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Identification of Ligand Analogues that Control c-di-GMP Riboswitches

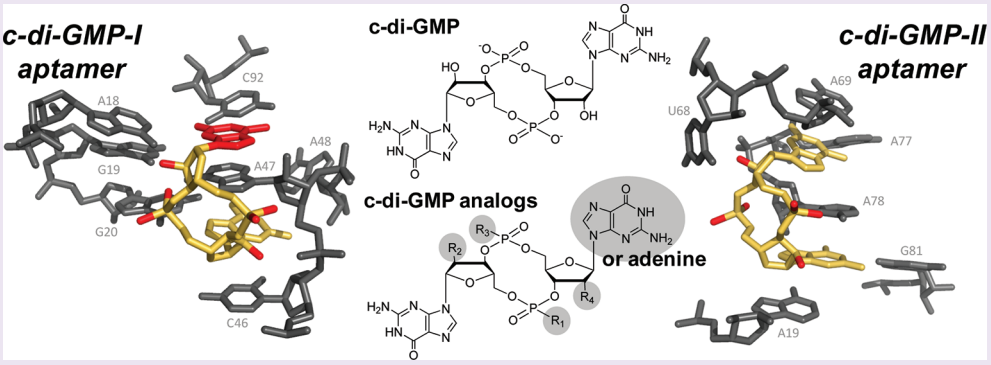
Kazuhiro Furukawa,[†] Hongzhou Gu,[‡] Narasimhan Sudarsan,[‡] Yoshihiro Hayakawa,[∥] Mamoru Hyodo,[⊥] and Ronald R. Breaker*[,†,‡,§]

[†]Department of Molecular, Cellular and Developmental Biology, [‡]Howard Hughes Medical Institute, and [§]Department of Molecular Biophysics and Biochemistry, Yale University, P.O. Box 208103, New Haven, Connecticut 06520-8103, United States

[∥]Department of Applied Chemistry, Faculty of Engineering, Aichi Institute of Technology, 1247 Yachigusa, Yakusa, Toyota 470-0392, Japan

[⊥]Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo, Hokkaido 060-0812, Japan

Supporting Information



ABSTRACT: Riboswitches for the bacterial second messenger c-di-GMP control the expression of genes involved in numerous cellular processes such as virulence, competence, biofilm formation, and flagella synthesis. Therefore, the two known c-di-GMP riboswitch classes represent promising targets for developing novel modulators of bacterial physiology. Here, we examine the binding characteristics of circular and linear c-di-GMP analogues for representatives of both class I and II c-di-GMP riboswitches derived from the pathogenic bacterium *Vibrio choleae* (class I) and *Clostridium difficile* (class II). Some compounds exhibit values for apparent dissociation constant (K_D) below 1 μM and associate with riboswitch RNAs during transcription with a speed that is sufficient to influence riboswitch function. These findings are consistent with the published structural models for these riboswitches and suggest that large modifications at various positions on the ligand can be made to create novel compounds that target c-di-GMP riboswitches. Moreover, we demonstrate the potential of an engineered allosteric ribozyme for the rapid screening of chemical libraries for compounds that bind c-di-GMP riboswitches.

The bacterial second messenger c-di-GMP (Figure 1, compound **1**) is a circular RNA dinucleotide that is biosynthesized from two GTP molecules by various GGDEF-domain diguanylate cyclase (DGC) enzymes and is degraded by various EAL- or HD-GYP-domain phosphodiesterases (PDE).[1,2] Numerous studies have shown that c-di-GMP in certain species activates extracellular polysaccharide production and biofilm formation but inhibits motility and virulence. To exert its function, c-di-GMP commonly binds and allosterically alters enzymes or other proteins that carry specialized receptor sites such as PilZ domains.[3,4] However, this second messenger is also bound by at least two distinct classes of riboswitches,[5,6] and these interactions directly control the expression of a diversity of genes involved in physiological changes brought about by increasing or decreasing c-di-GMP concentrations.

Riboswitches are structured RNA elements that commonly reside within the 5′ untranslated regions (UTRs) of bacterial mRNAs where they control gene expression by directly binding ions or small molecules such as metabolites, coenzymes, amino acids, and nucleobases.[7−10] Riboswitches typically consist of two different functional domains: a ligand-binding aptamer domain and an expression platform that is more directly involved in regulating gene expression.[9−11] Ligand binding stabilizes a specific structural state of the aptamer and subsequently alters folding of an expression platform usually located immediately downstream of the aptamer. Different expression platform structures control gene expression by regulating such processes as transcription termination,[9−11] translation initiation,[9,10] and RNA splicing.[6,12−14]

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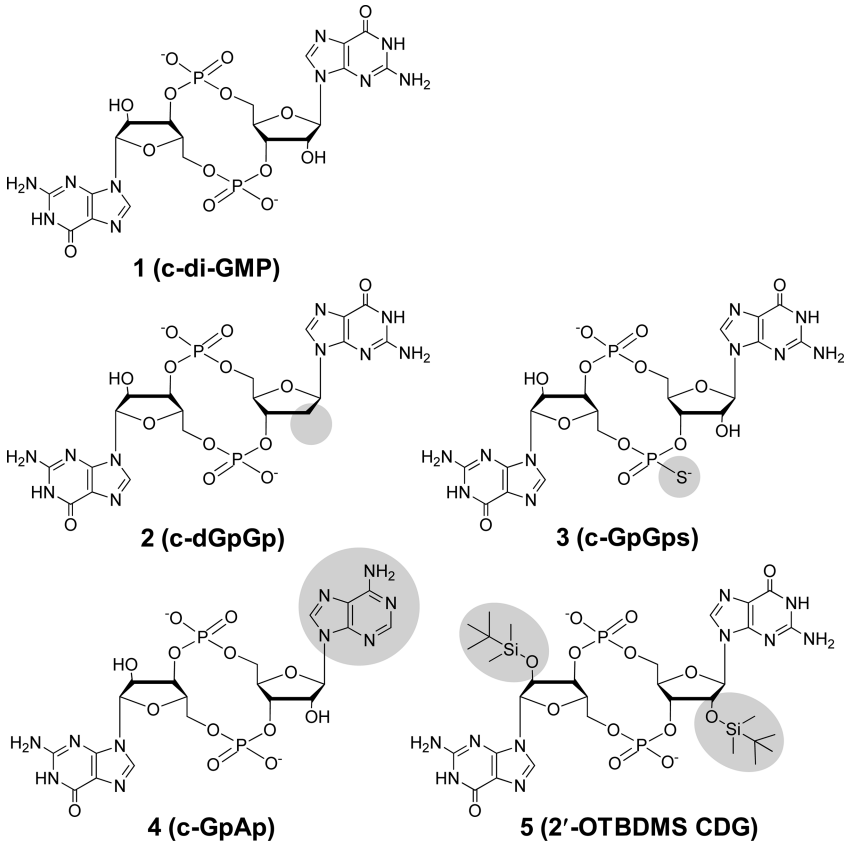

Figure 1. Chemical structures of c-di-GMP and several circular analogues.

We[15−19] and others[20−27] have reported the potential utility of riboswitches as the targets for antimicrobial compounds. For example, the thiamin analogue pyrithiamine has been shown to bind to TPP riboswitch and suppress bacterial[15] and fungal growth.[27] Most antibiotics currently used affect only a few cellular processes,[28] and bacteria have well-developed resistance mechanisms to protect these processes.[29] Therefore, riboswitches offer promising new targets for developing additional antibiotics classes with novel modes of action.

In this study, we focused on examining ligand analogues for representatives of two c-di-GMP riboswitches classes (Figure 2A,B). We examined the binding characteristics of sugar-modified (compounds **2** and **5**), backbone-modified (compound **3**), and base-modified (compound **4**) circular c-di-GMP analogues (Figure 1) for the riboswitches derived from the pathogenic bacteria *Vibrio choleae* and *Clostridium difficile*. Representatives of these riboswitch classes have been crystallized, and atomic-resolution models for their ligand-bound structures are available (Figure 2C,D).[30−32] Empirical binding data from this study and others[33,34] combined with modeling studies should permit the generation of analogues with potent riboswitch-modulating activities.

RESULTS AND DISCUSSION

Riboswitch Binding Affinities of Circular c-di-GMP Analogues. The thermodynamic binding characteristics of c-di-GMP analogues were evaluated by using a method called in-line probing,[35,36] wherein changes in RNA structure due to ligand binding are observed as changes in the levels of spontaneous RNA degradation. For these assays, the riboswitch aptamer regions from the 5′ untranslated region (UTR) of the Tfox-like gene in *V. choleae* and from a possible virulence gene in *C. difficile* were chosen as representatives of class I and II aptamers, respectively. RNA aptamer constructs 110 Vc2 RNA (Figure 2A) and 84 Cd RNA (Figure 2B) were 5′ ^{32}P-labeled, trace amounts were subjected to in-line probing reaction conditions, and the resulting products were separated by denaturing polyacrylamide gel electrophoresis (PAGE) and then visualized using a PhosphorImager (see Supplementary Data for all detailed methods).

Compounds **1**−**5** initially were examined at a ligand concentration of 50 μM to assess relative affinities (Supplementary Figure 1). These assays reveal patterns of spontaneous RNA cleavage and corresponding structural changes that are largely consistent with those observed previously,[5,6] indicating that analogue binding is similar to that of the natural ligand. The use of a range of ligand concentrations revealed the concentrations of ligand required to half-maximally modulate RNA structure (*e.g.*, see Figure 3A,C). These data were then plotted to yield binding curves and K_D values (*e.g.*, see Figure 3B,D).

The K_D values obtained for the circular analogues **2**−**5** for both class I and II aptamers (Table 1) reveal that the class I aptamer retains a strong affinity ($K_D \leq 10$ nM) to compounds **2** and **3** that have relatively minor modifications. However, compound **4**, wherein a single GMP is replaced by AMP, and compound **5**, whose 2′-OH group is modified by a bulky *tert*-butyldimethylsilyl (TBDMS) group, experience a 20,000-fold and 2,000,000-fold loss in binding affinity, respectively. The result with **4** is similar to that reported recently for a similar construct evaluated by different binding assays.[33] Note that the K_D value for **1** is so low that it cannot be accurately measured

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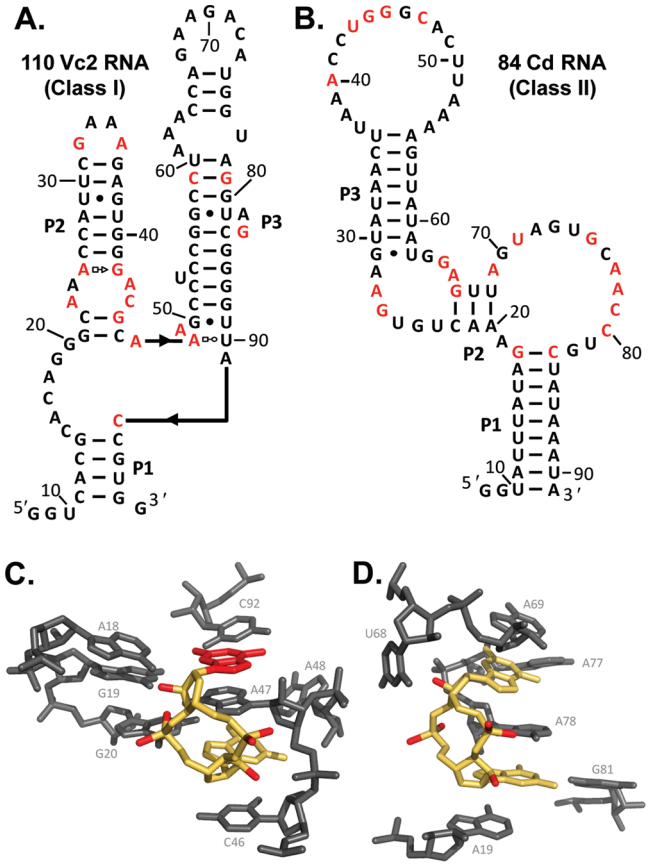

Figure 2. Sequences, secondary- and tertiary-structure models for representative class I and class II c-di-GMP aptamers. (A) A representative c-di-GMP-I aptamer called Vc2 110 RNA from *V. choleae*. (B) A representative c-di-GMP-II aptamer called 84 Cd RNA from *C. difficile*. Red nucleotides are conserved in at least 90% of the representatives identified by bioinformatics.[5,6] (C) Atomic-resolution model for the ligand-binding site of a c-di-GMP-I aptamer.[30,31] Aptamer and c-di-GMP structures are depicted in gray and yellow, respectively. Locations of chemical modifications examined in this study are highlighted in red. (D) Atomic-resolution model for the ligand-binding site of a c-di-GMP-II aptamer. [32] Other annotations are as described for panel C.

by in-line probing, and the number listed has been previously estimated[30] by determining the kinetic parameters (k_{on} and k_{off}) of ligand-aptamer interaction. Likewise, due to limitations of in-line probing, the K_D values for **2** and **3** listed in Table 1 should be considered an upper limit.

In contrast to the class I aptamer, the class II aptamer maintains strong interactions with all circular analogues (K_D values of less than or equal to 10 nM; Table 1). The results with both classes are consistent with their atomic-resolution structure models,[30−32] which reveal that class I aptamers make aptamer-ligand contacts *via* the 2′-hydroxyl groups of c-di-GMP (Supplementary Figure 2A), whereas class II aptamers do not (Supplementary Figure 2B). These differences in molecular recognition contacts permit the use of c-di-GMP analogues that carry bulky groups such as TBDMS at the 2′ ribose positions but retain function as class II riboswitch ligands. This finding highlights opportunities to introduce other chemical groups at these positions potentially to create compounds that have the characteristics needed to modulate gene expression in cells.

Notably, the patterns of RNA degradation products generated during in-line probing of the class I aptamer with 50 μM concentration of circular analogues (Supplementary Figure 1) reveal several differences between **4** and other analogues (Supplementary Figure 2). Specifically, nucleotides 13−17, 95, and 96 remain susceptible to spontaneous cleavage (unstructured) when **4** is bound, whereas these nucleotides become resistant to spontaneous cleavage (structured) when c-di-GMP or its other analogues are bound. This is consistent with the observation that both G bases are recognized by class I aptamers, and the change from G to A in one nucleotide of the ligand causes a loss of ligand affinity and a loss of folding by the P1 region of the aptamer.

Riboswitch Binding Affinities of Linear c-di-GMP Analogues. In addition to the circular analogues, we prepared 18 linear c-di-GMP analogues (Table 2) and subjected these to aptamer binding and riboswitch function assays. Linear analogues have two properties that are more attractive from a drug development perspective. First, these compounds generally are simpler in chemical structure and therefore less costly to synthesize. Second, linear compounds are structurally more dissimilar to c-di-GMP than certain circular derivatives and therefore might be less likely to interact with protein receptors. It is known[5,6,34] that c-di-GMP riboswitches from both classes retain some affinity for pGpG (compound **7**; K_D ~300 nM), which is the linear breakdown product of the second messenger. Thus, we expected that some linear analogues prepared for this study would have a high probability of binding to these aptamers.

Indeed, we find that all linear analogues interact well with the class II aptamer, while only six of these compounds are rejected at concentrations up to 100 μM by the more selective class I aptamer (Table 2). As an example of this in-line probing data, compound **8** with 110 Vc2 RNA (Figure 3C) exhibits robust structural modulation. A plot of the normalized fraction of RNA cleaved in three regions (Supplementary Figure 3A, regions a−c) of the RNA *versus* the logarithm of the concentration of **8** (Figure 3D) reveals a K_D value of ~10 μM (Table 2). Analyses for the other ligands with both aptamer classes (data not shown) were conducted in a similar manner.

Several specific modifications were included in the collection of linear analogues that address important questions. For example, removal of the 5′ phosphate (Table 2, G_α position R_1) eliminates a charged chemical group that otherwise is expected to decrease the compound's ability to cross bacterial cell membranes. Similarly, the introduction of more hydrophobic groups (*e.g.*, Me, TBDMS) at 2′ oxygen atoms (R_2 of G_α; R_4 of G_β) also might improve cell permeability. Also, the use of a phosphorothioate linkage should increase both the cellular permeability[37] and the resistance against phosphodiesterases.[38] Although methylphosphonate linkage analogues (R_3 = Me) were prepared, the compounds exhibited extremely low solubility in both water and dimethylsulfoxide and therefore were not subjected to further analysis.

Among the linear analogues, the natural breakdown product of c-di-GMP (**7**) exhibits the best K_D value for both riboswitch classes. For the class I riboswitch, the presence of the 5′ phosphate group causes very little change in affinity compared to the same analogues lacking this group. This is surprising since both phosphate groups are predicted to form contacts with functional groups of class I aptamers.[30,31] We speculate that the linearization of the ligand might disrupt this aptamer-ligand contact, and therefore removal of this terminal phosphate group in the linear form of the ligand has no further detrimental effect on affinity.

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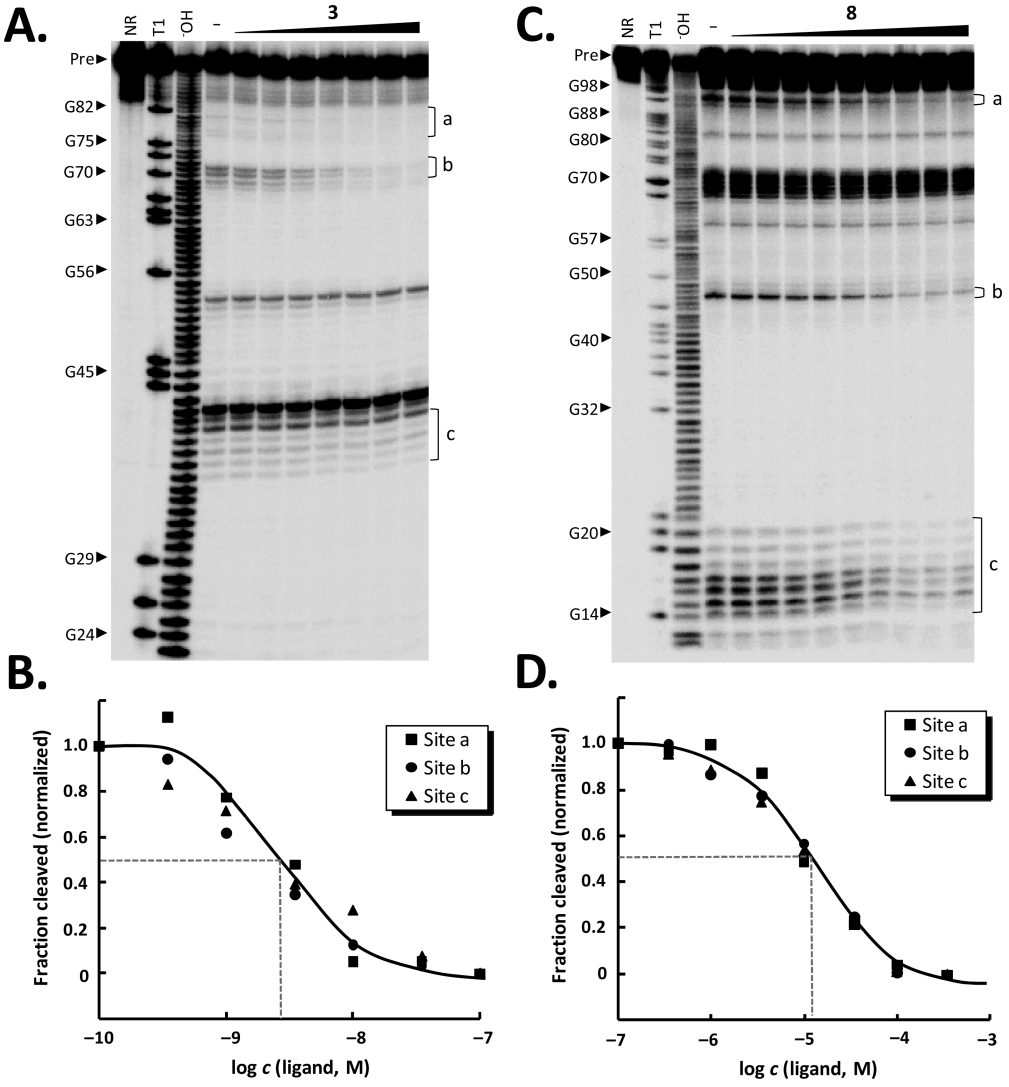

Figure 3. Representative in-line probing assay results and K_D determinations. (A) Autoradiogram of in-line probing products from 5′ ^{32}P-labeled 84 Cd RNA separated by denaturing 10% PAGE. RNAs were incubated with concentrations of **3** ranging from 100 pM to 100 nM. Sites a, b, and c indicate regions that undergo substantial structural modulation. NR, no reaction; T1, partial digest with RNase T1; ⁻OH, partial digest with alkali. Selected bands in the T1 lane are labeled to identify the G nucleotide located 5′ of the RNase T1 cleavage site. (B) Plot of the normalized fraction of 84 Cd RNA cleaved *versus* the concentration of **3**. (C) Autoradiogram of in-line probing products from 5′ ^{32}P-labeled 110 Vc2 RNA incubated with concentrations of **8** ranging from 100 nM to 350 μM. Other details are as described for panel A. (D) Plot of the normalized fraction of 110 Vc2 RNA cleaved *versus* the concentration of **8**.

Table 1. K_D Values and Transcription Modulation Rates for Circular c-di-GMP Analogues with Both c-di-GMP-I and -II Aptamers

	c-di-GMP-I		c-di-GMP-II	
	K_D (nM)[a]	terminated (%)[b]	K_D[a] (nM)	elongated (%)[b]
1	0.01[c]	52.0	≤0.2[d]	58.3
2	5	43.8	2	54.0
3	9	42.3	2	56.2
4	200	14.1	2	44.4
5	20,000	13.0	10	44.0

[a]Measured by the in-line probing assay. [b]Measured by the single round transcription termination assay. The percentage of the terminated products for the class I and the elongated products for the class II without any ligand are 5.2% and 13.7%, respectively (see Supplementary Figure 4 for detail). [c]Value taken from ref 30. [d]Value taken from ref 6.

In contrast, the class II K_D values for the compounds with 5′ phosphate were typically 3- to 10-fold better than those for the corresponding analogues lacking this group. Since a hydrogen bond is known to form between the exocyclic amine of an adenine moiety in the class II riboswitch and one of the nonbridging phosphate oxygen atoms of c-di-GMP,[32] the 5′ phosphate of each linear analogue might retain this interaction. However, the sulfur substitution of a nonbridging phosphate oxygen of our linear analogues (R_3) does not affect the binding affinity with the class II aptamer.

Another notable observation is that the removal of one or both of the 2′ oxygen atoms (H at R_2 and R_4) as in compounds **8**–**11** causes one or more orders of magnitude loss in affinity with the class I aptamer. The losses in affinity for compounds **8** and **9** are similar to the losses observed for compounds **12** and **13** that carry the TBDMS group at R_2 and for compounds **14** and **15** that carry Me groups at R_2. These results suggest that the larger modifications likely block the natural aptamer-ligand

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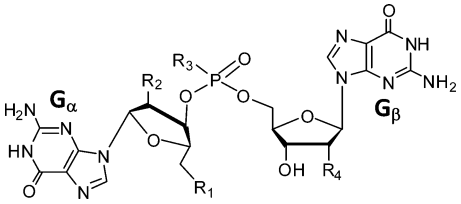
Table 2. Structures, K_D Values, and Transcription Termination Effects for Linear c-di-GMP Analogues with c-di-GMP-I (Vc2 110 RNA) and c-di-GMP-II (84 Cd RNA) Aptamers and Their Corresponding Riboswitches



					c-di-GMP-I		c-di-GMP-II	
	R_1	R_2	R_3	R_4	K_D (μM)[b]	terminated (%)[c]	K_D (μM)[b]	elongated (%)[c]
6	OH	OH	O^-	OH	1	8.4	2	19.3
7	PO_3^-H	OH	O^-	OH	0.3		0.3	
8	OH	H	O^-	OH	10		10	
9	PO_3^-H	H	O^-	OH	20		3	21.4
10	OH	H	O^-	H	>100	4.9	30	17.3
11	PO_3^-H	H	O^-	H	>100		30	
12	OH	O-TBDMS[a]	O^-	OH	10		8	
13	PO_3^-H	O-TBDMS	O^-	OH	8		0.5	24.2
14	OH	OMe	O^-	OH	20		10	
15	PO_3^-H	OMe	O^-	OH	50		3	20.3
16	OH	OH	S^-	OH	3	5.4	2	15.4
17	PO_3^-H	OH	S^-	OH	8		0.8	19.1
18	OH	H	S^-	OH	>100		9	
19	PO_3^-H	H	S^-	OH	>100		5	22.1
20	OH	O-TBDMS	S^-	OH	20		9	
21	PO_3^-H	O-TBDMS	S^-	OH	30		0.8	20.0
22	OH	OMe	S^-	OH	>100		10	
23	PO_3^-H	OMe	S^-	OH	>100		3	28.6

[a]TBDMS indicates a *tert*-butyldimethylsilyl group. [b]Measured by in-line probing assays. [c]Measured by single round transcription termination assays. The percentage of the terminated products for the class I and the elongated products for the class II riboswitches without any ligand are 5.2% and 13.7%, respectively (see Supplementary Figure 4 for details).

contacts, but otherwise these compounds are well accommodated in the aptamer binding pocket.

Riboswitch-Mediated Transcription Termination Triggered by c-di-GMP Analogues. The function of at least some riboswitches is dictated by kinetic factors such as the speeds of RNA folding, aptamer-ligand association, and RNA transcription rather than by the thermodynamic equilibrium between the aptamer and its target ligand.[39] The majority of riboswitches in Gram-positive bacteria including *C. difficile* control gene expression through ligand-dependent competition between a properly folded aptamer structure and an alternate mutually exclusive structure that prohibits or promotes formation of an intrinsic transcription termination stem.[10,11,40] Therefore, each riboswitch may have only a short time to bind its ligand before RNA polymerase reaches the terminator stem region and its genetic decision-making point. Some riboswitch aptamers do not have time to reach thermodynamic equilibrium with their ligand, and in these cases the rate constant for ligand association with the aptamer may be more important than its K_D value.

Given this distinction in kinetic *versus* thermodynamic functions of some riboswitches, we examined whether some of our analogues can trigger riboswitch function during transcription *in vitro*, thereby causing transcription termination (class I riboswitch) or transcription elongation (class II riboswitch). Specifically, *in vitro* transcription termination assays using *Escherichia coli* RNA polymerase were conducted with certain analogues (Supplementary Figure 4), and the percentages of full-length relative to total RNA transcripts were recorded (Tables 1, 2).

A terminator stem was not readily observed by bioinformatics analysis of the Vc2 riboswitch, and therefore we chose to use one of the 12 class I riboswitch representatives from *C. difficile*. DNA templates corresponding to the *C. difficile* c-di-GMP-I riboswitch associated with the flagellar operon is a genetic OFF switch, and therefore ligand binding should cause transcription termination. Indeed, near complete production of a full-length transcript is evident in the absence of ligand, but the reactions yielded close to 50% terminated products when incubated with c-di-GMP or analogues **2** and **3** (Supplementary Figure 4A). All other analogues tested triggered termination to a much lesser extent (Tables 1, 2), which is consistent with their lower binding affinities. However, the differences in K_D values between analogues are not perfectly reflected by the differences in transcription termination values (Table 1). This suggests that the kinetics of analogue association or other structural differences between the analogue-aptamer complexes influence the extent of transcription termination, rather than K_D values alone.

DNA templates corresponding to the c-di-GMP-II riboswitch used for our binding studies is a genetic ON switch, and therefore ligand binding should induce the production of full-length transcripts. As expected, only about 14% of transcripts are full length when the reaction lacks c-di-GMP, whereas this ligand and analogues such as **2**−**5** induce the production of full-length transcripts to near 50% or greater (Supplementary Figure 4B). Again, the extent of full-length transcription

production generally corresponds with the binding affinities for the circular analogues (Table 1). In contrast, nearly all of the linear analogues yield levels of full-length transcription that are within 2-fold of the value observed without ligand addition (Table 2). This finding suggests that these analogues at a concentration of 100 μM cannot bind with sufficient speed to promote transcription elongation efficiently, despite the fact that some of these compounds exhibit K_D values in the low micromolar range.

Preliminary experiments were conducted to determine if some analogues can trigger riboswitch-mediated gene regulation. This was achieved by using riboswitch-reporter fusion constructs carrying either a wild-type or a mutant class I riboswitch located upstream of the coding region for the *E. coli lacZ* gene similar to that described previously.[5] However, cells carrying the reporter construct coding for the mutant riboswitch that cannot bind c-di-GMP exhibits similar changes in expression upon analogue addition as that observed for cells carrying the wild-type construct (Supplementary Figure 5 and data not shown). Since c-di-GMP is involved in bringing about many changes in cell physiology and gene expression, the analogues might be influencing other cellular factors and causing gene expression changes that mask the effect of the riboswitch. Other constructs or methods will be needed to convincingly evaluate the effects of these and other analogues on riboswitch-mediated gene regulation.

Engineered Allosteric Ribozyme Assay for c-di-GMP Analogue Screening. We assessed whether an engineered allosteric hammerhead ribozyme constructed recently[42] can be utilized in a more rapid assay for analogue binding to and activation of a c-di-GMP riboswitch. This allosteric ribozyme (Figure 4A) was generated by conducting *in vitro* selection on a parental population of RNAs formed by fusing a c-di-GMP-I riboswitch aptamer derived from the Vc2 RNA to a hammerhead ribozyme *via* short bridging domains. [42] In a 15-min assay (Figure 4B), the RNA construct exhibits robust activity (79.5% cleavage) in the absence of c-di-GMP, whereas the addition of the second messenger strongly suppresses activity (7.5% cleavage).

Four additional compounds (**4**, **5**, **13**, and **22**) that exhibit different K_D values as determined by in-line probing also were examined for their effects on allosteric ribozyme activity. The results indicated that the levels of ribozyme inhibition correspond well to the K_D values measured for each compound (Figure 5). Furthermore, our findings are consistent with the molecular recognition characteristics of class I aptamers when examined using other c-di-GMP analogues and other binding assays.[32−34,42] Since the ribozyme assays can be conducted with small amounts of RNA with far less incubation time and could make use of fluorescence readouts, this method could be applied for the efficient and rapid screening of analogues that modulate c-di-GMP-I riboswitches. Although a corresponding allosteric hammerhead ribozyme has not been made for c-di-GMP-II aptamers, a natural allosteric group I ribozyme that undergoes self-splicing and is controlled by a c-di-GMP class II riboswitch exists.[6] This RNA should also be useful in screening for c-di-GMP analogues that bind to and activate class II riboswitches.

In summary, the aptamer binding characteristics for more than 20 circular or linear analogues of c-di-GMP were established, and several compounds were determined to modulate riboswitch-mediated transcription termination. Moreover, several compounds were evaluated for their ability to regulate an engineered allosteric ribozyme that can serve as a biosensor for screening chemical libraries for compounds that bind c-di-GMP-I riboswitches. This RNA or similar allosteric ribozyme constructs[41] have the functional characteristics needed for the rapid and high-throughput screening of c-di-GMP analogues. Such assays can be used in a manner similar to that demonstrated for *glmS* riboswitches.[43,44]

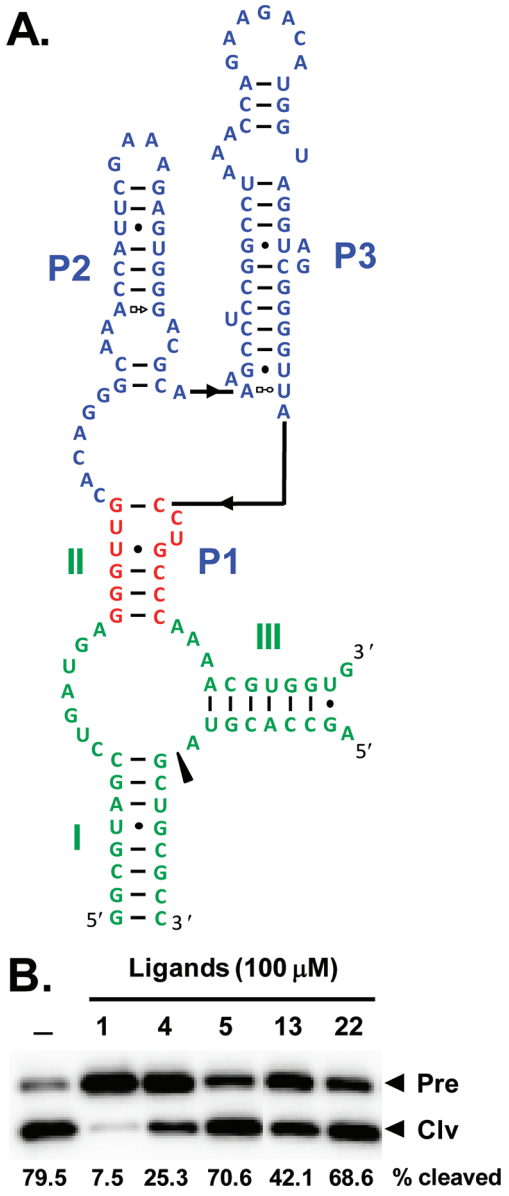


Figure 4. Allosteric hammerhead ribozyme assay. (A) Structure and sequence of allosteric hammerhead ribozyme (14-II). The allosteric ribozyme consists of the c-di-GMP class I aptamer (blue) and a hammerhead ribozyme (green) joined *via* a communication module bridge (red). The arrowhead indicates the cleavage site. Binding of c-di-GMP or its analogues to the aptamer triggers a conformational change within the bridge, and this structural reorganization dictates the activity of the adjoining hammerhead ribozyme. Ribozyme activity is inhibited by the binding of c-di-GMP or its analogues. (B) Representative gel image of allosteric ribozyme assays. Pre and Clv identify uncleaved RNA precursors and its 5′-cleavage products, respectively.

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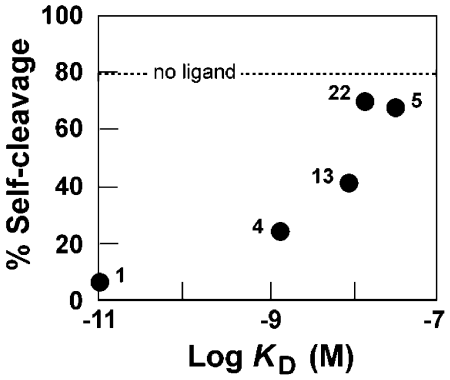

Figure 5. Modulation of self-cleavage of an allosteric hammerhead ribozyme by c-di-GMP and several analogues. Compounds designated are plotted on the basis of their K_D value *versus* the ribozyme cleavage yield in a 15-min reaction when present at 100 μM. Data (derived from the analysis depicted in Figure 4B) are a representative from two replicates wherein standard deviation is less than the size of the points.

METHODS

c-di-GMP and Analogues. c-di-GMP and its circular analogues (cyclic bis(3′-5′)-2′-deoxyguanylic/guanylic acid (c-dGpGp), monophosphothioic acid of c-di-GMP (c-GpGps), cyclic bis(3′-5′)guanylic/adenylic acid (c-GpAp), and 2′-*O*-di(*tert*-butyldimethylsilyl)-c-di-GMP) were chemically synthesized as described previously.[45−48] pGpG (**7**) was purchased from Biolog Life Science Institute. All linear analogues except for pGpG were purchased from the W. M. Keck Foundation Biotechnology Resource Laboratory at Yale University and were synthesized by solid-phase oligonucleotide synthesis methods. The identities of all analogues were confirmed by high-resolution mass spectrum analysis (Supplementary Table 1). The purities of these compounds were >95% as measured by analytical HPLC with an Agilent Eclipse XDB-C18 column (5 μm; 75 mm length and 4.6 mm internal diameter) using a gradient of acetonitrile (0−30%) in triethylamine-acetate buffer (pH 7.0) and analysis by absorbance at 260 nm.

In-Line Probing. The 110 Vc2 (class I) and the 84 Cd (class II) RNAs were prepared according to published methods[5,6] and subjected to in-line probing analyses as described previously.[35,36] Briefly, 5′ ^{32}P-labeled RNA (~50 pM) was incubated at room temperature (~23 °C) for approximately 40 h in 100 mM KCl, 50 mM Tris-HCl (pH 8.3 at 23 °C), and 20 mM $MgCl_2$. Cleavage products were separated by denaturing 10% PAGE, and the resulting gel was dried and imaged using a PhosphorImager (Molecular Dynamics). Analyses of band intensities were carried out using ImageQuant software (Molecular Dynamics). Areas of band-intensity modulation were identified and measured. The band intensity values were normalized and plotted against the logarithm of the concentration of ligand. Apparent K_D values were determined by quantitating the amount of RNA degradation for a given nucleotide position over a range of ligand concentrations.

Transcription Termination Assays. A DNA template encompassing the class I riboswitch sequence from *C. difficile* (5′ UTR of the flagellar operon) was fused to a sequence carrying the *B. subtilis lysC* promoter. Also, a DNA template harboring the class II riboswitch sequence from *C. difficile* (5′ UTR of the *ompR* gene) carrying its native promoter was prepared. Transcription termination assays were conducted using a method of single-round transcription adapted from that described previously.[5,6] All circular analogues and some linear analogues that showed relatively low values for K_D (less than ~5 μM) except for **7** and **10** were subjected to this assay.

Allosteric Ribozyme Assays. An allosteric ribozyme (14H-II) that responds to c-di-GMP binding[42] was prepared by *in vitro* transcription with T7 RNA polymerase. Briefly, the double-stranded DNA template including T7 promoter was incubated for 2 h at 37 °C in transcription mixture containing 50 mM Tris-HCl (pH 7.5 at 23 °C), 15 mM $MgCl_2$, 5 mM dithiothreitol, 2 mM spermidine, 0.1 mM EDTA, 2 mM each of the four NTPs, 1000 units of T7 RNA polymerase, and 10 μCi [α-^{32}P]UTP. Also, 100 μM c-di-GMP was added to the mixture to favor isolation of ribozymes that are inactive when bound to the second messenger. The full-length RNA product was then purified by denaturing 8% PAGE and isolated from the gel using elution buffer (50 mM Tris-HCl [pH 7.5 at 23 °C], 200 mM NaCl, and 1 mM EDTA [pH 8.0 at 23 °C]). Trace amounts of internally ^{32}P-labeled RNA were incubated with analogues at a concentration of 100 μM for 15 min at 23 °C. Reactions were terminated by the addition of stop buffer containing 95% (v/v) formamide and 25 mM EDTA. Products were separated by denaturing 8% PAGE, and the yields were quantitated by ImageQuant software (Molecular Dynamics).

ASSOCIATED CONTENT

Supporting Information

One table and five figures as described in the text. This material is available free of charge *via* the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*E-mail: ronald.breaker@yale.edu.

Notes

The authors declare the following competing financial interest(s): Dr. Breaker is a cofounder of BioRelix, a company that has licensed riboswitch intellectual property from Yale University.

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REFERENCES

(1) Hengge, R. (2009) Principles of c-di-GMP signaling in bacteria. *Nat. Rev. Microbiol. 7*, 263−273.

(2) Tamayo, R., Pratt, J. T., and Camilli, A. (2007) Roles of cyclic diguanylate in the regulation of bacterial pathogenesis. *Annu. Rev. Microbiol. 61*, 131−148.

(3) Amikam, D., and Galperin, M. Y. (2006) PilZ domain is part of the bacterial c-di-GMP binding protein. *Bioinformatics 22*, 3−6.

(4) Merighi, M., Lee, V. T., Hyodo, M., Hayakawa, Y., and Lory, S. (2007) The second messenger bis-(3′-5′)-cyclic-GMP and its PilZ domain-containing receptor Alg44 are required for alginate biosynthesis in *Pseudomonas aeruginosa*. *Mol. Microbiol. 65*, 876−895.

(5) Sudarsan, N., Lee, E. R., Weinberg, Z., Moy, R. H., Kim, J. N., Link, K. H., and Breaker, R. R. (2008) Riboswitches in eubacteria sense the second messenger cyclic di-GMP. *Science 321*, 411−413.

(6) Lee, E. R., Baker, J. L., Weinberg, Z., Sudarsan, N., and Breaker, R. R. (2010) An allosteric self-splicing ribozyme triggered by a bacterial second messenger. *Science 329*, 845−848.

(7) Roth, A., and Breaker, R. R. (2009) The structural and functional diversity of metabolite-binding riboswitches. *Annu. Rev. Biochem. 78*, 305−334.

(8) Dambach, M. D., and Winkler, W. C. (2009) Expanding roles for metabolite-sensing regulatory RNAs. *Curr. Opin. Microbiol. 12*, 161−169.

(9) Breaker, R. R. (2011) Riboswitches and RNA world. In *RNA Worlds: From Life's Origins to Diversity in Gene Regulation* (Atkins, J. F., Gesteland, R. F., and Cech, T. R., Eds.), pp 63−77, CSH Laboratory Press, New York.

(10) Bastet, L., Dubé, A. E., Massé, and Lafontaine, D. A. (2011) New insights into riboswitch regulation mechanisms. *Mol. Microbiol. 80*, 1148−1154.
(11) Hollands, K., Proshkin, S., Sklyarova, S., Epshtein, V., Mironov, A., Nudler, E., and Groisman, E. A. (2012) Riboswitch control of Rho-dependent transcription termination. *Proc. Natl. Acad. Sci. U.S.A.*,in press.
(12) Wachter, A., Tunc-Ozdemir, M., Grove, B. C., Green, P. J., Shintani, D. K., and Breaker, R. R. (2007) Riboswitch control of gene expression in plants by splicing and alternative 3′ end processing of mRNAs. *Plant Cell 19*, 3437−3450.
(13) Bocobza, S., and Aharoni, A. (2007) Riboswitch-dependent gene regulation and its evolution in the plant kingdom. *Genes Dev. 21*, 2874−2879.
(14) Croft, M. T., Moulin, M., Webb, M. E., and Smith, A. G. (2007) Thiamine biosynthesis in algae is regulated by riboswitches. *Proc. Natl. Acad. Sci. U.S.A. 104*, 20770−20775.
(15) Sudarsan, N., Cohen-Chalamish, S., Nakamura, S., Emilsson, G. M., and Breaker, R. R. (2005) Thiamine pyrophosphate riboswitches are targets for the antimicrobial compound pyrithiamine. *Chem. Biol. 12*, 1325−1335.
(16) Blount, K. F., Wang, J. X., Lim, J., Sudarsan, N., and Breaker, R. R. (2007) Antibacterial lysine analogs that target lysine riboswitches. *Nat. Chem. Biol. 3*, 44−49.
(17) Blount, K. F., and Breaker, R. R. (2006) Riboswitches as antibacterial drug targets. *Nat. Biotechnol. 24*, 1558−1564.
(18) Lee, E. R., Blount, K. F., and Breaker, R. R. (2009) Roseoflavin is a natural antibacterial compound that binds to FMN riboswitches and regulates gene expression. *RNA Biol. 6*, 187−194.
(19) Kim, J. N., Blount, K. F., Puskarz, I., Lim, J., Link, K. H., and Breaker, R. R. (2009) Design and antimicrobial action of purine analogues that bind Guanine riboswitches. *ACS Chem. Biol. 4*, 915−927.
(20) Mulhbacher, J., Brouillette, E., Allard, M., Fortier, L. C., Malouin, F., and Lafontaine, D. A. (2010) Novel riboswitch ligand analogs as selective inhibitors of guanine-related metabolic pathways. *PLoS Pathog. 6*, e1000865.
(21) Lünse, C. E., Schmidt, M. S., Wittman, V., and Mayer, G. (2011) Carba-sugars activate the glmS-riboswitch of Staphylococcus aureus. *ACS Chem. Biol. 6*, 675−678.
(22) Cressina, E., Chen, L., Moulin, M., Leeper, F. J., Abel, C., and Smith, A. G. (2011) Identification of novel ligands for thiamine pyrophosphate (TPP) riboswitches. *Biochem. Soc. Trans. 39*, 652−657.
(23) Deigan, K. E., and Ferré-D'Amaré, A. (2011) Riboswitches: discovery of drugs that target bacterial gene-regulatory RNAs. *Acc. Chem. Res. 44*, 1329−1338.
(24) Serganov, A., Polonskaia, A., Phan, A. T., Breaker, R. R., and Patel, D. J. (2006) Structural basis for gene regulation by a thiamine pyrophosphate-sensing riboswitch. *Nature 441*, 1167−1171.
(25) Edwards, T. E., and Ferré-D'Amaré, A. R. (2006) Crystal structures of the thi-box riboswitch bound to thiamine pyrophosphate analogs reveal adaptive RNA-small molecule recognition. *Structure 14*, 1459−1468.
(26) Thore, S., Frick, C., and Ban, N. (2008) Structural basis of thiamine pyrophosphate analogues binding to the eukaryotic riboswitch. *J. Am. Chem. Soc. 130*, 8116−8117.
(27) Kubodera, T., Watanabe, M., Yoshiuchi, K., Nishimura, A., Nakai, S., Gomi, K., and Hanamoto, H. (2003) Thiamine-regulated gene expression of Aspergillus oryzae thiA requires splicing of the intron containing a riboswitch-like domain in the 5′-UTR. *FEBS Lett. 555*, 516−520.
(28) Wolfson, W. (2006) Holding back the tide of antibiotic resistance. *Chem. Biol. 13*, 1−3.
(29) D'Costa, V. M., McGrann, K. M., Hughes, D. W., and Wright, G. D. (2006) Sampling the antibiotic resistome. *Science 311*, 374−377.
(30) Smith, K. D., Lipchock, S. V., Ames, T. D., Wang, J., Breaker, R. R., and Strobel, S. A. (2009) Structural basis of ligand binding by a c-di-GMP riboswitch. *Nat. Struct. Mol. Biol. 16*, 1218−23.
(31) Kulshina, N., Baird, N. J., and Ferré-D'Amaré, A. R. (2009) Recognition of the bacterial second messenger cyclic diguanylate by its cognate riboswitch. *Nat. Struct. Mol. Biol. 16*, 1212−1217.
(32) Smith, K. D., Shanahan, C. A., Moore, E. L., Simon, A. C., and Strobel, S. A. (2011) Structural basis of differential ligand recognition by two classes of bis-(3′-5′)-cyclic dimeric guanosine monophosphate-binding riboswitches. *Proc. Natl. Acad. Sci. U.S.A. 108*, 7757−7762.
(33) Shanahan, C. A., Gaffney, B. L., Jones, R. A., and Strobel, S. A. (2011) Differential analog binding by two classes of c-di-GMP riboswitches. *J. Am. Chem. Soc. 133*, 15578−15592.
(34) Smith, K. D., Lipchock, S. V., and Strobel, S. A. (2012) Structural and biochemical characterization of linear dinucleotide analogues bound to the c-di-GMP-I aptamer. *Biochemistry 51*, 425−432.
(35) Soukup, G. A., and Breaker, R. R. (1999) Relationship between internucleotide linkage geometry and the stability of RNA. *RNA 5*, 1308−1325.
(36) Regulski, E. E., and Breaker, R. R. (2008) In-line probing analysis of riboswitches. *Methods Mol. Biol. 419*, 53−67.
(37) Jaroszewski, J. W., and Cohen, J. S. (1991) Cellular uptake of antisense oligodeoxynucleotides. *Adv. Drug Delivery Rev. 6*, 235−250.
(38) Spitzer, S., and Eckstein, F. (1988) Inhibition of deoxyribonucleases by phosphorothioate groups in oligodeoxyribonucleotides. *Nucleic Acids Res. 16*, 11691−11704.
(39) Wickiser, J. K., Winker, W. C., Breaker, R. R., and Crothers, D. M. (2005) The speed of RNA transcription and metabolite binding kinetics operate an FMN riboswitch. *Mol. Cell 18*, 49−60.
(40) Barrick, J. E., and Breaker, R. R. (2007) The distributions, mechanisms, and structures of metabolite-binding riboswitches. *Genome Biol. 8*, R239.
(41) Gu, H., Furukawa, K., and Breaker, R. R. (2012) Engineered allosteric ribozymes that sense the bacterial second messenger c-di-GMP. *Anal. Chem. 84*, 4935−4941.
(42) Luo, Y., Zhou, J., Watt, S. K., Lee, V. T., Kwaku Dayie, T., and Sintim, H. O. (2012) Differential binding of 2′-biotinylated analogs of c-di-GMP with c-di-GMP riboswitches and binding proteins. *Mol. BioSyst. 8*, 772−778.
(43) Blount, K., Puskarz, I., Penchovsky, R., and Breaker, R. (2006) Development and application of a high-throughput assay for glmS riboswitch activators. *RNA Biol. 3*, 77−81.
(44) Mayer, G., and Famulok, M. (2006) High-throughput-compatible assay for *glmS* riboswitch metabolite dependence. *ChemBioChem 7*, 602−604.
(45) Kumagai, Y., Matsuo, J., Hayakawa, Y., and Rikihisa, Y. (2010) Cyclic di-GMP signaling regulates invasion by *Ehrlichia chaffeensis* of human monocytes. *J. Bacteriol. 192*, 4122−33.
(46) Mano, E., Hyodo, M., Sato, Y., Ishihara, Y., Ohta, M., and Hayakawa, Y. (2007) Synthesis of cyclic bis(3′-5′)-2′-deoxyguanylic/guanylic acid (c-dGpGp) and its biological activities to microbes. *ChemMedChem 2*, 1410−1413.
(47) Hyodo, M., Sato, Y., and Hayakawa, Y. (2006) Synthesis of cyclic bis(3′-5′)guanylic acid (c-di-GMP) analogs. *Tetrahedron 62*, 3089−3094.
(48) Hyodo, M., and Hayakawa, Y. (2004) An improved method for synthesis of cyclic bis(3′-5′)diguanylic acid (*c*-di-GMP). *Bull. Chem. Soc. Jpn. 77*, 2089−2093.