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Conservative Change to the Phosphate Moiety of Cyclic Diguanylic Monophosphate Remarkably Affects Its Polymorphism and Ability To Bind DGC, PDE, and PilZ Proteins

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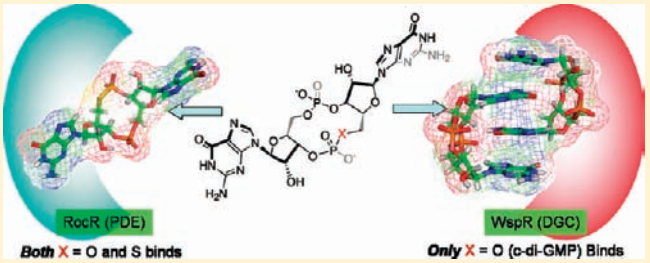
Supporting Information

ABSTRACT: The cyclic dinucleotide c-di-GMP is a master regulator of bacterial virulence and biofilm formation. The activations of c-di-GMP metabolism proteins, diguanylate cyclases (DGCs) and phosophodiesterases (PDEs), usually lead to diametrically opposite phenotypes in bacteria. Analogues of c-di-GMP, which can selectively modulate the activities of c-di-GMP processing proteins, will be useful chemical tools for studying and altering bacterial behavior. Herein we report that a conservative modification of one of the phosphate groups in c-di-GMP with a bridging sulfur in the phosphodiester linkage affords an analogue called endo-S-c-di-GMP. Computational, NMR (including DOSY), and CD experiments all reveal that, unlike c-di-GMP, endo-S-c-di-GMP does not readily form higher aggregates. The lower propensity of endo-S-c-di-GMP to form aggregates (as compared to that of c-di-GMP) is probably due to a higher activation barrier to convert from the "open" conformer (where the two guanines are on opposite faces) to the "closed" conformer (where the two guanines are on the same face). Consequently, endo-S-c-di-GMP has selectivity for proteins that bind monomeric but not dimeric c-di-GMP, which form from the "closed" conformer. For example, endo-S-c-di-GMP can inhibit the hydrolysis of c-di-GMP by RocR (a PDE enzyme that binds monomeric c-di-GMP) but did not bind to Alg44 (a PilZ protein) or regulate WspR (a DGC enzyme that has been shown to bind to dimeric c-di-GMP). This work demonstrates that selective binding to different classes of c-di-GMP binding proteins could be achieved by altering analogue conformer populations (conformational steering). We provide important design principles for the preparation of selective PDE inhibitors and reveal the role played by the c-di-GMP backbone in c-di-GMP polymorphism and binding to processing proteins.



INTRODUCTION

Cyclic diguanylic monophosphate (c-di-GMP), a 12-membered bisguanine dinucleotide, has attracted interest from researchers from varied fields since being discovered by Benziman more than two decades ago.[1] Originally discovered as a regulator of cellulose synthase in *Gluconacetobacter xylinus* (formerly called *Acetobacter xylinum*),[1] c-di-GMP has now emerged as a master regulator of several processes in bacteria, including the synthesis of biopolymers that are components of bacterial biofilms[2,3] and the expression of virulence-associated genes.[4,5] In the bacterium *Vibrio cholerae*, the causative agent of profuse watery diarrhea in regions with poor sanitation conditions, c-di-GMP has been shown to regulate *Vibrio* polysaccharide (*vps*) genes.[6,7] These genes are responsible for the formation of an extracellular matrix, which is required for the establishment of a biofilm structure. C-di-GMP also negatively regulates flagella activity[8,9] and twitching motility.[10−12] *Pseudomonas aeruginosa* is an opportunistic pathogen that is a model system for the studies of c-di-GMP, as all of the diguanylate cyclases (DGCs), phosphodiesterases (PDEs), and PilZ-domain-containing binding proteins have been systematically investigated.[13,14] The *P. aeruginosa* c-di-GMP system features WspR, one of the most potent DGCs,[13,15] RocR, one of the most potent PDEs,[13,16] and Alg44, one the best-characterized PilZ-domain-containing c-di-GMP binding proteins, which is required for the production of alginate and the mucoid phenotype that is often associated with negative clinical outcome in cystic fibrosis patients.[14]

C-di-GMP signaling has consequently become of interest for the development of anti-biofilm or anti-virulence drugs. Despite the central role that c-di-GMP plays in bacterial "lifestyle", several aspects of this signaling molecule remain far from being understood. For example, the so-called adaptor proteins that bind to c-di-GMP and transmit this binding event into processes that lead to biofilm formation are poorly characterized. Most of the adaptor proteins found so far (for example, the PilZ family) do not have any enzymatic activity of their own, suggesting that they probably relay the c-di-GMP binding event into allosteric

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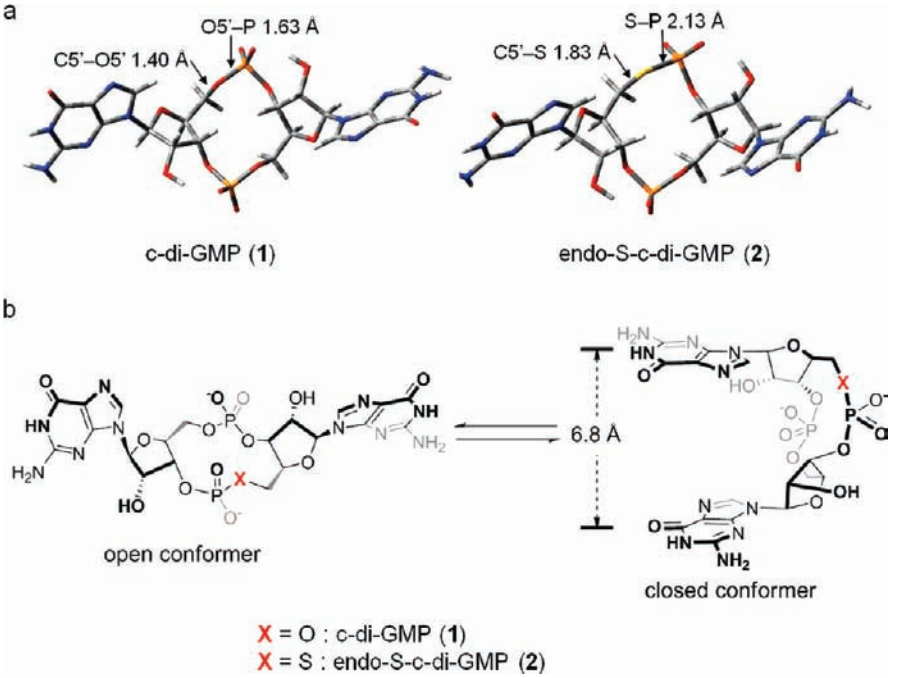

Figure 1. (a) Structures of c-di-GMP (**1**, left) and endo-S-c-di-GMP (**2**, right) monomer. The structure was optimized by Gaussian 09 software[19] with HF/6-31G(d) basis set. (b) Two general conformers of c-di-GMP and analogues (open and closed).

modulation of other enzymes with which they associate. However, these associated enzymes or factors of c-di-GMP adaptor proteins have largely not been found.

Analogues of c-di-GMP that selectively target one class of binding proteins would be excellent tools for studying c-di-GMP signaling in bacteria and could even become lead compounds for the design of anti-biofilm agents. Different classes of c-di-GMP binding proteins can promote opposite phenotypes in bacteria: PDEs promote biofilm dissolution, whereas DGCs facilitate biofilm formation.[2,3] Both classes of enzymes interact extensively with c-di-GMP, and it remains to be shown how one would design an analogue or any small molecule that will selectively target PDE and not DGC, or vice versa. C-di-GMP has been shown to readily form dimers, tetraplexes, and higher aggregates in the presence of cations.[17a,18] Divalent cations such as magnesium promote dimer formation in c-di-GMP, whereas monovalent cations such as potassium promote the formation of tetraplexes and octaplexes in c-di-GMP.[17a,18] This propensity of c-di-GMP to form tetraplexes or octaplexes (G-quadruplexes) at micromolar concentrations in the presence of cations (such as magnesium and potassium) is intriguing because simple nucleotides (such as cGMP, GTP, or pGpG) do not readily form G-quadruplex structures at micromolar concentrations. In the presence of aromatic intercalators, c-di-GMP has also been shown to form G-quadruplexes at physiological concentrations.[17b,c] Plausibly, the facile interconversion of c-di-GMP into different aggregation states (especially in the presence of cations) could be a means whereby bacteria regulate biofilm formation in the presence of different metals or metabolites.

Understanding the structural features that allow c-di-GMP to readily form aggregates would increase our fundamental understanding of how different nucleic acids adopt different architectures as well as provide insight into how this important bacterial signaling molecule achieves its interesting polymorphism. Additionally, one would ideally want to make molecules that have lower propensities to aggregate into inactive forms (as this lowers the effective molarity of the molecule); therefore, knowing which moieties on c-di-GMP facilitate aggregate formation could provide important design principles for the synthesis of non-aggregation-prone c-di-GMP-like molecules, which could be used to perturb biological signaling networks. We have been particularly interested in G-quadruplex and dimer formation by c-di-GMP and how this is so readily achieved when the dinucleotide pGpG does not so readily form intermolecular dimer or G-quadruplex complexes. As both c-di-GMP and pGpG contain guanine bases, which are required for the formation of the G-tetrad plane found in G-quadruplexes, it is reasonable to assume that other structural features found in c-di-GMP, but not in the linear pGpG, are responsible for the enhancement of G-quadruplex formation. In this paper, we reveal that small changes to the phosphodiester backbone of c-di-GMP (0.93 Å increase in the circumference of the backbone ring; see Figure 1a) remarkably decrease the propensity to form G-quadruplexes. We show that a c-di-GMP analogue that has one of the oxygens in the 5′-bridging phosphodiester linkage replaced by sulfur (endo-S-c-di-GMP (**2**); see Figure 1b) has altered biophysical and biochemical properties, distinct from those of c-di-GMP.

RESULTS AND DISCUSSION

One general strategy that is typically used by drug developers to discover antagonists of signaling molecules is to modify the signaling molecule to afford analogues[20] that still maintain the ability to bind the receptors to which the signaling molecule binds but are unable to activate the receptors for biological function. In order to develop effective c-di-GMP analogues that can be used to antagonize the actions of c-di-GMP, it is of interest to determine which functionalities on c-di-GMP could be modified to maintain binding to c-di-GMP processing proteins without triggering processes that lead to biofilm formation.

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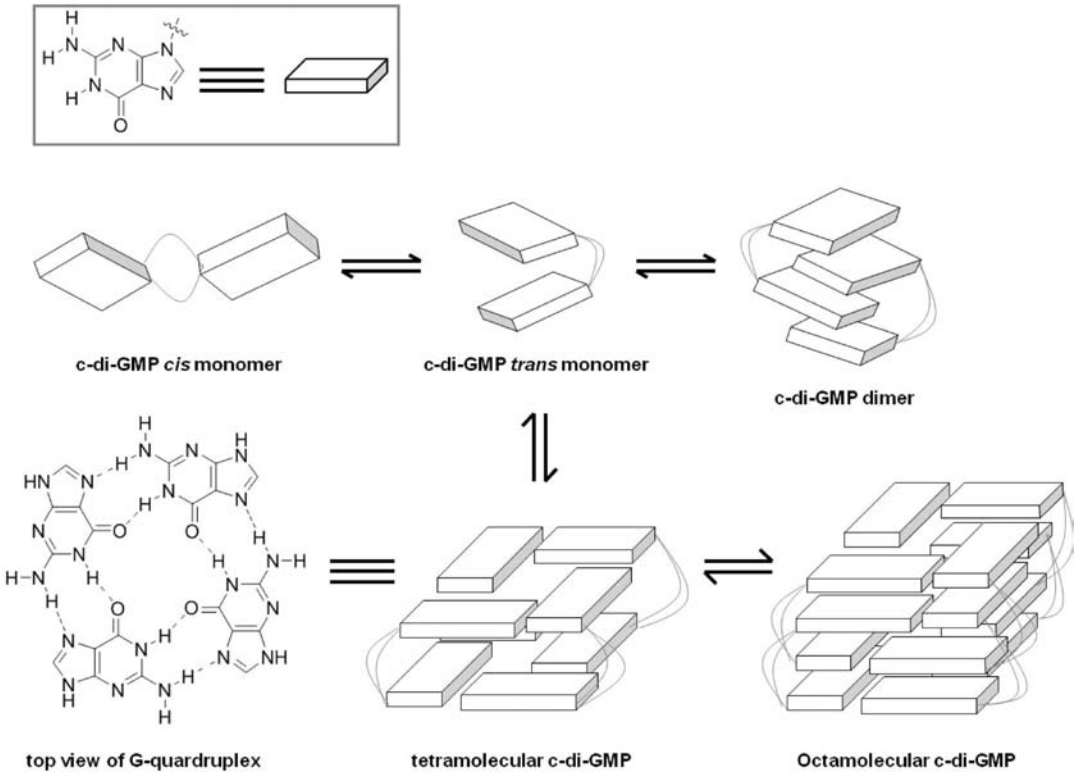


Figure 2. Polymorphism of c-di-GMP.[18]

The aggregation of a c-di-GMP antagonist into higher aggregates would reduce its effective molarity. Therefore, we initiated a program to determine which moieties on c-di-GMP facilitate aggregate formation. C-di-GMP is a 12-membered ring with limited conformational flexibility. The crystal structure of c-di-GMP reveals that the torsion angles found in this macrocycle are similar to those found in standard linear RNAs, implying that the ring structure imposes little or no torsional stress on this molecule.[21]

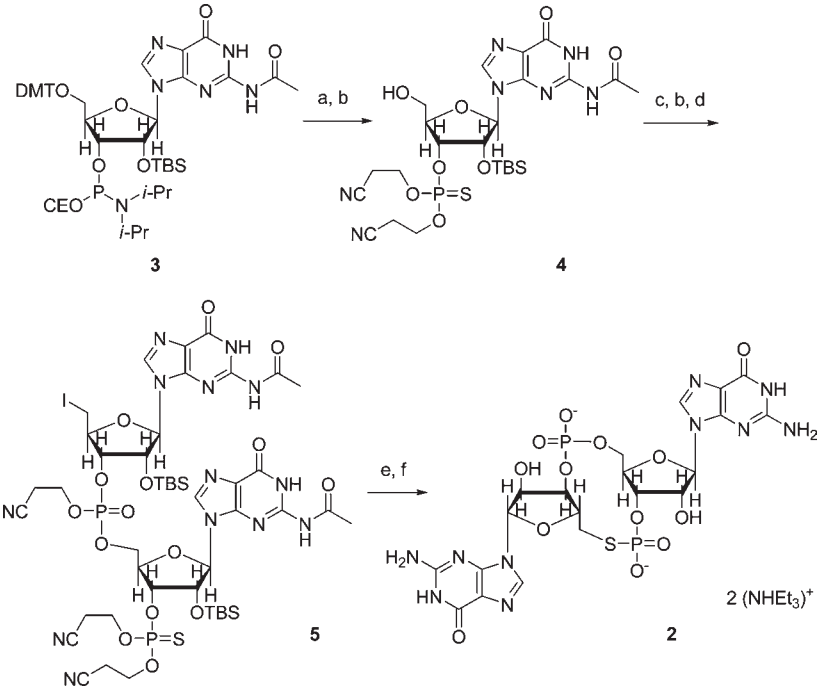
The ability of c-di-GMP but not linear pGpG to readily form dimers and G-quadruplexes at micromolar concentrations prompted us to hypothesize that the lack of conformational flexibility in c-di-GMP coupled with the low torsional stress in the macrocycle poise this molecule to make aggregates, such as G-quadruplexes or dimers (see Figure 2). As a starting point to determine if the 12-membered ring of c-di-GMP is critical to its biophysical (aggregate formation) as well as biochemical (binding to receptors) properties, we chose to study a very close analogue of c-di-GMP, referred in this paper as endo-S-c-di-GMP (**2**).

The replacement of oxygen at the bridging positions in phosphate linkages in nucleic acids can be considered conservative; Kool has shown that the thermal stabilities of DNAs containing phosphorodiester linkages are similar to those of native DNAs.[22] Enzymes, such as Klenow fragment of DNA polymerase I or T7 RNA polymerase, can utilize templates containing phosphorothioates as effectively as those containing native phosphodiester linkages, and no pauses were observed at the phosphorothioester sites when these replicative enzymes were used.[22]

Synthesis of Endo-S-c-di-GMP. The synthesis of endo-S-c-di-GMP (**2**) is summarized in Scheme 1 (22% overall yield from commercially available phosphoramidite **3**). The key step for the synthesis of **2** is the phosphorothioate−iodide macro-ring closure.[23] It has been shown by several researchers that the "bridging" positions in the phosphate linkages of both DNA[24−28] and RNA[29−32] can be replaced by sulfur to give phosphorothioester linkages. In DNA, 5′-phosphorothioester linkages are stable,[22] whereas for RNAs, 5′-phosphorothioester linkages are about 6-fold more labile than the natural phosphodiester linkages at pH 7.[29] Although endo-S-c-di-GMP (**2**) also contains 2′-OH (the functionality that is responsible for facilitating hydrolysis in RNA), it is stable at neutral pH because the cyclic structure positions the phosphate moiety more than 3.3 Å away from the 2′-OH, making it impossible for the 2′-OH in endo-S-c-di-GMP to participate in an in-line cleavage reaction.[33]

Polymorphism of C-di-GMP and Endo-S-c-di-GMP. C-di-GMP can exist in many conformations. These conformations can be generally categorized as "closed" (the two guanine bases are on the same face) or "open" (the two guanines are on opposite faces). Calculations using Gaussian 09 software with HF/6-31G(d) basis set, using Onsager's model in a self-consistent reaction field (a mimic for solution phase), revealed that the ground-state conformer of c-di-GMP in water is a closed conformer, in which the two guanines are parallel and on the same face. The energy of the open conformer, in which the C5's of the two guanines are 13.5 Å apart, is only 1.9 kcal/mol higher than that of the closed conformer, (Table 2) implying that c-di-GMP probably exists as a continuum of conformers in which the inter-guanine distances can range from 6.8 to 13.5 Å. The closed conformer of c-di-GMP, in which the two guanines are separated by 6.8 Å, is biologically relevant and found in the active site of many c-di-GMP binding proteins (for recent examples, see Protein Databank (PDB) crystal structures 3KYF,[34] 3KLO,[35] and 3I5A[36]). Computational studies revealed that the torsion angle[21] of endo-S-c-di-GMP is different from that of the native

Scheme 1. Synthesis of Endo-S-c-di-GMP (2)[a]



[a] Conditions: (a) cyanoethyl alcohol (5.0 equiv), imidazolium perchlorate (3.6 equiv), 6 h, then Beacauge reagent (2.5 equiv), 1 h, MeCN, rt; (b) dichloroacetic acid (11−14 equiv), CH_2Cl_2, 10 min, 60% over two steps; (c) phosphoramidite **3** (1.5 equiv), imidazolium perchlorate (4.7 equiv), then *t*-BuOOH (9.3 equiv), MeCN, rt, 6 h; (d) $Me(PhO)_3P^+I^-$ (5.3 equiv), 2,6-lutidine (20 equiv), DMF, rt, 1 h, 61% over three steps; (e) ammonia, rt, 24 h; (f) $NEt_3 \cdot 3HF$ (20 equiv), MeCN, rt, 12 h, 59% over two steps. DMF = dimethylformamide.

Table 1. Backbone Torsion Angles for Computed C-di-GMP and Endo-S-c-di-GMP Structures

		angle, degrees					
		α	β	γ	δ	ε	ζ
c-di-GMP[a]	open[d]	72.3	−163.9	50.4	96.1	−161.5	63.6
	closed[e]	79.7	−151.9	55.1	83.2	−179.4	64.9
endo-S-c-di-GMP[b]	open[f]	63.9	−146.6	58.9	94.3	−164.1	64.8
	closed[e]	68.5	−137.6	64.5	80.6	−179.9	65.9
linear RNA[c]		73.8	−168.2	62.2	81.6	−147.1	63.9

[a] The dihedral angular notations are as follow: $O3'-P^{\alpha}O5'^{\beta}C5'^{\gamma}C4'^{\delta}C3'^{\varepsilon}O3'^{\zeta}P-O5'$. [b] The dihedral angular notations are as follows $O3'-P^{\alpha}S^{\beta}C5'^{\gamma}C4'^{\delta}C3'^{\varepsilon}O3'^{\zeta}P-O5'$. [c] Aerage values, taken from PDB 3MXH. [d] C5's of the guanines are 13.5 Å apart. [e] C5's of the guanines are 6.8 Å apart. [f] C5's of the guanines are 11.7 Å apart as the most stable conformer.

Table 2. Energy Difference (in kcal/mol) between "Open" (Inter-guanine Distance = 13.5 Å) and "Closed" (Inter-guanine Distance = 6.8 Å) Forms of C-di-GMP and Endo-S-c-di-GMP

	ΔE^{sol} (open −closed)[a]	ratio open:closed[b]
c-di-GMP	1.9	1:25
endo-S-c-di-GMP	1.3	1:9

[a] The electronic energy was computed with Gaussian 09 software with HF/6-31G(d) basis set. Solvent effect (H_2O) was calculated using Onsager's model in a self-consistent reaction field (see Supporting Information for details). [b] The ratio was determined from the equilibrium constant *K*, obtained from the equation $\Delta E = -RT \ln K$ (T = 298 K).

c-di-GMP (see Table 1). From these computational studies, we predicted that the aggregative behavior of endo-S-c-di-GMP would be different from that of c-di-GMP.

It is expected that the "closed" conformers of c-di-GMP (**1**) or endo-S-c-di-GMP (**2**) would more readily form dimers or G-quadruplexes than the "open" conformers of c-di-GMP and endo-S-c-di-GMP (Figure 2). This is because in the closed conformations of both c-di-GMP and endo-S-c-di-GMP, the two guanines are suitably positioned for mutual intercalation (dimer, Figure 2) or for forming G-quadruplexes (Figure 2). One can therefore reliably predict the relative aggregative property of a c-di-GMP analogue by comparing the relative energy difference between the closed and open conformers of an analogue to that of c-di-GMP.

Computational studies done on both c-di-GMP and endo-S-c-di-GMP (using Onsager's solvent model in a self-consistent reaction field) revealed that c-di-GMP is more likely (~3 times) to form a closed conformer than endo-S-c-di-GMP in water (see Table 2). This prediction was verified experimentally; the ^{1}H NMR spectra of c-di-GMP and endo-S-c-di-GMP (both as triethylammonium salts) showed that both compounds exist as monomeric forms at 20 °C in the absence of monovalent cations such as K^+ (see Figures 3c and 4c). The ^{1}H NMR spectrum of c-di-GMP (which is C_2 symmetric) shows two singlets at 5.87 and 7.96 ppm, assigned to the anomeric H1′ and guanine H8 protons, respectively (Figure 3c). Endo-S-c-di-GMP is not C_2 symmetric, due to the presence of one "bridging" sulfur atom in the phosphorothioate moiety, and therefore the two anomeric

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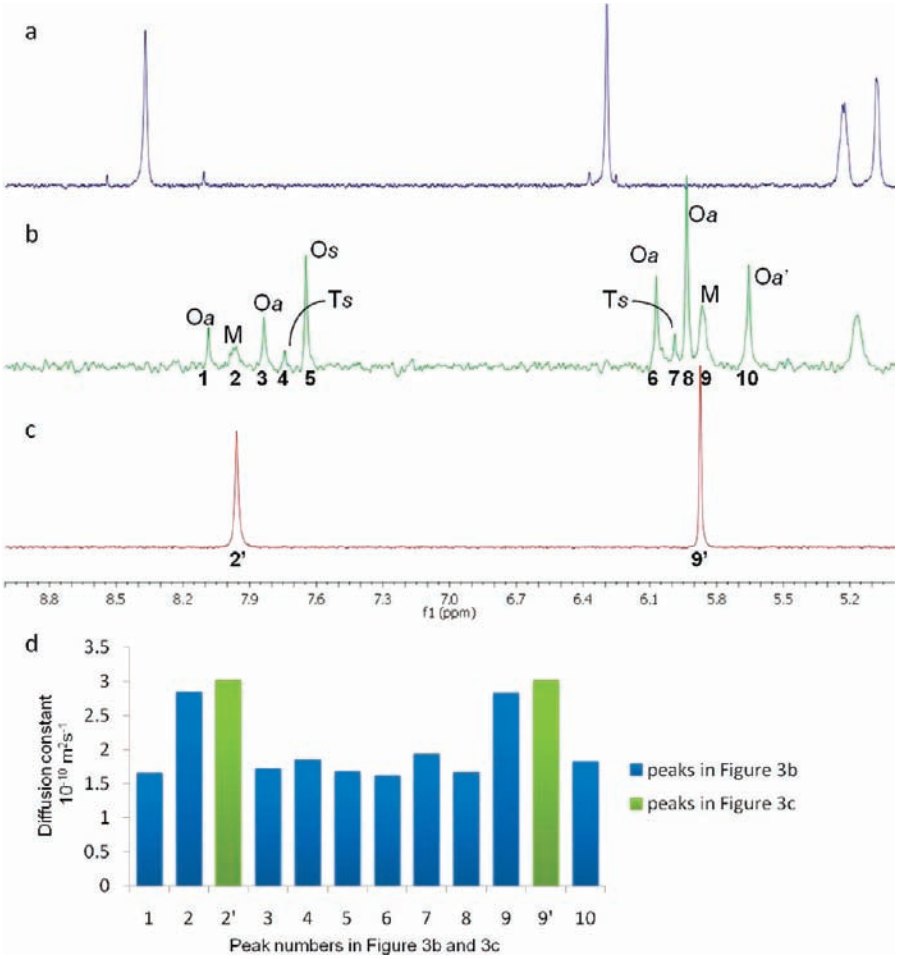

Figure 3. ^{1}H NMR stacked spectra of 1.0 mM c-di-GMP in D_2O. Conditions: (a) 100 mM KCl, 60 °C; (b) 100 mM KCl, 20 °C [the peaks were assigned on the basis of T_1/T_2 relaxation analysis and ref 18]; (c) no metal cation, 20 °C; (d) T_1/T_2 relaxation analysis (from DOSY experiments).

H1′ protons as well as the two guanine H8 protons in endo-S-c-di-GMP are chemically nonequivalent and have different chemical shifts (Figure 4c). The guanine H8 in endo-S-c-di-GMP appears as two singlets of equal intensities at 7.90 and 8.02 ppm, and the anomeric H1′ in endo-S-c-di-GMP appears as a singlet at 5.95 ppm and a doublet at 5.85 ppm (Figure 4c). Upon the addition of 100 mM K^+ to c-di-GMP, the intensities of the peaks at 7.96 and 5.87 ppm are reduced, and other peaks appear around 7.96 and 5.87 ppm (Figure 3b). These peaks are attributed to different aggregates of c-di-GMP because, upon heating of the sample to 60 °C (which will break all aggregates), the multiple peaks disappear and new singlet peaks at 8.39 and 6.30 ppm appear (corresponding to the guanine H8 and the anomeric H on c-di-GMP respectively, see Figure 3a). The shift in ppm values for monomeric c-di-GMP is expected, as temperature affects ppm values.[37] On the basis of integration of the peaks in the ^{1}H NMR spectrum of c-di-GMP in a buffer containing 100 mM potassium cations at 20 °C, we estimate that only 14% of c-di-GMP exists in the monomeric form under this condition (Figure 3b). On the other hand, upon the addition of 100 mM K^+ to endo-S-c-di-GMP, 43% of the monomeric form still remains in the solution (compare Figures 3b and 4b). It therefore appears that c-di-GMP has a higher propensity to form aggregates than endo-S-c-di-GMP, and it is remarkable that a single conservative substitution in the phosphate moiety can result in such drastic consequences.

In order to determine the nature of the aggregation states of both c-di-GMP and endo-S-c-di-GMP, DOSY experiments were conducted. Following literature precedent, the diffusion constants of the various c-di-GMP/endo-S-c-di-GMP were obtained via analysis of T_1/T_2 relaxation.[18] According to the Stokes–Einstein equation, the diffusion constant $D = kT/(6\pi\eta R)$, where k is the Boltzmann constant, T is the temperature, η is the solvent viscosity, and R is the radius of the molecular sphere. It therefore follows that the diffusion constant is inversely proportional to the radius of the molecule (or aggregate). The diffusion constants of c-di-GMP and endo-S-c-di-GMP in their monomeric forms can be obtained via DOSY experiments, in the absence of added metal cations (Figures 3c,d and 4c,d; see Supporting Information). On the basis of the obtained diffusion constants for monomeric c-di-GMP and endo-S-c-di-GMP, the diffusion constants for the dimeric, tetrameric, and octameric forms could be predicted, using calculated radii of these aggregates (Figures 3d and 4d; see Supporting Information). The monomer, dimer, tetramer, and octamer forms of c-di-GMP and endo-S-c-di-GMP are denoted as M, B (indicating "bimolecular"[18]), T, and O, respectively, in Figures 3b and 4b. For c-di-GMP, the majority of the aggregates in the presence of K^+ are T and O forms,[18] with predicted diffusion constants of 2.01×10^{-10} and 1.62×10^{-10} m^2/s respectively; the experimental diffusion constants for the T and O forms of c-di-GMP (obtained from the DOSY experiment) are

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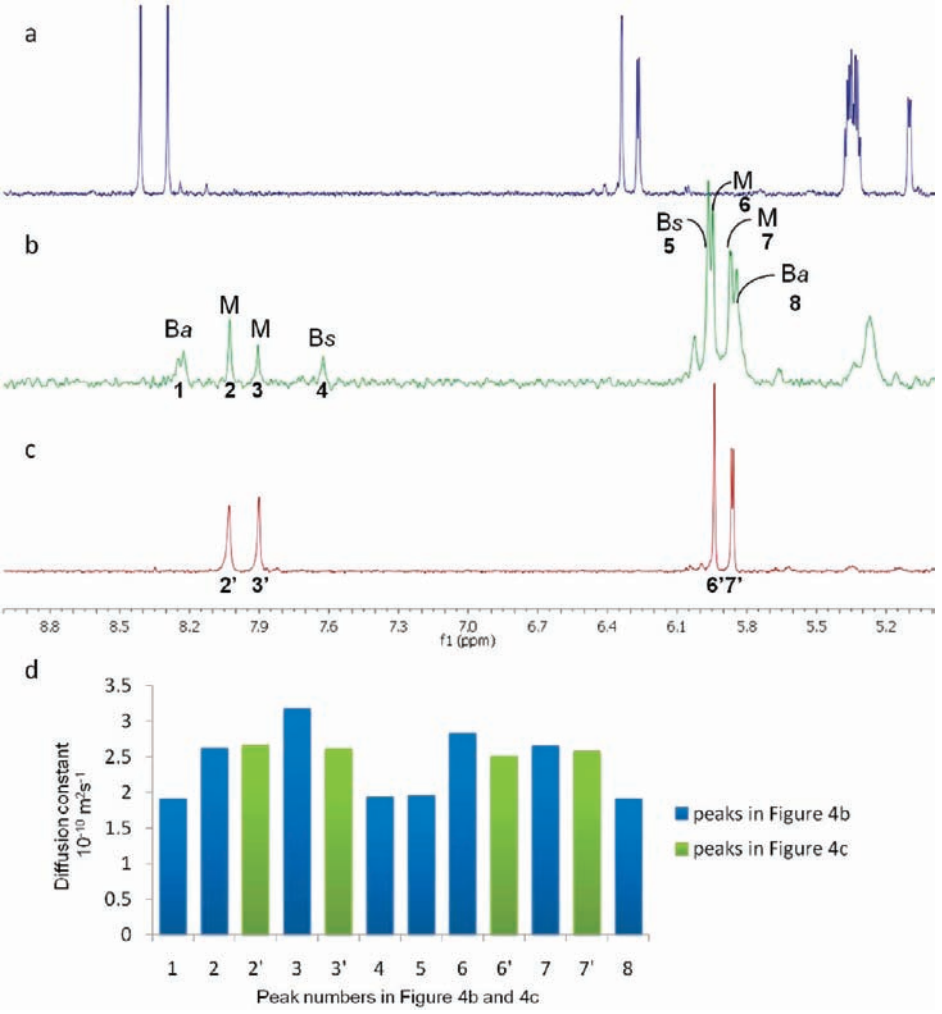

Figure 4. ^{1}H NMR stacked spectra of 1.0 mM endo-S-c-di-GMP in D_2O. Conditions: (a) 100 mM KCl, 60 °C; (b) 100 mM KCl, 20 °C [the peaks were assigned on the basis of T_1/T_2 relaxation analysis and NOE experiments (see Supporting Information)]; (c) no metal cation, 20 °C; (d) T_1/T_2 relaxation analysis (from DOSY experiments).

1.91×10^{-10} and 1.60×10^{-10} m^2/s, respectively. For endo-S-c-di-GMP, the only identified aggregate form that is present in the presence of K^+ cations is the B form, with predicted and experimental diffusion constants of 2.07×10^{-10} and 1.93×10^{-10} m^2/s, respectively (see Supporting Information). The relative conformations of the guanine H8 and anomeric H1′ were determined following the reported method.[18] Guanine H8 in the *syn* conformation and the anomeric H1 are expected to exhibit strong positive NOE effects, whereas guanine H8 in the *anti* conformation is expected to show a much weaker NOE effect with the anomeric H1 (see Supporting Information). The *syn* or *anti* relation is denoted as "*s*" or "*a*" in Figures 3b and 4b, respectively. For example, B*a* represents a dimeric endo-S-c-di-GMP with the H1′ and H8 in an *anti* conformation in Figure 4b.

The circular dichroism (CD) spectra of c-di-GMP (under different conditions) are also remarkably different from those of endo-S-c-di-GMP (Figure 5). In the presence of potassium cation, c-di-GMP (100 μM) forms G-quadruplexes (a positive CD peak around 310 nm is indicative of G-quadruplex formation in c-di-GMP; see Figure 5a and also ref 18). However, the CD spectra of endo-S-c-di-GMP (100 μM) in the presence of various monovalent cations (Na^+, K^+, Li^+) do not show any sign of G-quadruplex formation (no positive peak around 310 nm; see Figure 5b). Even when the concentration of endo-S-c-di-GMP is increased to 200 μM, no G-quadruplex formation is observed (Figure 5c).

C-di-GMP and endo-S-c-di-GMP Binding to Metabolism and "Adaptor" Proteins. Having established via NMR and CD studies that endo-S-c-di-GMP has a lower propensity to form aggregates (dimers and tetraplexes), we proceeded to investigate if endo-S-c-di-GMP would bind to proteins that have been previously shown to bind to the native c-di-GMP. Most of the crystal structures of c-di-GMP, bound to various proteins, reveal extensive interactions between the protein residues and the phosphate and nucleobase moieties of c-di-GMP (see Figure 6). For example, the phosphate moieties of c-di-GMP interact with Arg479 and Gln596 of the EAL domain of FimX[38,39] (from *P. aeruginosa*) and binds monomeric c-di-GMP (see Figure 6c). The majority of c-di-GMP binding proteins whose crystal structures have been deposited in the Protein Databank bind to dimeric c-di-GMP. In most of these structures, the protein residues interact with the phosphate moiety of c-di-GMP. The crystal structure of dimeric c-di-GMP bound to VpsT[35,40] (a transcriptional regulator from *V. cholerae*)

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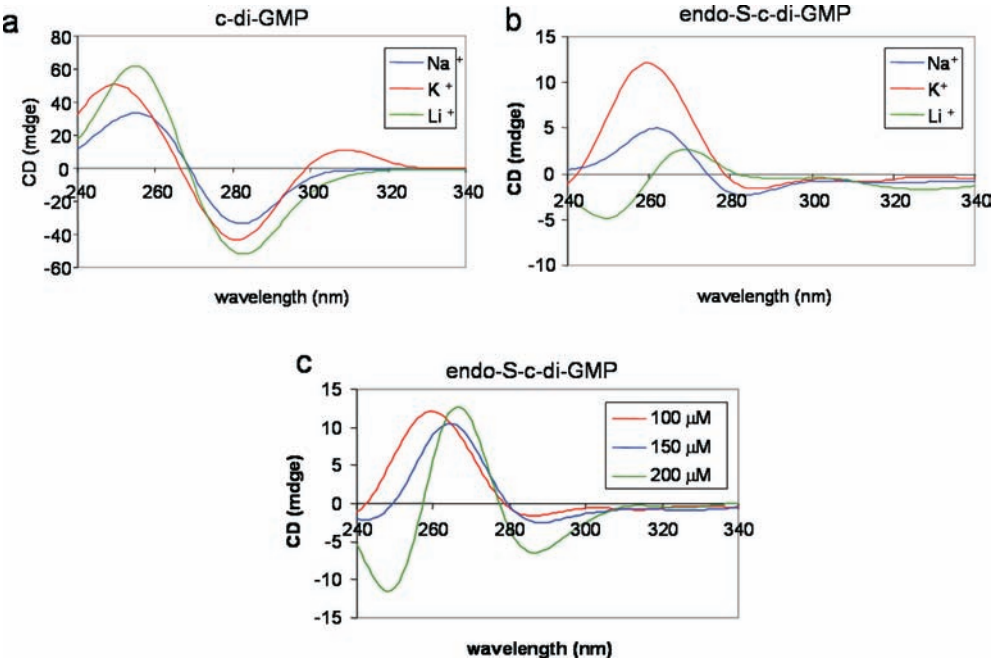

Figure 5. CD spectra of c-di-GMP and endo-S-c-di-GMP. Conditions: 10 °C, [MCl] (where M is Li, Na, or K) = 1.0 M, 10 mM Tris-HCl (pH 7.5); (a) 100 μM c-di-GMP; (b) 100 μM endo-S-c-di-GMP; (c) 100, 150, and 200 μM endo-S-c-di-GMP in 1.0 M of KCl.

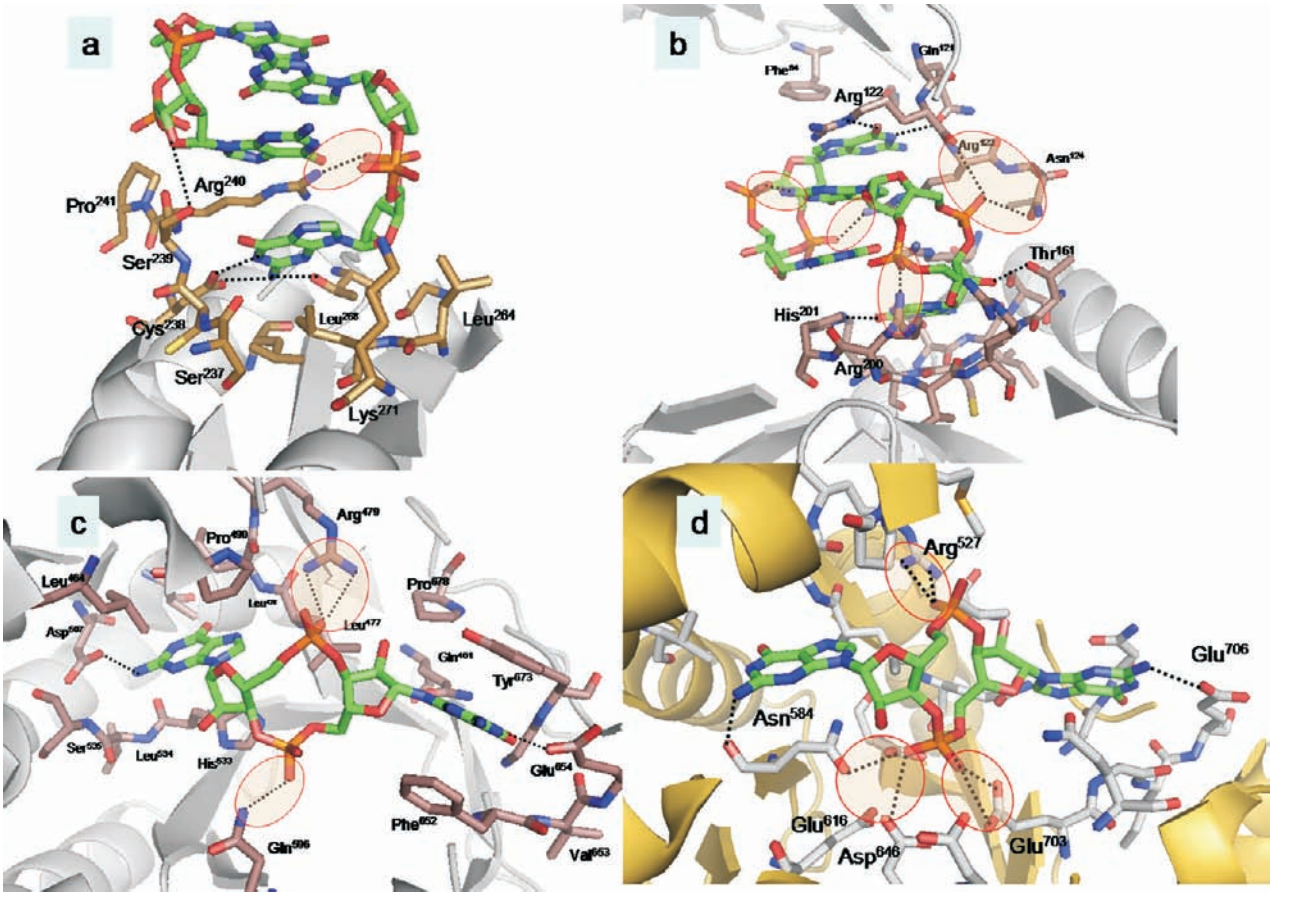

Figure 6. (a) Dimeric c-di-GMP, bound to WspR (DGC domain; PDB code 3I5A). (b) Dimeric c-di-GMP, bound to P4397 (PilZ domain; PDB code 3KYF). (c) Monomeric c-di-GMP, bound to FimX (EAL domain; PDB code 3HV8). (d) Monomeric c-di-GMP, bound to TBD1265 (EAL domain; PDB code 3N3T).

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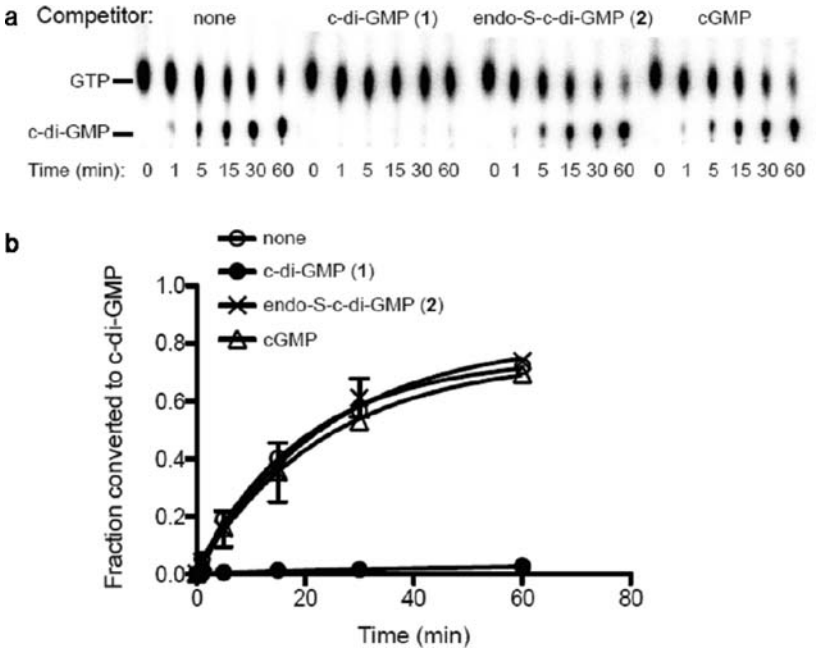

Figure 7. Inhibition of c-di-GMP synthesis by WspR with guanine-containing nucleotides. (a) TLC of reaction mixture at different time points. Initial reaction conditions: WspR, radio-labeled GTP, and unlabeled c-di-GMP/endo-S-c-di-GMP/cGMP or no added nucleotide inhibitor. (b) Graph showing fraction of radio-labeled GTP that was converted into radio-labeled c-di-GMP in the presence of inhibitor.

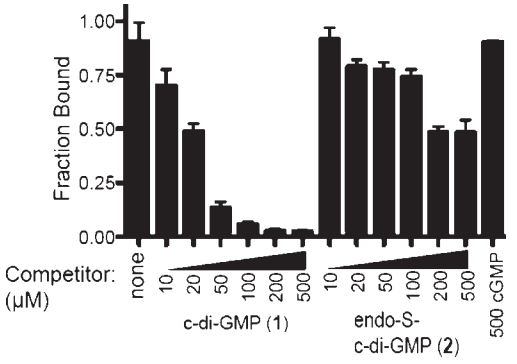

Figure 8. Displacement of radio-labeled c-di-GMP from Alg44 (PilZ protein) by competition with c-di-GMP, endo-S-c-di-GMP, or cGMP. Reduction of the "fraction bound" of radio-labeled c-di-GMP indicates binding of the unlabeled ligand to the same site on the Alg44 protein.

reveals that Thr133 and Arg134 as well as other residues make specific interactions with the phosphate groups of c-di-GMP. Similarly, Arg123 and Asn124 of P4397 (a c-di-GMP "adaptor" protein containing the PilZ domain[10,34]) interact with the phosphate moiety in dimeric c-di-GMP (Figure 6b). Most diguanylate cyclases contain an inhibitory site (I-site) that allosterically modulates the synthesis of c-di-GMP.[41] WspR, a DGC from *P. aeruginosa*, also contains the I-site, and analysis of a crystal structure of WspR bound to dimeric c-di-GMP (Figure 6a)[36] reveals specific interactions between the protein and the phosphate group found in c-di-GMP (Figure 6d).[42]

Are these phosphate−protein residue interactions important for c-di-GMP binding, and how much do these interactions contribute to the overall binding of c-di-GMP to metabolism and processing proteins? Additionally, different proteins bind to different conformers of c-di-GMP (open vs closed conformers; see Figure 6). Could one therefore achieve selectivity in the

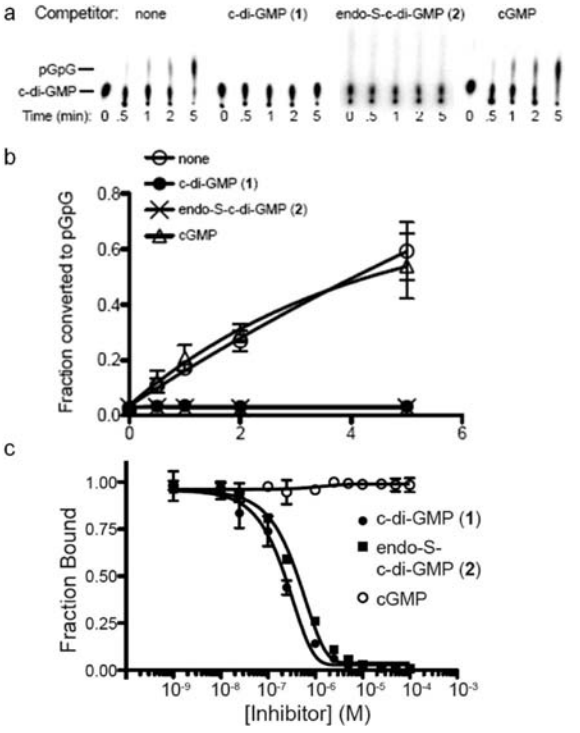

Figure 9. Inhibition of c-di-GMP hydrolysis by RocR with guanine-containing nucleotides. (a) TLC of reaction mixture at different time points. Initial reaction conditions: RocR, radio-labeled c-di-GMP, and unlabeled c-di-GMP/endo-S-c-di-GMP/cGMP or no added nucleotide inhibitor. (b) Graph showing fraction of radio-labeled c-di-GMP that was converted into radio-labeled pGpG in the presence of competitor nucleotide. (c) RocR binding to radiolabeled c-di-GMP in the presence of indicated concentrations of unlabeled c-di-GMP, endo-S-c-di-GMP, or cGMP competitors in the presence of Ca^{2+}.

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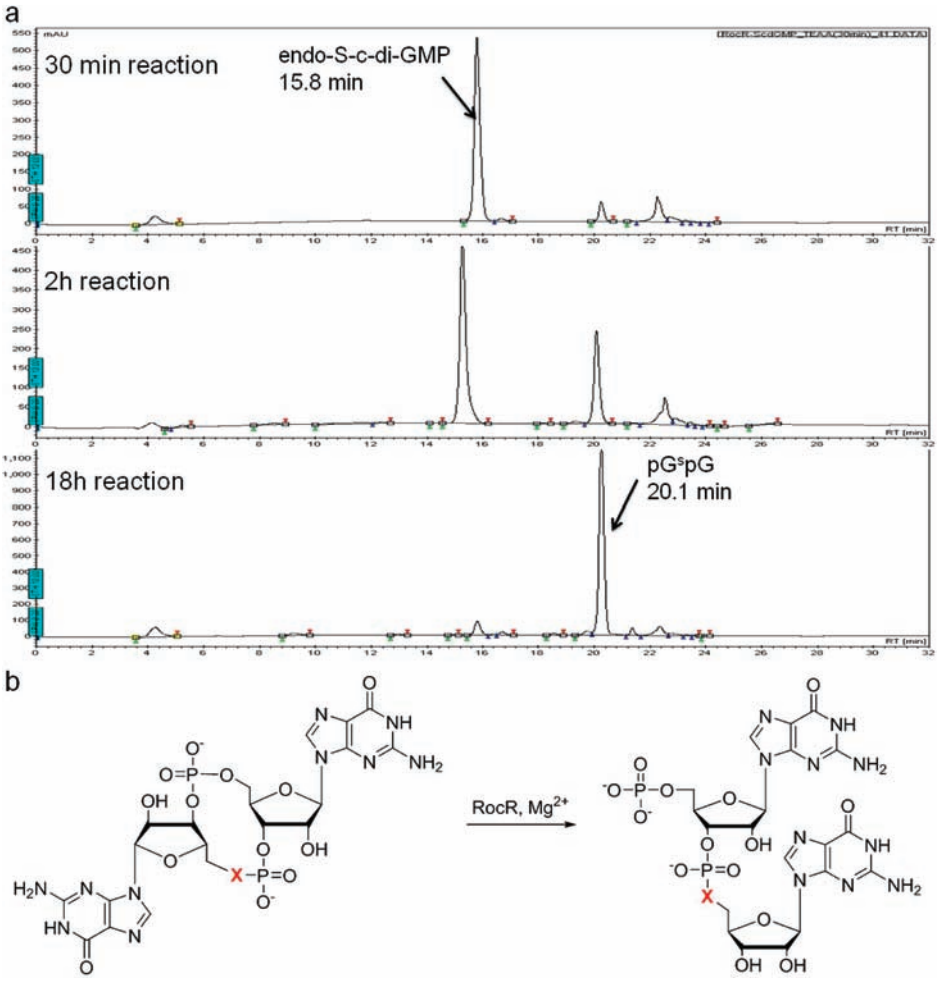

Figure 10. (a) HPLC analysis of endo-S-c-di-GMP after enzymatic cleavage by RocR phosphodiesterase. (b) Scheme for RocR cleavage products of c-di-GMP and endo-S-c-di-GMP (see Supporting Information for product characterization).

binding of c-di-GMP analogues to metabolism or "adaptor" proteins via conformational biasing (in other words, conformational steering)? To address these questions, we took advantage of the higher propensity of endo-S-c-di-GMP to exist in the open conformer (as compared to c-di-GMP) and also the small perturbation of the negative charge on the phosphate moiety, when the bridging oxygen is changed to a sulfur atom, to compare binding to each class of c-di-GMP proteins. We investigated the biological profile of endo-S-c-di-GMP binding toward well-characterized c-di-GMP interacting proteins from *P. aeruginosa*, including WspR (a DGC),[15] RocR (a PDE),[43] and PilZ-domain-containing protein Alg44.[14,44] WspR has been shown to be a potent DGC that promotes biofilm formation via synthesis of c-di-GMP. As with a number of DGCs, WspR has an I-site that binds c-di-GMP on the face opposite the catalytic site to inhibit the diguanylate cyclase activity.[16,41] RocR is a potent PDE that has been shown to inhibit the expression of chaperone-usher pili that participate in biofilm formation.[45] Alg44 is a gene encoded in the *alg* operon that binds c-di-GMP, and this binding is required for the production of the alginate polysaccharide. The interaction of Alg44 with c-di-GMP is well studied through isothermal calorimetry and filter binding assays.[14] To assess the effect of endo-S-c-di-GMP, the compound was tested for its ability to inhibit the DGC activity of WspR, to compete for cleavage by the PDE activity of RocR, and to compete for binding to Alg44 by thin-layer chromatography (TLC) or filter binding assay. The effect of endo-S-c-di-GMP was compared to those of c-di-GMP, which interacts with all three proteins, and cGMP, which does not. In the presence of endo-S-c-di-GMP (1 mM), WspR (5 μM) converted 70% of GTP (8 nM) into c-di-GMP, whereas 1 mM c-di-GMP was able to bind the I-site and inhibit WspR from converting GTP into c-di-GMP (see Figure 7), indicating that endo-S-c-di-GMP does not alter the DGC activity of WspR. Similarly, displacement of radio-labeled c-di-GMP from Alg44 required greater than 10-fold concentrations of endo-S-c-di-GMP as compared to c-di-GMP (see Figure 8). Endo-S-c-di-GMP did, however, bind to RocR, evident by the inhibition of RocR cleavage of radio-labeled c-di-GMP in the presence of endo-S-c-di-GMP (Figure 9a,b). Since endo-S-c-di-GMP was almost as effective as unlabeled c-di-GMP at inhibiting RocR cleavage of radio-labeled c-di-GMP, we tested the ability of endo-S-c-di-GMP to compete with radio-labeled c-di-GMP for RocR active site. In the presence of Ca^{2+}, PDEs do not cleave c-di-GMP.[1] Therefore, to determine the binding affinities of c-di-GMP/endo-S-c-di-GMP for RocR, we added Ca^{2+} (5 mM) to the binding buffer. Unlabeled c-di-GMP could compete with radio-labeled c-di-GMP (5 nM) binding to RocR (5 μM) with an IC_{50} of 236 nM, whereas endo-S-c-di-GMP competed with radio-labeled c-di-GMP with an IC_{50} of 431 nM (Figure 9c).

Because endo-S-c-di-GMP blocked RocR activity, we wondered if endo-S-c-di-GMP is itself cleaved by the phosphodiesterase enzyme. This was tested by exposing endo-S-c-di-GMP to RocR. At various times of enzymatic reaction, aliquots were taken and analyzed by HPLC (Figure 10a). This HPLC analysis showed that the endo-S-c-di-GMP molecule was linearized by RocR. The cleavage site of endo-S-c-di-GMP was at the natural phosphodiester but not at the endo-S-phosphorothioate site (Figure 10b; also see Supporting Information).

The studies presented herein reveal that proteins that bind to c-di-GMP utilize the phosphate moiety of c-di-GMP as an important recognition element. The crystal structure of c-di-GMP bound to the EAL domain of FimX shows the dinucleotide bound in the "open" conformer (Figure 6c,d), whereas c-di-GMP is bound to most DGCs and PilZ proteins in the "closed" conformer (Figure 6a,b). RocR has been crystallized, but its structure has not been solved.[46] However, Rao et al. have computed the structure of RocR and shown that the computed structure binds to monomeric c-di-GMP which is in the "open" conformer.[47] Interestingly, endo-S-c-di-GMP (**2**) (which has a lower propensity to form a "closed" conformer) could only inhibit an EAL-containing protein, RocR, and not DGC- or PilZ-containing proteins (WspR and Alg44, respectively).

CONCLUSION

Synthetic cyclic nucleotides that are resistant to hydrolysis and inhibit key proteins have the potential to be used to inhibit proteins that are important for the onset of the diseased state.[48] C-di-GMP analogues that are hydrolytically stable and able to selectively inhibit c-di-GMP metabolism or "adaptor" proteins will become useful tools for modulating c-di-GMP signaling in bacteria. In this work, we reveal that a conservative modification of one of the phosphate moieties in c-di-GMP (by replacing only one of the "bridging" oxygens in the phosphate linkages in c-di-GMP with sulfur) gives an analogue, endo-S-c-di-GMP, which is remarkably different (biophysically and biochemically) from c-di-GMP. This suggests that the phosphate moieties in c-di-GMP play important roles in aggregate formation as well as the binding of c-di-GMP to metabolism and processing proteins. The crystal structures of the majority of c-di-GMP binding proteins (DGC, PDE, and "adaptor" proteins) reveal that the residues in these proteins make extensive interactions with both the guanine and phosphate moieties in c-di-GMP. We show that one can still discriminate between these proteins by taking advantage of the fact that the spatial orientations of c-di-GMP is distinguished by the different classes of c-di-GMP binding proteins. By modifying c-di-GMP with moieties that facilitate the formation of the open conformer, we achieved the selective inhibition of PDE (which binds to the open conformer of c-di-GMP) but not PilZ or DGCs (which bind to the closed conformer of c-di-GMP). So far, a general paradigm for the design of small molecules that can target only one class of c-di-GMP binding proteins has not emerged. This work provides a good starting point for the design of small-molecule inhibitors that act only on specific proteins in the c-di-GMP pathway. Future studies may also reveal selective inhibition of the proteins that bind the closed form of c-di-GMP.

EXPERIMENTAL SECTION

Synthesis. See Supporting Information for the details of the synthesis of endo-S-c-di-GMP. For the synthesis of c-di-GMP, see ref 49.

Sample Preparation for Spectrometric Measurements. c-di-GMP or endo-S-c-di-GMP, water, buffer solution (pH 7.5), and metal solution were mixed, heated at 95 °C for 5 min, cooled to room temperature for 15 min, and then incubated in a refrigerator (4 °C) for 12 h.

Optical Measurements. NMRs were measured on a Bruker 600 MHz spectrometer, absorbance spectra were obtained on a JASCO V-630 spectrophotometer with a 1 cm path length cuvette, and CD experiments were performed on a JASCO J-81 spectropolarimeter with a 1 cm path length cuvette. The concentrations of stock solutions of c-di-GMP and endo-S-c-di-GMP were determined by measuring absorbance at 260 nm for c-di-GMP and endo-S-c-di-GMP and using 21 600 M^{-1} cm^{-1} as molar extinction coefficient for both compounds.

Enzymatic Assays. Alg44, RocR, and WspR were purified by histidine chromatography and then passed through a Q-sepharose anion-exchange column. Proteins were dialyzed into a 10 mM Tris, 100 mM NaCl solution. α-^{32}P-c-di-GMP was generated from purified WspR.[50] For binding assay of Alg44, 5 μM protein was mixed with 4 nM α-^{32}P-c-di-GMP and indicated competitor (c-di-GMP or endo-S-c-di-GMP) in a buffer containing 10 mM Tris (pH 8.0), 100 mM KCl, and 5 mM $MgCl_2$. This mixture was allowed to equilibrate for 10 min. The c-di-GMP binding assay was analyzed via filter binding assay using nitrocellulose membranes.[50] For RocR and WspR enzymatic assays, 5 μM enzyme was added to 4 nM α-^{32}P-c-di-GMP (for RocR) or 4 nM α-^{32}P-GTP (for WspR) and indicated competitor (500 μM for endo-S-c-di-GMP, 1 mM for cGMP, and 1 mM for c-di-GMP) in the reaction buffer, containing 10 mM Tris (pH 8.0), 100 mM NaCl, and 5 mM $CaCl_2$ (for RocR binding assay) or 5 mM $MgCl_2$ (for WspR or RocR cleavage assay). One microliter of sample was spotted on polyethyleneimine cellulose TLC plates at indicated times after addition of the enzyme. Samples were dried and separated using a mobile phase consisting of 1:1.5 of saturated $(NH_4)_2SO_4$ and 1.5 M KH_2PO_4. TLC plates were dried, exposed on a phosphorimager screen, and visualized with a Fujifilm FLA-7000 instrument.

ASSOCIATED CONTENT

Supporting Information. Complete ref 19, synthetic methods, NMR spectra of endo-S-c-di-GMP, and computational details. This material is available free of charge via the Internet at http://pubs.acs.org.

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