COOH$, CH_2Cl_2, 0 °C; 5) NaI, acetone, reflux; 6) 2,4,6-triisopropylbenzenesulfonyl chloride, *N*-methylimidazole, THF, 28 °C; 7) concd aq. NH_3–CH_3OH (1:1 v/v), 50 °C.

calculated.[31] However, the cyclization proceeded similarly, as judged by the overall yield of **1a** (vide infra). The two-step yields (43–46%) of **6** from **4** are inferior to that of the 2′-*O*-TBDMS analog (57%) in *c*-di-AMP synthesis.[20]

Finally, the benzoyl (Bz) and cyanoethyl (CE) protecting groups of **6** were removed by treatment with concentrated aqueous ammonia and methanol to afford the target *c*-di-AMP analogs **1** as triethylammonium salts. Figure 1 shows the HPLC profiles of the final products obtained after purification by reversed-phase preparative HPLC. The profiles indicate that the purities were satisfactory. The scales and overall yields of the synthesis as well as molecular ion peaks in ESI-MS analyses are summarized [Table S1 in Supporting Information (SI)[32]]. The observed molecular ion peaks are consistent with the calculated values. Moreover, the fragmentation patterns of the analogs **1** in the ESI tandem mass measurements are the same as those of *c*-di-AMP (Figures S1–S4 in SI[32]), confirming that the final products possess the cyclic dinucleotide structures.[6,20] In addition, the triethylammonium salt of **1a** was converted into the corresponding sodium salt by treatment with a cation-exchange resin. The ^1H and ^{31}P NMR spectra of the obtained sample are consistent with the reported data (see SI[32]).[14]

The biological activity of *c*-di-AMP was investigated as follows. The culture of *C. reinhardtii* was started using 4×10^5 cells per plastic dish (2.0×10^5 cells mL^{-1}) at time zero. After culturing for 3 days, the cell numbers of *C. reinhardtii* were estimated. Based on the dose-response curve from 0 to 100 μM

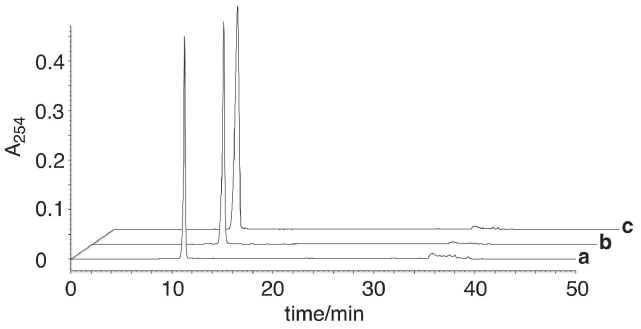


Figure 1. Reversed-phase HPLC profiles of purified (**a**) **1a**, (**b**) **1b**, and (**c**) **1c** under the following conditions: column, COSMOSIL 5C_{18}-AR-II [4.6 (diameter) mm × 250 (height) mm]; flow rate, 1.0 mL min^{-1}; detection, 254 nm; eluent and gradient [A: 100 mM $CH_3COO^-NH_4$ in H_2O, B: a 20:80 mixture of H_2O and CH_3CN, 0–2 min A 100%, 2–32 min with a linear gradient from A 100% to A 70%/B 30%, 32–50 min B 100%]; temperature, 40 °C.

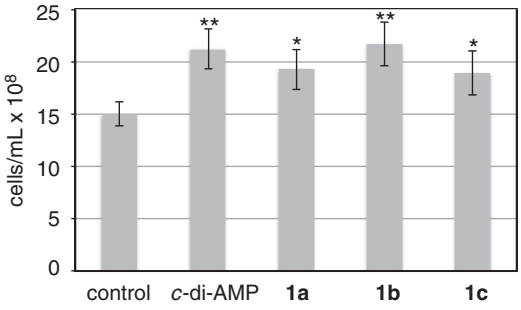


Figure 2. The 2′-substitution effects of *c*-di-AMP on the cell division of *Chlamydomonas reinhardtii*. *C. reinhardtii* cells were cultured in the presence and absence of *c*-di-AMP (10 μM), **1a** (10 μM), **1b** (10 μM), or **1c** (10 μM) for 3 days under a light condition of 17 μmol m^{-2} s^{-1} at 27 °C. Vertical bars represent the means ± S.E. ($n = 50$). The asterisks indicate a significant difference in the cases of *c*-di-AMP, **1a**, **1b**, and **1c** compared with the control at $P < 0.01$ (**) and $P < 0.05$ (*) by Student's *t*-test.

of *c*-di-AMP, the optimum concentration was found to be 10 μM (Figure S6 in SI[32]). The cell division (cell number) was promoted to 19.04×10^8 cells mL^{-1}, or increased approximately 42%, by culture with 10 μM *c*-di-AMP, and this effect was statistically significant (Figure 2). Other adenine nucleotides such as cyclic adenosine 3′,5′-monophosphate (*c*AMP) and adenosine 5′-monophosphate (5′-AMP) under the same conditions showed lesser promotions with 17% and 14%, respectively (Figure S7 in SI[32]).

The 2′-substitution effects of *c*-di-AMP on the cell division of *C. reinhardtii* (2.3×10^5 cells mL^{-1} at time zero) were similarly investigated. Figure 2 shows that all of the analogs enhanced the cell division. The cell numbers after culture increased in the following order: **1b** (increased by 45%), *c*-di-AMP (41%), **1a** (28%), and **1c** (26%), which probably reflected several factors of the *c*-di-AMP derivatives, such as phosphodiesterase stability, cell permeability (or lipophilicity), affinity to the receptor, and so on. The lipophilicities of the *c*-di-AMPs decreased in the following order: **1b** > **1c** > **1a** > *c*-di-AMP, as

estimated by the retention times in the reversed-phase HPLC analysis (Figure 1 and SI[32]).[20] Thus, the lipophilicity is not the only dominant factor for the biological activity: the other factors might contribute as well. Consequently, the 2′-substituents did not interfere with the enhancement of cell division by *c*-di-AMP.

In summary, based on the practical synthesis of *c*-di-AMP and its 2′-modified analogs, we revealed that the *c*-di-AMPs promote the cell division of *C. reinhardtii*, which is the first such finding in planta. The three 2′-modified analogs from the commercially available adenosine phosphoramidites were synthesized in overall yields of 28–32%. The key intermediates are the linear dinucleotide 3′-phosphodiester sodium salts, which can be cyclized by the phosphotriester method to give fully protected cyclic products. Our method provides a general route for various 2′-modified analogs regardless of the electronic properties of 2′-substituents. Among the analogs, di(2′-*O*-methyl)-*c*-di-AMP showed the highest activity (albeit comparable to that of *c*-di-AMP), and all the 2′-substituents positively influenced the cell division. We believe that the structure–activity relationship studies including the 2′-substitution effects will lead to an understanding of the signaling mechanism of *c*-di-AMP.

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# These authors contributed equally to this work.

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31 This is due to a substantial amount of *N*-methylimidazolium 2,4,6-triisopropylbenzenesulfonate, which is unable to separate from **6a** by column chromatography.

32 Supporting Information is available electronically on the CSJ-Journal Web site, http://www.csj.jp/journals/chem-lett/index.html.

