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Identification of c-di-GMP derivatives resistant to an EAL domain phosphodiesterase

Carly A. Shanahan[†], **Barbara L. Gaffney**[§], **Roger A. Jones**[§], and **Scott A. Strobel**[†,‡,*]

[†]Department of Chemistry, Yale University, New Haven, Connecticut 06520

[‡]Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06520

[§]Department of Chemistry and Chemical Biology, Rutgers, The State University of New Jersey, Piscataway, New Jersey 08854

Abstract

The bacterial second messenger signaling molecule bis-(3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) controls important biological processes such as biofilm formation, virulence response, and motility. This second messenger is sensed by macromolecular targets inside the cell, both protein and RNA, which induce specific phenotypic responses critical for bacterial survival. One class of enzymes responsible for regulating the intracellular concentration of c-di-GMP, and therefore the physiological behavior of the cell, is the EAL domain phosphodiesterases, which degrade the second messenger to its linear form, pGpG. Here, we investigate how base and backbone modifications to c-di-GMP affect the rate of cyclic dinucleotide degradation by an EAL domain protein (CC3396 from *Caulobacter crescentus*). The doubly substituted thiophosphate analog is highly resistant to hydrolysis by this metabolizing enzyme but can still bind c-di-GMP riboswitch targets. We used these findings to develop a novel ribosyl-phosphate modified derivative of c-di-GMP containing 2′-deoxy and methylphosphonate substitutions that is charge neutral and demonstrate that this analog is also resistant to EAL domain catalyzed degradation. This suggests a general strategy for designing c-di-GMP derivatives with increased enzymatic stability that also possess desirable properties for development as chemical probes of c-di-GMP signaling.

Bacteria have the ability to sense different external signals present in diverse environments and translate these cues into physiological responses that can be critical for their survival[1]. One mechanism bacteria have evolved to facilitate such behavioral adaptation is through the use of the second messenger signaling molecule bis-(3′-5′)- cyclic dimeric guanosine monophosphate (c-di-GMP) (for recent reviews see[2–5]). c-di-GMP is ubiquitous within the bacterial domain and is vital for regulating the transition between a sessile, biofilm-forming state and a motile, planktonic existence[6]. This second messenger also controls the virulence response of pathogenic organisms[7] and has been linked to quorum sensing, the process by which bacteria detect and communicate with one another[8].

c-di-GMP is synthesized from two molecules of GTP by diguanylate cyclases (DGCs), which contain a GGDEF domain[9], and is degraded to the linear 5′-phosphate dinucleotide pGpG by EAL domain phosphodiesterases (PDEs) (Figure 1a)[10]. A second class of c-di-

*To whom correspondence should be addressed. Phone (203) 432-9772. Fax: (203) 432-5767. scott.strobel@yale.edu.

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GMP phosphodiesterases known as the HD-GYP domain proteins have also been identified in some bacterial species, though these enzymes are much less common than the EAL domain proteins[11,12]. GGDEF and EAL domain proteins are widely distributed throughout the bacterial domain[13], suggesting that this second messenger plays an essential biological role in many different species. The opposing activities of these enzymes tightly regulate the concentration of c-di-GMP in the cell, which is directly sensed by downstream targets of the second messenger that act to induce the appropriate phenotypic response[2–5].

Although c-di-GMP is prevalent in bacteria, both the macromolecular targets that sense this second messenger and the specific mechanisms responsible for the observed behavioral transitions are only beginning to emerge and are highly diverse. Several classes of proteins including PilZ domain proteins[14], transcription factors[15–17], and degenerate DGCs and PDEs[18–20], as well as two structurally distinct classes of riboswitch RNAs[21,22], have been identified as c-di-GMP effectors, underscoring the complexity of this key bacterial signaling pathway.

Understanding the metabolism of c-di-GMP is essential to elucidate how different bacterial phenotypes are triggered in response to varying intracellular concentrations of the second messenger. Structural characterization of this class of metabolizing enzymes indicates that c-di-GMP is bound in the extended conformation wherein the guanine bases (termed G1 and G2) are splayed apart from one another (Figure 1b,c)[23–25], rather than the eclipsed conformation that is observed for the c-di-GMP binding riboswitches[26,27] and many effector proteins[15,28,29]. Few specific interactions are made to the guanine bases of the second messenger. The most highly conserved residues are involved in coordination of active site metals, the proposed nucleophilic water molecule and the c-di-GMP phosphates (Figures 1b,c). Crystal structures of catalytically active EAL domain proteins reveal two metals in the active site that each contact a different oxygen atom of the scissile phosphate (P1, Figure 1b), suggesting that c-di-GMP hydrolysis is achieved via a two-metal ion mechanism and that both oxygens are critical to catalysis[23,25]. Several inactive EAL domain proteins that can bind c-di-GMP, but cannot catalyze degradation, have been identified that function as downstream signaling molecules[19,30]. These proteins contain mutations to the amino acids coordinating the metals, phosphate P1, or the nucleophilic water molecule, highlighting the essential role these residues play in catalysis. The inactivity of such mutants suggests that changes to the c-di-GMP phosphate linkages may also impact reactivity.

Before the EAL domain was identified as the specific enzyme responsible for c-di-GMP degradation, Benziman and coworkers demonstrated that membrane extracts possessing c-di-GMP specific PDE-activity were able to degrade doubly substituted 2′- deoxy and inosine analogs and a singly substituted thiophosphate modified analog[31]. This suggests that these particular modifications to c-di-GMP are unlikely to yield EAL domain resistant analogs, but these single time point studies did not provide information on how these modifications affect the rate of second messenger degradation or, in some cases, what effect double substitution has on activity. Therefore, we sought to determine which c-di-GMP modifications have the largest effect on catalysis by an EAL domain phosphodiesterase and identify those that render the second messenger resistant to degradation.

Here, we test a series of base, ribose and phosphate modified c-di-GMP derivatives to investigate the specificity of the EAL domain proteins for its cognate ligand. We identified modifications that render the second messenger highly resistant to EAL domain catalyzed degradation and used these findings to develop a charge neutral, EAL domain resistant methylphosphonate c-di-GMP derivative.

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MATERIALS AND METHODS

Materials

Radiolabeled c-di-GMP (*c-di-GMP) and unlabeled c-di-GMP were synthesized enzymatically using purified PleD* and tDGC diguanylate cyclase proteins respectively, as previously described[32,33]. Base and ribose modified analogs were synthesized on solid phase as previously described[34]. Mono and dithiophosphate analogs were synthesized in solution as previously described[35]. The absolute stereochemistry of the thiophosphate c-di-GMP analogs was assigned as previously described based on the specificity of P1 and venom phosphodiesterases[36]. 3′- phosphate CPG beads, guanosine methylphosphonamidite precursors, and Poly-Pak II desalting columns were purchased from Glen Research. 4-(dimethylamino)pyridine (DMAP), tetrahydrofuran (THF), acetic anhydride, anhydrous pyridine, anhydrous acetonitrile (ACN), triethylamine (TEA) and 1-(2-mesitylenesulfonyl)-3-nitro-1*H*-1,2,4- triazole (MSNT) were purchased from Sigma. The 100 nucleotide class I Vc2 riboswitch RNA from *Vibrio cholerae* containing 2-aminopurine at position 94 (G94(2AP) RNA) was prepared as previously described[34]. The wild-type class II aptamer from *Clostridium acetobutylicum* was cloned and transcribed *in vitro* using T7 RNA polymerase as previously described[27,34].

Expression and purification of the phosphodiesterase protein CC3396 from *Caulobacter crescentus*

The 6×-histidine tagged EAL domain phosphodiesterase protein CC3396 was purified by affinity chromatography using Ni-NTA agarose (Qiagen) as previously described[37]. Overnight cultures of *E. coli* BL21 cells harboring the expression plasmid (pET21) encoding CC3396 were grown at 37°C. The overnight culture was diluted into fresh LB medium and cells were grown to an OD_{600} of 0.6. Protein expression was then induced for 45 min. at 37°C by adding 0.5 mM isopropyl 1- thio-β-D-galactopyranoside (IPTG). Cells were pelleted and resuspended in lysis buffer (50 mM sodium phosphate pH 7.0, 300 mM NaCl, 5 mM BME) and lysed by passing through a microfluidizer at 15,000 psi. The lysate was cleared by centrifugation (20,000 g) and the cleared lysate was incubated with Ni-NTA agarose for 1 hour at 4°C to allow the protein to bind the column. The column was washed with lysis buffer containing 20 mM imidazole followed by washing with lysis buffer containing 50 mM imidazole. The protein was eluted from the column in 250 mM imidazole and the pure protein fractions were pooled and dialyzed into 25 mM Tris-HCl pH 8.0, 250 mM NaCl, 5 mM BME. Dialyzed protein was concentrated and stored in 10% glycerol.

Phosphodiesterase assays to evaluate degradation rates of c-di-GMP and analogs

Phosphodiesterase activity was evaluated under multiple turnover conditions with saturating concentrations of substrate. Pure CC3396 protein (1 μM) was incubated with 100 μM GTP and 100 μM c-di-GMP (or analog) in 1×PDE buffer (10 mM $MgCl_2$, 25 mM Tris-HCl pH 8.0, 250mM NaCl) at room temperature (22°C). Aliquots were removed at each time point and the reaction was quenched by heating to 95 °C for 5 minutes. Degradation was monitored in the range where the reaction was still linear to measure the initial velocity. Samples were diluted in 50 mM TEAAc buffer (pH 6.0) and compound degradation was analyzed by HPLC on a reverse-phase C8 column using a gradient of 0 to 4% ACN in 50 mM TEAAc pH 6.0 over 15 minutes for most compounds. A steeper gradient was used for the following compounds as indicated: c-(R_PR_P)-di-G_{ps} 0 to 15% ACN; c-(R_PS_P)-di-G_{ps} 0 to 10% ACN; c-(R_P)-G_{ps}-GMP 0 to 10% ACN; c-di$dG_{p(Me)}$-I and c-di-$dG_{p(Me)}$-II 0 to 20% ACN. The area under peaks corresponding to linear and cyclic compounds was determined by integration (HPChem Software) and the fraction of linear (F_L) product formed was calculated using the following equation:

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$$F_L = \frac{Area_L}{Area_L + Area_C}$$

where $Area_L$= area of the linear product and $Area_C$= area of the cyclic compound. F_L was multiplied by the substrate concentration to obtain the amount of product formed, which was plotted against time. The data was fit to a line and the slope of the line was used to determine the initial rate (k_{cat}).

Competition phosphodiesterase assays

The degradation of radiolabeled c-di-GMP (*c-di-GMP) to pGpG was monitored by determining the fraction of pGpG formed by separating cyclic and linear products by polyethyleneimine-cellulose thin layer chromatography (PEI-cellulose TLC). For the competition assays, CC3396 (10 nM) was incubated at room temperature for 5 minutes with GTP (100 μM) in 1× PDE buffer before the simultaneous addition of trace amounts of radiolabeled c-di-GMP and an excess of unlabeled competitor analog (100 μM). Aliquots were removed at each timepoint and quenched by the addition of an equal volume of 0.5 M EDTA. Reactions were extracted with a mixture of phenol:chloroform:isoamyl alcohol (25:24:1) to remove the protein before analysis by PEI-cellulose TLC. PEI-cellulose TLC plates were run in a 1:1.5 (v/v) mixture of saturated NH_4SO_4: 1.5 M KH_2PO_4, pH 3.6 and the amount of c-di-GMP and pGpG quantified as previously described[37].

Chemical synthesis of the methylphosphonate analog

The methylphosphonate c-di-GMP analog was synthesized on solid support (3′-phosphate CPG beads) on a 1 μmole scale as previously described[38] with the following adaptations (Figure 2a). The 5′-dimethoxytrityl (DMTr) protected guanosine methylphosphonamidite (50 mM in THF) was coupled to the solid support using 5-benzyl mercaptotetrazole (125 mM in ACN) as the activator. Oxidation was performed using a 0.02 M solution of iodine in THF/pyridine/water (88:10:2) and unreacted sites were capped using a 1:1 mixture of 6.5% DMAP/THF and 10% acetic anhydride/THF. The 5′-DMTr group was removed using 3% dicholoracetic acid in dichloromethane followed by coupling, oxidation, capping and detritylation of the second nucleotide as described above. The linear dinucleotide was cleaved from the solid support by incubating with 10% TEA/ACN and co-evaporated with anhydrous pyridine. The dinucleotide was cyclized in solution using 0.1 M MSNT in pyridine under argon for approximately 96 hours. Global deprotection was afforded by evaporating the cyclization solution and resuspending the dry product in a 45:45:10 mixture of ACN:EtOH:NH_4OH. After a 30 min room temperature incubation in the deprotection mixture, an equal volume of ethylenediamine was added and the room temperature incubation was allowed to proceed for an additional 6 hours. The mixture was diluted with water and the pH was adjusted to 7.0 using 6 M HCl. The product was desalted on a Poly-Pak II column (Glen Research) according to the manufacturer's instructions. The desalted product was lyophilized to dryness and then purified by high performance liquid chromatography (HPLC) on a C18 reverse phase column using a gradient of 0% to 30% acetonitrile in 50 mM TEAAc, pH 6.0 over 30 minutes (Figure 2b). Two of the three possible diastereomers were synthesized and product identity was confirmed by ESI-MS in negative ion and positive ion mode (exact mass= 654.15; observed mass c-di-$dG_{p(Me)}$-I [M-H]/1= 653.144, [M+H]/1= 655.1473, [M+Na]/1= 677.1346; observed mass c-di-$dG_{p(Me)}$-II [M-H]/1= 653.1306, [M+H]/1= 655.1335, [M+Na]/1= 677.1207). The major impurity present is the non-cyclized linear intermediate (Figure 2b). Based on the integration of peaks corresponding to the linear and cyclic compounds, the cyclization reaction proceeded with

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approximately 50% efficiency. The overall yield of cyclic compound was 10% (c-di-$dG_{p(Me)}$-I = 7% and c-di$dG_{p(Me)}$-II= 3%).

Affinity measurements of the methylphosphonate analog for c-di-GMP binding riboswitches

Dissociation constants (K_d's) of c-di-GMP analogs for the class I and class II riboswitches were determined as previously described by competition gel-shift assay with radiolabeled c-di-GMP[34]. Radiolabeled c-di-GMP was incubated with riboswitch aptamer RNA (25 nM class I (G94(2AP) variant) or 50 nM class II) and increasing concentrations of analog in folding buffer containing 10 mM $MgCl_2$, 10 mM NaCl, and 10 mM sodium cacodylate pH 6.8 until equilibrium was achieved. Free c-di- GMP and RNA-bound c-di-GMP were separated by native PAGE (100 mM Tris/HEPES pH 7.5, 0.1 mM, 10 mM $MgCl_2$) at 4°C. The amounts of free and bound c-di-GMP were quantified and fit to an equation for competitive binding as previously described to determine the K_d of the competitor analog[34].

RESULTS

Monitoring the enzymatic hydrolysis of c-di-GMP and analogs by HPLC

We employed an HPLC-based assay to measure the rate of hydrolysis for c-di-GMP and its analogs by the EAL domain PDE CC3396 from *C. crescentus*. CC3396 is a prototypical PDE and the activity of this protein has been well characterized *in vitro*[37]. It is a GGDEF-EAL protein containing a degenerate GGDEF domain that possesses no diguanylate cyclase activity, but instead acts to allosterically activate the phosphodiesterase by binding to GTP[37]. Sequence alignment with other structurally and biochemically characterized PDEs indicates that the most highly conserved residues necessary for catalysis are conserved in this protein (supplemental info., Table S1)[23,25,39]. Using this assay we measured the rate of cyclic dinucleotide degradation for the singly substituted (c-GMP-IMP, c-(R_P)-G_{ps}-GMP, c-(S_P)-G_{ps}-GMP, c-dG-GMP) and doubly substituted analogs (c-di-IMP, c-di-AMP, c-di-dGMP) previously tested in single time point studies [31]. We also tested analogs whose susceptibility to EAL domain catalyzed degradation had not been previously investigated (c-GMP-AMP, c-N^1mGGMP, c-di-2′F-GMP, c-di-2′OMe-MP, c-(R_PR_P)-di-G_{ps}, c-(R_PS_P)-di-G_{ps}, c-di-$dG_{p(Me)}$) (Figure 3). With the exception of c-di-IMP, c-di-AMP, and c-di-dGMP, the previous biochemical work only looked at the degradation of singly substituted analogs. Because c-di-GMP is a symmetric molecule, single substitutions are likely to leave the dinucleotide susceptible to degradation by targeting the unsubstituted position.

The rate of c-di-GMP degradation was measured under saturating conditions of both the second messenger (100 μM, see below) and GTP (100 μM) and the conversion of c-di-GMP to pGpG was detected via HPLC by monitoring the appearance of a peak corresponding to pGpG (retention time = 7.5 minutes) and the disappearance of the peak corresponding to c-di-GMP (retention time= 10 minutes) (Figure 4a). Co-injection of the reaction with a chemically synthesized standard of pGpG confirmed that the peak appearing at 7.5 min. was the correct breakdown product. Under these conditions, the second messenger was completely degraded within 10 min., with the rate of c-di-GMP hydrolysis measured to be 15 mol/(min*mol enzyme) (Figure 4, Table 1). To confirm that we were using saturating concentrations of c-di-GMP, we measured the rate of degradation using 200 μM c-di-GMP and found it to be the same as that measured using 100 μM c-di-GMP (15.3 mol/(min*mol enzyme) at 200 μM c-di-GMP). This indicates that the enzyme is saturated with substrate and that the observed rate corresponds to the k_{cat}. This was also confirmed for the ribosyl-phosphate modified analogs that were susceptible to degradation (Table 1), indicating that all rates measured for these analogs were done under saturating conditions. The rate of c-di-GMP hydrolysis by CC3396 that we measured is in reasonable agreement with that

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previously reported (115 mol/(min*mol enzyme) at 30°C)[37] and the different temperature (37°C vs 30°C) and method used to carry out the two studies likely account for the 8-fold difference in measurements.

Enzymatic hydrolysis of base modified analogs

Previous biochemical studies have demonstrated that mutations to EAL domain residues contacting the c-di-GMP bases do not significantly affect enzymatic activity[25,39]. This suggests that modifications to the c-di-GMP bases would not be predicted to render the second messenger resistant to enzymatic degradation. We first tested the effects of modifying the c-di-GMP guanine bases with inosine, N^1methyl guanine, and adenine substitutions on hydrolysis by the EAL domain protein CC3396 to determine which guanine analogs are most tolerated by the EAL domain (Figure 3a).

With the exception of c-di-AMP, all base modified analogs tested were susceptible to EAL domain catalyzed degradation (Table 1). Second messenger analogs containing either one inosine (c-GMP-IMP) or N1-methyl guanine (c-N^1mG-GMP) base in place of a single guanine were both degraded to their corresponding linear products at a rate within 2–3 fold of that for the native second messenger. In contrast, both c-di-IMP and c-GMP-AMP were hydrolyzed approximately 30-fold slower than c-di-GMP (Table 1). However, for the base modified analogs tested, we did not determine if the substrate concentration (100 μM) was saturating and it is likely that the observed decrease in rate for c-di-IMP and c-GMP-AMP is a binding effect rather than a catalytic effect. These data indicate that the EAL domain can accommodate structurally related analogs of guanine in the active site and suggests that specific recognition of only one guanine base is sufficient for second messenger binding and degradation.

In the HPLC profile of c-N^1mG-GMP after treatment with CC3396, two product peaks with retention times differing from that of the cyclic dinucleotide were observed (Figure S1a). These peaks likely correspond to the two possible linear products p- N^1mG-pG and pGpN^1mG. They were formed in unequal amounts, suggesting that CC3396 preferentially binds the modified dinucleotide in one orientation. For c-GMP-IMP, a major product peak and a much smaller minor peak were observed suggesting a preference for binding orientation for that analog as well (Figure S1b). In contrast, only one product peak was observed for c-GMP-AMP (Figure S1c). This suggests there is a preference for binding orientation between the G and the A. These observations are consistent with structural analysis, which predicts that one guanine base is more extensively recognized than the other[23,25].

Enzymatic hydrolysis of analogs containing ribose modifications

Based on structural analysis, no specific contacts are made to the 2′-OH's of the c-di-GMP ribose sugars by the EAL domain (Figure 1b,c)[23–25], suggesting that hydroxyl modification would not significantly affect the rate of catalysis. To test this prediction, we first measured the rate of degradation for the 2′-deoxy ligands, c-di-dGMP and c-dG-GMP (Figure 3b). We found no significant effect on degradation for c-dG-GMP (2-fold) however, c-di-dGMP was hydrolyzed approximately 14-fold slower than c-di-GMP (Table 1), suggesting that modification of both ribose rings increases the resistance of the second messenger towards EAL domain catalyzed degradation. To further test this observation, we measured the rate of hydrolysis for 2′-fluoro (c-di-2′F-GMP) and 2′- OMethyl (c-di-2′OMe-GMP) modified analogs (Figure 3). These ribose modifications have been shown to increase the stability of nucleic acids towards nucleases that cleave phosphodiester bonds[40]. We found that both analogs were hydrolyzed at a slower rate than c-di-GMP, with c-di-2′F-GMP degraded 5-fold slower and c-di-2′-OMe- GMP degraded 17-fold slower (Table 1). The 2′-OMethyl

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substitutions have a slightly larger effect on hydrolysis than 2′-fluoro substitutions, but both substitutions were degraded with reasonably good efficiency.

Enzymatic hydrolysis of analogs containing phosphate modifications

In the proposed mechanism for c-di-GMP hydrolysis by EAL domain proteins, both nonbridging oxygens of the scissile phosphate are contacted by an active site metal ion (Figure 1b)[23,25]. Furthermore, it was previously demonstrated that a c-di-GMP analog containing one phosphorothioate linkage is hydrolyzed by membrane extracts possessing c-di-GMP specific PDE activity to only a single linear product, with the modified linkage contained at the internal phosphate of the dinucleotide product[31]. This suggests that phosphorothioate linkages are resistant to enzymatic hydrolysis by EAL domain proteins and that a second messenger analog with phosphorothioate substitutions in place of both phosphodiester bonds may be resistant to enzymatic degradation. To investigate this hypothesis, we tested mono and dithiophosphate c-di- GMP derivatives for degradation by the EAL protein.

Based on previous reports in the literature, we anticipated that the monothiophosphate c-di-GMP derivatives, c-(R_P)-G_{ps}-GMP and c-(S_P)-G_{ps}-GMP (Figure 3), would be efficiently degraded[31]. As expected, both analogs were degraded at a rate within two-fold of that for c-di-GMP (Table 1), confirming that modification to only one of the c-di-GMP phosphates does not significantly affect catalysis. Furthermore, only a single degradation product was detected by HPLC analysis for both the R_P and S_P derivatives, consistent with the previous observations that incubation of these analogs with membrane extracts containing c-di-GMP specific PDE activity also only yielded a single linear product[31].

We next tested the ability of the protein to degrade the dithiophosphate analogs, c-(R_PR_P)-di-G_{ps} and c-(R_PS_P)-di-G_{ps} (Figure 3). In contrast to the monothiophosphate derivatives, we observed no detectable degradation for either dithiophosphate analog even after a 24-hour incubation with enzyme (Figure 5a,b). Even if 2% of the compound was hydrolyzed after 24 hours, which is the limit of our detection, the rate of hydrolysis would be estimated as 0.001 mol/(min*mol enzyme), which is at least 12,000-fold slower than that of c-di-GMP. This indicates that substitution of either phosphate oxygen of the scissile phosphate for sulfur renders the second messenger highly resistant to EAL domain catalyzed degradation. To ensure that the linear hydrolysis product was not co-eluting with the cyclic starting material, we collected the peak and analyzed it by ESI-MS to determine the mass of the corresponding compound. For both diastereomers, the mass of the cyclic, but not the linear product, was detected (supplemental info, Table S2).

Because nuclease activity against phosphorothioate containing nucleic acids is often recovered in the presence of manganese[41,42], we next tested both dithiophosphate analogs for degradation by the EAL domain PDE with 10 mM manganese in place of 10 mM magnesium in the reaction buffer. Under these conditions, we found that c-(R_PS_P)- di-G_{ps} was approximately 20% degraded after 24 hours and estimate that the rate of degradation is approximately 1000-fold slower than c-di-GMP (Figure 5b). In contrast, no degradation for c-(R_PR_P)-di-G_{ps} was observed under these conditions (Figure 5a). This indicates that manganese is able to rescue the activity of the protein, but only for one of the phosphorothioate diastereomers. Taken together, these data demonstrate that replacing both phosphodiester linkages with phosphorothioate linkages renders the second messenger highly resistant to EAL domain catalyzed degradation.

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Synthesis and EAL domain catalyzed degradation of a backbone neutral c-di-GMP analog

The enzymatic degradation studies of the dithiophosphate c-di-GMP derivatives suggest that modification of both phosphate linkages renders the second messenger resistant to degradation by EAL domain proteins. To further explore this observation, we synthesized a second backbone modified c-di-GMP derivative containing methylphosphonate linkages in place of both canonical phosphodiester bonds. The methylphosphonate derivative of c-di-GMP, termed c-di-$dG_{p(Me)}$, substitutes a non-bridging phosphate oxygen of each phosphodiester linkage with an isosteric methyl group, resulting in a molecule with a neutral backbone (Figure 3). Because the methylphosphonamidite monomers are only commercially available in the 2′-deoxy version due to the instability of ribo-methylphosphonate linkages[43–45], we synthesized the di-2′-deoxy derivative and tested this molecule in comparison to the di-2′-deoxy parental dinucleotide with standard phosphate linkages, c-di-dGMP (Figure 3). We modified the solid phase method for c-di-GMP reported by Kiburu *et al* to access this cdi- GMP derivative [38]. We took advantage of the fact that methyl phosphonamidites starting monomers do not possess any phosphate protecting groups. This allowed for solid-phase dinucleotide synthesis, followed by selective cleavage of the linear dimer from the solid support under mild basic conditions without removing any phosphate protecting groups, or the protecting groups on the bases (Figure 2a). After coupling of both guanosine methyl phosphonamidites to the solid support, the linear dinucleotide was cleaved from the bead via β-elimination under basic conditions, and cyclized in solution.

Replacing one of the non-bridging oxygens on each phosphate renders both phosphates chiral and results in three different possible diastereomers (R_PR_P, R_PS_P, or S_PS_P). The fourth diastereomer (S_PR_P) is equivalent to the R_PS_P isomer because of the two fold symmetry within the cyclic dinucleotide. Using solid-phase chemistry as described above, we were able to synthesize and isolate two of the three possible diastereomers after HPLC purification (Figure 2b). This is similar to what was achieved for the synthesis of the phosphorothioate analogs. In that case the cyclization reaction proceeded with stereochemical specificity[35,46]. Thus, the absence of the third isomer is likely due to the cyclization step producing a linkage of only one chirality (most likely the R_P, see discussion). Due to the small amount of product generated from solid phase synthesis, we were not able to make enough material to assign absolute stereochemistry. We have designated these analogs c-di-$dG_{p(Me)}$-I and c-di-$dG_{p(Me)}$-II based on their order of elution during HPLC purification (Figure 2b). However, tentative stereochemical assignments are possible based upon their relative affinities to riboswitch targets (binding data suggest isomer I is R_PR_P and isomer II is R_PS_P ; see discussion).

To determine if methylphosphonate c-di-GMP derivatives are nuclease resistant, we tested both diastereomers for degradation by the EAL domain protein CC3396. Similar to what we observed for the phosphorothioate derivatives, neither analog was degraded even after a 24 hour incubation period with the protein (Figure 5d). In contrast, the parental dinucleotide, c-di-dGMP, is nearly completely hydrolyzed (> 85%) after 5 hours (Figure 5c). Assuming the maximum possible level of degradation of 2% after 24 hours, the rate of degradation is more than 1000-fold slower than the parental dinucleotide (1.1 mol*min^{-1}*mole $enzyme^{-1}$, Table 1). Methyl substitution of both phosphodiester bonds results in a c-di-GMP analog that has high resistance to EAL domain catalyzed degradation.

We next sought to determine if the EAL domain resistance of the dithiophosphate and methylphosphonate c-di-GMP analogs results from a weaker affinity for the protein for these analogs or if the protein is unable to perform catalysis on these modified phosphate centers. To differentiate between these two possibilities, we looked at the ability of these analogs to competitively inhibit the degradation of radiolabeled c-di-GMP (*c-di-GMP)[47]. In the presence of trace amounts of *c-di-GMP and a large excess of an unlabeled

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competitor analog that is able to bind the EAL domain, *c-di-GMP binding and degradation should effectively be blocked. We found that in the absence of any competitor analog, *c-di-GMP was completely degraded to pGpG after approximately 15 min, whereas in the presence of a large excess of unlabeled c-di-GMP, no degradation was observed as expected (Figure 6a,b). However, in the presence of 100 μM c-di- AMP, which does not bind the EAL domain, we found that *c-di-GMP is degraded at nearly the same rate as in the absence of a competitor (Figure 6c). This establishes that only molecules able to bind the EAL domain can prevent *c-di-GMP degradation under these conditions.

To determine if the dithiophosphate analogs bind the EAL domain, we tested their ability to block degradation of *c-di-GMP as described above. In the presence of either 100 μM c-(R_PR_P)-di-G_{ps} or c-(R_PS_P)-di-G_{ps}, we found that *c-di-GMP degradation was completely inhibited (Figure 6c). This indicates that the EAL domain can recognize and bind both dithiophosphate diastereomers and suggests that their nuclease resistance results from the inability of the protein to catalyze hydrolysis on these phosphatemodified substrates. We estimated the affinity (K_i) of both the R_PR_P and R_PS_P diastereomers for the EAL domain to be within 10-fold of the K_m of c-di-GMP (measured as ~ 110 nM), indicating that these analogs may function as effective competitive inhibitors of this enzyme class (c-(R_PR_P)-di-G_{ps} K_i~ 480 nM; c-(R_PS_P)-di-G_{ps} K_i~ 820 nM).

For the methylphosphonate derivatives, we found that isomer-I was unable to block second messenger degradation even though the analog concentration was approximately 6-orders of magnitude greater than that of *c-di-GMP. This indicates that c-di-$dG_{p(Me)}$-I has very little affinity for the EAL protein. For isomer-II, we observed that only 30% of c-di-GMP was degraded to pGpG under the same conditions in which 95% of the substrate was degraded in the absence of any competitor analog (Figure 6c). This indicates that this analog retains some affinity for the EAL domain. Based on the affinity measurements for the dithiophosphate analogs as indicated above along with the competition studies indicating that isomer-II retains some affinity for the EAL domain, we estimate the K_i for isomer-II to be > 10-fold above the K_m of c-di-GMP. Taken together, these data suggest that for isomer-I, EAL domain resistance is primarily due to the inability of this compound to bind the protein whereas for isomer-II, resistance stems from a combination of both weaker binding and chemical effects.

Effects of methylphosphonate substitutions on c-di-GMP binding by the class I and class II riboswitches

We previously published a structure-function study of c-di-GMP analog binding to both the class I and class II riboswitches[34]. With the exception of the novel methylphosphonate derivative developed here, all other analogs tested for EAL domain susceptibility were tested for riboswitch binding, indicating which analogs with increased enzymatic resistance still retain affinity for a known c-di-GMP target. Therefore, we next sought to determine the effects of methylphosphonate substitution of c-di-GMP on the affinity for its second messenger riboswitches.

Structural characterization of c-di-GMP binding to the class I and class II riboswitches has revealed that class I more extensively recognizes the ribosylphosphate backbone compared to class II [26,27] (Figure 7a,c). We measured the affinity (K_d) of both methylphosphonate diastereomers for the class I riboswitch (Figure 7b). For c-di-$dG_{p(Me)}$-I, only slight binding was detectable at high analog concentrations, suggesting that the K_d is > 100 μM (Table 2). In contrast, this RNA bound the second diastereomer, c-di-$dG_{p(Me)}$-II, with surprisingly good affinity (540 nM). This is 5-fold tighter than the parental dinucleotide c-di-dGMP (Table 2) and indicates that binding of c-di-$dG_{p(Me)}$-II resulted in improved affinity (−0.9 kcal/mol, Table 1) relative to the dideoxy cyclic dinucleotide analog.

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Similar studies were performed on the class II riboswitch aptamer (Figure 7d). This RNA bound c-di-$dG_{p(Me)}$-I significantly weaker than c-di-$dG_{p(Me)}$-II. The K_d of isomer-I was approximately 1 μM, 90-fold weaker than that of c-di-dGMP (Table 2). As with class I, we found that c-di-$dG_{p(Me)}$-II analog bound the class II RNA 5-fold tighter than c-didGMP with a K_d nearly identical to that of c-di-GMP (2.4 nM, Table 2). For both riboswitch classes, a gain of 0.9 kcal/mol upon binding isomer-II was observed relative to the parental double deoxy analog (Table 2).

DISCUSSION

The enzymes responsible for the metabolism of c-di-GMP regulate the intracellular concentration of the second messenger, which ultimately controls diverse behaviors in bacteria including biofilm formation, motility and virulence response[2,6,9,10]. Here, we probed the specificity of the c-di-GMP specific EAL domain PDE CC3396 for its cognate ligand to identify analogs that are resistant to enzymatic hydrolysis. We found that modification of both GMP units of c-di-GMP is necessary to significantly affect the stability of the compound to hydrolysis. Substitution of both phosphates with either phosphorothioate or methylphosphonate linkages yields an EAL domain resistant second messenger analog. In addition, one of the two methylphosphonate diastereomers synthesized and tested (isomer-II) had increased affinity for the c-di- GMP riboswitches that function as downstream effectors in this signaling pathway relative to its parental dinucleotide c-di-dGMP. These results suggest that modifying both c-di-GMP phosphate linkages is a general strategy for designing second messenger analogs that are stable to EAL domain catalyzed degradation yet can still bind known downstream RNA effectors in this signaling pathway.

c-di-GMP has a two-fold axis of symmetry and the second messenger can bind the active site of the EAL domain in two possible orientations, with the modified GMP moiety placed either distal (P2) or proximal (P1) to the site where catalysis is taking place (Figure 1b). As a result, for asymmetric ribose or phosphate modified second messenger analogs in which one GMP moiety remains unmodified, the rate of enzyme catalyzed degradation by the EAL domain PDE was not significantly affected. For example, monothiophosphate and single 2′-deoxy modified ligands are degraded by the PDE at nearly the same rate as the native second messenger whereas the doubly modified ligands with these same substitutions are degraded significantly slower than cdi- GMP.

With the exception of c-di-AMP, modification to the guanine bases does not render the second messenger resistant to EAL domain catalyzed degradation. Substitution of one c-di-GMP base with either inosine or N^1methyl guanine, which both are close structural analogs of guanine, had little to no effect on the rate of degradation. An analog with one adenosine substitution, c-GMP-AMP, was still recognized and degraded whereas the doubly substituted analog c-di-AMP was not. This suggests that specific recognition of only one c-di-GMP base is sufficient for binding to the PDE albeit with weaker affinity if the second site is substituted.

Interestingly, c-GMP-AMP has been discovered as a new second messenger in *Vibrio cholerae*, synthesized by a novel class of dinucleotide cyclases that preferentially produces this dinucleotide over c-di-GMP and c-di-AMP[56]. The ability of the EAL domain protein studied here to degrade c-GMP-AMP, as discussed above, suggests that specific recognition of only one guanine base is necessary for c-di-GMP recognition and subsequent degradation. However, sequence alignments of EAL domain proteins indicate that guanine base recognition is achieved primarily through highly conserved residues involved in G_2 binding, whereas those residues involved in G_1 binding are highly variable (Table S1). Therefore, in organisms that utilize both c-di-GMP and potentially c-GMP-AMP, such as *Vibrio cholerae*,

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recognition of G_1 by endogenous EAL domain phosphodiesterases may be more stringent to specifically discriminate against c-GMP-AMP.

Dithiophosphate c-di-GMP analogs can bind the EAL domain, but what is the molecular basis of their nuclease resistance? Crystal structures of active EAL domain proteins reveal that two metal ions are bound in the active site and that both coordinate the proposed nucleophilic water molecule[23,25]. In addition, each metal ion coordinates a different non-bridging phosphate oxygen of the c-di-GMP scissile phosphate (P1; Figure 1b). This is different than the classic two metal ion nucleases in which both metal ions coordinate the same phosphate oxygen[48] and often show stereochemical specificity for degrading phosphorothioate linkages (R_P or S_P)[49–51]. For many systems, making sulfur substitutions in place of oxygen at the site of metal coordination destroys enzymatic activity[52]. In the case of c-di-GMP, sulfur substitution may simply displace one of the active site metals, significantly affecting the ability of the protein to hydrolyze the scissile phosphate linkage. However, unlike some systems where one oxygen is strongly affected by sulfur substitution and the other is not[49–51], both oxygens show strong effects for c-di-GMP degradation. Although dithiophosphate analogs are resistant to enzymatic hydrolysis, these derivatives retain the ability to bind the EAL domain with reasonable affinity and therefore have the potential to function as competitive inhibitors of these c-di-GMP metabolizing enzymes.

Similar to phosphorothioate substitution of c-di-GMP, methylphosphonate substitution also rendered the second messenger resistant to EAL domain catalyzed degradation. However, the methylphosphonate analogs were weaker inhibitors of the EAL domain than the phosphorothioate derivatives. In contrast, both the phosphorothioate and methylphosphonate substituted derivatives of c-di-GMP bound both the class I and class II c-di-GMP riboswitches with good affinity, and in the case of one methylphosphonate diastereomer, with an affinity better than the parental analog. This suggests that these enzymatically stable versions of second messenger analogs may be able to persist longer in the cell and may therefore be useful chemical tools for probing RNA-mediated c-di-GMP signaling *in vivo*.

While the absolute stereochemistry of the methylphosphonate derivatives are unknown, the data are consistent with the possibility that c-di-$dG_{p(Me)}$-I is the R_PR_P diastereomer based on the affinity for riboswitch targets. The interaction between the non-bridging phosphate oxygen of $P_{G\alpha}$ and the adenosine that intercalates between the c-di-GMP bases is the only conserved backbone contact between the otherwise distinct modes of ligand recognition by the two classes of riboswitches[27] (Figure 7a,c). This is the only phosphate contact made to c-di-GMP by the class II riboswitch[27]. Therefore, substitution of the pro-R_P oxygen of $P_{G\alpha}$ is expected to have negative effects on binding for both RNAs. Because methyl groups are isosteric with oxygen and the pro-R_P oxygen of $P_{G\beta}$ is not recognized by either riboswitch, replacing this phosphate oxygen with a methyl group is not expected to significantly impact ligand affinity. The R_PS_P derivative can bind in two possible orientations, one in which the highly conserved contact to the pro-R_P oxygen of $P_{G\alpha}$ is not affected. In contrast, the R_PR_P diastereomer retains the two-fold symmetry axis and presents a pro-R_P oxygen substitution at $P_{G\alpha}$ in both binding orientations. The large loss in affinity for c-di-$dG_{p(Me)}$-I by both the class I and class II riboswitch suggests that this contact is being disrupted and that isomer-I is the R_PR_P isomer. Due to the stereochemical specificity of the cyclization step, only two of the three possible diastereomers are produced and each must contain at least one R_P linkage. This suggests that c-di-$dG_{p(Me)}$-II is the R_PS_P isomer.

While methylphosphonate modification of both c-di-dGMP phosphate linkages had negative effects on binding and catalysis by the EAL domain, positive effects on binding by riboswitch targets of the second messenger were observed. The affinity of one of the methylphosphonate diastereomers (c-di-$dG_{p(Me)}$-II) for the class II riboswitch was almost

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identical to that of c-di-GMP and tighter than that of its parental dinucleotide, c-di-dGMP. This suggests that this analog could affect the function of its RNA target molecule to the same extent as the native second messenger, while remaining more stable to degradation by EAL domain PDEs. Although the other methylphosphonate diastereomer (c-di-$dG_{p(Me)}$-I) bound to the class II riboswitch with weaker affinity, the EAL domain was unable to recognize this compound even at very high analog concentrations. Thus, this EAL domain resistant c-di-GMP derivative shows a strong preference for binding the class II c-di-GMP riboswitch over the EAL domain and could be useful for specifically perturbing RNA mediated signaling processes without affecting the function of phosphodiesterases.

The class I riboswitch aptamer is also able to bind c-di-$dG_{p(Me)}$-II tighter than the parental dinucleotide c-di-dGMP, despite the significant number of contacts made to the phosphate backbone. Interactions with the 2′-hydroxyl groups make a large contribution to ligand binding by the class I riboswitch[34] however, removal of this functional group in the context of the methylphosphonate substitution did not have as large an effect on binding as in the background of the native, charged molecule. This suggests that alleviating electrostatic repulsion between c-di-GMP and the riboswitch RNA increases the affinity of the second messenger for its RNA targets. In addition, previous studies have suggested that a large portion of the binding energy for the c-di-GMP riboswitches results from base stacking interactions[34,53]. Collectively, these observations indicate that it may be possible to completely replace the ribosyl-phosphate backbone of the second messenger with neutral linkers and maintain affinity for riboswitch targets, provided that the guanine bases are held at the appropriate distance to maintain these high affinity stacking interactions.

Although c-di-$dG_{p(Me)}$-II binds tighter to c-di-GMP riboswitches than the parental dinucleotide c-di-dGMP, the ribose version of this analog is expected to have even higher affinity, particularly for the class I riboswitch. Although methylphosphonate linkages next to ribose hydroxyls are unstable[43–45], it may be feasible to synthesize the ribo-methylphosphonate derivative of c-di-GMP due to the limited conformational flexibility of this cyclic dinucleotide. Wang *et al.* recently reported the synthesis of a c-di-GMP derivative containing a sulfur substitution in place of a bridging phosphate oxygen that was stable at neutral pH[47]. When incorporated into linear RNA oligonucleotides, these linkages are highly labile due to in-line attack from the neighboring 2′-hydroxyl. However, when incorporated into c-di-GMP, this linkage proved to be stable because the cyclic backbone of the second messenger constrains the molecule and effectively prevents the 2′-hydroxyl from attacking the scissile phosphate[47]. Ribo-methylphosphonate linkages incorporated into c-di-GMP may be stable provided that the 2′-OH group is protected until after the cyclization reaction is complete. Improved synthetic methods will be necessary to fully explore the potential of this analog as a chemical tool to study c-di-GMP signaling.

Given that the EAL domain cannot efficiently hydrolyze second messenger analogs with methlyphosphonate or phosphorothioate substitutions, which closely resemble the canonical phosphodiester linkage, it is unlikely that a c-di-GMP derivative with a non-native backbone would be susceptible to enzyme catalyzed degradation. Modifying the phosphate backbone provides not only the opportunity to increase the enzymatic stability of the second messenger but to also increase its cell permeability. Neutral molecules often have increased cell permeability and therefore a greater chance of entering cells via passive diffusion as compared to charged compounds[54,55]. Because c-di-GMP signaling is used by many pathogenic organisms to control lifestyle changes that often allow the bacteria to infect a host or form biofilms, which are highly resistant to current antibiotic treatments, the ability to target this pathway for therapeutic purposes is desirable. Thus, the degradation resistant phosphorothioate and neutral methyphosphonate derivatives studied here have desirable

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properties that may make them useful for studying c-di-GMP signaling *in vivo* and manipulating the biological processes under control of this second messenger.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

c-di-GMP bis-(3′-5′)-cyclic dimeric guanosine monophosphate

REFERENCES

1. Camilli A, Bassler BL. Bacterial small-molecule signaling pathways. Science. 2006; 311:1113–1116. [PubMed: 16497924]
2. Hengge R. Principles of c-di-GMP signalling in bacteria. Nat. Rev. Microbiol. 2009; 7:263–273. [PubMed: 19287449]
3. Mills E, Pultz IS, Kulasekara HD, Miller SI. The bacterial second messenger c-di-GMP: mechanisms of signalling. Cell. Microbiol. 2011; 13:1122–1129. [PubMed: 21707905]
4. Schirmer T, Jenal U. Structural and mechanistic determinants of c-di-GMP signalling. Nat. Rev. Microbiol. 2009; 7:724–735. [PubMed: 19756011]
5. Sondermann H, Shikuma NJ, Yildiz FH. You've come a long way: c-di-GMP signaling. Curr. Opin. Microbiol. 2012; 15:140–146. [PubMed: 22226607]
6. Simm R, Morr M, Kader A, Nimtz M, Romling U. GGDEF and EAL domains inversely regulate cyclic di-GMP levels and transition from sessility to motility. Mol. Microbiol. 2004; 53:1123–1134. [PubMed: 15306016]
7. Tamayo R, Pratt JT, Camilli A. Roles of cyclic diguanylate in the regulation of bacterial pathogenesis. Annu. Rev. Microbiol. 2007; 61:131–148. [PubMed: 17480182]
8. Waters CM, Lu W, Rabinowitz JD, Bassler BL. Quorum sensing controls biofilm formation in Vibrio cholerae through modulation of cyclic di-GMP levels and repression of vpsT. J. Bacteriol. 2008; 190:2527–2536. [PubMed: 18223081]
9. Ryjenkov DA, Tarutina M, Moskvin OV, Gomelsky M. Cyclic diguanylate is a ubiquitous signaling molecule in bacteria: insights into biochemistry of the GGDEF protein domain. J. Bacteriol. 2005; 187:1792–1798. [PubMed: 15716451]
10. Schmidt AJ, Ryjenkov DA, Gomelsky M. The ubiquitous protein domain EAL is a cyclic diguanylate-specific phosphodiesterase: enzymatically active and inactive EAL domains. J. Bacteriol. 2005; 187:4774–4781. [PubMed: 15995192]
11. Galperin MY, Natale DA, Aravind L, Koonin EV. A specialized version of the HD hydrolase domain implicated in signal transduction. J. Mol. Microbiol. Biotechnol. 1999; 1:303–305. [PubMed: 10943560]
12. Ryan RP, et al. Cell-cell signaling in Xanthomonas campestris involves an HD-GYP domain protein that functions in cyclic di-GMP turnover. Proc. Natl. Acad. Sci. U.S.A. 2006; 103:6712–6717. [PubMed: 16611728]
13. Galperin MY, Nikolskaya AN, Koonin EV. Novel domains of the prokaryotic two-component signal transduction systems. FEMS Microbiol. Lett. 2001; 203:11–21. [PubMed: 11557134]

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14. Amikam D, Galperin MY. PilZ domain is part of the bacterial c-di-GMP binding protein. Bioinformatics. 2006; 22:3–6. [PubMed: 16249258]
15. Krasteva PV, et al. Vibrio cholerae VpsT regulates matrix production and motility by directly sensing cyclic di-GMP. Science. 2010; 327:866–868. [PubMed: 20150502]
16. Hickman JW, Harwood CS. Identification of FleQ from Pseudomonas aeruginosa as a c-di-GMP-responsive transcription factor. Mol. Microbiol. 2008; 69:376–389. [PubMed: 18485075]
17. Leduc JL, Roberts GP. Cyclic di-GMP allosterically inhibits the CRPlike protein (Clp) of Xanthomonas axonopodis pv. citri. J. Bacteriol. 2009; 191:7121–7122. [PubMed: 19633082]
18. Duerig A, et al. Second messenger-mediated spatiotemporal control of protein degradation regulates bacterial cell cycle progression. Genes Dev. 2009; 23:93–104. [PubMed: 19136627]
19. Newell PD, Monds RD, O'Toole GA. LapD is a bis-(3',5')-cyclic dimeric GMP-binding protein that regulates surface attachment by Pseudomonas fluorescens Pf0-1. Proc. Natl. Acad. Sci. U.S.A. 2009; 106:3461–3466. [PubMed: 19218451]
20. Qi Y, et al. Binding of cyclic diguanylate in the non-catalytic EAL domain of FimX induces a long-range conformational change. J. Biol. Chem. 2011; 286:2910–2917. [PubMed: 21098028]
21. Sudarsan N, et al. Riboswitches in eubacteria sense the second messenger cyclic di-GMP. Science. 2008; 321:411–413. [PubMed: 18635805]
22. Lee ER, Baker JL, Weinberg Z, Sudarsan N, Breaker RR. An allosteric self-splicing ribozyme triggered by a bacterial second messenger. Science. 2010; 329:845–848. [PubMed: 20705859]
23. Barends TRM, et al. Structure and mechanism of a bacterial lightregulated cyclic nucleotide phosphodiesterase. Nature. 2009; 459:1015–1018. [PubMed: 19536266]
24. Minasov G, et al. Crystal structures of YkuI and its complex with second messenger cyclic Di-GMP suggest catalytic mechanism of phosphodiester bond cleavage by EAL domains. J. Biol. Chem. 2009; 284:13174–13184. [PubMed: 19244251]
25. Tchigvintsev A, et al. Structural insight into the mechanism of c-di-GMP hydrolysis by EAL domain phosphodiesterases. J. Mol. Biol. 2010; 402:524–538. [PubMed: 20691189]
26. Smith KD, et al. Structural basis of ligand binding by a c-di-GMP riboswitch. Nat. Struct. Mol. Biol. 2009; 16:1218–1223. [PubMed: 19898477]
27. Smith KD, Shanahan CA, Moore EL, Simon AC, Strobel SA. 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. 2011; 108:7757–7762. [PubMed: 21518891]
28. Benach J, et al. The structural basis of cyclic diguanylate signal transduction by PilZ domains. EMBO J. 2007; 26:5153–5166. [PubMed: 18034161]
29. Ko J, et al. Structure of PP4397 reveals the molecular basis for different cdi-GMP binding modes by Pilz domain proteins. J. Mol. Biol. 2010; 398:97–110. [PubMed: 20226196]
30. Navarro MVAS, De N, Bae N, Wang Q, Sondermann H. Structural analysis of the GGDEF-EAL domain-containing c-di-GMP receptor FimX. Structure. 2009; 17:1104–1116. [PubMed: 19679088]
31. Ross P, et al. The cyclic diguanylic acid regulatory system of cellulose synthesis in Acetobacter xylinum. Chemical synthesis and biological activity of cyclic nucleotide dimer, trimer, and phosphothioate derivatives. J. Biol. Chem. 1990; 265:18933–18943. [PubMed: 2172238]
32. Paul R, et al. Cell cycle-dependent dynamic localization of a bacterial response regulator with a novel di-guanylate cyclase output domain. Genes Dev. 2004; 18:715–727. [PubMed: 15075296]
33. Rao F, et al. Enzymatic synthesis of c-di-GMP using a thermophilic diguanylate cyclase. Anal. Biochem. 2009; 389:138–142. [PubMed: 19328769]
34. Shanahan CA, Gaffney BL, Jones RA, Strobel SA. Differential analogue binding by two classes of c-di-GMP riboswitches. J. Am. Chem. Soc. 2011; 133:15578–15592. [PubMed: 21838307]
35. Gaffney BL, Veliath E, Zhao J, Jones RA. One-flask syntheses of c-di-GMP and the [Rp,Rp] and [Rp,Sp] thiophosphate analogues. Org. Lett. 12:3269–3271. [PubMed: 20572672]
36. Zhao J, Veliath E, Kim S, Gaffney BL, Jones RA. Thiophosphate analogs of c-di-GMP: impact on polymorphism. Nucleosides Nucleotides Nucleic Acids. 2009; 28:352–378. [PubMed: 20183589]

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37. Christen M, Christen B, Folcher M, Schauerte A, Jenal U. Identification and characterization of a cyclic di-GMP-specific phosphodiesterase and its allosteric control by GTP. J. Biol. Chem. 2005; 280:30829–30837. [PubMed: 15994307]
38. Kiburu I, Shurer A, Yan L, Sintim HO. A simple solid-phase synthesis of the ubiquitous bacterial signaling molecule, c-di-GMP and analogues. Mol. Biosyst. 2008; 4:518–520. [PubMed: 18493648]
39. Rao F, Yang Y, Qi Y, Liang ZX. Catalytic mechanism of cyclic di-GMP-specific phosphodiesterase: a study of the EAL domain-containing RocR from Pseudomonas aeruginosa. J. Bacteriol. 2008; 190:3622–3631. [PubMed: 18344366]
40. Manoharan M. RNA interference and chemically modified small interfering RNAs. Curr. Opin. Chem. Biol. 2004; 8:570–579. [PubMed: 15556399]
41. Basu S, Strobel SA. Thiophilic metal ion rescue of phosphorothioate interference within the Tetrahymena ribozyme P4-P6 domain. RNA. 1999; 5:1399–1407. [PubMed: 10580468]
42. Narlikar GJ, Herschlag D. Mechanistic aspects of enzymatic catalysis: lessons from comparison of RNA and protein enzymes. Annu. Rev. Biochem. 1997; 66:19–59. [PubMed: 9242901]
43. Dertinger D, Uhlenbeck OC. Evaluation of methylphosphonates as analogs for detecting phosphate contacts in RNA-protein complexes. RNA. 2001; 7:622–631. [PubMed: 11345440]
44. Pritchard CE, et al. Methylphosphonate mapping of phosphate contacts critical for RNA recognition by the human immunodeficiency virus tat and rev proteins. Nucleic Acids Res. 1994; 22:2592–2600. [PubMed: 8041622]
45. Kulshina N, Baird NJ, Ferre-D'Amare AR. Recognition of the bacterial second messenger cyclic diguanylate by its cognate riboswitch. Nat. Struct. Mol. Biol. 2009; 16:1212–1217. [PubMed: 19898478]
46. Battistini C, Fustinoni S, Brasca MG, Borghi D. Stereoselective synthesis of cyclic dinucloetide phosphorothioates. Tetrahedron. 1993; 49:1115–1132.
47. Wang J, et al. Conservative change to the phosphate moiety of cyclic diguanylic monophosphate remarkably affects its polymorphism and ability to bind DGC, PDE, and PilZ proteins. J. Am. Chem. Soc. 2011; 133:9320–9330. [PubMed: 21612220]
48. Yang W, Lee JY, Nowotny M. Making and breaking nucleic acids: two-Mg2+-ion catalysis and substrate specificity. Mol. Cell. 2006; 22:5–13. [PubMed: 16600865]
49. Burgers PM, Eckstein F, Hunneman DH. Stereochemistry of hydrolysis by snake venom phosphodiesterase. J. Biol. Chem. 1979; 254:7476–7478. [PubMed: 224029]
50. Connolly BA, Potter BV, Eckstein F, Pingoud A, Grotjahn L. Synthesis and characterization of an octanucleotide containing the EcoRI recognition sequence with a phosphorothioate group at the cleavage site. Biochemistry. 1984; 23:3443–3453. [PubMed: 6087894]
51. Potter BVL, Connolly BA, Eckstein F. Synthesis and configurational analysis of a dinucleoside phosphate isotopically chiral at phosphorus. Stereochemical course of Penicillium citrinum nuclease P1 reaction. Biochemistry. 1983; 22:1369–1377. [PubMed: 6301546]
52. Brautigam CA, Steitz TA. Structural principles for the inhibition of the 3'-5' exonuclease activity of Escherichia coli DNA polymerase I by phosphorothioates. J. Mol. Biol. 1998; 277:363–377. [PubMed: 9514742]
53. Smith KD, Lipchock SV, Strobel SA. Structural and biochemical characterization of linear dinucleotide analogues bound to the c-di-GMP-I aptamer. Biochemistry. 2012; 51:425–432. [PubMed: 22148472]
54. Varma RS. Synthesis of oligonucleotide analogues with modified backbones. Synlett. 1993; 1993:621–637.
55. Rozners E. Carbohydrate chemistry for RNA interference: Synthesis and properties of RNA analogues modified in sugar-phosphate backbone. Curr. Org. Chem. 2006; 10:675–692.
56. Davies BW, Bogard RW, Young TS, Mekalanos JJ. Coordinated Regulation of Accessory Genetic Elements Produces Cyclic Di-Nucleotides for *V. cholerae* Virulence. Cell. 2012; 149:358–370. [PubMed: 22500802]

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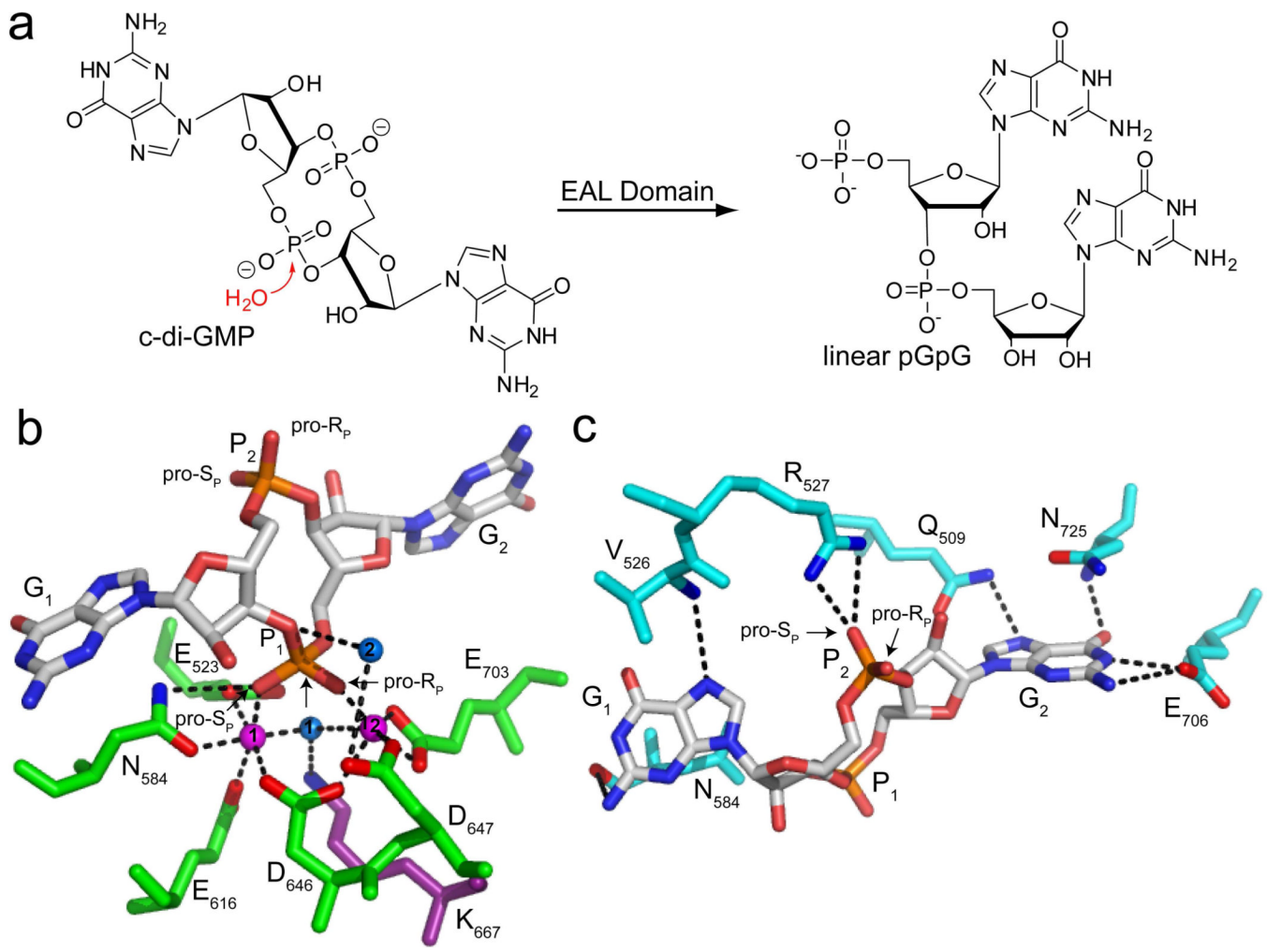

Figure 1.

Recognition and enzymatic hydrolysis of c-di-GMP by EAL domain proteins. (a) Schematic reaction. In the presence of an EAL domain protein, a nucleophilic water molecule attacks one of the c-di-GMP phosphodiester bonds to yield the linear 5′- phosphate product, pGpG. (b) c-di-GMP bound to the active site of the EAL domain protein TBD1265 from *Thiobacillus denitrificans* (PDB ID 3N3T). c-di-GMP is colored by atom with carbon shown in white, oxygen in red, nitrogen in blue and phosphorous in orange. Water molecules are shown as blue spheres and magnesium ions are shown as magenta spheres. The protein residues in the active site involved in metal coordination are colored in green and oxygens and nitrogens making contacts to c-di-GMP are colored in red and blue, respectively. The highly conserved lysine that stabilizes the negative charge of water 1 and positions it for nucleophilic attack on the scissile phosphate, P1, is shown in purple. Water 2 is predicted to donate a proton to the leaving group. The pro-R_P and pro-S_P oxygens of P1 and P2 are labeled for sulfur substitution. (c) Recognition of the c-di-GMP bases and the phosphate distal to the site of catalysis, P2 (PDB ID 3N3T). Coloring of c-di-GMP is the same as in (b). Residues in contact with G1 and G2 are colored in cyan and the highly conserved arginine that makes electrostatic contacts to P2 is shown in white. Oxygens and nitrogens of protein side chains are colored as in part (b). Residues involved in G2 recognition are conserved while those involved in G1 recognition are highly variable. The residue corresponding to N_{725} is most often aromatic (F, W, or Y).

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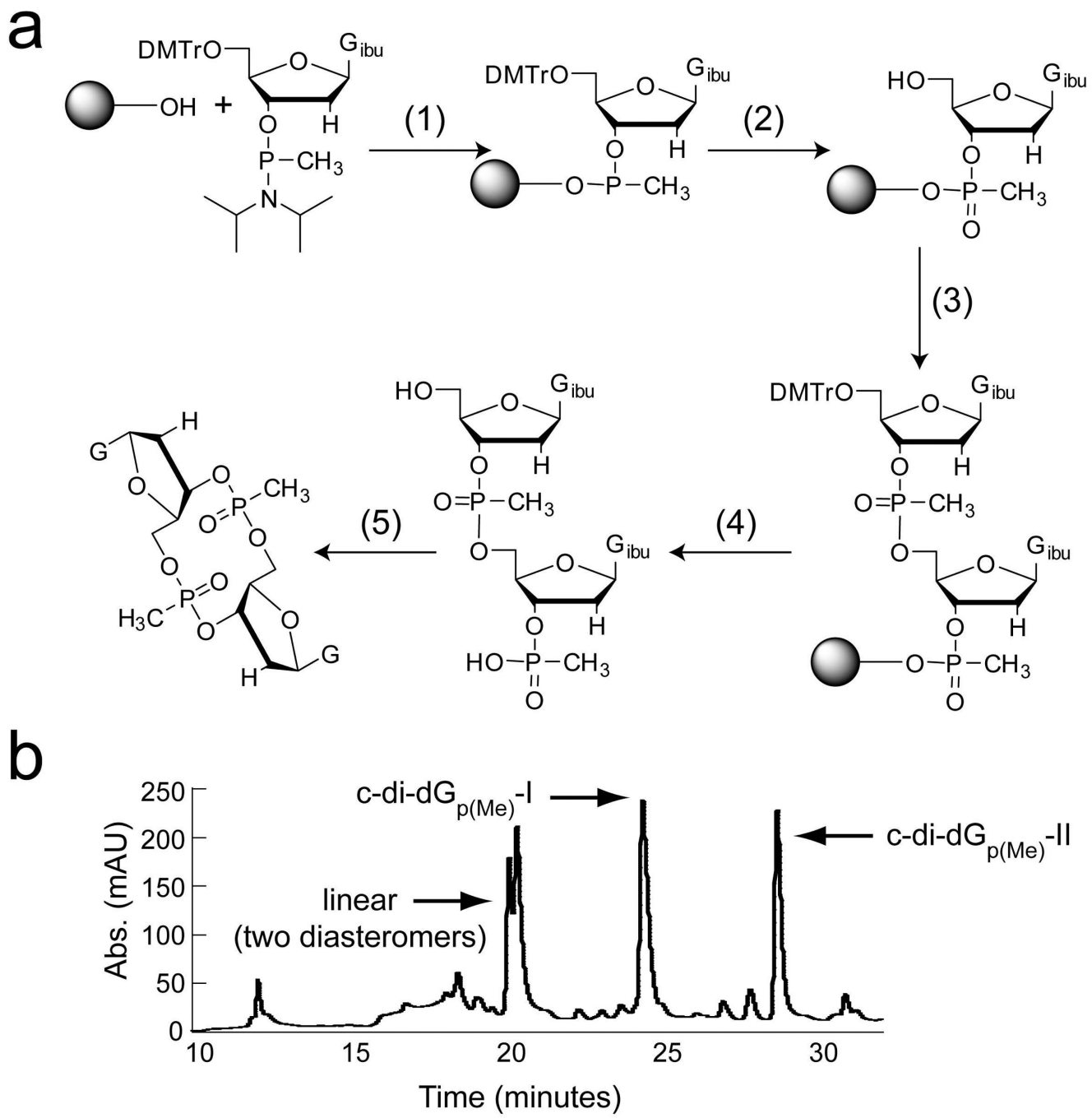

Figure 2.

Synthesis of the double methylphosphonate c-di-GMP analog. (a) Synthetic scheme. The linear dinucleotide was synthesized on solid support and then cyclized in solution. (1) (i) tetrazole/ACN; (2) (i) 3% DCA/DCM (ii) I_2/THF/pyridine, (iii) acetic anhydride/DMAP, (iv) 3% DCA/DCM; (3) tetrazole/ACN + guanosine methylphosphonamidite/THF; (4) (i) 3% DCA/DCM, (ii) 10% TEA/ACN; (5) (i) 0.1 M MSNT/pyridine, (ii) ACN/EtOH/NH_4OH, (iii) ethylenediamine.

(b) HPLC trace monitored at 254 nm showing purification of the crude reaction synthesis for the methylphosphonate c-di-GMP analog c-di-$dG_{p(Me)}$. Peaks corresponding to the two cyclized diastereomers and the linear, uncyclized molecule are labeled.

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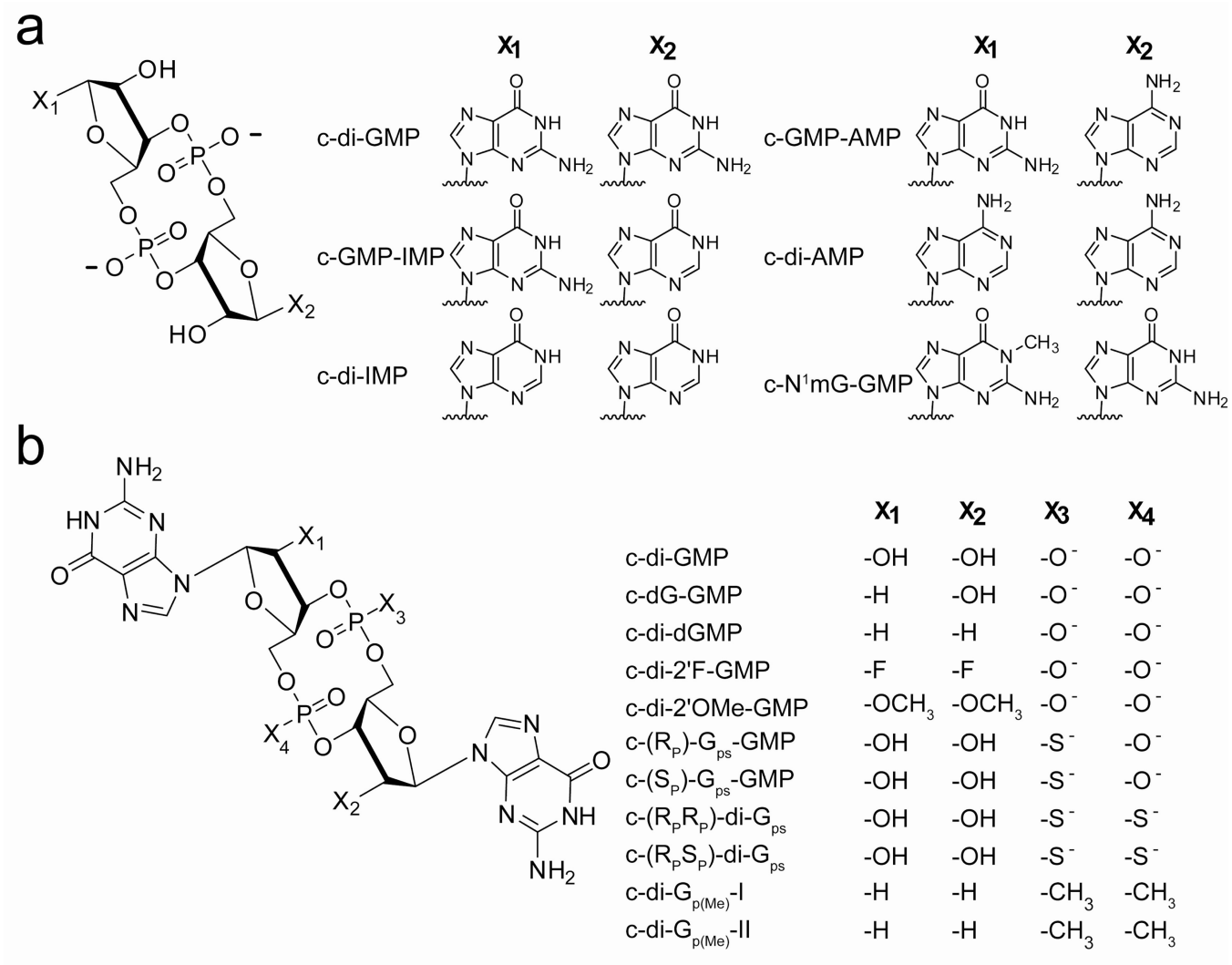

Figure 3.

Structures of c-di-GMP analogs. (a) Base modified c-di-GMP analogs. Guanine was replaced with inosine, adenosine, or N^1-methyl guanosine as indicated by X_1 and X_2. (b) Ribose and phosphate modified analogs. Modifications made to the ribose rings are indicated by X_1 and X_2 and those made to the phosphate linkages are indicated by X_3 and X_4.

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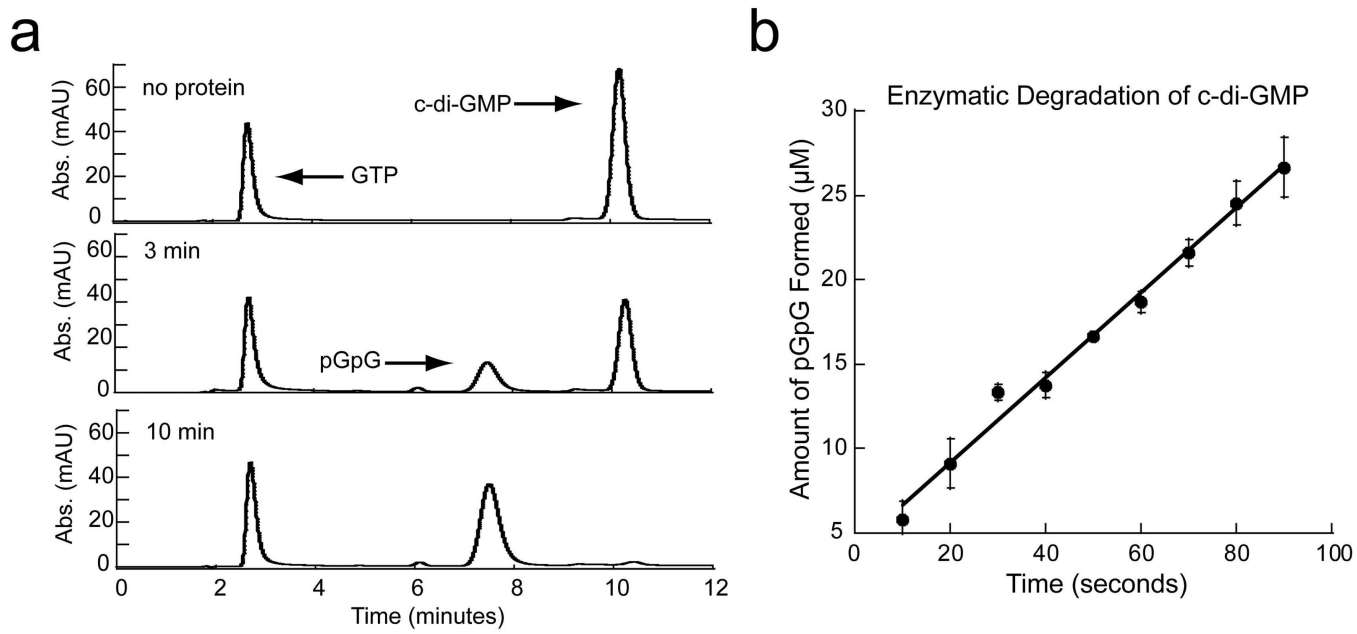

Figure 4.

Monitoring the enzymatic degradation of c-di-GMP by HPLC. (a) HPLC traces showing elution of GTP, pGpG, and c-di-GMP. Peaks corresponding to each of these compounds are labeled. After 10 minutes under the experimental conditions used, c-di-GMP is completely degraded. (b) The amount of pGpG formed was measured in the linear range of the reaction to determine the initial velocity of c-di-GMP degradation. Data was collected and analyzed in the same manner for those analogs that were susceptible to degradation.

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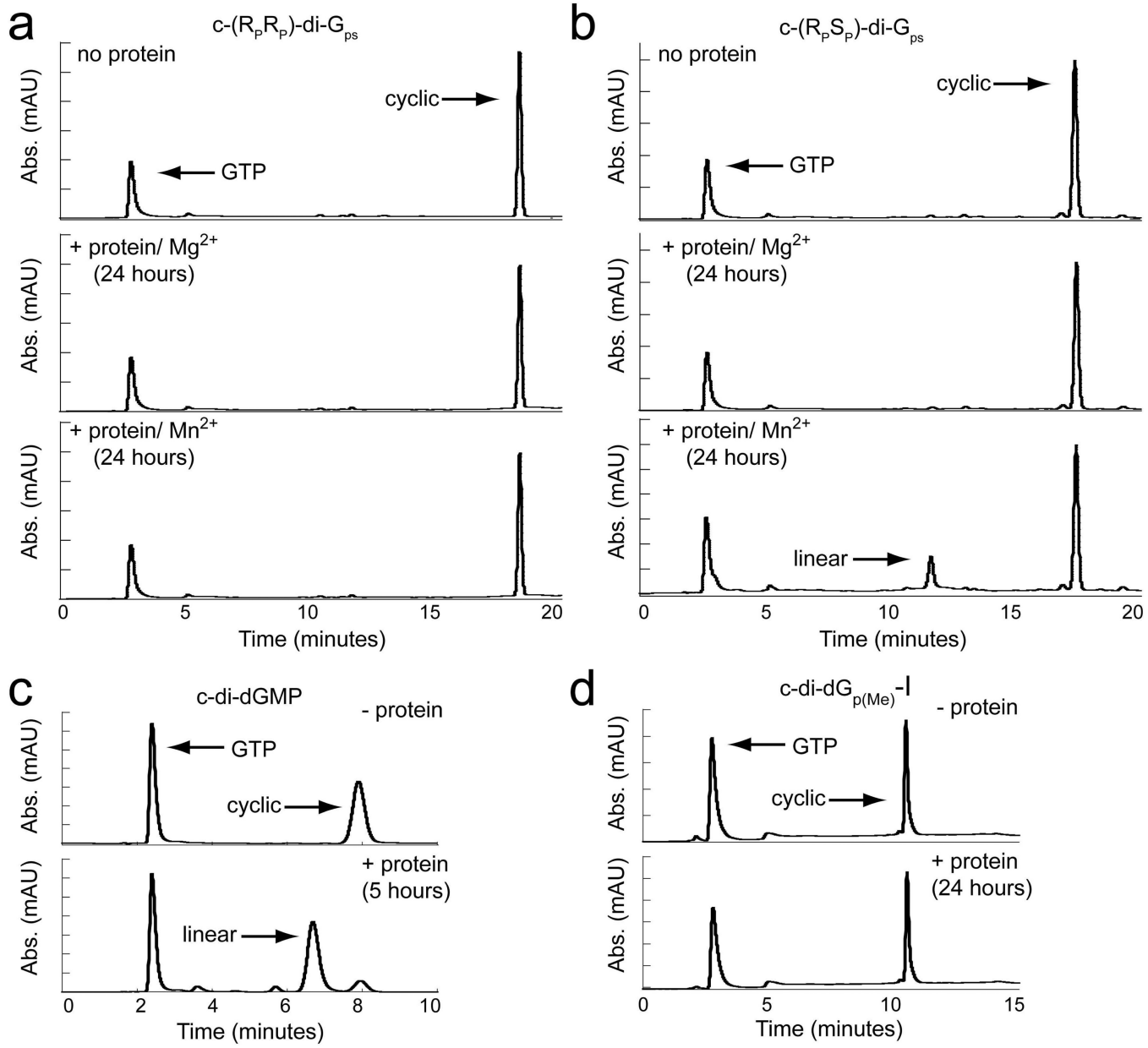

Figure 5.

HPLC traces monitored at 254 nm showing degradation of backbone modified analogs by the EAL domain protein CC3396. Peaks corresponding to GTP, cyclic and linear dinucleotides are labeled. (a) In the absence of protein (top panel) a single peak corresponding to c-(R_PR_P)-di-G_{ps} is observed. Only a peak corresponding to the cyclic dinucleotide is detected after a 24 hour incubation period with the protein in the presence of either 10 mM $MgCl_2$ (middle panel) or 10 mM $MnCl_2$ (bottom panel). (b) HPLC analysis of c-(R_PS_P)-di-G_{ps} in the absence (top) and presence of protein (middle and bottom). No degradation product is observed with $MgCl_2$, whereas a peak corresponding to the linear product is observed in the presence of $MnCl_2$.

Approximately 20% of the cyclic product is hydrolyzed after 24 hours. (c) HPLC traces of the c-di-dGMP degradation reaction. The top panel shows the reaction mixture in the absence of protein and the bottom panel is in the presence of protein. After a 5 hour

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incubation with CC3396, c-di-dGMP is almost completely hydrolyzed (> 85%). (d) HPLC traces of c-di-$dG_{p(Me)}$-I in the absence (top) and presence (bottom) of CC3396. No hydrolysis products are detectable even after a 24 hour incubation with the protein. The same lack of reactivity was observed for c-di-$dG_{p(Me)}$-II after treatment with the protein for 24 hours (data not shown).

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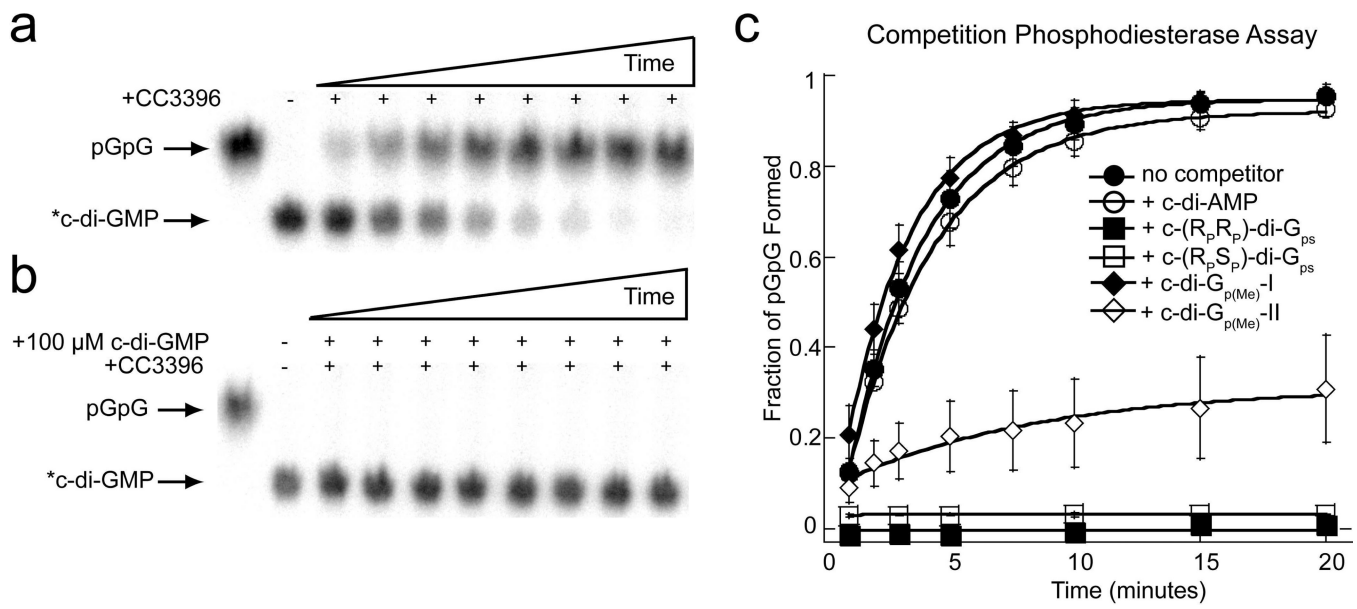

Figure 6.

Degradation of radiolabeled c-di-GMP (*c-di-GMP) in the presence of EAL domain resistant c-di-GMP analogs. (a) PEI-cellulose TLC showing degradation of *c-di-GMP to pGpG by CC3396 over time. (b) Degradation of *c-di-GMP in the presence of unlabeled c-di-GMP. (c) Fraction of pGpG formed from degradation of *c-di-GMP by CC3369 over time in the presence of unlabeled c-di-AMP, dithiophosphate, and methylphosphonate analogs.

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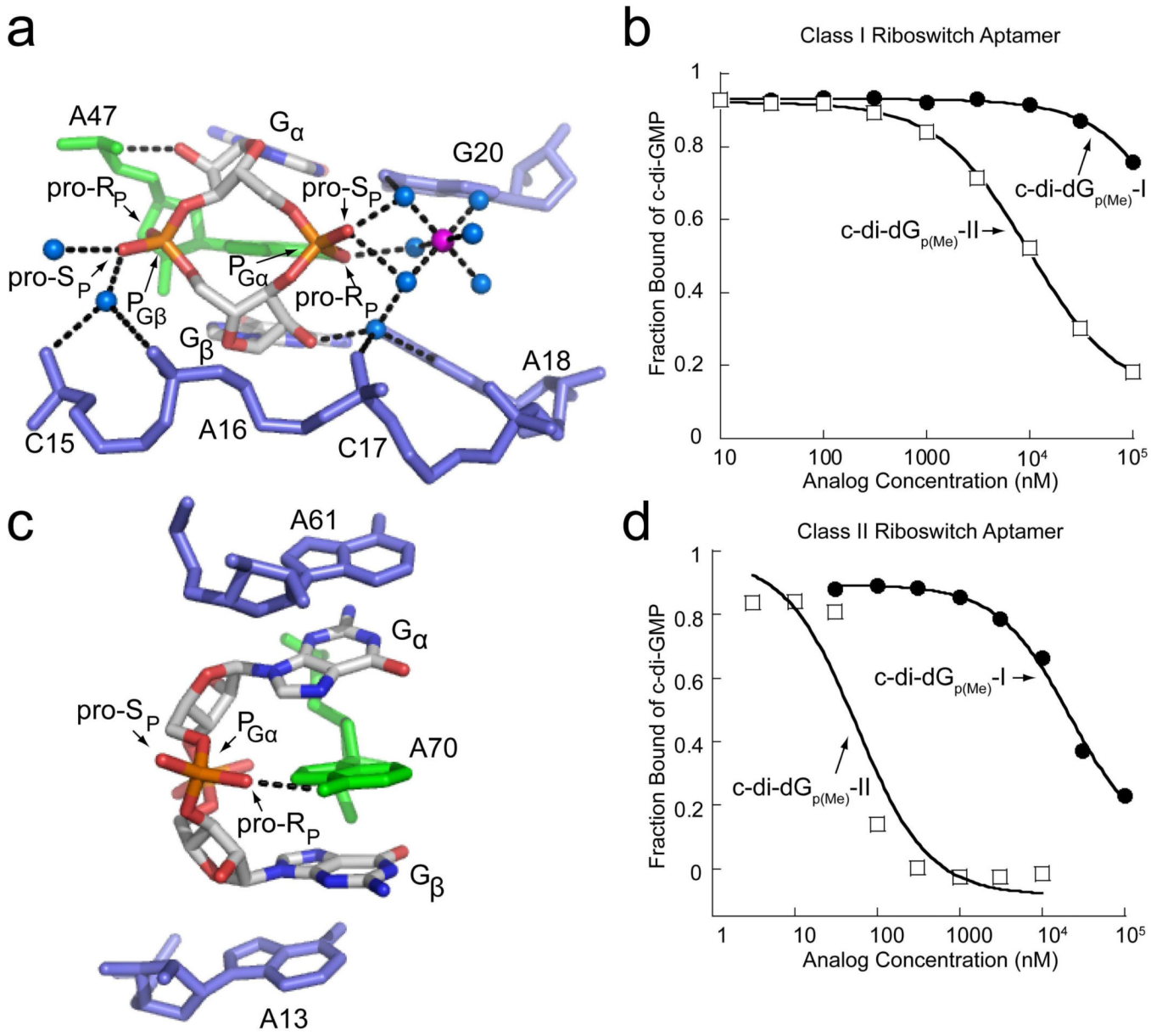

Figure 7.

Backbone recognition of c-di-GMP and binding of methylphosphonate analogs by the class I and class II riboswitch aptamers. (a) c-di-GMP bound to the class I aptamer (PDB ID 3MXH). c-di-GMP is colored by atom with carbon shown in white, nitrogen in blue, oxygen in red, and phosphorous in orange. The highly conserved adenosine that intercalates between the guanine bases of c-di-GMP is colored in green and additional RNA residues are colored in blue. Water molecules are shown as blue spheres and magnesium ions as purple spheres. The pro-R_P and pro-S_P oxygens are indicated to show the sites of methyl substitutions. Hydrogen bonds are indicated by black dashed lines. (b) Binding curves of methylphosphonate derivatives to the class I riboswitch. The c-di-$dG_{p(Me)}$-I binding curve is shown as black circles and the c-di-$G_{p(Me)}$-II curve is shown as open squares. (c) c-di-GMP bound to the class II aptamer (PDB ID 3Q3Z). Coloring and labeling is the same as in part (a). (d) Binding curves of the methylphosphonate derivatives to the class II riboswitch. Labeling is the same as in part (b).

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Table 1

Rates of enzymatic hydrolysis for c-di-GMP and its nucleotide analogs by the EAL domain phosphodiesterase protein CC3396. All data reported is the average of at least three independent trials.

Modification	Analog	Rate (k_{cat}) (mol*min^{-1}*mol enzyme^{-1})	Fold Change
	c-di-GMP[a]	15 ± 1.6	–
Base[b]	c-GMP-IMP	7.0 ± 0.4	2.1
	c-N^{1}mG-GMP	4.5 ± 0.7	3.3
	c-di-IMP	0.51 ± 0.06	29
	c-GMP-AMP	0.48 ± 0.04	31
	c-di-AMP	n.d.[c]	–
Ribose[a]	c-dG-GMP	6.4 ± 0.2	2.3
	c-di-dGMP	1.1 ± 0.6	14
	c-di-2′F-GMP	2.9 ± 0.4	5.2
	c-di-2′OMe-GMP	0.9 ± 0.2	17
Phosphate[a]	c-(S_P)-G_{ps}-GMP	29 ± 1.8	0.5
	c-(R_P)-G_{ps}-GMP	8.3 ± 0.6	1.8
	c-(R_PR_P)-di-G_{ps}	n.d.	–
	c-(R_PS_P)-di-G_{ps}	n.d.	–
	c-di-$dG_{p(Me)}$-I	n.d.	–
	c-di-$dG_{p(Me)}$-II	n.d.	–

[a] Rates were measured under saturating conditions of substrate and therefore correspond to k_{cat}

[b] Rates measured under multiple turnover conditions with 100 μM substrate and 1μM enzyme.

[c] n.d. indicates no degradation under the conditions tested after 24 hour incubation with CC3396.

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Table 2

K_d measurements of methylphosphonate analogs for the class I and class II c-di-GMP binding riboswitches determined by the competition gel-shift assay.

	Class I			Class II		
Analog	K_d (nM)	Fold Change[a]	$\Delta\Delta G_{bind}$ (kcal/mol)[a]	K_d (nM)	Fold Change[a]	$\Delta\Delta G_{bind}$ (kcal/mol)[a]
c-di-dGMP[b]	2600 ± 88	–	–	11 ± 1.2	–	–
c-di-GMP[b]	1.4 ± 0.1	[1900][c]	−4.4	2.2 ± 0.2	[5]	−0.9
c-di-$dG_{P(Me)}$-I	> 100,000	>38	>2.2	1000 ± 93	91	2.7
c-di-$dG_{P(Me)}$-II	540 ± 57	[5]	−0.9	2.4 ± 0.3	[5]	−0.9
c-(R_PR_P)-di-G_{ps}	150 ± 33	[17]	−1.7	3.6 ± 0.6	[3]	−0.7
c-(R_PS_P)-di-G_{ps}[b]	750 ± 63	[3]	−0.7	4.0 ± 0.8	[3]	−0.6

[a]Calculated relative to the parental dinucleotide c-di-dGMP.

[b]Binding data for these compounds was previously reported[34].

[c]Brackets indicate fold increase in binding.