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Synthesis of Cyclic Bis(3′-5′)-2′-deoxyguanylic/guanylic Acid (*c*-dGpGp) and Its Biological Activities to Microbes

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Cyclic bis(3′-5′)diguanylic acid (*c*-di-GMP) is an important compound with various biological activities, including regulation of cellulose synthesis in the bacterium *Acetobacter xylinum*,[1,2] acceleration of DNA synthesis and retardation of cell division in Molt4 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] reduction of the virulence of biofilm-forming *S. aureus* strains in a mouse model of mastitis infection,[7] and activation of the immune response.[8] Furthermore, *c*-di-GMP is considered to play an important role in regulating exopolysaccharide production, biofilm formation, and other phenotypes.[9] These attractive biological properties of *c*-di-GMP prompted us to carry out a systematic study of the bioactivity of *c*-di-GMP-related compounds, including derivatives with modified nucleoside bases, carbohydrates, or internucleotide bonds. This study may lead not only to the discovery of new bioactive compounds, but also to an elucidation of the mechanism by which *c*-di-GMP affects the cell receptors. As a part of this study, we prepared cyclic bis(3′-5′)-2′-deoxyguanylic/guanylic acid (*c*-dGpGp) (**6**) and investigated its effect on the biofilm formation and motility of several bacteria.

c-dGpGp (**6**) was previously synthesized by van Boom and coworkers.[2] However, as they did not describe the experimental details of the synthesis, we could not successfully perform the synthesis according to their method. Therefore, we developed a novel synthetic method for *c*-dGpGp as shown in Scheme 1. The nucleoside phosphoramidite **1**[10] was reacted with allyl alcohol by using imidazolium perchlorate (IMP)[11] as a promoter in the presence of molecular sieves 3A (MS 3A)[12] in acetonitrile (30 min) and the resulting phosphite product was oxidized with a 5 M *tert*-butyl hydroperoxide (TBHP)/decane solution[13] (30 min) to give the nucleoside phosphotriester in 96% yield. This product was treated with a 20% dichloroacetic acid/dichloromethane solution (30 min) to remove the 5′-*O*-*p*,*p*′-dimethoxytrityl (DMTr) protector, giving the nucleoside 3′-phosphate **2** in 82% yield. The product **2** was reacted with the phosphoramidite **3** by the aid of IMP in acetonitrile containing MS 3A (30 min), followed by a 5 M TBHP/decane solution (30 min), and then the 5′-*O*-DMTr group of the resulting product was deblocked by a 20% dichloroacetic acid/dichloromethane solution (30 min) to afford the linear (3′-5′)-linked diguanylate **4** in 76% overall yield. Subsequently, the cyanoethyl group on the 3′-terminal phosphotriester moiety of **4** was removed by exposure to diisopropylamine in methanol[14] (2 h), and the resulting product was subjected to intramolecular cyclization using a mixture of 2,4,6-trisipropylbenzenesulfonyl chloride (TPSCl) (5 equiv) and *N*-methylimidazole (5 equiv) (36 h) in THF using a previously reported method[15] to provide fully protected *c*-dGpGp **5** in 91% overall yield. Finally, **5** was reacted with a 1:1 (*v*/*v*) mixture of conc. aqueous ammonia and methanol at 50 °C (12 h) to eliminate the dimethylformamidine (dmf)-protecting group and the allyl-protecting group, followed by $(C_2H_5)_3N{\cdot}3\,HF$[16] to deblock the *tert*-butyldimethylsilyl (TBDMS)-protecting groups (12 h) to afford the target compound **6** in 40% overall yield.

To consider the biological effects of *c*-dGpGp, we first examined its inhibitory effect (at a high concentration) on the biofilm formation of *Pseudomonas aeruginosa*, *Vibrio parahaemolyticus*, and *S. aureus* and compared these levels of inhibition with those by *c*-di-GMP. Experiments were carried out using strains of *P. aeruginosa* PAO1, *S. aureus* MS2507, and *V. parahaemolyticus* ATCC17802. Approximately 10^5 CFUs/mL of *P. aeruginosa* and *S. aureus*, and 10^7 CFU mL^{-1} of *V. parahaemolyticus* were incubated in L broth for *P. aeruginosa* and *S. aureus* and heart infusion broth for *V. parahaemolyticus* supplemented with 200 μM of *c*-dGpGp and 200 μM of *c*-di-GMP, respectively, in the wells of a polystyrene microtiter plate without shaking at 30 °C for 24 h. Subsequently, dishes were washed with saline twice and stained with crystal violet as described previously.[6,17] As control, aliquots of the microbes were incubated under the same conditions but without the nucleic acid treatment. Measurements were performed in triplicate. Experiments were repeated at least twice.

The effect of *c*-dGpGp and *c*-di-GMP on bacterial biofilm formation is shown in Figure 1. Both cyclic dinucleotides suppressed the biofilm formation of the three different kinds of bacteria, although the activity of *c*-dGpGp was less than that of *c*-di-GMP. The difference in the inhibitory activity should not be due to the growth suppression of bacteria, as the number of viable bacteria (colony forming unit, CFU) after the treatment with *c*-dGpGp and *c*-di-GMP were not different from those of control experiments (data not shown).

According to a previous study[18] using a microbe with flagella, motility and the ability to form biofilm are closely related. Therefore, we subsequently investigated the effect of *c*-dGpGp and *c*-di-GMP on the motility of the three bacteria, which belong to different groups in the bacterial taxonomy. The examinations were performed using *P. aeruginosa* PAO1, *Salmonella enterica* serovar Typhimurium LT2, and *V. parahaemolyticus* ATCC17802 according to the methods described in a previous study.[19,20] The motility of *P. aeruginosa* and *S. typhimurium* were measured in the presence of 200 μM of *c*-di-GMP and *c*-

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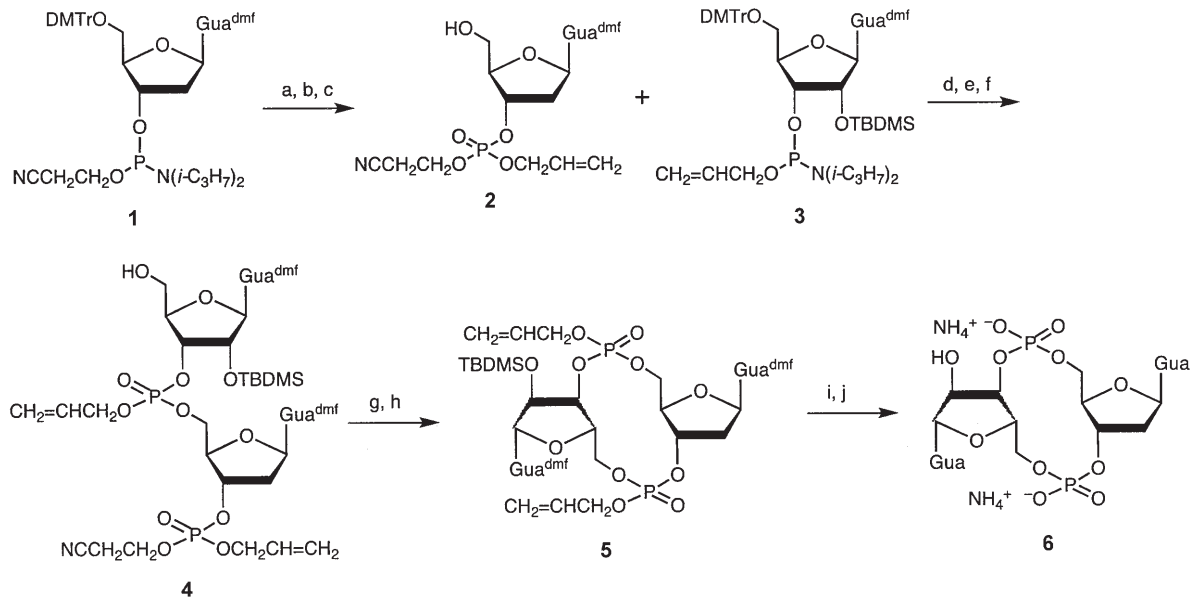

Scheme 1. a) allyl alcohol, IMP, MS 3A, CH_3CN, 25 °C, 30 min; b) 5–6 M TBHP/decane solution, 25 °C, 30 min; c) 20% $Cl_2CHCOOH/CH_2Cl_2$ solution, 25 °C, 30 min; d) IMP, MS 3 A, CH_3CN, 25 °C, 30 min; e) 5–6 M TBHP/decane solution, 25 °C, 30 min; f) 20%$Cl_2CHCOOH/CH_2Cl_2$ solution, 25 °C, 30 min; g) $(iPr)_2NH$-CH_3OH (1:1 *v/v*), 25 °C, 1 h; h) TPSCl, *N*-methylimidazole; 25 °C, 36 h; i) conc. aq. NH_3-CH_3OH (1:1 *v/v*), 50 °C, 12 h; j) $(C_2H_5)_3N{\cdot}3HF$, 25 °C, 12 h.

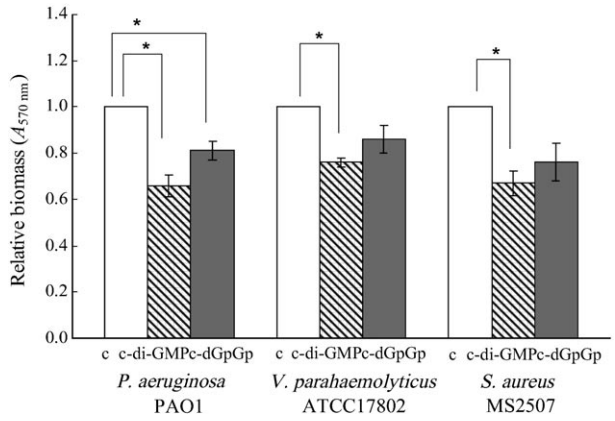

Figure 1. Effect of cyclic-dinucleotides on biofilm formation. Biofilm was stained with crystal violet and solubilized with dimethyl sulfoxide to measure at OD_{570}. Longitudinal axis expresses relative biomass to control. * $P < 0.05$.

Table 1. Effect of c-di-GMP and c-dGpGp on motility of some bacteria.

Strain	Change of motility[a]	
	treatment with *c*-di-GMP	treatment with *c*-dGpGp
P. aeruginosa PA01	8% promotion[b]	17% repression[b]
S. typhimurium LT2	37% repression[b]	29% repression[b]
V. parahaemolyticus ATCC17802	60% promotion[c]	10% repression[c]

[a] Comparison with the sample untreated with the cyclic dinucleotide. [b] Treated with 200 mM c-di-GMP and c-dGpGp. [c] Treated with 100 mM c-di-GMP and c-dGpGp.

dGpGp, and that of *V. parahaemolyticus* was measured in the presence of 100 μM of *c*-di-GMP and *c*-dGpGp. These bacteria were incubated on plates of 0.3% agar L broth containing each concentration of c-di-GMP and c-dGpGp at 30 °C for 20 h and the diameter of the swimming was measured as the index of motility. As control, aliquots of the three microbes were incubated under the same conditions but without the cyclic dinucleotides. The resulting motility patterns are shown in Figure 2. Further, Table 1 summarizes the mobility of the treated bacteria relative to that of the untreated bacteria. Thus, these examinations revealed that *c*-di-GMP promoted the motility of *P. aeruginosa* and *V. parahaemolyticus*, but repressed the motility of *S. typhimurium*; on the other hand, *c*-dGpGp weakly repressed the motility of all of the bacteria.

We next examined why *c*-dGpGp and *c*-di-GMP should exhibit different biological activities despite their similar structures. Although a number of explanations are conceivable, the most feasible one is that *c*-dGpGp and *c*-di-GMP have different three-dimensional conformations and thus different binding affinity to receptors (target molecules). Thus, the most stable conformations of *c*-dGpGp and *c*-di-GMP were analyzed by the B3LYP/6-31 + G(d,p)level MO calculation method using Spartan 04. Figure 3 exhibits the results. Comparison of the resulting structures indicated that the conformation of one of the two guanine groups was quite different between *c*-dGpGp and *c*-di-GMP. As the obtained conformations were not those in water, we propose—but cannot definitively state—that the conformational difference in *c*-dGpGp and *c*-di-GMP may be one of the factors causing their different biological properties.

As a part of our ongoing investigation of the biological activity of *c*-di-GMP and its analogues, we prepared *c*-dGpGp (**6**) and investigated the biological activities of this compound in comparison with those of *c*-di-GMP. The investigation disclosed that both *c*-dGpGp and *c*-di-GMP inhibit the biofilm formation

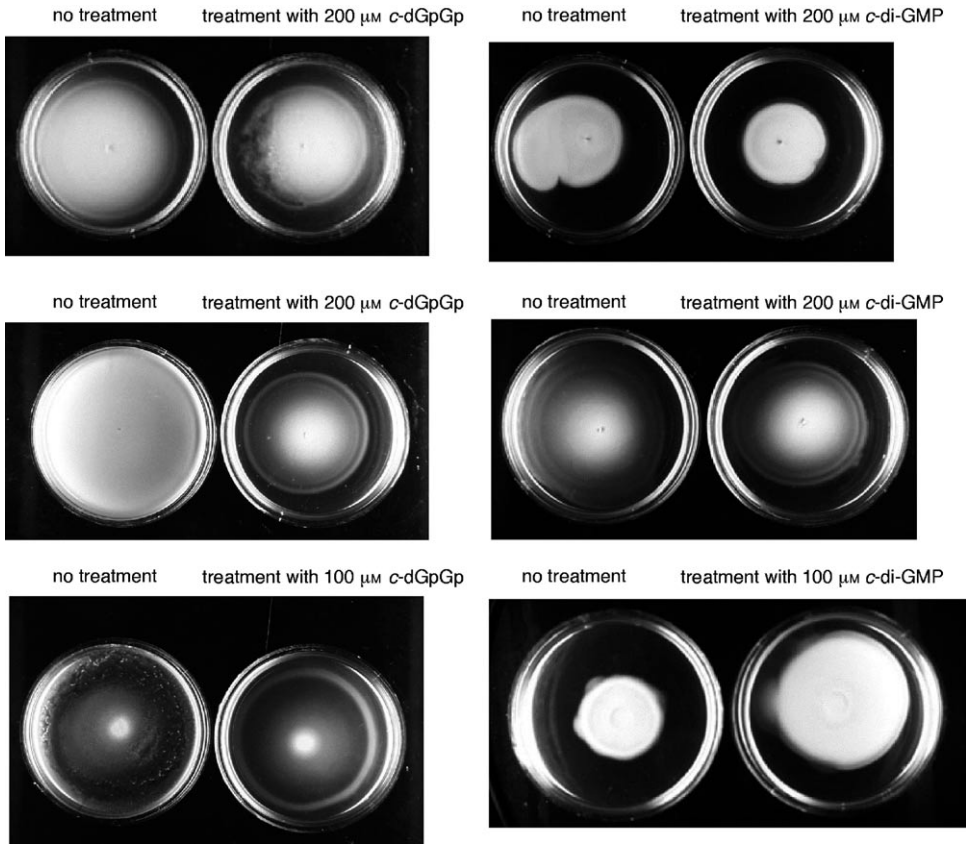

Figure 2. Motility of *P. aeruginosa*, *S. typhimurium*, and *V. parahaemolyticus* treated with and without c-dGpGp and c-di-GMP. Upper panel: *P. aeruginosa*; middle panel: *S. typhimurium*; lower panel: *V. parahaemolyticus*.

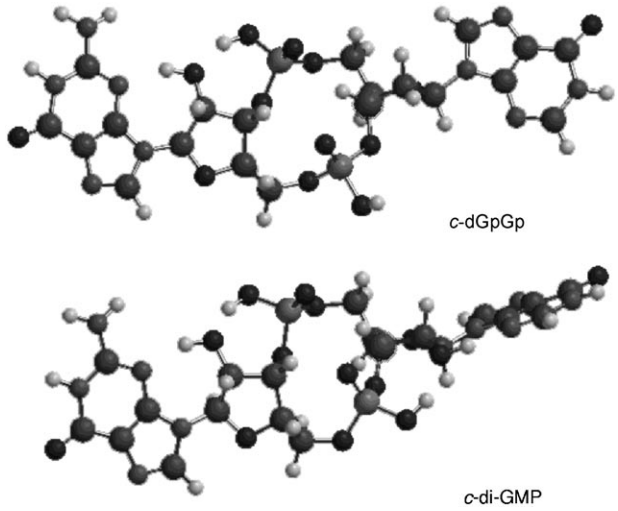

Figure 3. The most stable comformations of c-dGpGp and c-di-GMP.

of *P. aeruginosa*, *V. parahaemolyticus*, and *S. aureus*, and the inhibitory effect of *c*-di-GMP is higher, though not significant, than that of *c*-dGpGp. Further, it was revealed that *c*-dGpGp slightly represses the motility of *P. aeruginosa*, *V. parahaemolyticus*, and *S. typhimurium*. The observed activities for *P. aeruginosa* and *V. parahaemolyticus* were different from those of *c*-di-GMP. That is, *c*-di-GMP promoted the motility of *P. aeruginosa* and *V. parahaemolyticus*. These findings suggest that certain analogues of *c*-di-GMP, much like those of *c*-di-GMP, may have good potential as antibacterial agents, and thus further investigations on the biological properties of various *c*-di-GMP analogues are necessary. Among the discoveries in the present work, the fact that both the motility and biofilm-formation ability were depressed in *V. parahaemolyticus* treated with *c*-dGpGp may be the most attractive and important because previous examinations have suggested that enzymes that produce biofilm and those that enhance the motility of microbes do not work simultaneously. Thus, when biofilm formation occurs, the microbe stops its movement. Conversely, when the microbe moves, biofilm formation is stopped. Therefore, microbe motility should be promoted in microbes, in which biofilm formation is inhibited. However, the present experiments were not designed to examine this hypothesis. This result has motivated us to carry out extensive and systematic investigations for elucidating the true relationship between biofilm-forming ability and motility of microbes with flagella.

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Keywords: biological activity · *c*-di-GMP · nucleotides · structure–activity relationships

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