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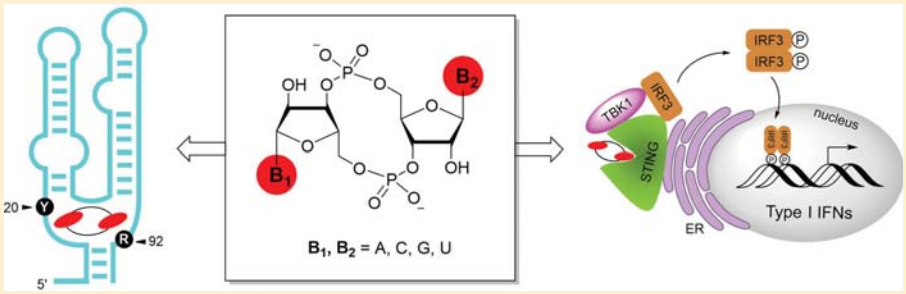
Synthesis of All Possible Canonical (3′−5′-Linked) Cyclic Dinucleotides and Evaluation of Riboswitch Interactions and Immune-Stimulatory Effects

Changhao Wang,[†,‡] Malte Sinn,[†,§] Julia Stifel,[†,§] Anna C. Heiler,[†,§] Annette Sommershof,[∥] and Jörg S. Hartig*[,†,§]

[†]Department of Chemistry, University of Konstanz, Konstanz 78457, Germany
[‡]Key Laboratory of Applied Surface and Colloid Chemistry, Ministry of Education, School of Chemistry and Chemical Engineering, Shaanxi Normal University, Xi'an 710119, China
[§]Konstanz Research School Chemical Biology (KoRS-CB), University of Konstanz, Konstanz 78457, Germany
[∥]Department of Biology, University of Konstanz, Konstanz 78457, Germany

Supporting Information



ABSTRACT: The cyclic dinucleotides (CDNs) c-di-GMP, c-di-AMP, and c-AMP-GMP are widely utilized as second messengers in bacteria, where they signal lifestyle changes such as motility and biofilm formation, cell wall and membrane homeostasis, virulence, and exo-electrogenesis. For all known bacterial CDNs, specific riboswitches have been identified that alter gene expression in response to the second messengers. In addition, bacterial CDNs trigger potent immune responses, making them attractive as adjuvants in immune therapies. Besides the three naturally occurring CDNs, seven further CDNs containing canonical 3′−5′-linkages are possible by combining the four natural ribonucleotides. Herein, we have synthesized all ten possible combinations of 3′−5′-linked CDNs. The binding affinity of novel CDNs and GEMM riboswitch variants was assessed utilizing a spinach aptamer fluorescence assay and in-line probing assays. The immune-stimulatory effect of CDNs was evaluated by induction of type I interferons (IFNs), and a novel CDN c-AMP-CMP was identified as a new immune-stimulatory agent.

INTRODUCTION

Cyclic dinucleotides (CDNs) have gained increasing interest in the past few years.[1] In bacteria, 3′−5′-linked CDNs were found to play key roles in intracellular signaling pathways.[2] Furthermore, the innate immune response in mammalian cells is triggered by CDNs.[3] About 30 years ago, Benziman and co-workers identified c-di-GMP (Figure 1) when studying the regulation of cellulose biosynthesis in *Gluconacetobacter xylinus*.[4] Since then, c-di-GMP was found to be involved in controlling motility,[5] the transition between motility and sessility,[6] biofilm formation,[7] and virulence.[8]

More recently, the second CDN, c-di-AMP (Figure 1), has been discovered. C-di-AMP regulates bacterial sporulation, reports DNA damage,[9] and is linked to the synthesis of fatty acids,[10] antibiotic resistance,[11] and cell wall homeostasis.[12] In contrast, the third CDN, c-AMP-GMP (Figure 1), was discovered even more recently by finding that it promotes the intestinal colonization of the human host in *Vibrio cholerae*.[13] In addition, c-AMP-GMP has been demonstrated to regulate exo-electrogenesis in certain *Geobacter* species.[14,15]

For all known bacterial CDNs, riboswitches have been discovered. Riboswitches are *cis*-regulatory RNAs that bind to small-molecule ligands. They fold into complex tertiary structures and control transcription and translation of associated genes in response to their specific ligand. They are typically located in 5′-UTRs (5′-untranslated regions) of mRNA and usually consist of a sensor domain (termed aptamer) and an expression platform.[16] Upon binding of the ligand, the aptamer undergoes a conformational change that induces a rearrangement of the expression platform, resulting in altered gene expression.[17] Two classes of riboswitches responding to c-di-GMP are known.[18,19] They are also termed GEMM riboswitches because they control genes related to

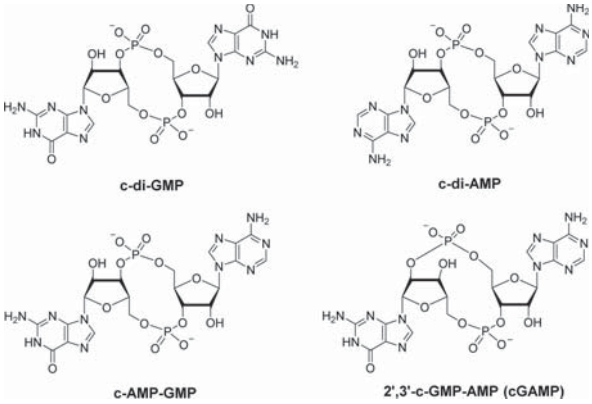


Figure 1. Structures of known naturally occurring CDNs.

environment, membranes, and motility. GEMM riboswitches also regulate genes associated with the synthesis and degradation of c-di-GMP (diguanylate cyclases and phosphodiesterases).[18,20] GEMM class I and class II riboswitches both have very high affinities for c-di-GMP, ranging from 10 pM for class I riboswitches[20] to 2 nM for class II riboswitches,[19] but recognize their ligand through distinct mechanisms.[19,21]

Three helices forming a y-shaped GEMM-I aptamer are responsible for ligand binding at the central three-way junction. Each of the two guanine bases is recognized predominantly by two nucleotides, G20 and C92.[20,22] The c-di-GMP G_α nucleotide forms hydrogen bonds to the Hoogsteen face of G20 of the aptamer, whereas the second guanine G_β in c-di-GMP is contacted by Watson−Crick base pairing to C92 (Figure S1).[20−22] Besides hydrogen bonds, the ligand is involved in base stacking interactions that bridge the P1 and P2 helices, and the highly conserved base A47 further stabilizes the binding by intercalating between the two guanine bases of c-di-GMP.[20,23] It was shown that the mutation of G20 to an adenine among other characteristic changes in the GEMM-I aptamer leads to specific binding of c-AMP-GMP.[24] Bioinformatics analyses revealed that this G20A mutation occurs in natural riboswitch sequences. Subsequent experiments showed that riboswitches with a high selectivity for c-AMP-GMP exist in *Geobacter* species, where they regulate expression of genes associated with exo-electrogenesis. In contrast to these highly discriminatory riboswitches found in *Geobacter*, the majority of tested aptamers that carry the G20A mutation bind both c-di-GMP and c-AMP-GMP with similar affinities.[14,15]

A bioinformatically conducted search for GEMM-I riboswitches revealed several sequence variations that differ at other positions than the described G20A mutations. Some sequences carry a G20Y mutation (with Y being C or U), and others show sequence variations at C92. Breaker and co-workers have speculated that these sequences could bind other CDNs than the three known bacterial second messengers.[25] Considering the four natural ribonucleotides and the three known CDNs, seven more CDNs containing canonical 3′−5′-linkages are possible (Figure 2). Herein, we have chemically synthesized all ten CDNs and assayed binding toward all combinations of GEMM-I riboswitches with variations at positions G20 and C92.

In addition to the function of c-di-GMP, c-di-AMP, and c-AMP-GMP as signaling molecules in bacteria, an isomer of c-AMP-GMP containing a 2′−5′-linkage (2′,3′-c-GMP-AMP or cGAMP, Figure 1) triggers immune responses in mammalian

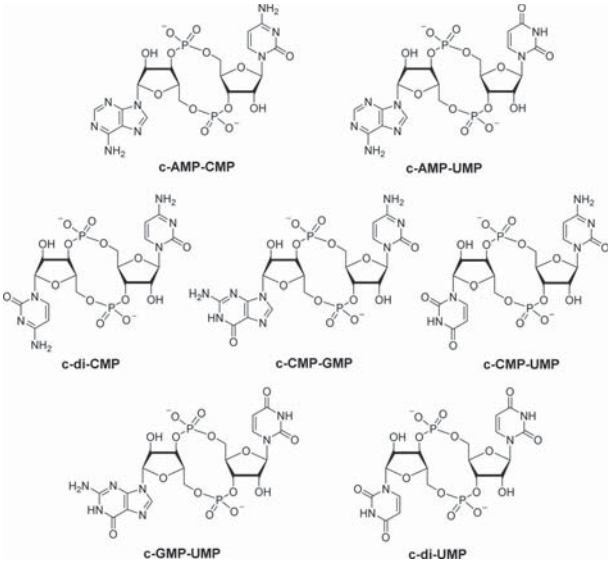


Figure 2. Structures of CDNs so far unknown in nature.

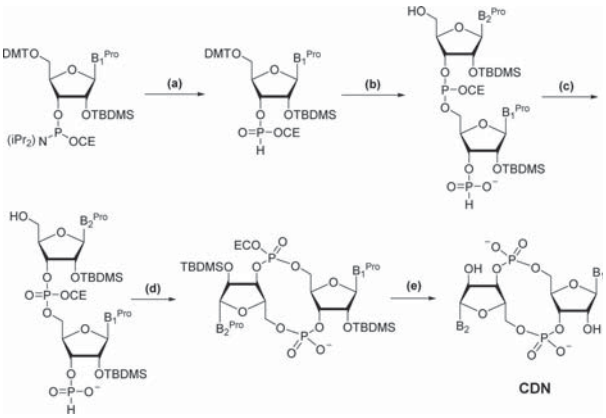
cells.[26] There, invasion of a pathogen can be sensed by recognition of cytosolic DNA via the cyclic GMP-AMP synthase (cGAS) that is cyclizing ATP and GTP to produce cGAMP.[26−28] cGAMP acts as second messenger that triggers the stimulator of interferon genes (STING) pathway, resulting in the production of type I interferons (IFNs). Importantly, all known bacterial CDNs also stimulate interferon production via binding to STING. These very pronounced stimulatory effects have made the CDNs very attractive as adjuvants in vaccination strategies.[29] This interesting application has prompted our interest in the immune-stimulatory potential of the novel CDNs introduced in this work.

RESULTS AND DISCUSSION

Synthesis and Characterization of CDNs. Starting from the commercially available RNA phosphoramidites, we have synthesized all ten canonical CDNs bearing 3′−5′-linkages adopted from a one-pot strategy for the synthesis of c-di-GMP.[30] The general synthesis route is shown in Scheme 1.

The RNA phosphoramidite was first hydrolyzed by pyridinium trifluoroacetate (TFA-pyridine) and water in acetonitrile, and the cyanoethyl group was deprotected with *tert*-butylamine (*t*-$BuNH_2$) followed by the detritylation using dichloroacetic acid (DCA) and water in dichloromethane (step a). Then the solution was quenched and concentrated prior to changing the solvent to dry acetonitrile, followed by coupling with the second RNA phosphoramidite (step b). After an instant oxidation by *tert*-butylhydroperoxide (*t*-BuOOH) and the second detritylation by DCA, a linear dimer was obtained (step c). The cyclization was promoted by 2-chloro-5,5-dimethyl-1,3,2-dioxaphosphorinane-2-oxide (DMOCP), yielding the protected CDN after oxidation by iodine (step d). After deprotection of the cyanoethyl group by *t*-$BuNH_2$, the nucleobase protection groups by methylamine, and the *tert*-butyldimethylsilyl (TBDMS) groups by triethylamine (TEA)-HF, the crude CDN was obtained as a TEA salt (step e). The previously described procedure was modified regarding step d when performing crystallization: fully protected CDN was precipitated instantly by adding diethyl ether instead of

Scheme 1. Representative Synthesis Route for CDN[a]



[a](a) TFA-pyridine, H_2O, CH_3CN; *t*-$BuNH_2$; DCA in CH_2Cl_2, H_2O; (b) B_2^{pro}-RNA phosphoramidite in pyridine; (c) *t*-BuOOH; DCA in CH_2Cl_2, H_2O; (d) DMOCP; I_2, H_2O; (e) *t*-$BuNH_2$; $MeNH_2$; TEA·HF. Abbreviations: DMT, 4,4′-dimethoxytrityl; TBDMS, *tert*-butyldimethylsilyl; CE, 2-cyanoethyl; B_1^{pro} and B_2^{pro}, protected RNA nucleobases A, C, G, or U.

crystallization in dichloromethane overnight, enabling the completion of CDN synthesis within one day.

Apart from the naturally occurring CDNs c-di-GMP, c-di-AMP, and c-AMP-GMP, the synthesis of c-di-CMP, c-GMP-UMP, and c-di-UMP has been mentioned in the literature but lacks analytical characterizations.[31,32] To the best of our knowledge, c-AMP-CMP, c-AMP-UMP, c-CMP-GMP, and c-CMP-UMP were synthesized for the first time. In order to fully characterize these novel CDNs, one- and two-dimensional NMR and high-resolution mass spectrometry (HRMS) were employed. Protons and carbons in CDNs were assigned based on the integrated analysis of ^{1}H NMR, ^{13}C NMR, ^{1}H−^{1}H correlation spectroscopy (COSY), ^{1}H−^{13}C heteronuclear single quantum correlation (HSQC), and ^{1}H−^{13}C heteronuclear multiple-bond correlation (HMBC). Phosphorus in CDNs possessing two different RNA bases was assigned by applying ^{1}H NMR, ^{31}P NMR, and ^{1}H−^{31}P HMBC; see the Supporting Information for details.

We utilized the CDNs in order to detect novel signaling compounds in bacterial extracts. For this purpose, CDNs were co-injected as standards together with small-molecule extracts from 12 diverse bacteria in an HPLC-MS setup; see the Supporting Information for details. However, we did not detect novel CDNs in the tested bacterial extracts (data not shown). Since c-di-GMP could form stable G-quadruplex structures under certain conditions, circular dichroism (CD) was employed to investigate whether the novel CDNs form unusual conformations or higher structural assemblies. In the presence of K^+, we found CD signatures typical of quadruplex formation for c-di-GMP (a strong positive peak at 215 nm and a weak positive peak at 309 nm) in accordance with published reports,[33] whereas the presence of Li^+, Na^+, Rb^+, NH_4^+, and Mg^{2+} did not support quadruplex formation (Figure S2). CD spectra of the other CDNs displayed different CD profiles as compared to that of c-di-GMP in the presence of K^+ (Figure S3). Some CDNs such as c-CMP-UMP displayed significant signals in the CD spectra that could result from higher order structures (Figure S3). As cytosine-rich oligonucleotides are able to form *i*-motif structures especially under acidic conditions, we decreased the pH and observed induction of additional structure for c-di-CMP that could result from a mirrored *i*-motif with a positive peak at 268 nm and a negative peak at 290 nm (Figure S4).[34] However, ^{1}H NMR of c-di-CMP did not detect imino signals between 15 and 16 ppm that are typical for the formation of C−C$^+$ base pairs (Figure S5).[35,36] Taken together, the novel CDNs do not show pronounced structure formation at neutral pH. The formation of a higher order structure is observed for c-di-CMP at acidic conditions.

GEMM Riboswitch Binding Assays. Breaker and co-workers have speculated before that certain GEMM riboswitch variants that are not able to bind to either one of the three known CDNs could bind to a so-far unknown CDN.[14] In order to test whether GEMM class I riboswitch variants bind to novel CDNs, we characterized binding interactions of the CDNs and GEMM-I riboswitches that comprise mutations at position G20 and/or position C92. Representatives for all prevalent mutations at positions G20 and C92 were chosen by examining published sequence variations;[14,15] see Table 1 and Figure S6.

Table 1. GEMM Riboswitches Tested for CDN Binding

abbreviation	species	G20 variant	C92 variant	binding specificity[a]
Ece-1-1	*Eubacterium cellulosolvens*	G20G	C92C	c-di-GMP
Env-13	*Clostridia Environmental Sample 13*		C92(C)A	NB
Awo-1-3	*Acetobacterium woodii*		C92(U)A	NB
Nca-1-1	*Neptuniibacter caesariensis*		C92(C)U	NB
Gs1945b	*Geobacter sulfurreducens*		C92G	c-AMP-GMP
Gm0292	*Geobacter metallireducens*	G20A	C92C	c-AMP-GMP
Bce-8−1	*Bacillus cereus*		C92G	NB
Car-1-1	*Clostridium arbusti*		C92U	NB
Cbo-1-1	*Clostridium bolteae*		C92(C)A	NB
Toc-1-1	*Thermosediminibacter oceani*	G20U	C92C	NB
Rso-1-1	*Ralstonia solanacearum*		C92U	NB
Cbu-1-1	*Clostridium butyricum*	G20C	C92C	NB

[a]NB (no binding detected): Neither enhanced fluorescence in spinach aptamer assay nor modulated cleavage pattern in in-line probing detected. The sequences of Gs1945b and Gm0292 are taken from ref 15 and the remaining sequences are from ref 14; see Figure S6 for details. Nucleotides in parentheses: additional nucleotides that are found in the vicinity of position 92 compared to the canonical sequence.

In order to evaluate binding affinities, we performed two different assays. First, riboswitches of interest were coupled via their P1 stem directly to a spinach aptamer as described before to obtain a fusion RNA that enables detection of ligand binding by an increase in fluorescence.[24] Upon binding of CDNs to the riboswitch, the structural rearrangements are transduced to the spinach aptamer, which is stabilized and enabled to bind to 3,5-difluoro-4-hydroxybenzylidene imidazolinone (DFHBI), which then fluoresces; see Figure 3A. Second, in-line probing assays were carried out that are frequently used in order to characterize riboswitch binding affinity and selectivity.[37]

First, we examined whether fusions of GEMM-I sequences and spinach aptamers detect binding of CDNs to RNAs. Of the analyzed riboswitches, only three were found to bind either c-di-GMP or c-AMP-GMP (Figure 3B and Figure S7). Binding

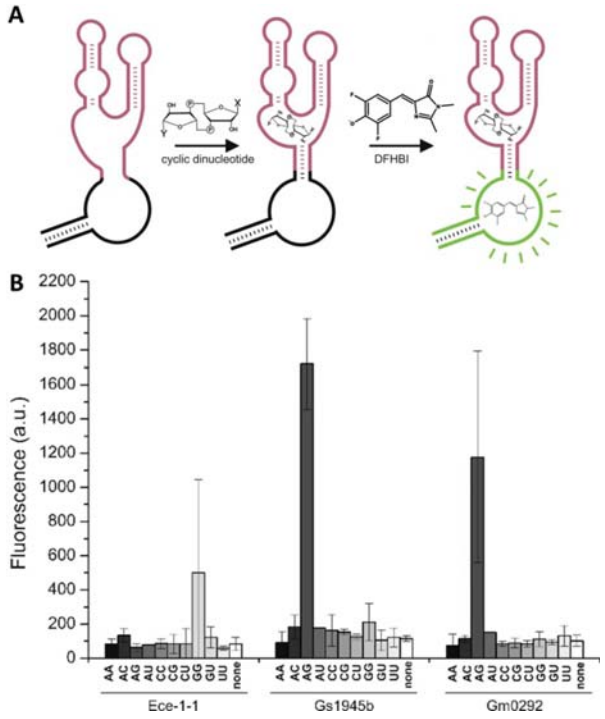


Figure 3. CDN binding of GEMM riboswitches assessed utilizing spinach aptamer fusion constructs. (A) Schematic representation for CDN sensing GEMM riboswitch (red) that induces the spinach aptamer (black) to bind DFHBI and generate fluorescence. (B) GEMM-I riboswitch variants (Ece-1-1, Gs1945b, and Gm0292) were fused to spinach aptamers, and fluorescence was measured after incubation of the RNAs with 100 μM CDN ("c-di-AMP" denoted as "AA", c-AMP-CMP denoted as "AC", and so on) and 10 μM fluorescent spinach ligand DFHBI. Errors represent standard deviation of three independent experiments.

could be observed for c-AMP-GMP to Gm0292 (G20A, C92C) from *Geobacter metallireducens* and Gs1945b (G20G, C92G) from *Geobacter sulfurreducens* and c-di-GMP to Ece-1-1 (G20G, C92C) from *Eubacterium cellulosolvens*. In contrast to the report of Kellenberger et al., who did not observe binding for Gm0292, we elongated the riboswitch sequence in a way that the P1 stem is stabilized and the P1 internal loop is formed (Figure S8), which is an important feature of c-AMP-GMP binding GEMM-I variants.[15] Apparently, these changes stabilized the fold of the riboswitch.

In order to confirm our results and to determine binding affinities, in-line probing assays were performed. In a first screen, all ten CDNs were tested at 10 μM concentration. Binding of the same CDNs to the three already identified riboswitches was confirmed by the spinach fusion assay. For Gm0292 we observed binding of c-AMP-GMP, c-CMP-GMP, c-di-GMP, and c-GMP-UMP (Figure S9A). Apparent K_d values were determined for these four CDNs to evaluate the binding specificity (Figure 4A). The results demonstrate that the Gm0292 riboswitch sequence discriminates c-AMP-GMP from c-di-GMP approximately 1000-fold, whereas it discriminates against c-CMP-GMP and c-GMP-UMP about 100-fold.

Preferential binding of c-AMP-GMP is in accordance with the results obtained with the spinach fusion assay. This finding could be explained by *Geobacter metallireducens* making use of both c-di-GMP and c-AMP-GMP as second messengers. Hence, discrimination between both related compounds is highly advantageous, whereas strong discrimination against c-CMP-GMP and c-GMP-UMP is apparently not that necessary. Structural discrimination of the cognate ligand occurs through base pairing and stacking interactions with the riboswitch. Gm0292 exhibits a canonical sequence of the P2 stem, which is directly involved in ligand binding.[38] This feature explains why Gm0292 is a selective c-AMP-GMP riboswitch rather than a promiscuous one. Although in the in-line probing assay c-CMP-GMP and c-GMP-UMP bind with nanomolar dissociation constants, both compounds do not show effects in the spinach assay. A possible reason for this lack of functional effects could be a discrepancy between binding and triggering the riboswitch which has been observed before, e.g., for the tetrahydrofolate (THF) riboswitch.[39] This finding highlights the benefits of utilizing two independent methods for assaying molecular interactions.

The sequence Ece-1-1 also binds both c-AMP-GMP and c-di-GMP at 10 μM in the in-line probing assay (Figure S9B). Concentration-dependent investigation showed that the *Eubacterium cellulosolvens* GEMM-I aptamer is able to discriminate between c-di-GMP and c-AMP-GMP 30-fold (Figure 4B). However, the affinity for c-di-GMP is rather low (~30 nM) compared to already described GEMM-I riboswitches (1 nM).[18] Eubacteria are also known to use c-di-AMP.[25] It could be speculated that a strong discrimination between c-di-GMP and c-di-AMP evolved, but since c-AMP-GMP is not utilized by these bacteria, no specific discrimination is necessary.

In addition to investigating CDN interactions with described GEMM-I variants, we decided to characterize binding of all ten CDNs to a representative of a GEMM class II riboswitch aptamer. The GEMM-II aptamers also display frequent variants that in most cases have not been assayed for interactions with c-di-GMP or related compounds. We chose a representative from the GEMM-II GAG subgroup identified bioinformatically in *Deinococcus peraridilitoris*.[40] The GAG subgroup is the most abundant of the so far uncharacterized groups of the GEMM-II riboswitches. The examined sequence derived from *Deinococcus peraridilitoris* is shown in Figure 4C. At 10 μM CDN concentration binding could be observed for c-AMP-GMP, c-di-GMP, and c-GMP-UMP (Figure S9C). A concentration-dependent investigation showed high-affinity binding for c-di-GMP. However, the discrimination is only 3- and 6-fold compared to c-AMP-GMP and c-GMP-UMP, respectively (Figure 4C).

Previously, c-di-GMP has been shown to form stable quadruplex structures.[41] Quadruplexes are strongly stabilized by potassium ions, while the buffers utilized for in-line probing and the spinach aptamer fusion assays contain high amounts of potassium ions. In order to exclude the possibility that binding of c-di-GMP was underestimated due to the formation of G-quadruplexes by c-di-GMP, in-line probing assays were repeated in the presence of Na^+ instead of K^+ (Figure S10). The nature of the ion did not significantly influence binding affinities.

To conclude the riboswitch binding studies, the majority of sequences tested do not bind to one or more of the ten canonical CDNs. These sequences could represent degenerated motifs that are no longer able to recognize their cognate ligand. However, since some sequence variations occur at relatively high frequencies, it could also be possible that the respective ligand for these sequences is still unknown. In this case, it

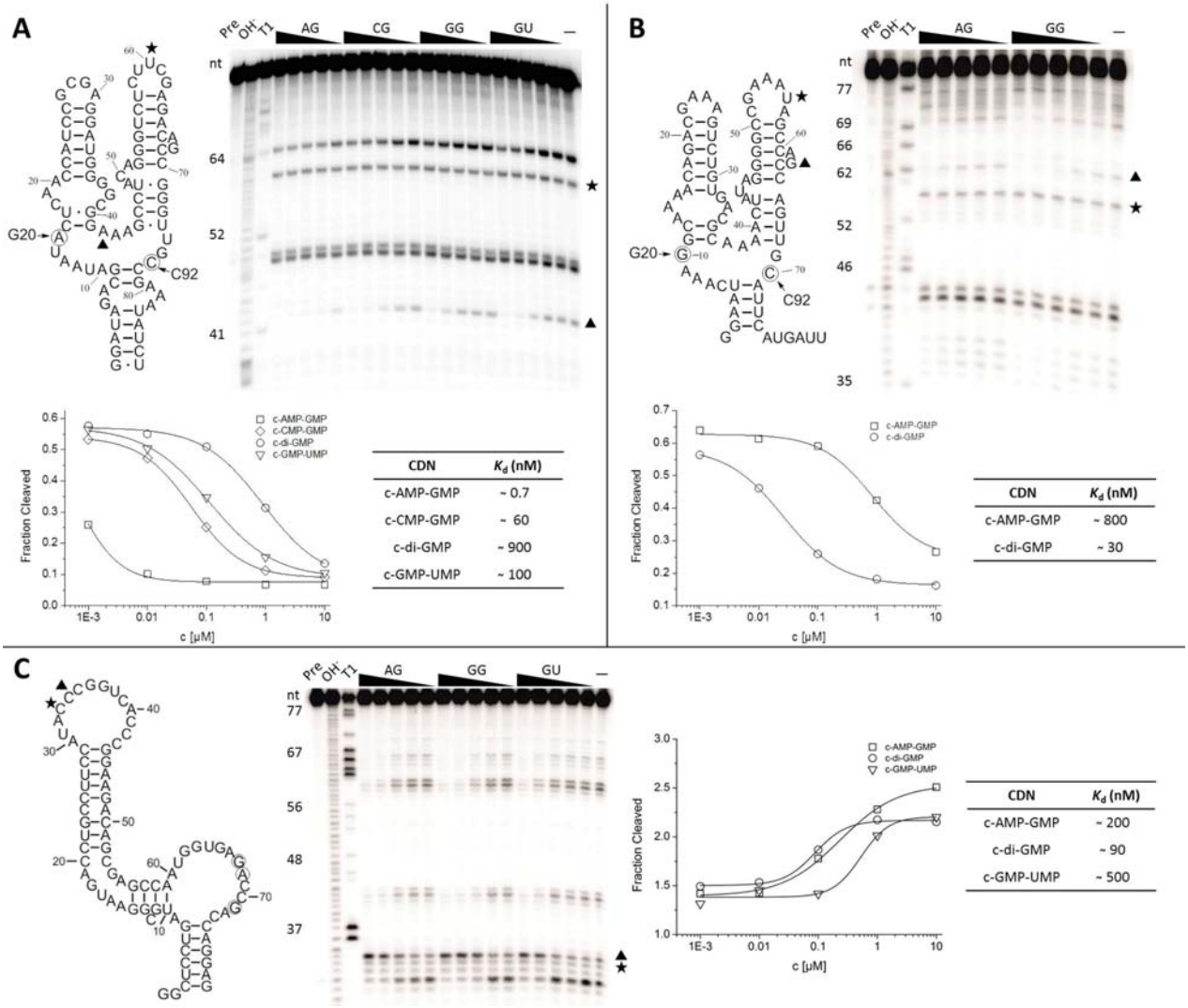


Figure 4. Determination of binding affinity by in-line probing assay. (A) Results for Gm0292: In-line probing was conducted in the presence of c-AMP-GMP (AG, □), c-CMP-GMP (CG, ◇), c-di-GMP (GG, ○), and c-GMP-UMP (GU, ▽). (B) Results for Ece-1-1: In-line probing was conducted in the presence of c-AMP-GMP (AG, □) and c-di-GMP (GG, ○). (C) Results for a representative of a GEMM-II riboswitch (GAG-group): In-line probing was conducted in the presence of c-AMP-GMP (AG, □), c-di-GMP (GG, ○), and c-GMP-UMP (GU, ▽). In-line probing, secondary structure of the probed motifs and a plot of the modulated band (▲) intensity normalized to intensity of an unchanged band (★) against ligand concentration are displayed. In-line probing was conducted in the absence (−) or presence of 10 μM, 1 μM, 100 nM, 10 nM, and 1 nM CDN, respectively. As controls precursor RNA (Pre), alkaline (OH^-), and RNase T1 digestion (T1) of the respective RNA are shown. Several G residues of the RNase T1 reaction are indicated. Positions G20 and C92 used to select riboswitch variants are marked in the structure together with the cleavage sites used for the determination of the apparent K_d. For additional data and a discussion regarding the reproducibility of the in-line probing experiments see the Supporting Information.

seems likely that the cognate ligand is structurally related to a cyclic dinucleotide.

Immune-Stimulatory Effects. The three known bacterial CDNs elicit strong immune-stimulatory effects and hence are characterized as promising adjuvants in immunotherapeutic strategies.[42−44] To investigate the type I IFN-stimulatory potency of the novel CDNs, we used RAW264.7-derived interferon regulatory factor (IRF) reporter cells and examined type I IFN induction after cytoplasmic delivery of the respective CDNs. In line with previous reports, cytosolic delivery of c-di-AMP, c-AMP-GMP, and c-di-GMP induced robust type I IFN responses in RAW 264.7 macrophage IRF reporter cells after transfection (Figure 5). Transfection of RAW cells with c-AMP-CMP also induced significant levels of type I IFN responses compared to nontransfected cells, although to a much smaller extent compared to the known CDNs. In contrast, c-AMP- UMP, c-di-CMP, c-CMP-GMP, c-CMP-UMP, and c-GMP-UMP failed to induce type I IFN production in RAW IRF reporter cells.

The STING pathway is reported as the mechanism by which CDNs activate type I interferon responses.[45] To evaluate the contribution of STING to the c-AMP-CMP-mediated IFN-β response, we further transfected RAW 264.7 IRF reporter cells lacking STING (STING-KO) with the respective CDNs. As shown in Figure 5, ablation of STING completely abolished type I IFN production in response to CDN transfection,

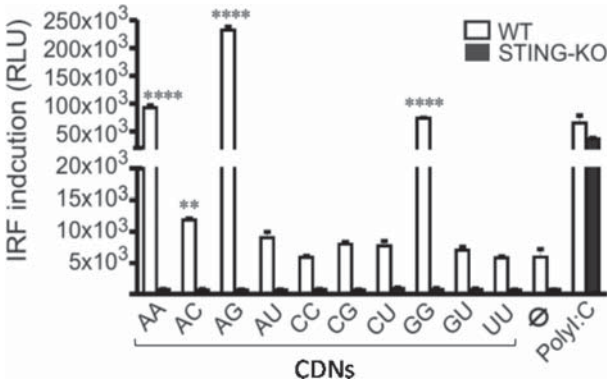


Figure 5. Type I IFN induction after cytoplasmic delivery of the respective CDNs. CDN ("c-di-AMP" denoted as "AA", c-AMP-CMP denoted as "AC", and so on) and a TLR3 ligand (poly I:C) induced type I IFN responses were measured in mouse RAW264.7-derived IRF reporter cells via STING. RAW-Lucia ISG and RAW-Lucia ISG-KO-STING cells were transfected with 3.3 μg/mL of the indicated CDN. IRF pathway activation was measured by luciferase reporter assay 18 h after stimulation. Luminescence data are mean relative light units (RLU) ± SEM of at least three experiments (n = 3). Significant differences were calculated between cells transfected with CDN and cells treated with FuGENE HD transfection reagent. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001 by Student's t test (two tailed).

whereas the RNA ligand poly I:C that stimulates type I IFN through STING-independent pathways induced significant IFN activation also in STING-KO cells. Collectively, our results describe c-AMP-CMP as a weak immune stimulator that induces a significant but weaker type I IFN response in murine macrophages via the same signaling axis as the already described CDNs.

CONCLUSIONS

We have synthesized a series of novel canonical CDNs by a modified one-pot strategy that allows for the preparation of CDNs within one day. The novel CDNs were evaluated regarding their binding to naturally occurring GEMM riboswitch aptamers containing sequence variations. We found that sequences binding to c-di-GMP or c-AMP-GMP discriminate against their noncognate CDNs to varying degrees, and some novel CDNs such as c-CMP-GMP and c-GMP-UMP could bind moderately to the GEMM-I riboswitch variant. With regard to recognition of the novel CDNs by the STING receptor, we found that a novel CDN c-AMP-CMP could be a potential immune-stimulatory compound, although its induction of type I IFN responses is much less pronounced compared to the three naturally occurring CDNs. However, it still seems possible that nature has exploited more diversity for signaling purposes by dimerizing further NTPs to so-far undescribed CDNs. The introduced CDNs will enable an increased effort in order to identify such novel signaling compounds in nature.

MATERIALS AND METHODS

Synthesis of CDNs. All ten CDNs were chemically synthesized from a one-pot strategy as reported previously.[30] The crude CDNs were purified by reversed phase high-performance liquid chromatography (RP-HPLC) on a Nucleodur C18 Pyramid column with a gradient of 2−14% acetonitrile in triethylammonium acetate buffer (50 mM, pH 7.0) or ammonium bicarbonate buffer (50 mM, pH 7.0). The detailed synthesis procedure and full characterization data are presented in the Supporting Information.

Riboswitch Binding Assays. Two different assays were applied to characterize binding of CDN riboswitch variations to the novel CDNs. First, a spinach aptamer was fused to the GEMM riboswitch aptamer in a way that allows detection of ligand binding by an increase in fluorescence in the presence of the spinach aptamer ligand (DFHBI).[15,24] Second, in-line probing assays were carried out that are based on the detection of a changed RNA conformation upon ligand binding.[37]

Spinach Aptamer Fusion Riboswitches. Generation of GEMM-I-spinach aptamer fusion constructs: Different motifs were inserted into stem-loop II of the spinach aptamer by whole plasmid PCR; see Tables S1 and S2. The plasmid used (pET11a-Spinach) carries the spinach aptamer sequence (24−2)[46] under the control of a T7 promoter. RNAs were prepared as follows: Template DNA was amplified by PCR and subsequently *in vitro* transcribed using T7 RNA polymerase. The resulting RNA was purified by preparative PAGE and dissolved in spinach reaction buffer (40 mM HEPES, 125 mM KCl, 3 mM $MgCl_2$). The spinach fluorescence assay was carried out as follows: RNA samples were heated to 70 °C for 3 min and then cooled to room temperature within 5 min to fold the RNA. The concentration of RNA was adjusted to 100 nM for each measurement. The concentration of CDNs was 100 μM, and DFHBI was added to a final concentration of 10 μM. Each reaction was performed in a 100 μL reaction volume with triplicate experiments. Samples were incubated at 37 °C in a black 96-well microtiter plate until an equilibrium was reached and recorded on a Tecan Infinite M200 plate reader (excitation: 460 nm, emission: 500 nm).

In-Line Probing. Generation of 5′-end-labeled RNA and subsequent in-line probing was performed as described by Breaker and Soukup.[47] DNA templates containing the riboswitch sequence were generated from plasmids by PCR using primer containing the T7 promoter (Tables S1 and S3). DNA templates were used for *in vitro* transcription with T7 RNA polymerase (NEB) according to the manufacturer. RNase Inhibitor (NEB) and PPase were added for more efficient transcription. RNA was purified by PAGE. A 20 pmol amount of RNA was dephosphorylated with rSAP (NEB), and ^{32}P-labeling was achieved by T4 PNK (NEB) with γ-[^{32}P]-ATP. Radiolabeled RNA was PAGE purified. In-line probing reactions contained the respective CDNs in Tris-HCl buffer (50 mM pH 8.3, 20 mM $MgCl_2$, 100 mM KCl or NaCl if described) at 25 °C. $PEG_{20\,000}$ was added to a final concentration of 1 g/L to support RNA folding.[48] The RNA was incubated for 40 h at 25 °C. For nucleotide assignment partial digestion of the RNA with T1 RNase and alkaline digestion were performed as described.[37] Controls and in-line probing reactions were analyzed by PAGE and imaged using a Typhoon FLA 7000 phosphorimager.

Immune-Stimulatory Effects: Cell Culture. RAW-Lucia ISG or RAW-Lucia ISG-KO-STING (InvivoGen) cells were derived from the murine RAW 264.7 macrophage cell line by stable integration of an IRF-inducible luciferase reporter construct and used to assay the capacity of CDNs to trigger induction of type I IFN. RAW-Lucia ISG or RAW-Lucia ISG-KO-STING were maintained in Dulbecco's modified Eagle's medium (DMEM) (supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 U/mL penicillin/streptomycin, 200 mg/mL Zeocin) at 37 °C and 5% CO_2.

Cell Stimulation and Luciferase Assay. For cytosolic stimulation RAW-Lucia ISG or RAW-Lucia ISG-KO-STING cells were transfected with CDNs using FuGENE HD transfection reagent according to the manufacturer's protocol. Briefly, CDNs were mixed with 5 μL of DMEM medium and 0.5 μL of FuGENE HD reagent, followed by incubation at room temperature for 5 min, and then added dropwise to cells at a final concentration of 3.3 μg/mL in 96-well plates. After incubation for 18 h, supernatants were collected and IRF induction was analyzed by luminescence using QUANTI-Luc (InvivoGen) according to the manufacturer's instructions.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.7b06141.

Detailed experimental procedures, 1-D (^{1}H, ^{13}C, ^{31}P), 2-D (^{1}H–^{31}P HMBC, ^{1}H–^{1}H COSY, ^{1}H–^{13}C HSQC, ^{1}H–^{13}C HMBC) NMR spectra, CD spectra, primers and RNA sequences, and additional riboswitch binding data (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*joerg.hartig@uni-konstanz.de

ORCID

Jörg S. Hartig: 0000-0001-6601-7217

Notes

The authors declare no competing financial interest.

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