ABSTRACT

Title of Document:	CYCLIC DINUCLEOTIDE POLYMORPHISM: APPLICATIONS IN CATALYSIS AND DETECTION.
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Cyclic dinucleotides such as cyclic dimeric guanosine monophosphate (c-di-GMP,) cyclic dimeric adenosine monophosphate (c-di-AMP) and cyclic guanosine-adenosine monophosphate (cGAMP) are found in both prokaryotes and eukaryotes and are important in cell signaling processes. The work discussed herein aims at understanding the aggregative properties of these cyclic dinucleotides for interest in the biological implications of aggregation and for the purpose of various applications of these higher order structures. The applications include simple detection methods for these molecules as well as a unique G-quadruplex peroxidase that can be used for catalysis. To that end, a new fluorescent cyclic dinucleotide which incorporated the fluorescent purine analog, 2-aminopurine, was synthesized. This analog is capable of forming intercalated dimers with natural cyclic dinucleotides containing guanine which allows for a simple turn-off fluorescent method for these second messengers. Additionally, ligands containing four guanine bases were utilized to bind c-di-GMP

and leading to a fluorescent turn-on fluorescent detection method. The c-di-GMP octamer was also studied for its ability to enhance the peroxidation ability of the iron-containing cofactor hemin. Its proficient ability to enhance catalysis suggests that DNA loops found in other G-quadruplex forming molecules are not necessary for catalytic enhancement of hemin.

CYCLIC DINUCLEOTIDE POLYMORPHISM: APPLICATIONS FOR
CATALYSIS AND DETECTION.

By

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Dedication

It is with the greatest respect and humility that I dedicate this thesis to my beautiful wife, Leslie, and my parents, James and Holly. Their spiritual, emotional and educational advice has been invaluable during this undertaking. It is my hope that this small token of gratitude will help them understand how much they mean to me.

Acknowledgements

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List of Abbreviations

2',3'-cGAMP	2',3'-Cyclic guanosine-adenosine monophosphate
ABTS	2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid
ABTS$^{\cdot+}$	2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid radical cation
AMP	Adenosine monophosphate
A-site	Active site
ATP	Adenosine triphosphate
c-di-AMP	Cyclic dimeric adenosine monophosphate
c-di-GMP	Cyclic dimeric guanosine monophosphate
cGAMP	Cyclic guanosine-adenosine monophosphate
cGAS	Cyclic GMP-AMP synthase
CTD	C-terminal domain
DAC	Diadenylate cyclase
DBD	DNA binding domain
DGC	Diguanylate cyclase
DMZ	Diminazene aceturate
DOTA	1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid
GMP	Guanosine monophosphate
GTP	Guanosine triphosphate
HOMO	Highest occupied molecular orbital
HRP	Horseradish peroxidase
iPET	Intramolecular photoinduced electron transfer
I-site	Inhibitory site
LUMO	Lowest unoccupied molecular orbital
PDE	Phosphodiesterase
TASQ	Template-assembled synthetic G-quartets

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Chapter 1

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Chapter 4

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Chapter 5

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Chapter 1: Introduction

1.1.   Nucleotide Messengers

   Linear and cyclic nucleotides are used in eukaryotes and prokaryotes alike to

convey messages about the extracellular environment to different parts of the cell.

The newest of these classes of messenger molecules are known as cyclic

dinucleotides. Currently there are four known natural cyclic dinucleotides, which are

cyclic dimeric guanosine monophosphate (c-di-GMP, **1**),[1] cyclic dimeric adenosine

monophosphate (c-di-AMP, **2**),[2] cyclic guanosine-adenosine monophosphate

(cGAMP, **3**)[3] and 2',3'-cyclic guanosine-adenosine monophosphate[4] (2',3'-cGAMP,

4, see Figure 1.1 for structures).[5, 6] Although structurally similar, these nucleotides

control very different biological processes.
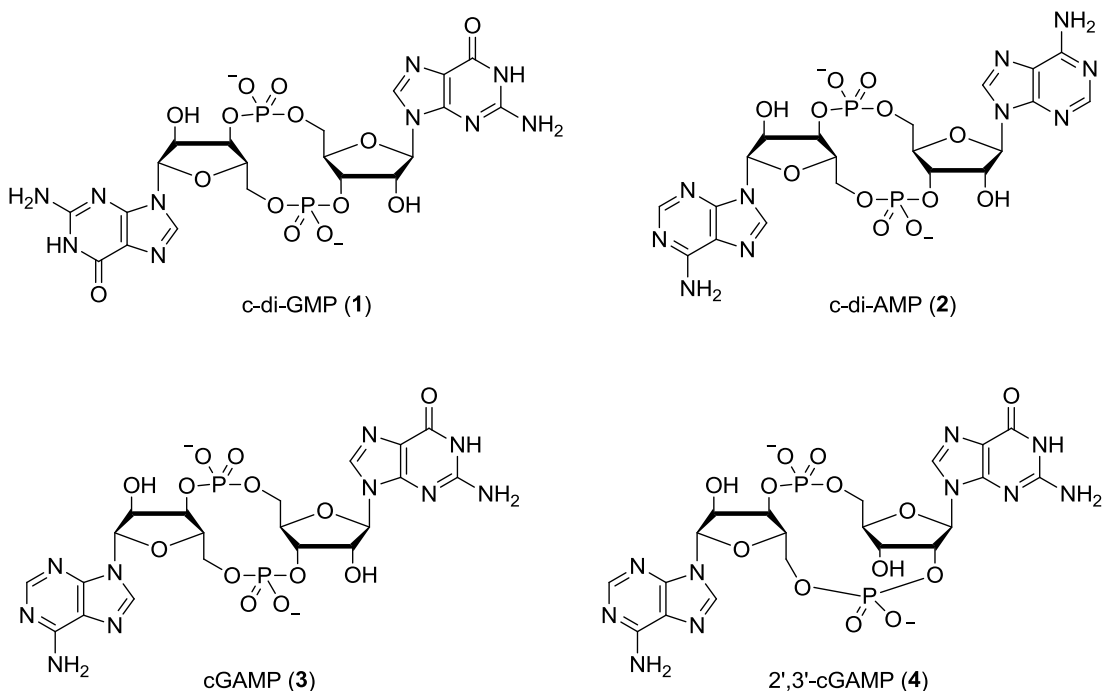
Figure 1.1. Structures of natural cyclic dinucleotides used as second messengers in biological systems.

Because cyclic dinucleotides affect a plethora of biological processes, cyclic dinucleotides and the binding targets which they affect have become targets for which to develop therapeutics. Each nucleotide is reviewed separately below, as they are typically found in different species and/or target different processes.

1.2. C-di-GMP

C-di-GMP (**1**) was first discovered in 1987 by Benziman and coworkers as a modulator of cellulose synthase in *G. xylinus* (previously known as *A. xylinum*).[1] Despite its discovery almost 30 years ago, the implications of c-di-GMP in other biological systems were not well understood or studied until recently. In the last

decade however, research involving c-di-GMP (and more generally cyclic

dinucleotides) has exploded as it has been determined that c-di-GMP regulates a

plethora of processes involved in the lifestyle of bacteria (for a comprehensive review

see ref. 5). Generally, when intracellular concentrations of c-di-GMP are elevated

bacteria adopt a surface-attached, non-motile lifestyle known as biofilm. Conversely,

when c-di-GMP concentrations are diminished, bacteria adopt a planktonic, motile

state.[7]

The intracellular concentration of c-di-GMP is controlled by the activity of

enzymes known as diguanylate cyclases (DGC, Scheme 1.1). These enzymes

condense two equivalents of guanosine triphosphate (GTP) into one equivalent of c-

di-GMP. C-di-GMP specific phosphodiesterases (PDE) on the other hand, are

responsible for the degradation of c-di-GMP into either linear 5'-pGpG-3' or into two

equivalents of guanosine monophosphate (GMP, Scheme 1.1).
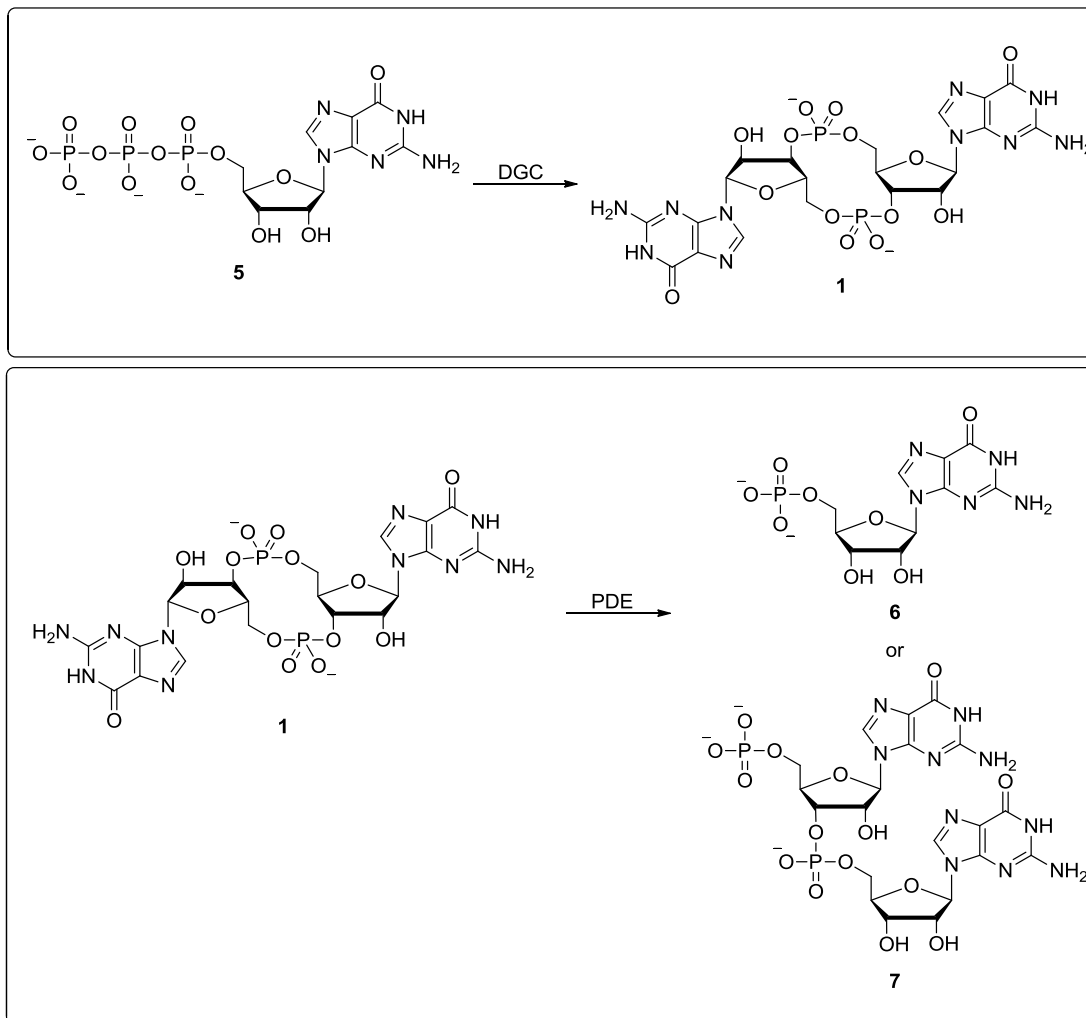
Scheme 1.1. Synthetic and degradation pathways of c-di-GMP.

DGC contains a well conserved GGEEF or GGDEF active site (A-site) motifs.[8] Any point mutation in these amino acids eliminates catalytic activity of these synthases.[9] To prevent excessive synthesis of c-di-GMP and overconsumption of GTP, DGCs often contain an inhibitory site (I-site).[10] The I-site contains a RxxD motif. Binding of c-di-GMP to this I-site allosterically regulates the activity of the enzyme and turns off production of c-di-GMP.[10] The first example of this type of allosteric regulation in a DGC was that of PleD (pleiotropic) protein in *C.*

crescentus.[10] PleD is a dimer which requires free rotation of the two DGC domains to incur synthesis of c-di-GMP. However, upon binding of c-di-GMP dimer to the I-site, the DGC domains are separated and immobilized preventing the chemical condensation of two equivalents of GTP.[11]

There are two known varieties of c-di-GMP PDE. The first contains an HD-GYP domain.[12] HD-GYP describes the active site residues essential for the catalytic activity of the PDE. HD-GYP domain PDE degrades c-di-GMP directly into GMP.[12] Another type of PDE with an EAL domain degrades c-di-GMP in a stepwise manner, breaking it down into 5'-pGpG-3' first followed by a second, slower cleavage into GMP.[13] Both classes of PDE require the presence of Mg^{2+} or Mn^{2+}.[13, 14] If these cations are not present or replaced with other divalent cations such as Ca^{2+}, Ni^{2+}, Fe^{2+} or Zn^{2+} the activity of the enzymes are inhibited.[13-15]

In order to regulate bacterial phenotypes, c-di-GMP must bind to receptors. Many receptors contain mutated EAL, HD-GYP, or GGDEF domains which bind c-di-GMP but do not have catalytic activity. In turn c-di-GMP can modulate some other activity of the protein through the binding process. This topic will be discussed in greater detail, *vide supra*.

1.3.   *Polymorphism of c-di-GMP*

C-di-GMP is known to adopt several polymorphs some of which have biological implications (see section 1.4). The first polymorph of c-di-GMP to be discovered was the intercalated dimer which was crystallized by the Frederick group in the presence

of Mg^{2+} (Figure 1.2).[16] The Jones,[17, 18] Sintim,[19] and Grzesiek[20] groups have studied, in-depth, the ability of c-di-GMP to aggregate *in vitro*. Through various spectroscopic techniques (^{1}H-NMR, ^{1}H-^{13}C HMBC/HMQC, ^{1}H-^{1}H NOESY, CD, UV-vis) the Jones group showed that c-di-GMP can adopt more complex structures known as G-quadruplexes (structures made up of several stacked G-tetrads) in which the guanines of the c-di-GMP Hoogsteen base pair to form large aromatic planar structures know as G-tetrads (Figure 1.2).[17, 18] The Sintim group showed several years later, that an analog of c-di-GMP in which one of the bridging oxygens in the phosphodiester linkage was replaced with a sulfur, has a much lower propensity to form higher order aggregates.[19] Grzesiek's group expanded on this research even further by determining rate constants of formation and degradation of c-di-GMP aggregates.[20]
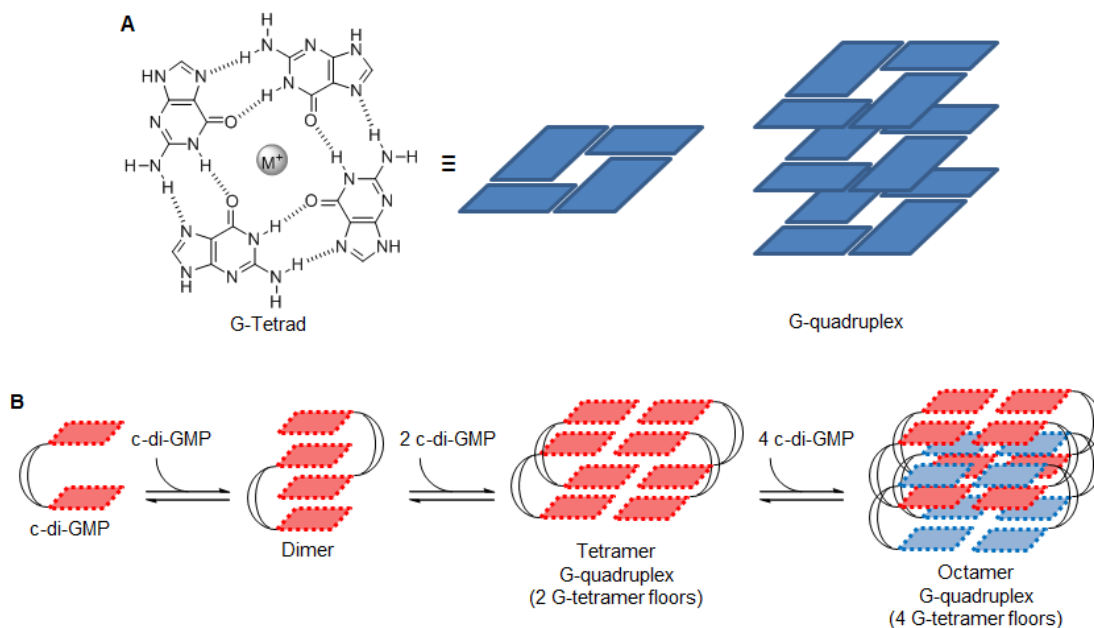
Figure 1.2. A) Chemical structure of G-tetrad and cartoon representation of a G-quadruplex. B) Cartoon representation of c-di-GMP aggregates first investigated by the Jones group.[17, 18]

As is with other intermolecular and intramolecular G-quadruplexes, c-di-GMP G-quadruplexes are stabilized in the presence of monovalent cations such as K^+.[18, 21] However, in the presence of divalent cations like Mg^{2+} or Mn^{2+} the intercalated dimer is favored.[17, 18, 22] It has been argued that the c-di-GMP G-quadruplexes are biologically irrelevant because at biological concentrations c-di-GMP exists predominantly as a monomer.[20] It has however, been shown that c-di-GMP can form G-quadruplexes at low concentrations in the presence of planar aromatic intercalators such as proflavine,[23] thiazole orange[24] and diminazene aceturate (DMZ).[25] This is interesting because linear nucleotides such as GMP, GTP or 5'-pGpG-3' are not capable of forming these higher order aggregates at micromolar concentrations.[19, 24, 26]

1.4. *Polymorphism of c-di-GMP in vivo*

The ability of c-di-GMP to form these higher order structures is exploited by nature to sense c-di-GMP. In order to convey information such as environmental cues to the cell, c-di-GMP must bind receptors.[5, 7] Several c-di-GMP receptors are now known, as pull down assays using surface bound c-di-GMP have become more widely available.[27] These receptors are numerous and include several classes of biomolecules with myriad functions. Examples of these receptors are the effector protein PilZ (so named because it controls pilus formation),[28] degenerate GGDEF domain proteins,[29] transcription factors, which regulate gene expression[30, 31] and RNA riboswitches, which regulate transcription or translation.[32]

Each one of these c-di-GMP receptors is known to bind one of the three distinct polymorphs of c-di-GMP (see Figure 1.3). The three polymorphs include the c-di-GMP monomer, intercalated dimer and hydrogen bonded tetramer (hydrogen bonded dimer of dimers). For instance, in the case of the DGC, PleD, which contains both an A-site and an I-site, c-di-GMP binds the protein both in its dimeric form and its monomeric form (Figure 1.4).[11] The I-site senses c-di-GMP dimer (this exists at elevated c-di-GMP concentrations[20]) and shuts off DGC activity.

c-di-GMP
Monomer

c-di-GMP
Dimer

c-di-GMP
Tetramer
(Dimer of dimers)
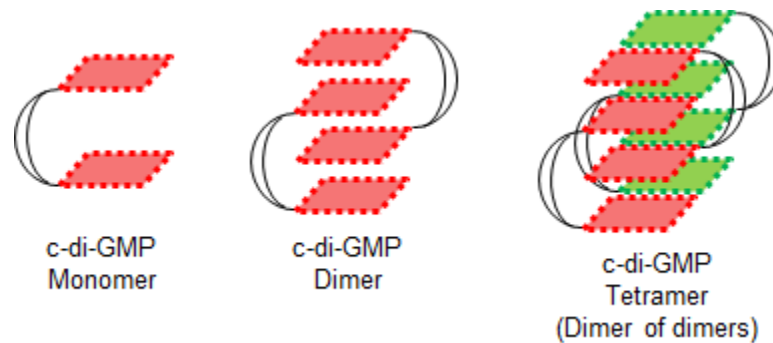
Figure 1.3. The three distinct c-di-GMP polymorphs which have been demonstrated to bind various biomolecules.
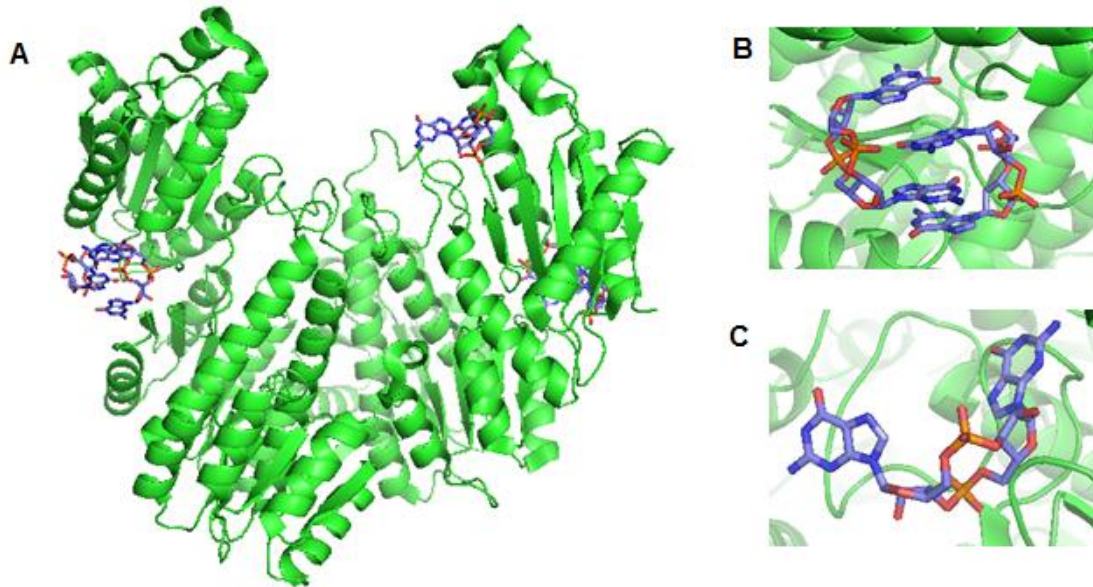
Figure 1.4. A) C-di-GMP bound to the DGC, PleD,[33] in B) monomer form (A-site) and C) dimeric form (I-site). Images constructed in PyMol.

Most recently, a new binding mode has been found for the transcription factor, BldD which controls the sporulation and development of *Streptomyces coelicolor*.[31] A unique c-di-GMP tetramer (two hydrogen bonded c-di-GMP intercalated dimers, Figure 1.5) serves to dimerize the BldD C-terminal domains (CTD) allowing a DNA binding domain (DBD) to bind a target promoter region thereby regulating transcription of several genes.
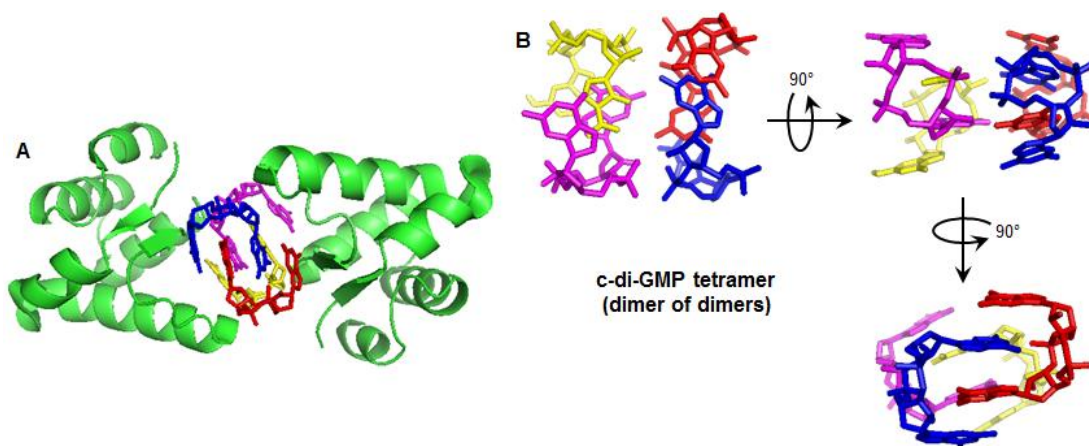
Figure 1.5. A) C-di-GMP tetramer bound to BldD[34] and B) analysis of c-di-GMP
tetramer structure. Images constructed in PyMol.

1.5. *Detection of c-di-GMP*

In order to understand the environmental cues which affect c-di-GMP levels
detection methodologies must be developed. Initially methodologies, utilized LC-
MS.[35] Although these or similar methods are still used, they are time consuming and
require expensive equipment not available to many research laboratories.[35, 36]
Conversely, the Miller lab used recombinant fluorescent proteins to detect c-di-GMP
in vivo, but this technique can be equally difficult to execute.[37]

To improve and simplify detection methodology, the Sintim group has developed
techniques whereby the polymorphism of c-di-GMP is exploited. The first example of
this used the cyanine dye, thiazole orange (**8**, Figure 1.6) to form a predicted c-di-
GMP tetramer.[24] Upon formation of the c-di-GMP tetramer-thiazole orange complex,
the thiazole orange becomes fluorescent thereby providing a simple turn-on
fluorescent probe for the detection of c-di-GMP. The simple detection system allows

10

detection of as low as 5 µM c-di-GMP.[24] Similarly, other aromatic ligands such as proflavine, **9**, and diminazene aceturate (**10**, DMZ), are capable of forming the putative c-di-GMP tetramer, although these ligands do not provide a useful signal output for detection purposes (Figure 1.6).[23, 25]

However, because G-quadruplex-hemin complexes are known to catalyze peroxidation reactions,[38] the Sintim group used the G-quadruplex formed in the presence of proflavine to catalyze the peroxidation of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS, **11**, Figure 1.7) to the colored ABTS radical cation (ABTS$^{\cdot+}$). This provides a simple colorimetric readout for the detection of c-di-GMP.[26]
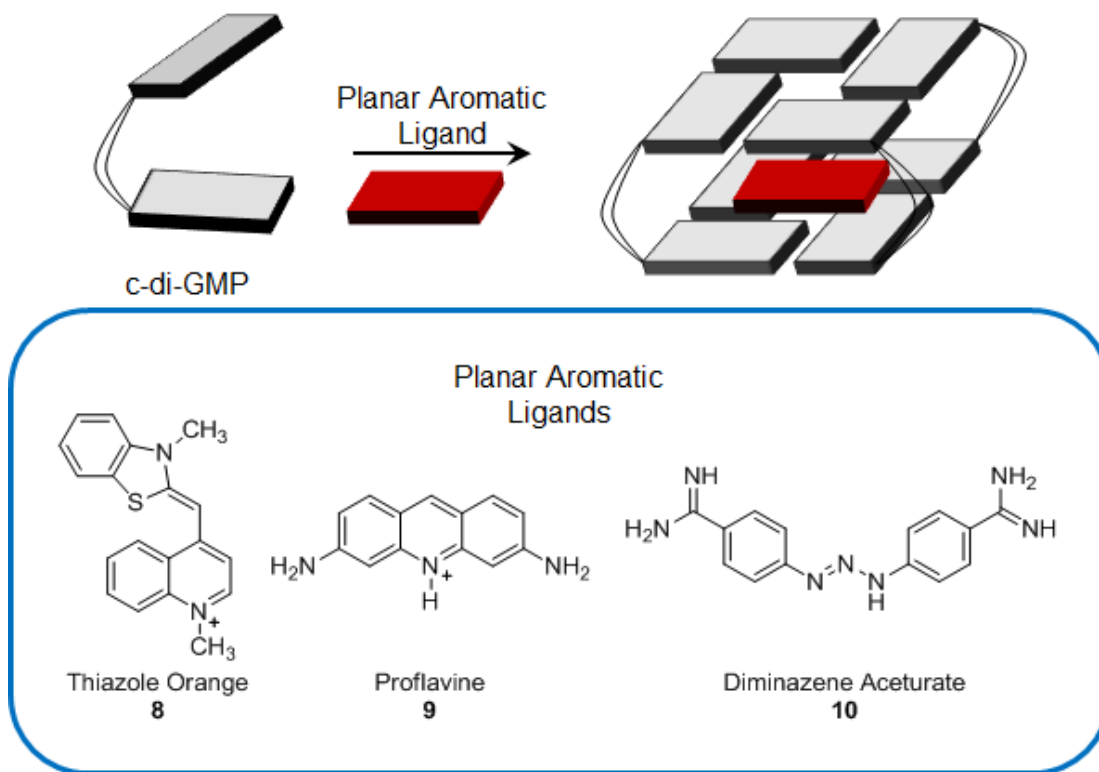
Figure 1.6. Formation of c-di-GMP G-quadruplex in the presence of planar aromatic intercalators.
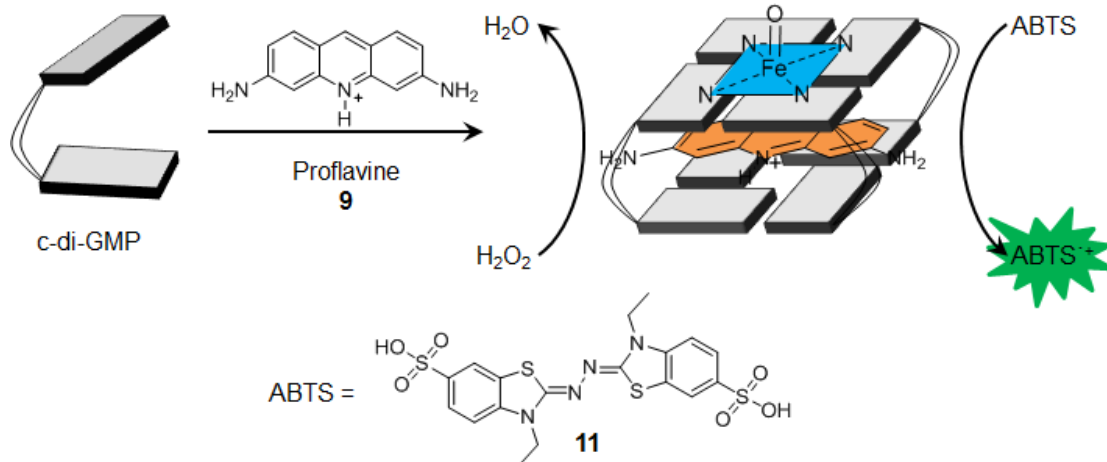
Figure 1.7. C-di-GMP-hemin-proflavine complex has catalytic activity as a peroxdiase and can oxidize ABTS to the green colored ABTS$^{\cdot+}$ radical cation.

Despite the ease of these aggregate forming ligands, these techniques have significant drawbacks. Each requires the formation of c-di-GMP complex made up of at least four c-di-GMP monomers. This limits the capability for a sensitive detection as the complex will inherently be four times more dilute than c-di-GMP monomer. Furthermore, the formation of this complex is slow[20] and requires overnight incubation in buffer with high monovalent salt concentrations.[20] This precludes its use as an *in vivo* or real-time detection modality. Additionally these ligands are known to interact with duplex DNA in addition to G-quadruplexes which would limit availability of thiazole orange in complex solutions (e.g. cell lysates) as well as create high background.

To address these issues, the Sintim group attempted to make a spinach RNA aptamer[39] conjugate with a c-di-GMP riboswitch to create a turn-on probe with high sensitivity and selectivity.[40] Because of the high binding affinity (K_d ~1 nM) of c-di-

GMP to the RNA riboswitch[32] the synthetic conjugate made by the Sintim group provides an extremely low limit of detection ($\sim 10^{-7}$ M).[40] This detection modality was even applied later by the Hammond group to detect both c-di-GMP and cGAMP in live cells.[41] Unfortunately, the on rate of c-di-GMP to the Vc2 riboswitch is 1×10^6 $M^{-1}s^{-1}$ which is well below diffusion controlled reaction rate ($\sim 1 \times 10^{10}$ $M^{-1}s^{-1}$). This prevents real-time detection of c-di-GMP (an important technique for finding inhibitors of metabolic enzymes).

1.6. C-di-AMP

C-di-AMP (**2**) has more recently been discovered as a regulator in Gram positive (and some Gram negative[42]) bacteria.[2] It was first discovered in *B. subtilis* bound to a DNA binding protein, DisA which scans the chromosome for double strand breaks.[2, 43] DisA was found to have diadenylate cyclase (DAC) activity allowing it to synthesize c-di-AMP from two equivalents of adenosine triphosphate (ATP). Because putative DAC genes were found in several species of bacteria several groups began studying c-di-AMP in hopes that c-di-AMP has an equally important role in prokaryotic cell function as c-di-GMP.[44] C-di-AMP was found to have regulatory function in cell size,[45] cell wall homeostasis,[46] stress response,[47] bacterial growth[48] and virulence.[49] Additionally secreted c-di-AMP from *L. monocytogenes* and *M. tuberculosis* activates host immune response.[49, 50]

Similar to c-di-GMP, c-di-AMP has several metabolic proteins which are responsible for its synthesis and degradation. The synthases are known as diadenylate

cyclases.[2] C-di-AMP specific PDEs (enzymes which degrade c-di-AMP) cleave it into 5'-pApA-3' (**14**) or two equivalents of adenosine monophosphate (AMP, **13**, Scheme 1.2).[51]
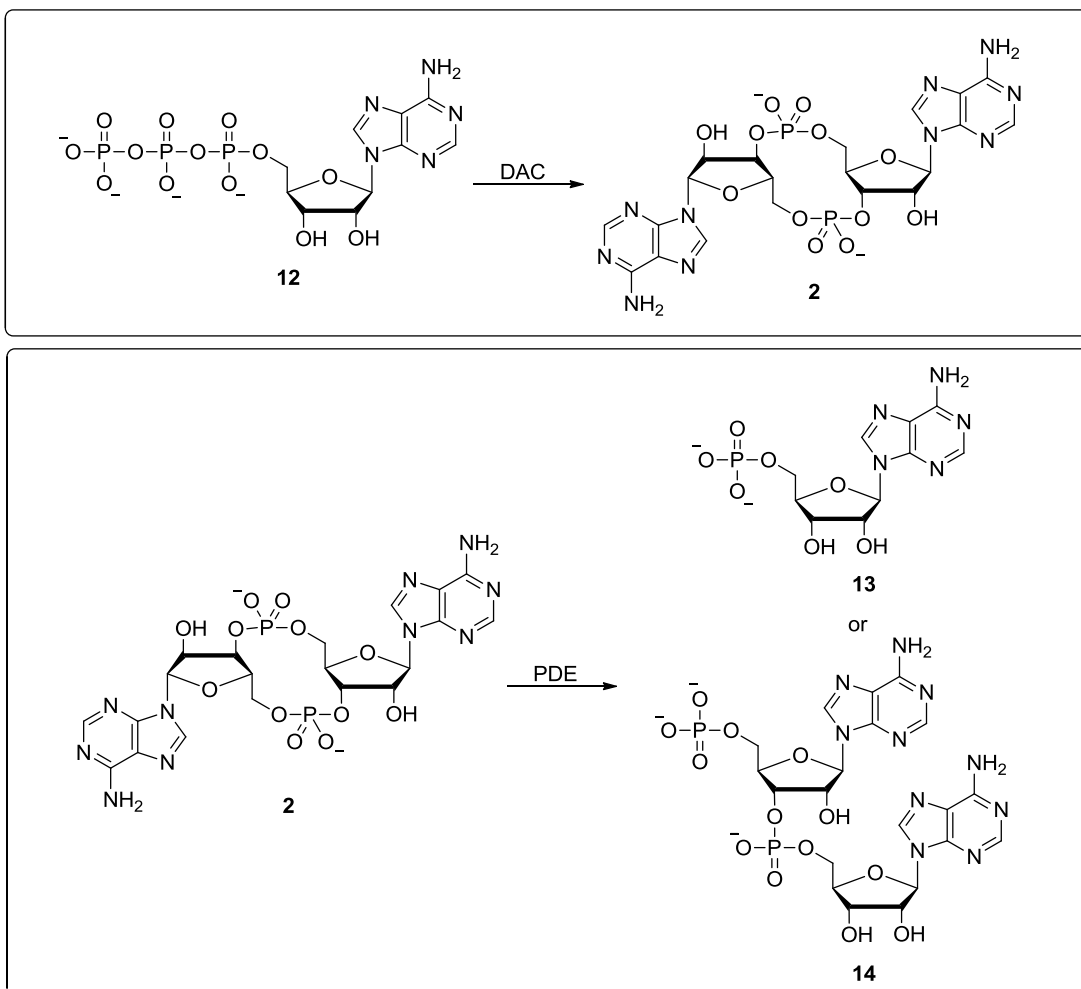
Scheme 1.2. Synthetic and degradation pathways for c-di-AMP.

Because of its more recent discovery less is known about c-di-AMP and its receptor molecules in the cell. However, targets have been found which identify c-di-AMP binding pockets and biochemical analysis have reveled several roles for these molecules. These receptors include proteins[47, 52] and RNA riboswitches.[53]

Interestingly, with one known exception,[52] c-di-AMP binds to the receptors only its

monomeric form which is strikingly different from c-di-GMP (Figure 1.8). The

protein, LmPC, a pyruvate carboxylase, was found to bind and be modulated by c-di-

AMP.[52] A crystal structure of this protein showed a c-di-AMP trimer bound to two

neighboring proteins. The trimer was made up of two monomers of c-di-AMP bound

to the I-site of each of the proteins with a single c-di-AMP bridging between the two

bound c-di-AMPs. It was hypothesized however, that the bridging c-di-AMP was

merely an artifact as another crystallization of the protein yielded an identical

structure without the bridging nucleotide.[52]
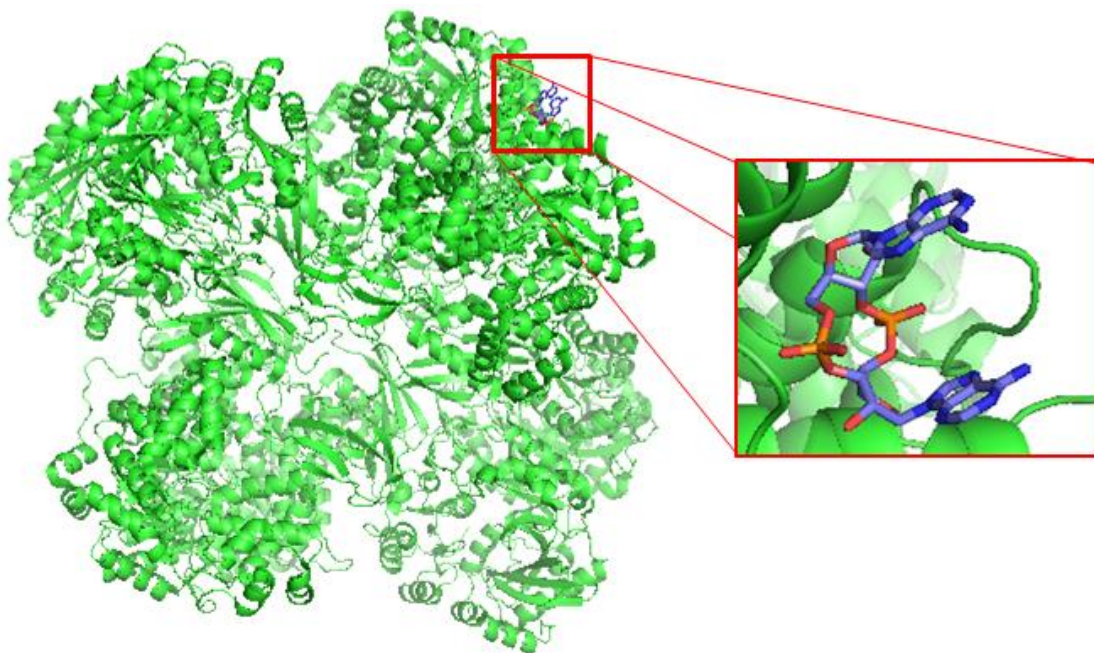
Figure 1.8. c-di-AMP monomer bound to LmPC.[54] Image made in PyMol.

1.7.   Detection of c-di-AMP

Few detection modalities are available for the c-di-AMP. Recently, the Sintim group showed that c-di-AMP is capable of forming a higher order complex with coralyne.[55] Coralyne was known to bind poly-A sequences of DNA.[56] The c-di-AMP-coralyne complex is still not well understood as it could not be studied by NMR. The complex is however, fluorescent thereby providing a convenient fluorescent readout to detect the presence of c-di-AMP. This detection method was even employed by the Sintim group to discover an inhibitor of the DAC, DisA.[57]

Another detection assay was described by the Bai group, using a competitive ELISA assay.[58] The assay utilizes a surface attached CabP (**c**-di-**A**MP binding **p**rotein) to bind a mixture of biotinylated-c-di-AMP (introduced by experimenter) and natural c-di-AMP (introduced from the biological sample of interest).[58] The surface is subsequently exposed to a horseradish peroxidase-streptavidin conjugate protein which will bind to the biotinylated-c-di-AMP. Treatment with hydrogen peroxide and *o*-phenylenediamine yields a colorimetric signal at 490 nm. Due to the nature of the ELISA assay, as more c-di-AMP (from the biological sample) is introduced the signal decays as less biotinylated-c-di-AMP is captured by the surface bound CabP. Other, more traditional, detection methods have been utilized which include HPLC, MS and TLC (TLC utilizes ^{32}P labeled c-di-AMP).[42]

1.8. cGAMP and 2',3'-cGAMP

Seminal work by the Mekalanos group showed that the DncV protein in *V. Cholerae* preferentially produces a novel cyclic dinucleotide with mixed bases known as cGAMP (**3**).[59] This protein was also found to be essential for intestinal colonization of *V. cholerae*.[59] Less than a year later, the Chen group showed that a cytosolic DNA sensor in humans was also a cyclic GMP-AMP synthase (cGAS).[3, 60] By synthesizing cGAMP, the protein activates the type I interferon pathway. This discovery opened the door for cyclic dinucleotide signaling in the animal kingdom. Shortly thereafter, the Tuschi and Patel groups showed that the cGAS previously described by Chen and coworkers, in fact, synthesized a cyclic dinucleotide with a noncanonical 2'-5' linkage (2',3'-cGAMP, **4**).[6]

1.9. New Detection Modalities

The emerging importance and diversity of cyclic dinucleotides make them good targets for new therapies which treat disease caused by bacteria. Detection of these molecules will allow new understanding of the biology which they regulate as well as discovery of inhibitors of the metabolic enzymes which synthesize or degrade them. These inhibitors would give scientists a good starting point from which to develop new therapies to battle bacteria. Therefore the aims of my dissertation are twofold: a) To use cyclic dinucleotides and their ability to form higher order aggregates to study factors that affect nucleic acid polymorphism and catalysis and; b) to improve and

expand upon the detection assays that exist for these important cyclic dinucleotides as well as to better understand the ability of these unique molecules to aggregate both *in vivo* and *in vitro*. Detection assays discussed herein will address the issue of slow formation of detected complexes which have been previously described as well as discuss the application of c-di-GMP G-quadruplex in the study of G-quadruplex peroxidase catalysts which have found use in other detection modalities.

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Chapter 2: Octameric G8 c-di-GMP is an Efficient Peroxidase and this Suggests that an Open G-Tetrad Site Can Effectively Enhance Hemin Peroxidation Reactions

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B. T. Roembke carried out all peroxidase reactions, kinetic analysis of catalysts, synthesis and purification of hemin analogs and binding studies of hemin analogs with G-quadruplexes. J. Wang was responsible for previously developing the synthesis of hemin analogs used in this study. S. Nakayama performed CD measurements of octameric c-di-GMP, UV-vis measurements of hemin analogs and assisted with binding studies of hemin analogs with G-quadruplexes. J. Zhou synthesized c-di-GMP used in the study.

2.1.   Introduction

Guanine rich DNA/RNA sequences and other small molecules that form tertiary structures, known as G-quadruplexes[1], play important roles in both biology and nanotechnology (see Figure 2.1).[2] G-quadruplexes are found at the end of chromosomes (in humans these are tandem telomeric TTAGGG repeats) and in the promoter regions of important oncogenes, presumably protecting genomic DNA as well as playing key regulatory roles at transcriptional level. Recently there have been enormous interests in the use of G-quadruplexes/hemin complexes as peroxidase mimics for biotechnological applications.[3-5] Apart from sensing applications, nucleic acid peroxidases, have also been proposed as green catalysts for the syntheses of important fine chemical intermediates, such as epoxides and sulfoxides.[6] As the potential uses of G-tetrad peroxidases increase, efforts to decipher the mechanism

employed by nucleic acid peroxidases to enhance the peroxidation reaction of hemin as well as develop more robust and highly efficient DNA-based peroxidases, including the addition of additives (such as ATP) have also increased.[7-9]

The Sen group has studied these DNA-based peroxidases in order to understand the mechanism by which they catalyze the oxidation of organic substrates. The Sen group was the first to show that DNA G-quadruplexes are capable of enhancing the peroxidation of the hemin cofactor.[4] The catalytic cycle of these G-quadruplex-hemin assemblies are dependent on three reaction steps (see Figure 2.2).[10] In the first reaction step, the Fe(III) hemin (complexed with DNA) readily reacts with hydrogen peroxide to yield a Fe(IV) hemin radical cation known as Compound I.[10] Subsequent reaction with the organic reductant yields the more stable Fe(IV) hemin intermediate known as Compound II.[10] Reaction with another equivalent with a organic reductant completes the catalytic cycle, yielding the intial Fe(III) hemin porphyrin.[10]

It has been suggested that the one of the guanines which constitute the G-quadruplex provides the fifth coordination necessary for catalytic activity.[11] In order position itself for iron coordination, the guanine flips out of the G-tetrad plane, effectively freeing the N7 from the hydrogen bond network of the G-tetrad.[11] In proteins, the fifth coordination is provided by a histidine residue which coordinates the iron via the imidazole base.[11, 12]

The active site has been a source of controversy in the field of DNA based peroxidases. The Sintim group has investigated whether the hemin cofactor remains bound the G-quadruplex during the entire catalytic cycle. One possibility is that the Fe(IV) hemin radical cation species (Compound I) dissociates from the G-quadruplex

and carries out the oxidation of the organic reductant.[8] Conversely, it is possible that the hemin cofactor remains bound through all steps of the catalytic cycle.[8] It was found that DNA peroxidation catalysts oxidize organic substrates of various sizes at different relative rates.[8] Therefore it was hypothesized that the most probable model is the one in which hemin remains bound throughout the catalytic cycle, otherwise the size of the organic substrate would be insignificant if oxidation occurred in bulk solution (i.e. outside of a structured binding pocket).[8]

One of the unresolved issues regarding nucleic acid enhancement of hemin peroxidation is the architecture of the hemin binding site.[13] Some reports have suggested an open binding site whereby hemin π-stacks with a solvent-exposed G-tetrad[6] whereas other reports have hypothesized a more secluded active site[14] or an active site with defined geometry.[8] A study by Yamamoto and coworkers showed via NMR, that hemin binds an intramolecular G-quadruplex in a 2:1 ratio in which a single hemin cofactor is sandwiched between two G-quadruplexes.[13] This study, however, did not show whether this complex was capable of peroxidation.

An ideal model to resolve this issue of whether the peroxidation proficiency of hemin could be significantly enhanced when bound to a water-exposed and open G-tetrad would be a guanine-based catalyst that is devoid of loops. DOTASQ, introduced by Monchaud[15], satisfies this requirement but unfortunately the catalytic efficiency of DOTASQ is significantly less than that of DNA-based peroxidases and so raises the question whether loop sequences or more than one G-tetrad are really needed for catalytically proficient nucleic acid-based peroxidases.[14] Recently, we proposed that in the presence of intercalators, c-di-GMP potentially forms G-

26

quadruplexes that enhance hemin peroxidation activity.[16, 17] The presumed c-di-GMP-G-quadruplex had a lower peroxidation efficiency than DNA G-quadruplex.[17] It has been shown that the loops of a DNA G-quadruplex (see Figures 2.2A and 2.2B) are important for ligand binding[18] and so a lower peroxidation efficiency of nucleotide- or nucleoside-based peroxidases, such as c-di-GMP nucleotidezyme[17] or DOTASQ[15] could be the result of loose hemin binding, due to the absence of loops, or some other subtle catalytic effect that the loops provide (see Figure 2.2).

c-di-GMP (**2**) when R = OH
2'-OMe-c-di-GMP (**3**) when R = OMe
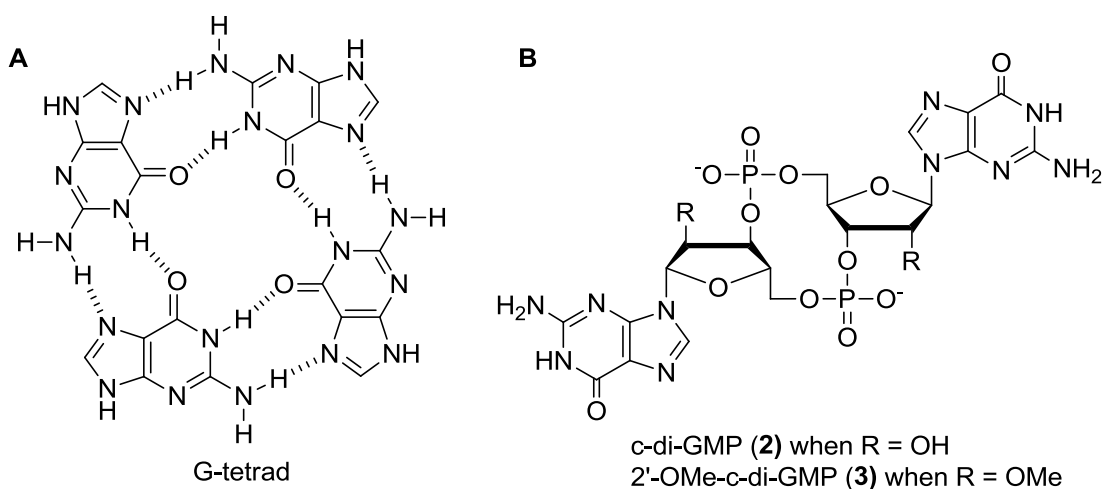
G-tetrad

Figure 2.9. Structure of A) G-tetrad and B) the cyclic dinucleotide analogs used in this study.
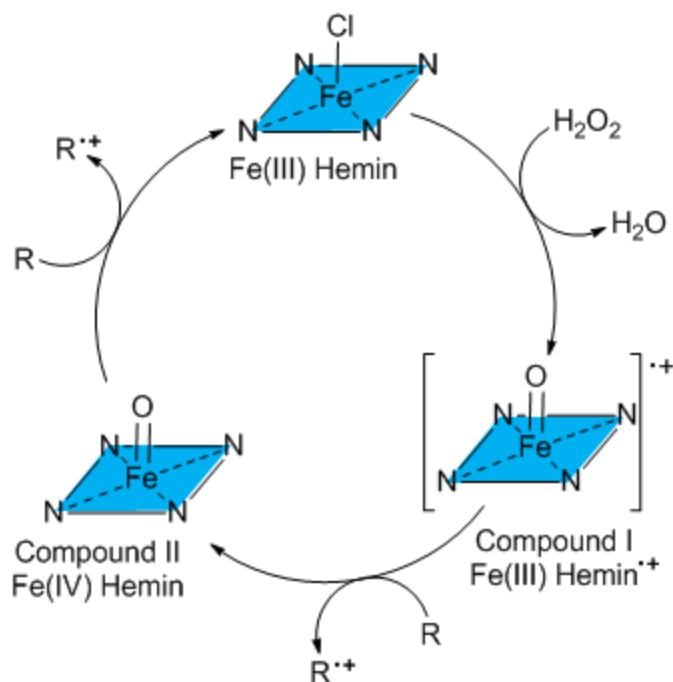
Figure 2.10. Catalytic cycle of DNA and protein based peroxidases.
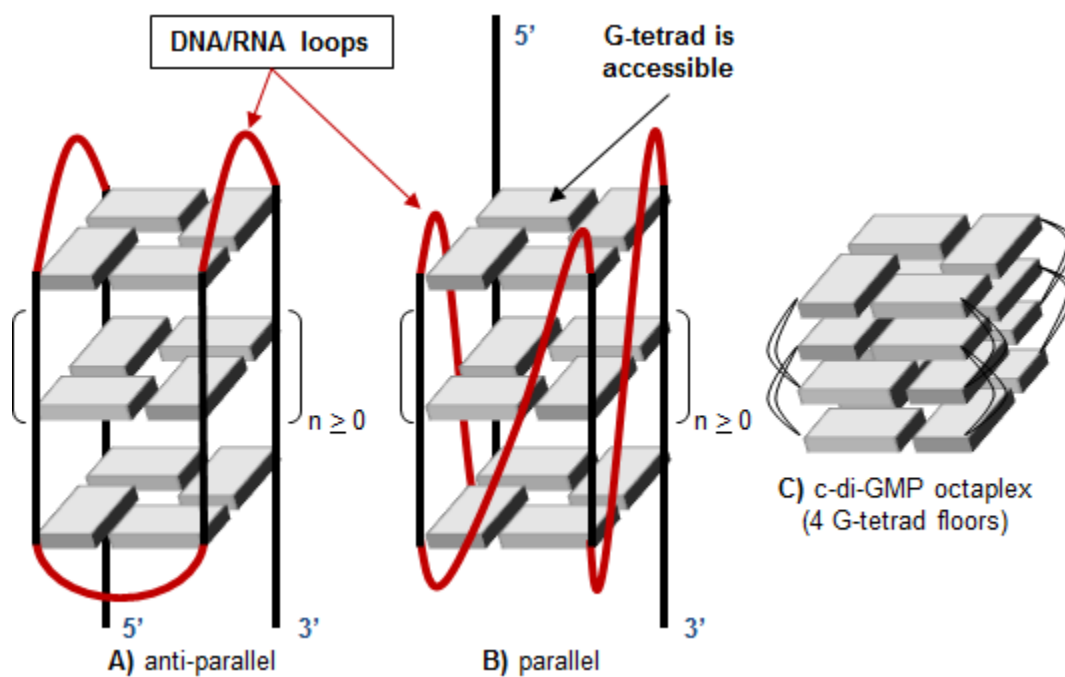
Figure 2.11. Cartoon representations of A) an anti-parallel DNA/RNA G-quadruplex, B) parallel DNA/RNA G-quadruplex, C) c-di-GMP octaplex.

Studies by Jones, Sintim and Grzesiek revealed that c-di-GMP octaplexes (see Figure 2.2C), formed in the presence of high concentration of potassium (250 mM), have unusual stability.[19, 20] C-di-GMP octaplex formation is kinetically controlled but once formed these octameric species remain intact, even when diluted into low monovalent cation-containing buffers.[19] These stable G-quadruplexes are therefore excellent species to study the roles that G-quadruplex loops have on peroxidation reactions.

2.2.   *Results and Discussion*

We prepared octameric solutions of **2** and **3** (for the synthesis of c-di-GMP see references 21 and 22) and tested the stability and peroxidation profile of these species (see Figures 2.3 and 2.4). Interestingly dilute solutions (40 μM) of octameric species of c-di-GMP **2** and 2'-OMe-c-di-GMP **3** were stable up to 24 h, as evidenced by the positive CD peak at approximately 300 nm (see CD spectra, Figure 2.3). In the presence of NH_4^+, octameric c-di-GMP and 2'-OMe-c-di-GMP exhibited peroxidation activity that was comparable to one another, although in the absence of cations the 2'-OMe analog showed slightly better peroxidation rates than the natural analog **2**. Later, we compared this peroxidation ability to various G-quadruplex forming DNA and showed that octameric **2** was better than these DNA peroxidases (see Figure 2.5). To the best of our knowledge, this is one of the very few

demonstrations of a small molecule guanine-based peroxidase that has higher

peroxidase capability than DNA G-quadruplex peroxidase.
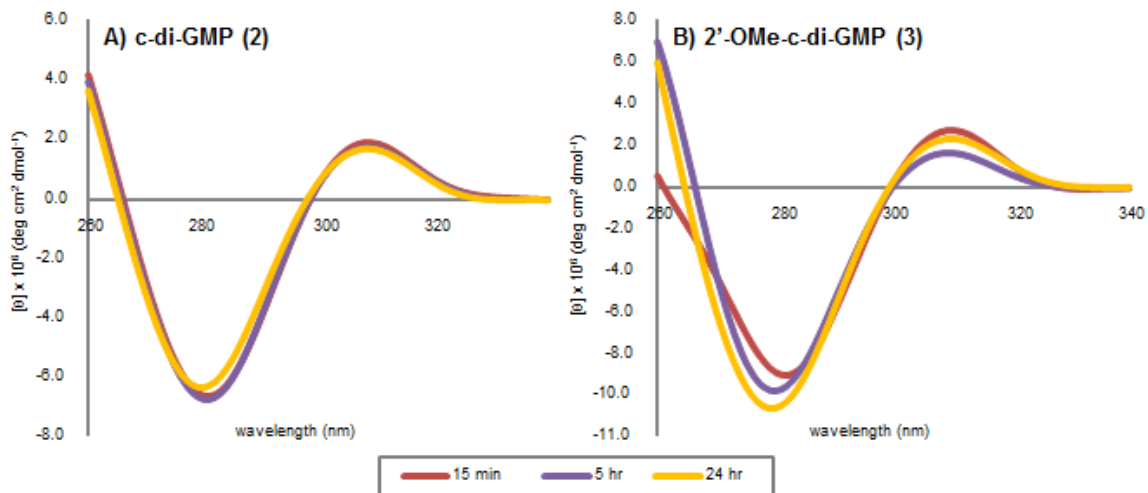
Figure 2.12. CD data of c-di-GMP and the 2'-OMe analog. Each compound was

prepared as the octamer (see Experimental Section) and diluted to 40 μM in 50 mM

Tris-HCl (pH = 7.5). The spectra were measured at 10 °C at different time intervals

after dilution. Samples were kept at 0 °C before measurement.
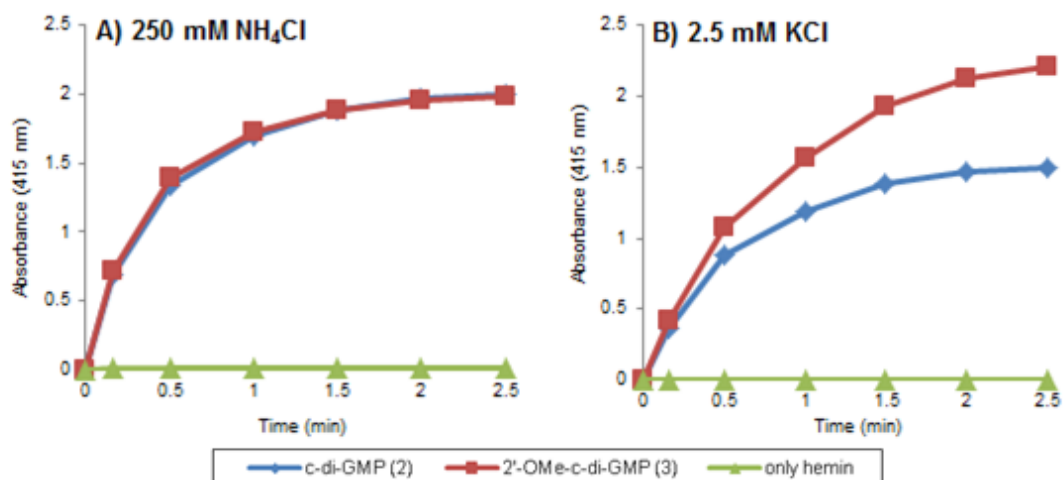
Figure 2.13. Peroxidation reaction using c-di-GMP and the 2'-OMe analog in octameric form. Peroxidation was started immediately after addition of hemin in other samples. Conditions: [octameric c-di-GMP/octameric 2'-OMe-c-di-GMP] = 2.5 µM ([monomeric c-di-GMP] = 20 µM), [Hemin] = 0.5 µM, [ABTS] = 2 mM, [H_2O_2] = 2 mM, Buffer: 50 mM Tris-HCl (pH = 7.9). See above for salt concentrations. An additional 2.5 mM KCl is present from c-di-GMP/analog stock solution. Peroxidation measurements shown are single trials.
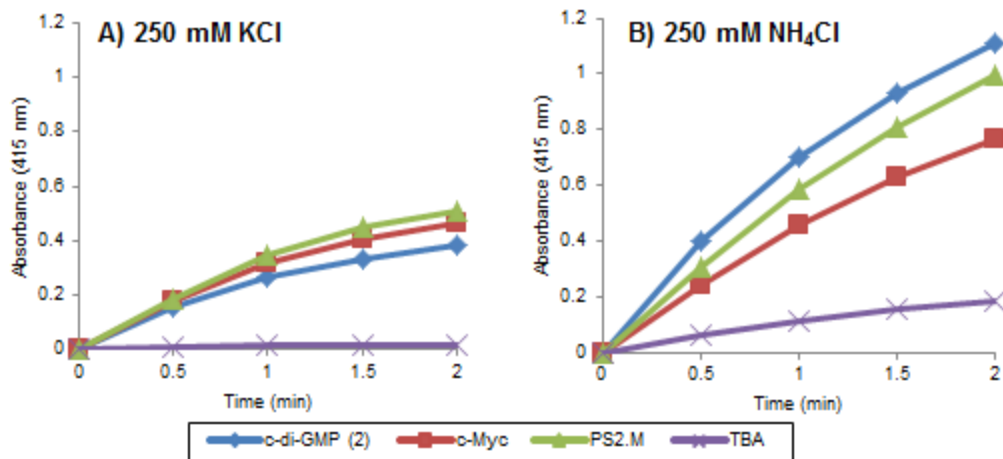
Figure 2.14. Peroxidation reactions with c-di-GMP octamer and various G-quadruplex forming DNAs. Conditions: [octameric c-di-GMP/DNA] = 10 µM ([monomeric c-di-GMP] = 80 µM), [Hemin] = 0.1 µM, [ABTS] = 2 mM, [H_2O_2] = 2 mM, Buffer: 50 mM Tris-HCl (pH = 7.9). See above for salt concentrations. An additional 10 mM KCl is already present from c-di-GMP/DNA stock solution. Peroxidation measurements shown are single trials.

Cations, especially monovalent ones such as K^+, Na^+ and NH_4^+, are important for both G-quadruplex formation and the peroxidation reaction.[4] However, because the monovalent cations are also important for the formation of the G-tetrad motif, it is unclear if the role of the cation is structural (i.e. promoting the G-quadruplex, which is the active catalyst) or catalytic (i.e. involved in the actual peroxidation catalysis). Because octameric c-di-GMP is stable, even at very low cation concentration (2.5 mM), we investigated if the octameric c-di-GMP and the octameric 2'-OMe-c-di-GMP showed peroxidation enhancement even at low cation concentration. At low cation concentration (2.5 mM K^+), octameric species of **3** (the 2'-OMe analog of c-di-GMP) produced about 50% more peroxidation product (ABTS$^{+•}$) than c-di-GMP **2**

after 2 min (see Figure 2.4B). A recent report also demonstrated that 2'-OMe analogs of guanine-rich DNAs were better peroxidases than unmodified guanine-rich DNAs.[9] In agreement with another previous study, peroxidation in the presence of NH_4^+ was higher than in the presence of K^+ (compare Figures 2.5A and 2.5B).[5] Octameric c-di-GMP, when bound to hemin, oxidizes ABTS using H_2O_2 with a k_{cat} of 4 s^{-1} (see Table 2.1). This is comparable to DNA peroxidases, PS2.M and c-Myc, which have a k_{cat} of 5 and 11 s^{-1} respectively (see Table 2.1). Interestingly amongst these three peroxidases, c-di-GMP has the lowest K_m for H_2O_2 (1.8 mM) compared to the DNA peroxidases tested, which have K_m for H_2O_2 ranging from 3.3 to 14 mM (see Table 2.1). Catalytic efficiency (k_{cat}/K_m) is highest for c-di-GMP (2×10^3 $M^{-1}s^{-1}$) and lowest for the anti-parallel G-quadruplex, TBA (0.1×10^3 $M^{-1}s^{-1}$; see Table 2.1). Earlier efforts to design small-molecule guanine-based peroxidases turned out inefficient peroxidases, so the demonstration that c-di-GMP octaplex is as efficient a peroxidase as DNA G-quadruplexes is significant.

Table 2.1. Kinetic parameters for peroxidation catalyzed by various G-quadruplex forming entities. [octameric c-di-GMP or DNA] = 10 μM, [Hemin] = 0.1 μM, [ABTS] = 2 mM, [H_2O_2] = 0.5, 1, 2, 3 and 4 mM, [NH_4Cl] = 250 mM in addition to 10 mM KCl already present from c-di-GMP/DNA stock solution. Buffer: 50 mM Tris-HCl (pH = 7.9). Kinetic analysis for some assembled catalysts show high error. It is expected that a more precise kinetic analysis could be performed with stop flow instrumentation.

G-Quadruplex	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)/10^3
Octameric c-di-GMP	1.8 ± 0.6	4 ± 1	2 ± 1
c-Myc	8 ± 5	11 ± 7	1 ± 1
PS2.M	3.3 ± 0.8	5 ± 1	1.4 ± 0.4
TBA	14 ± 7	2 ± 1	0.1 ± 0.1

Nucleic acid peroxidases, as well as the hemin co-factor, are anionic (hemin **4**, contains two ionizable carboxylic acid moieties, see Figure 2.6). We wondered if a co-factor with less negative charge or even better, with positive charge, would bind better to these anionic peroxidases and promote the peroxidation reaction better than the dianionic hemin co-factor. Towards this end, we prepared new hemin analogs **5** and **6** (see Figure 2.6). Modification of one of the carboxylic acid in hemin with an ammonium group, compound **5**, actually slowed the peroxidation reaction rate by 43-74% (compare Figures 2.7A and 2.7B) whereas modification of both carboxylic groups with two ammonium groups, compound **6**, nearly eliminated peroxidase activity. It therefore appears that at least one carboxylate is required to effectively catalyze the peroxidation reaction of nucleic acid peroxidases (see Figure 2.7). This is

similar to natural protein-based peroxidases, in which it has also been shown that the carboxylic acid moieties in the porphyrin co-factor are necessary for efficient catalysis. In horseradish peroxidase (HRP), the carboxylic groups are required for the proper alignment of heme in the binding pocket.[23] Substitution of the carboxylic acids with methyl esters resulted in a dramatic drop in oxidation rate of HRP.[23] The lower peroxidation rate obtained when analogs **5** or **6** was used (compared to hemin) is not due to an inability for the hemin analogs to bind to G8 c-di-GMP or DNA quadruplex. In fact, the positively charged hemin analogs bind to octameric c-di-GMP better than hemin (see Table 2.2 and Figures 2.8 and 2.9). The dissociation constants of c-Myc G-quadruplex/analog **5** or **6** complexes are similar to the hemin complex (Table 2.2).

4 X = OH Y = OH
5 X = NH(CH$_2$)$_2$N$^+$(CH$_3$)$_3$ Y = OH
6 X = NH(CH$_2$)$_2$N$^+$(CH$_3$)$_3$ Y = NH(CH$_2$)$_2$N$^+$(CH$_3$)$_3$
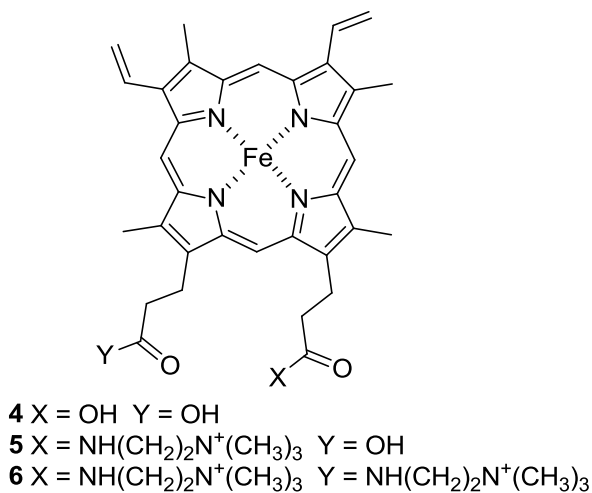
Figure 2.15. Hemin and carboxylate modified analogs.
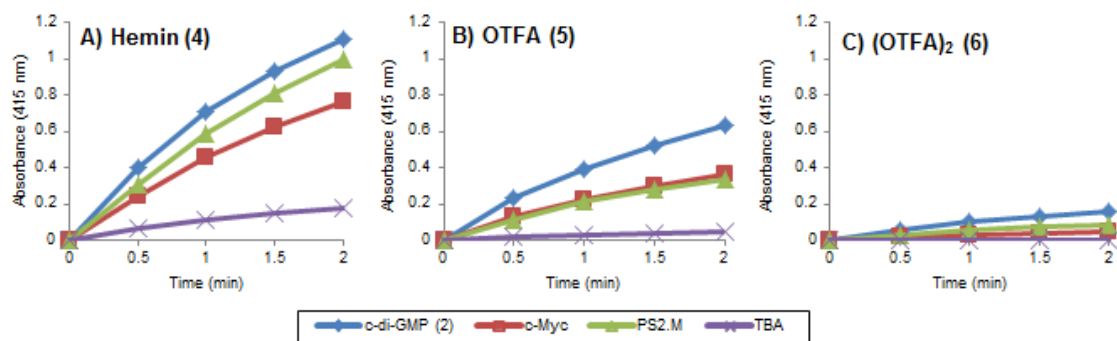
Figure 2.16. Peroxidation in the presence of different hemin analogs. Conditions: [octameric c-di-GMP] = 10 μM ([monomeric c-di-GMP] = 80 μM), [Hemin]/[Hemin analog] = 0.1 μM, [ABTS] = 2 mM, [H_2O_2] = 2 mM, [NH_4Cl] = 250 mM in addition to 10 mM KCl already present from c-di-GMP/DNA stock solution. Buffer: 50 mM Tris-HCl (pH = 7.9). Measurements shown are single trials.
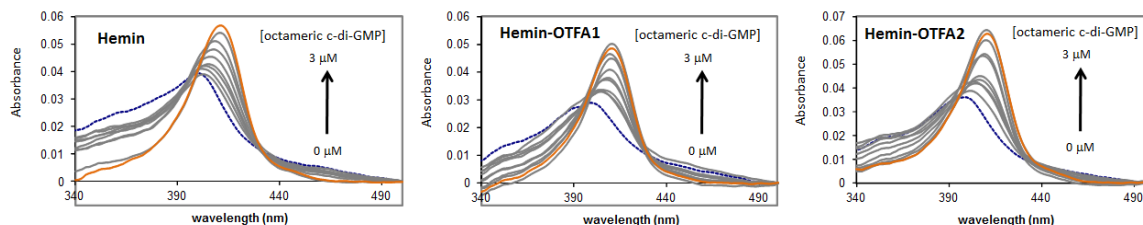
Figure 2.17. Dissociation constant determination of hemin and hemin analogs to octameric c-di-GMP. Conditions: [octameric c-di-GMP] = 0, 0.1, 0.15, 0.2, 0.25, 0.3, 0.4, 0.5, 1.0, 1.5 and 3 μM, [Hemin/Hemin analog] = 1 μM, buffer: 50 mM Tris-HCl (pH = 7.9), [NH_4Cl] = 250 mM, [KCl] = 3 mM, Triton = 0.05%. Dotted navy blue line = no added c-di-GMP; Orange line = 3 μM c-di-GMP. Samples contained a total concentration of 2% (v/v) DMSO. Spectra were measured in triplicate.

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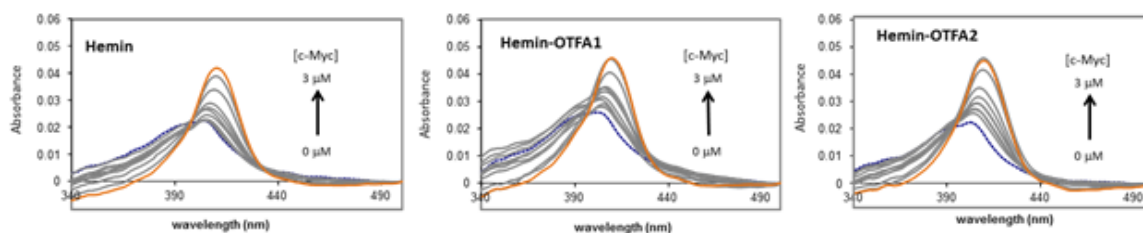
Figure 2.18. Dissociation constant determination of hemin and analogs to c-Myc.

Conditions: [c-Myc] = 0, 0.1, 0.15, 0.2, 0.25, 0.3, 0.4, 0.5, 1.0, 1.5 and 3 μM,

[Hemin/Hemin analog] = 1 μM, buffer: 50 mM Tris-HCl (pH = 7.9), [NH$_4$Cl] = 250

mM, [KCl] = 3 mM, Triton = 0.05%. Dotted navy blue line = no added c-Myc;

Orange line = 3 μM c-Myc. Samples contained a total concentration of 2% (v/v)

DMSO. Spectra were measured in triplicate.

Table 2.2. Dissociation constants (K$_d$) of G-quadruplexes (octameric c-di-GMP and c-

Myc) and hemin/analogs. Due to the insensitivity of UV-visible spectroscopy and

high signal to noise ratio of the measurements made, high errors are observed for

dissociation constants of hemin/analogs and G-quadruplexes.

G-Quadruplex	Hemin 4	Hemin-OTFA 5	Hemin-(OTFA)$_2$ 6
Octameric c-di-GMP	90 ± 30 nM	50 ± 20 nM	12 ± 7 nM
c-Myc	30 ± 10 nM	40 ± 30 nM	30 ± 40 nM

2.3   Conclusion

Octameric c-di-GMP, a "loopless" model for parallel structure G-quadruplex, has

been used to investigate the effects of DNA G-quadruplex loops on catalysis. Our

data shows that the loops in DNA/RNA peroxidases are not necessary for catalysis.

This work also supports the hypothesis of an open hemin binding site whereby the

hemin is end-stacked on a G-quadruplex moiety. Furthermore, it has been shown that the carboxylic functionalities of the hemin play an important role in efficiency of the nucleotide-based peroxidases. Green catalysts that work in water, such as G-quadruplex-based catalysts, are desirable and a few reports have shown that G-quadruplex-based catalysts could be used for the synthesis of fine chemicals in water.[6, 24] C-di-GMP, can be synthesized and purified on a larger scale[21] than DNA and so it is exciting that supramolecular aggregates of c-di-GMP could mimic DNAzymes. We anticipate that c-di-GMP G8 would be able to catalyze other reactions, similar to what has been done with DNA G-quadruplexes.[6, 24, 25]

2.4. *Experimental*

Materials

ABTS[2-] (2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) diammonium salt and hemin (chloro[3,7,12,17-tetramethyl-8,13-divinylporphyrin 2,18-dipropanoato(2-)]iron(III)) were purchased from Sigma Life Science. C-di-GMP and analog were made using the Jones protocol.[21] DNA was ordered from Integrated DNA Technologies and was used without further purification. DNA sequences were as follows: TBA: 5'-GGT TGG TGT GGT TG-3'; c-Myc: 5'-TGA GGG TGG GGA GGG TGG GG AA-3'; PS2.M: 5'- GTG GGT AGG GCG GGT TGG-3'.

Instrumentation

UV measurements were performed on a JASCO V-630 equipped with a Peltier-type temperature controller. CD measurements were performed on a JASCO V-810.

General preparation of octameric and monomeric c-di-GMP

Octameric c-di-GMP was prepared following literature protocol.[19] Briefly, a 2 mM stock solution of c-di-GMP or 2'-OMe analog in a buffer containing 250 mM KCl, 5 mM sodium phosphate and 10 mM EDTA (pH = 7.5) was kept at 4 °C for 24 h to form the octameric species (> 95%). Since 8 molecules of c-di-GMP would form an octameric species, the concentration of octameric c-di-GMP was estimated to be approximately 250 μM. Once formed, octameric c-di-GMP is stable, even in diluted concentrations, for a few hours without disaggregating into monomeric form.[19]

For the preparation of monomeric c-di-GMP, 20 or 40 μM of c-di-GMP was heated to 95 °C and then cooled to room temperature.

Colorimetric peroxidation assay

The experiment was performed in a solution consisting of c-di-GMP/2'-OMe-c-di-GMP or DNA (diluted before the measurement and used within 1 h) and hemin (0.5 or 0.1 μM), H_2O_2 (2 mM), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt ($ABTS^{2-}$, 2 mM), in buffer (50 mM Tris-HCl (pH = 7.9) containing no extra salt). For experiments containing salt, salt solutions were added separately to each sample. Absorbance changes at 415 nm were monitored to characterize the rate of oxidation of $ABTS^{2-}$. Measurements were taken in either a 0.1 cm or 1 cm cuvette at 20 °C.

Circular dichroism experiments

CD experiments were performed at 10 °C in a 1 cm path length cuvette. The concentration of c-di-GMP and the 2'-OMe analog in 250 mM Tris-HCl buffer containing no extra salt (pH = 7.5) was 40 μM. CD measurements from 220 to 340

nm were taken. The data pitch was 1 nm. The scan speed was 50 nm/min. The response was 8 sec. The band width was 1 nm. Each spectrum was corrected by subtracting the CD of the buffer.

Calculation of kinetic parameters

The peroxidation rate of ABTS was measured using different concentrations of H_2O_2 (0, 0.5, 1, 2, 3 and 4 mM) and plotted using the Lineweaver-Burke plot to obtain K_m and V_{max}. The k_{cat} was subsequently calculated using:

$$K_{cat} = V_{max}/[E] \qquad \text{Eq. 1}$$

Where [E] is active enzyme which was taken as the concentration of hemin as the [DNA or c-di-GMP] >>> [hemin], implying that greater than 98% of the hemin was bound to the nucleic acid.

Calculation of Dissociation constants (K_d)

Dissociation constant (K_d) was determined by the method described by Wang et al.[26] The absorbance change in the Soret band was plotted as a function of DNA concentration and fitted by the equation:

$$[DNA]_0 = K_d \frac{A - A_0}{A_\infty - A} + [P_0] \frac{A - A_0}{A_\infty - A_0} \qquad \text{Eq. 2}$$

Where $[DNA]_0$ is the initial concentration of DNA/c-di-GMP, $[P]_0$ is the initial concentration of hemin/hemin analog, A_∞ is the hemin absorbance at saturating DNA

concentration and A_0 is the hemin absorbance in the absence of DNA. Samples were allowed to equilibrate for 30 min after addition of hemin to DNA samples.

Synthesis and purification of hemin analogs

Hemin (0.37 g, 0.545 mmol) was dissolved in 15 mL anhydrous DMF to which was added N,N'-dicyclohexylcarboiimide (0.21 g, 1.0 mmol), N-hydroxysuccinimide (0.15 g, 1.3 mmol) and (2-aminoethyl)trimethylammonium chloride hydrochloride (0.17 g, 0.99 mmol). The reaction was allowed to stir for 6 h. The reaction mixture was then filtered to remove solids and vacuum evaporated to remove solvent. Purification was carried out on a Varian Prostar HPLC equipped with a 250 x 10 mm Varian Pursuit Diphenyl column. Solvent used were 0.1% (v/v) TFA in water (solvent A) and 0.1% (v/v) TFA, 9.9% (v/v) water in acetonitrile. Solvent gradient utilized is outlined in Table 2.3.

Table 2.3. Solvent gradient for purification of hemin analogs synthesized in this study.

Time (min.)	Temperature (°C)	Flow Rate (ml/min.)	% A	% B
0	65	3	100	0
2	65	3	65	35
32	65	3	35	65
34	65	3	0	100
44	65	3	0	100

The monosubstituted hemin-(OTFA), **5**, eluted at 17.8 minutes whereas the disubstituted hemin-(OTFA)$_2$, **6**, eluted at 12.2 minutes as the respective TFA salts. Because the iron in hemin is paramagnetic and would broaden NMR peaks, the

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identity of the analogs were confirmed by high resolution electrospray mass

spectrometry (positive mode) and UV spectroscopy (by comparing the UV profile of

hemin with that of analogs. The hemin moiety of the analogs was not modified so it

was expected that the UV profiles (see Figure 2.10) of the analogs and hemin would

be similar. MS characterization, ESI positive mode: Hemin-(OTFA), expected:

1012.8600 observed: 1012.7316; hemin-$(OTFA)_2$, expected: 814.6730, observed:

814.5472)
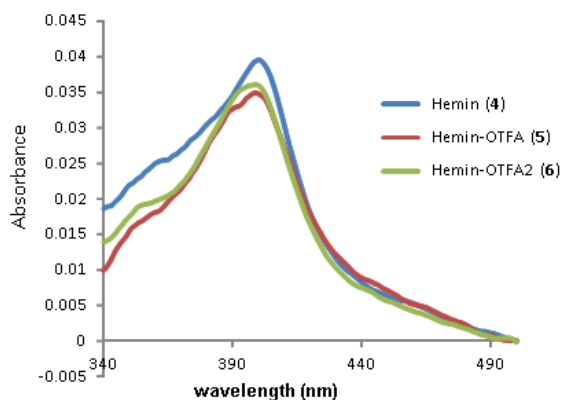
Figure 2.19. UV-vis spectra of hemin and synthesized hemin analogs **5** and **6**.

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Chapter 3: A cyclic dinucleotide containing 2-aminopurine is a general fluorescent sensor for c-di-GMP and 3',3'-cGAMP

B. T. Roembke carried out synthesis of 3',3'-cG(d2AP)MP and other cyclic dinucleotides, CD measurements and all fluorescent studies described herein. J. Zhou, D. Sayre and L. Bernard synthesized and purified c-di-GMP, cGAMP and c-di-AMP used in this study. Y. Zheng expressed and purified WspR and carried out HPLC cleavage assays. A. Lizardo assisted in synthesis of 3',3'-cG(d2AP)MP.

3.1.   Introduction

Cyclic dinucleotides (Figure 3.1) have piqued the interests of scientists of different disciplines in the last few years due to the increasingly diverse processes that they regulate in both prokaryotes and eukaryotes. In bacteria, c-di-GMP and c-di-AMP have been shown to regulate multitudes of processes, including antibiotic resistance,[1] cell wall remodeling,[2] biofilm formation,[3] virulence or toxin production,[4] resistance to heavy metals and others.[5] These bacterial second messengers achieve their biological effect via binding to proteins[5] and RNA riboswitches.[6] Recently it was shown that bacteria also produce the mixed cyclic dinucleotide, 3',3'-cGAMP (3',3' implies that both G and A have a 3'5'-phosphodiester linkage; hereinafter referred to as cGAMP) and that cGAMP affects bacterial chemotaxis and colonization.[7] Eukaryotes have not been shown yet to produce c-di-GMP or c-di-AMP but nonetheless, these molecules affect eukaryotic physiology via binding to the immune modulator STING.[8] Recently it has been shown that eukaryotes also produce 2',3'-

cGAMP (containing 2',5' and 3',5' linkages).[9] cGAMP production by cGAMP

synthase is initiated when DNA is present in the cytosol (which usually implies the

presence of a foreign genetic material or even self DNA, both of which signifies

danger). The produced cGAMP then binds to STING to activate the production of

type I interferons.[10] The newly discovered roles for cyclic dinucleotides in both

prokaryotes and eukaryotes have provided new targets to develop therapeutics against

diseased states ranging from cancer, autoimmune disease, bacterial infection and even

viral infection, such as HIV.[11] Consequently new tools that would facilitate the

specific detection of these cyclic dinucleotides would aid studies that aim to

understand how environmental conditions (e.g. nutrients and antibiotics) affect the

intracellular production or degradation of these nucleotides and also could be used to

discover new small molecules that inhibit cyclic dinucleotide metabolism proteins.
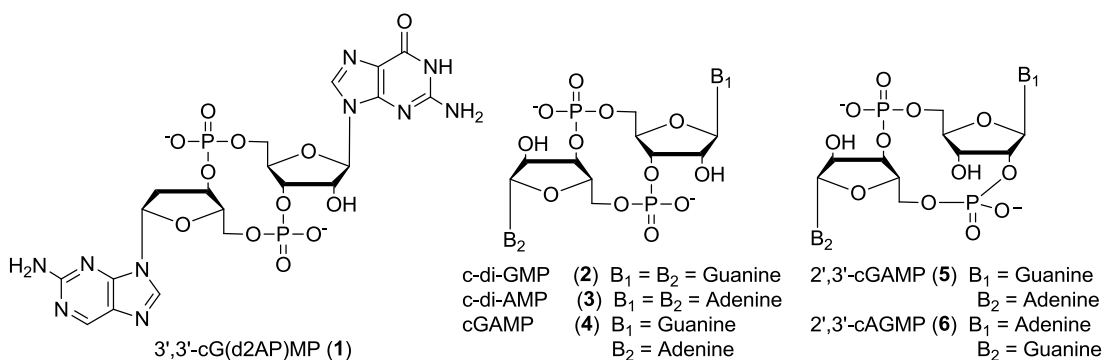
Figure 3.20. Structure of cyclic dinucleotides used in this study.

Few methods have been developed for the detection of cyclic dinucleotides or to

monitor the synthesis or degradation of cyclic dinucleotides.[1, 12-21] Furthermore, these

assays utilize radioactive TLC (for all dinucleotides for which the ^{32}P-labeled form

can be synthesized via enzymatic means), HPLC-MS/MS (c-di-GMP/c-di-

AMP/cGAMP),[12] circular dichroism (c-di-GMP),[13] conditional riboswitch (c-di-GMP),[14, 15, 20] or intercalator-based methods that utilize the ability of c-di-GMP to form G-quadruplexes.[16-18] The aforementioned methods, although sensitive, either use expensive instrumentation that might not be readily accessible for biologists researching nucleotide signaling or are difficult/inconvenient to use. The thiazole orange assay, which was disclosed by the Sintim group, is simple to use but requires high concentrations of monovalent cations and a long incubation time to form the fluorescent c-di-GMP aggregate so it is most appropriate for end-point, but not real-time, assay. A fluorescent riboswitch assay, using Spinach riboswitch, to detect c-di-GMP was also disclosed by Sintim and coworkers[14] and this method has been adapted for the in vivo detection of c-di-GMP by others.[20] Although this assay is sensitive, it utilizes an RNA sensor, which has to be handled carefully to avoid adventitious degradation by RNAses. Additionally, time required for adequate c-di-GMP binding it lengthy, requiring overnight incubation. An interesting whole-cell assay to detect c-di-GMP has also been described.[21] A complementary assay that does not utilize proteins or transformation (since not all bacterial strains can be readily transformed with plasmids) and which can be used in real-time to monitor enzymatic activity could be broadly useful for the cyclic dinucleotide signaling field.

3.2.   *Results and Discussion*

Herein we describe a convenient assay for the detection of cyclic dinucleotides that can be used in real-time to monitor the enzymatic activities of cyclic dinucleotide

synthases or phosphodiesterases and hence, has the potential to be used in high-throughput screening assays for inhibitors of these enzymes. Cyclic dinucleotides are known to form self-intercalated dimers and divalent cations are known to promote the dimerization of cyclic dinucleotides.[22] We therefore hypothesized that the two nucleobases of a 2-aminopurine (2AP) analog of cGAMP (3',3'-cG(d2AP)MP, **1**) cannot interact with each other because the distance between the nucleobases of a 3',3'-cyclic dinucleotide is ~6.7 Å,[23] which is too far for effective orbital overlap for π-π stacking interaction. However upon dimerization with self or other cyclic dinucleotides, the 2AP nucleobase in 3',3'-cG(d2AP)MP would π-stack with another nucleobase and the fluorescence of the 2AP would be quenched (see Figure 3.2).[24] Therefore at low concentrations of 3',3'-cG(d2AP)MP, when it will mainly exist in the monomeric form, it would form heterodimers with other natural cyclic dinucleotides and the heterodimerization could be detected via fluorescence reduction.
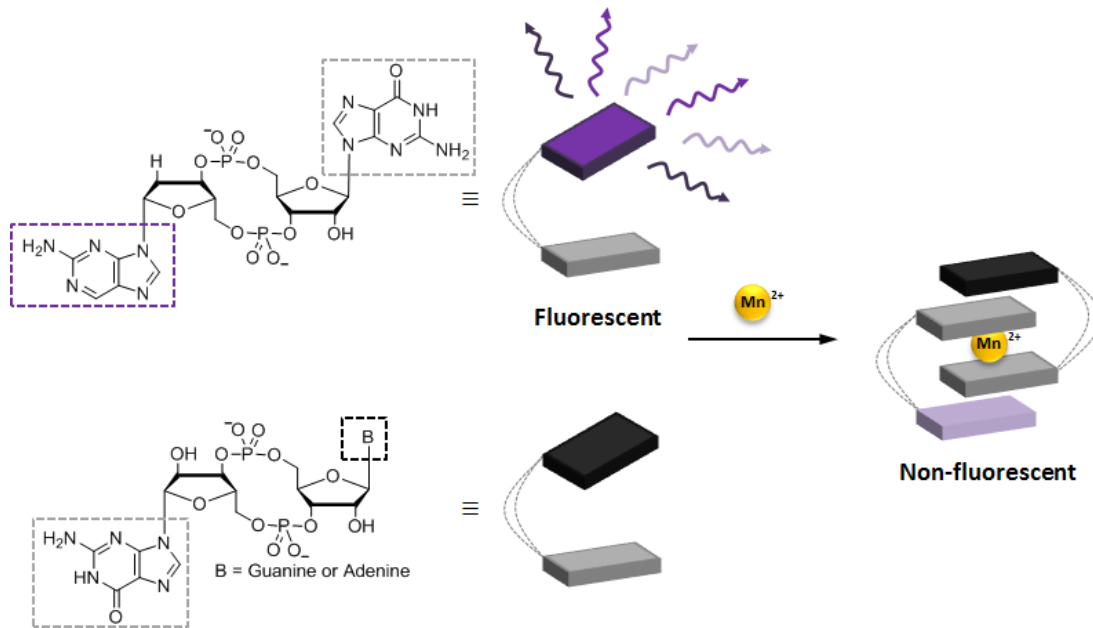
Figure 3.21. Our working hypothesis is that the 2AP cyclic dinucleotide would form heterodimers with natural cyclic dinucleotide resulting in fluorescence quenching.
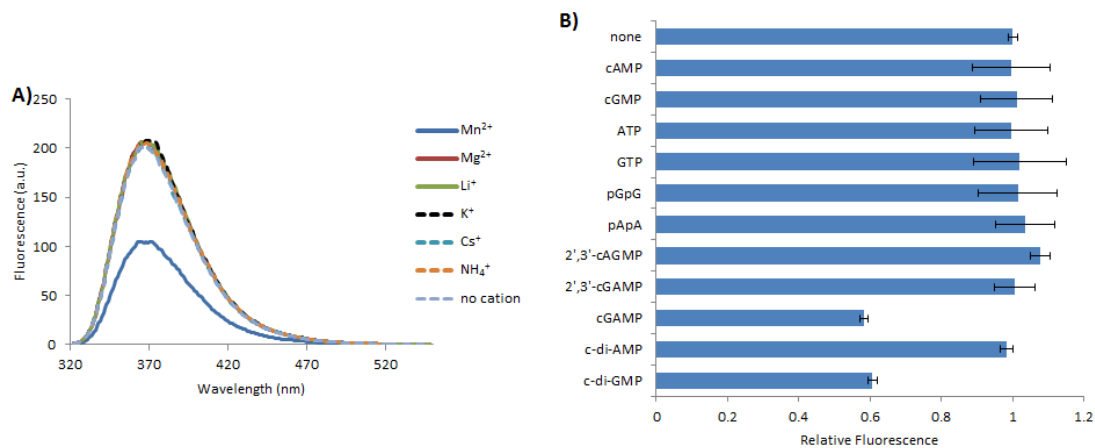
Figure 3.22. A) The fluorescence of 3',3'-cG(d2AP)MP in the presence of different cations: B) Relative fluorescence intensity of 3',3'-cG(d2AP)MP in the presence of different linear and cyclic nucleotides. Conditions: A) 10 µM 3',3'-cG(d2AP)MP, 15 mM divalent salt or 250 mM monovalent salt in 50 mM Tris-HCl buffer (pH = 7.5). B) 1 µM 3',3'-cG(d2AP)MP, 10 µM cyclic or linear nucleotide, 15 mM MnCl$_2$ in 50

mM Tris-HCl buffer (pH = 7.5). Excitation = 310 nm. Fluorescence emission was recorded at 369 nm and compared to the emission of 3',3'-cG(d2AP)MP without any added nucleotide to give relative fluorescence. All data was measured in triplicate.

The fluorescence of 10 µM 3',3'-cG(d2AP)MP in the presence of Mn^{2+} (15 mM) was 50% less than that in the presence of monovalent cations (Li^+, K^+, NH_4^+, Cs^+) or Mg^{2+} or when no cation was present (Figure 3.3A), suggesting that Mn^{2+} promoted the dimerization of 3',3'-cG(d2AP)MP. This was in line with earlier observation by Cutruzzola and co-workers who showed that c-di-GMP also forms self-intercalated dimer, which could be observed in a CD spectrum, in the presence of Mn^{2+}.[13] Without direct evidence, we cannot conclude with certainty that the inability of Mg^{2+} or monovalent cations to cause fluorescent quenching in 3',3'-cG(d2AP)MP is because they do not promote dimerization. It is however known that the fluorescence of 2-aminopurine is quenched upon stacking[25] so it is probably that at the tested concentration (10 µM), neither Mg^{2+} nor the tested monovalent cations facilitated significant dimerization of 3',3'-cG(d2AP)MP. Secondly, Gentner et al. have demonstrated, using NMR, that the dissociation constant of a similar dinucleotide, c-di-GMP, in the presence of Mg^{2+} is 520 ± 10 µM, which implies that cyclic dinucleotides that associate with similar association constants would mainly exist as monomers at 10 µM, in the presence of Mg^{2+}.[26]

Next, we investigated the fluorescence of 1 µM 3',3'-cG(d2AP)MP in the presence of 10 µM of several biologically relevant nucleotides (both linear and cyclic mono- and dinucleotides; see Figure 3.3B). Interestingly both c-di-GMP and cGAMP,

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but not 2',3'-cGAMP, 2',3'-cAGMP or c-di-AMP, decreased the fluorescence of 3',3'-cG(d2AP)MP (see Figure 3.3B). Linear dinucleotides, pGpG and pApA, as well as all mononucleotides tested (cGMP, cAMP, ATP and GTP) did not reduce the fluorescence of the 2AP analog of cGAMP. It appears that at least one guanine nucleobase is required for the formation of the heterodimer with 3',3'-cG(d2AP)MP. In addition to the requirement for at least one guanine, circularization of the dinucleotide is also a requirement for dimerization because the linear pGpG, which contains the same number of guanines as c-di-GMP, did not affect the fluorescence of 3',3'-cG(d2AP)MP. We have previously noted that circularization remarkably affects the propensity of dinucleotides to form polymorphs. For example, whereas c-di-GMP could form G-quadruplexes in the presence of aromatic intercalators at low micromolar concentrations, the linear form (pGpG) did not form G-quadruplexes at similar conditions or even when the concentration of the linear form was raised by orders of magnitude.[16, 17, 27] CD spectroscopy has been proposed as a good tool to investigate the dimerization of cyclic dinucleotides. Cutruzzola and co-workers demonstrated that increase in CD intensity, especially between the 260-280 nm region is an indication of dimerization.[13] We therefore used CD to investigate if the reduction of the fluorescence of 3',3'-cG(d2AP)MP by cyclic dinucleotides correlated with propensity to form self-dimers as judged by hyperchromicity in the CD bands between 260 and 280 nm. In the presence of Mn^{2+}, cGAMP, c-di-GMP and the fluorescent analog 3',3'-cG(d2AP)MP formed self-intercalated dimers (compare CD bands in the presence and absence of Mn^{2+}; see Figures 3.4A, 3.4B and 3.4C). On the other hand, at 40 μM nucleotide concentration, the CD spectra of c-di-AMP, 2',3'-

cGAMP or 2',3'-cAGMP did not show major differences between presence and absence of Mn^{2+} (Figures 3.4D, 3.4E and 3.4F), implying that these dinucleotides do not form significant amounts of dimers at the tested concentration (40 µM). Although, c-di-AMP has been shown to form dimers at millimolar concentrations[28] our data confirms earlier observation[13] that in the presence of Mn^{2+} cyclic dinucleotides that contain at least one guanine seem to have a higher propensity to form intercalated dimers than those lacking guanine but contain adenine. Analysis of the crystal structure of dimeric c-di-GMP, in complex with Mn^{2+}, explains why at least one guanine is needed for the effective dimerization in the presence of divalent cations. In dimeric c-di-GMP, Mn^{2+} coordinates with the 06 of guanine via water molecules.[13] Proteins that bind to self-intercalated dimers of c-di-GMP are known[29] but c-di-AMP has not been shown to be bound to any protein in the dimer form. This could be due to the fact that because c-di-AMP is a recently discovered dinucleotide,[30] in contrast to c-di-GMP that was discovered more than two decades ago.[31] Many receptors for c-di-AMP have not been structurally characterized and it may be just a matter of time before a receptor that binds to dimeric c-di-AMP is found.
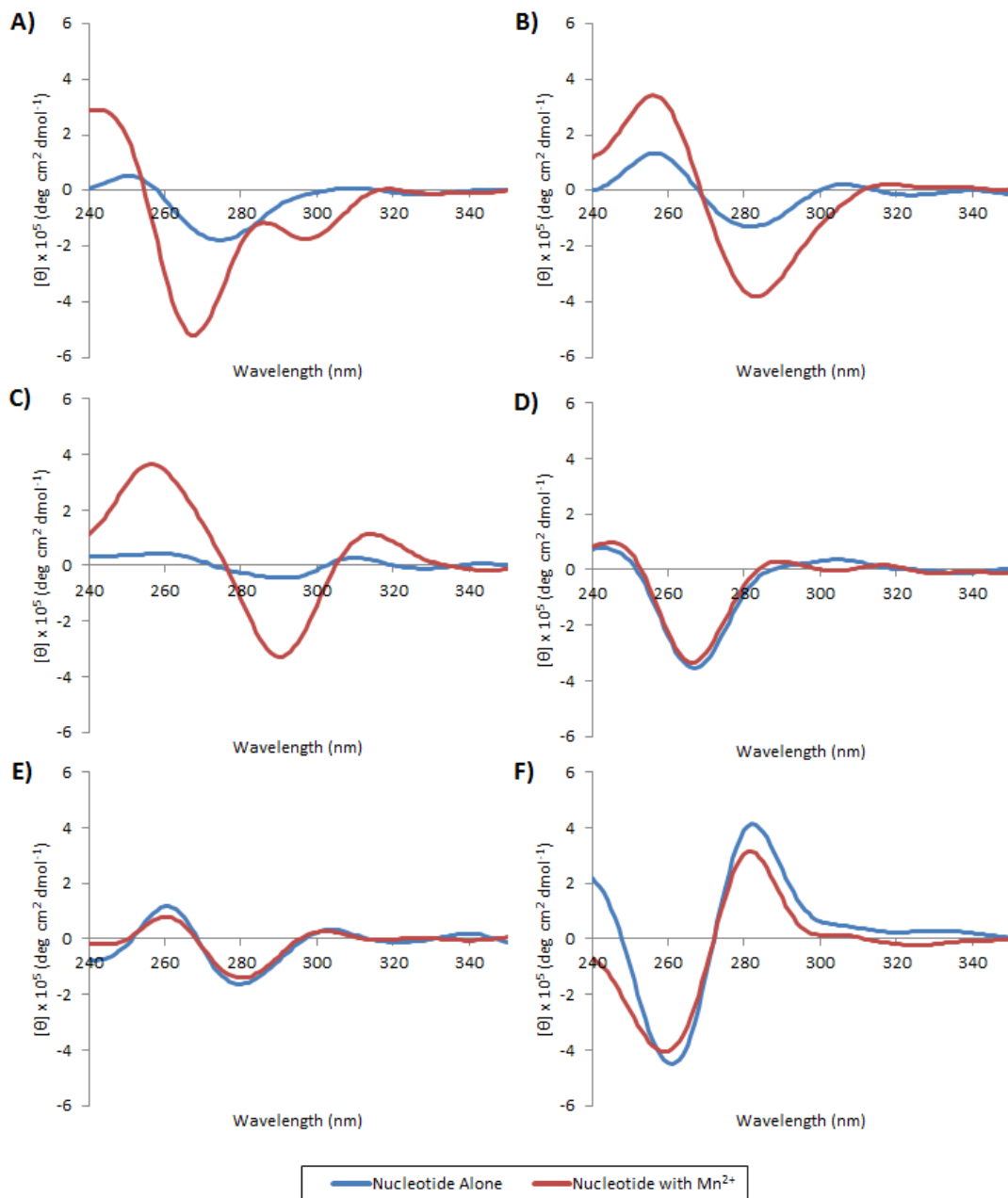
Figure 3.23. CD spectra of cyclic dinucleotides in the presence and absence of Mn^{2+}.

A) GAMP; B) c-di-GMP; C) 3',3'-cG(dAP)MP; D) c-di-AMP; E) 2',3'-cAGMP; F) 2',3'-cGAMP. Conditions: 40 μM cyclic nucleotide, 15 mM Mn^{2+} (where applicable) and 50 mM Tris-HCl (pH = 7.5)

Having established that 3',3'-cG(d2AP)MP can report the presence of c-di-GMP and cGAMP, we proceeded to determine the association/dissociation constant of this cyclic dinucleotide (see Figure 3.5). UV was found not to be a very sensitive method for the determination of dimerization constants of the cyclic dinucleotides studied, so we opted to use the fluorescence of 3'3-cG(d2AP)MP to determine both the homodimerization constant of 3',3'-cG(d2AP)MP and the IC_{50} for the heterodimerization of c-di-GMP and cGAMP with 3',3'-cG(d2AP)MP, *vide infra*.
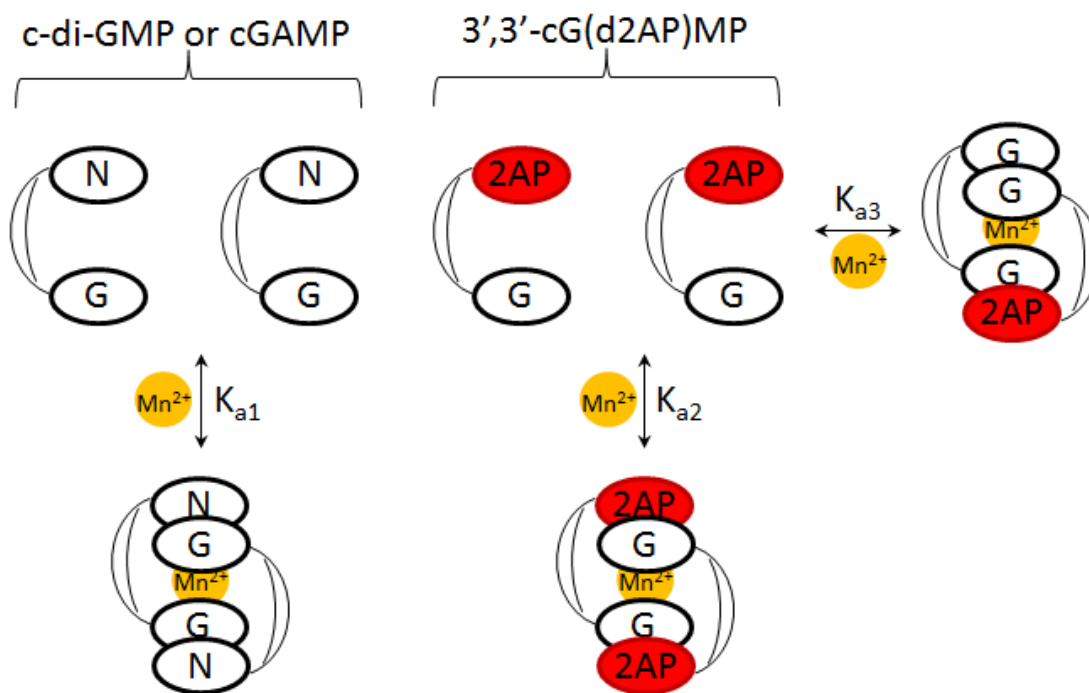
Figure 3.24. Cyclic dinucleotide binding equilibria.

The association constant of 3',3'-cG(d2AP)MP can be defined as follows:

$$K_{a2} = \frac{[2AP_2]}{[2AP]^2} \qquad \text{Eq. 1}$$

Where [2AP] is the equilibrium concentration of 3',3'-cG(d2AP)MP and $[2AP_2]$ is the equilibrium concentration of 3',3'-cG(d2AP)MP dimer. The $[Mn^{2+}]$ can be left out of the expression because $[Mn^{2+}]>>>[2AP]_T$ (the total concentration of 3',3'-cG(d2AP)MP). If $[Mn^{2+}]$ is included however, K_{a2} can be expressed as such:

$$K_{a2}{}^* = \frac{[2AP_2]}{[2AP]^2[Mn^{2+}]^n}$$

Eq. 1a

$$K_{a2} = K_{a2}{}^*[Mn^{2+}]^n$$

Eq. 1b

$$\ln K_{a2} = \ln K_{a2}{}^* + n\ln[Mn^{2+}]$$

Eq. 1c

Therefore, a plot of $\ln K_{a2}$ versus $\ln[Mn^{2+}]$ will give the number of Mn^{2+} associated with the dinucleotide dimer. The total fluorescence of the system (F_T) can be decomposed into two parts, see Eq. 2.

$$F_T = f_1[2AP] + f_2[2AP_2]$$

Eq. 2

Where f_1 and f_2 are fluorescence constants of 3',3'-cG(d2AP)MP and 3',3'-cG(d2AP)MP dimer respectively. Making appropriate substitutions (see experimental section),

$$F_T = \left(\frac{-1 + \sqrt{1 + 8K_{a2}[2AP]_T}}{4K_{a2}[2AP]_T}\right)\left([2AP]_T f_1 - \frac{[2AP]_T f_2}{2}\right) + \frac{[2AP]_T f_2}{2}$$

Eq. 3

Where $[2AP]_T$ is the total concentration of 3',3'-cG(d2AP)MP. Using Eq. 3 and curve fitting software (OriginPro 8), f_1, f_2 and K_{a2}, the homodimerization constant for 3',3'-cG(d2AP)MP, could be determined as 2.1×10^7, ~0 and $1.2 \pm 0.3 \times 10^5$ M^{-1} respectively (see Figure 3.6 and Table 3.2 for K_a and K_d values).
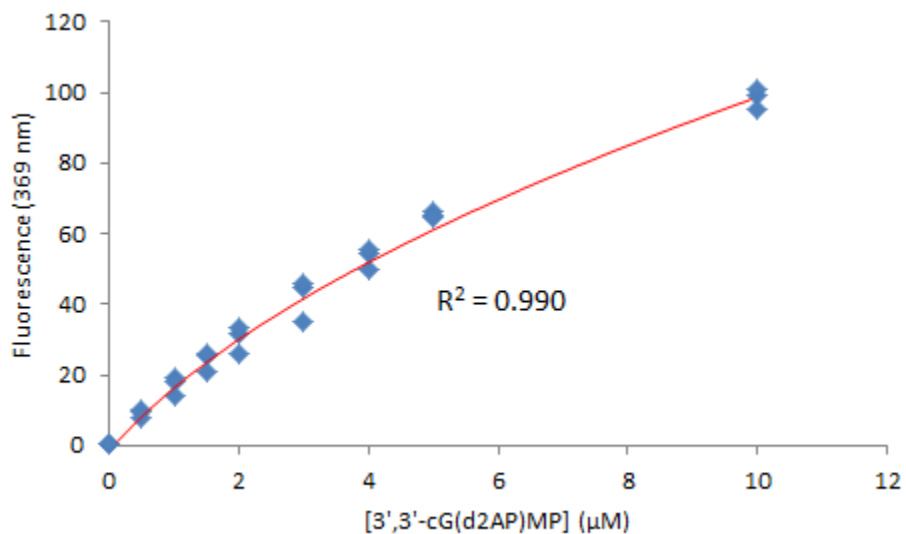
Figure 3.25. A plot of fluorescence intensity versus concentration of 3',3'-cG(d2AP)MP in the presence of 15 mM $MnCl_2$ and fitted using Eq. 3 to obtain the homodimerization constant. Conditions: Varying concentrations of 3',3'-cG(d2AP)MP, 15 mM $MnCl_2$ in 50 mM Tris-HCl (pH = 7.5). Measurements at each concentration were done in triplicate. All fluorescent measurements are shown.
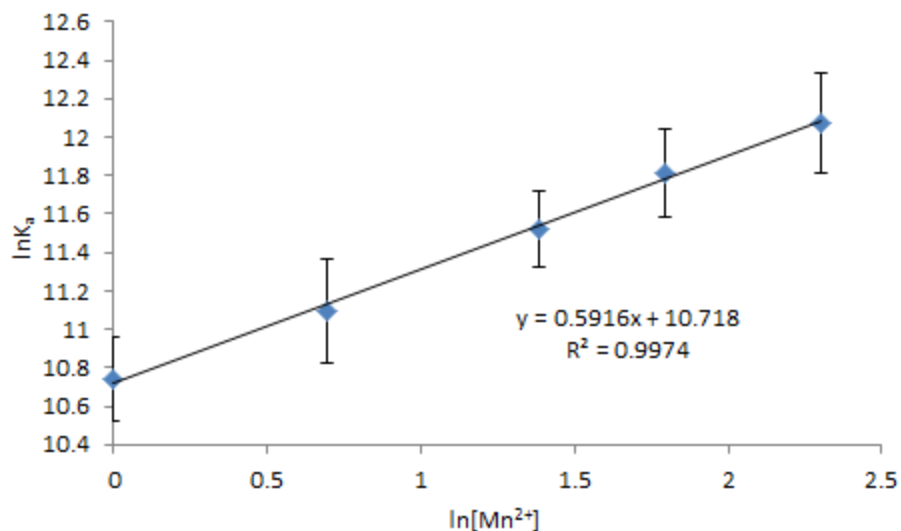
Figure 3.26. A plot giving the binding stoichiometry of Mn^{2+} as the slope. Conditions: Each binding constant was determined using varying concentrations of cG(d2AP)MP, 1, 2, 4, 6 or 10 mM $MnCl_2$ in 50 mM Tris-HCl (pH = 7.5). Association constants were determined in triplicate. Error bars are representative of a single standard deviation of association constants at each concentration of Mn^{2+}.

A plot of lnK_{a2} versus $[Mn^{2+}]$, see Figure 3.7, gave a linear graph with slope 0.59 $\pm$ 0.01 (i.e. the stoichiometry of bound Mn2+) and lnK_{a2}^{*} intercept of 10.72 $\pm$ 0.03. $e^{(lnKa2^{*})}$ = 45468 M^{-1}, is the association constant of 3',3'-cG(d2AP)MP which includes the $[Mn^{2+}]$ term. The value n =0.59 showed a slight deviation from the value obtained using X-ray crystallography (n = 1).[13] One possible explanation of this deviation is the necessary mathematical simplification of the complex equilibria that occurs with cyclic dinucleotides in solution. Additionally, instrumental error associated with fluorescence measurements needed to calculate the association constants at different Mn^{2+} concentrations may have affected the calculated value.

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More recently, however, it has been discovered that a c-di-GMP tetramer (hydrogen bonded dimer of dimers) binds and dimerizes a protein in *Streptomyces coelicolor* known as BldD (Figure 1.4).[32] It is therefore possible that a similar tetramer could be constructed after formation of a single 3',3'-cG(d2AP)MP dimer in the presence of Mn^{2+}. However, *in vitro*, this dimer of dimers lacks the hydrogen bonding contacts provided by the protein structure of BldD.[32] Specifically, an Arginine residue (R114) from each protein monomer facilitates a hydrogen bonding network between the top and bottom pairs of guanine bases in adjacent c-di-GMP dimers.[32] This Arginine accounts for six hydrogen bonds in the c-di-GMP tetramer.[32] Therefore, the tetrameric structure with one coordinated Mn^{2+} (leading to n =0.5) is unlikely. Future research with 3',3'-cG(d2AP)MP may clarify the disparity between the observed Mn^{2+} stoichiometry in the X-ray crystal structure and the fluorescent dimer investigated herein.
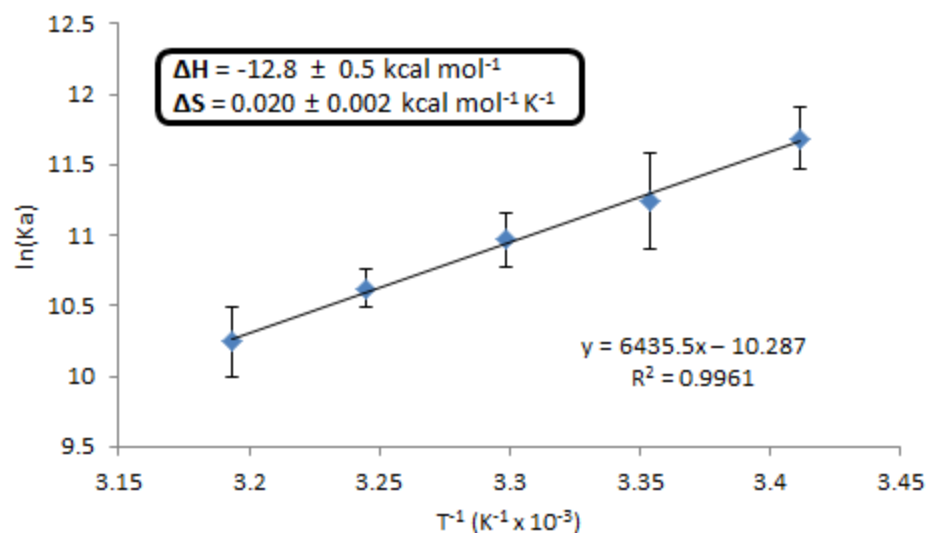
Figure 3.27. Van't Hoff plot of the association constants for 3',3'-cG(d2AP)MP

dimerization at different temperatures. Conditions: Varying concentrations of 3',3'-

cG(d2AP)MP, 15 mM $MnCl_2$ in 50 mM Tris-HCl (pH = 7.5). Each equilibrium

constant was measured in triplicate. Samples were allowed to incubate in the cuvette

at the given temperature for at 15 min prior to measurement.

Table 3.4. Association and dissociation constants for the dimerization of 3',3'-

cG(d2AP)MP. Experiments to determine association and dissociation constants were

performed in triplicate.

T (K)	K_a (M^{-1} x 10^5)	K_d (µM)	ΔG (kcal·mol^{-1})
293.15	1.2 ± 0.3	8 ± 2	-6.8
298.15	0.8 ± 0.3	13 ± 4	-6.7
303.15	0.6 ± 0.1	17 ± 3	-6.6
308.15	0.41 ± 0.06	24 ± 3	-6.5
313.15	0.28 ± 0.07	36 ± 9	-6.4

A Van't Hoff plot revealed a binding enthalpy of -12.8 ± 0.5 kcal·mol^{-1} and entropy of 0.020 ± 0.002 kcal·mol^{-1}·T^{-1} for 3',3'-cG(2AP)MP dimerization (see Figure 3.8 and Table 3.1). The reaction is exergonic and enthalpy driven.

Table 3.5. Thermodynamic parameters at 20 °C of cyclic dinucleotides used in this study. (N.D. = not determined)

Nucleotide	Homodimerization		Heterodimerization [a]
	K_d (μM)	K_a (M^{-1})	IC$_{50}$ (μM)
3',3'-cG(d2AP)MP	8 ± 2	$1.2 \pm 0.3 \times 10^5$	N/A
c-di-GMP	N.D.	N.D.	11 ± 1
cGAMP	N.D.	N.D.	9 ± 1

a) Concentration of 3',3'-cG(d2AP)MP = 1 μM and at this concentration over 91% of this fluorescent probe exists as monomer. The IC$_{50}$ value is a composite value of homodimerization and heterodimerization constants.

When another cyclic dinucleotide, apart from 3',3'-cG(d2AP)MP, is present, the total fluorescence intensity from the 2AP unit becomes,

$$F_T = f_1[2AP] + f_2[2AP_2] + f_3[2AP \cdot cNMP] \qquad \text{Eq. 4}$$

Where f_3 is the fluorescence constant of the heterodimer between c-di-GMP or cGAMP and 3',3'-cG(d2AP)MP. f_2 has already been determined to be ~0 (from curve fitting in Figure 3.6). Both f_2 and f_3 are low (the dimer forms are "darker" than the monomer form due to fluorescence quenching from $\pi-\pi$ stacking of the 2AP fluorophore. The dimer and monomer forms of c-di-GMP or cGAMP are also "dark"

so it is non-trivial to tease out the separate association constants in Figure 3.5 (K_{a1} and K_{a3}), using fluorescence data alone. Attempts to use UV titration to independently determine K_{a1} failed due to the insensitivity of UV. Because Mn^{2+} is paramagnetic, we could not apply NMR methods to determine K_{a1}, as was previously demonstrated for c-di-GMP dimer formation in the presence of Mg^{2+}.[26] We therefore proceeded to determine the IC_{50} of c-di-GMP or cGAMP binding to 1 µM 3',3'-cG(d2AP)MP. We calculated that at 1 µM, over 91% of 3',3'-cG(d2AP)MP would exist as monomer so the IC_{50} values (see Table 3.2) could give a reasonable guide of the concentration ranges that these non-fluorescent analogs form dimers in the presence of Mn^{2+}.
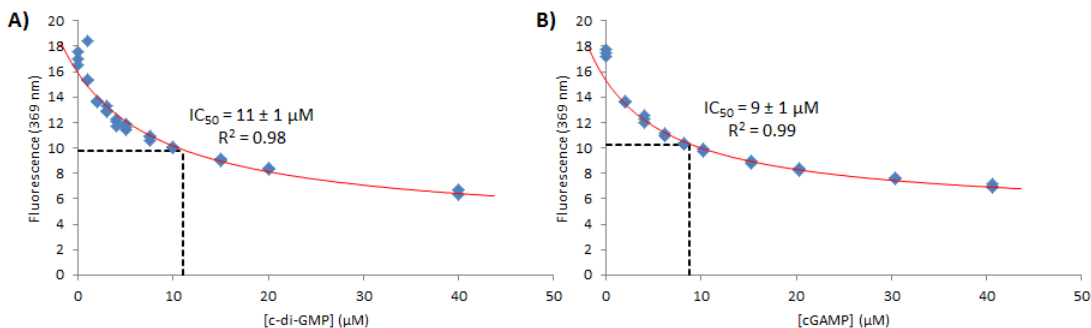
Figure 3.28. A plot of fluorescence intensity (from 3',3'-cG(d2AP)MP) versus concentration of cyclic dinucleotide in the presence of 15 mM MnCl$_2$ and fitted using Eq. 12 to obtain IC_{50} values of A) c-di-GMP and B) cGAMP binding with 3',3'-cG(d2AP)MP.[42] Conditions: 1 µM 3',3'-cG(d2AP)MP, 15 mM MnCl$_2$, 50 mM Tris-HCl (pH = 7.5) and varying concentration of cyclic dinucleotide. All measurements were performed in triplicate. All data is shown.

The c-di-GMP/cGAMP concentration dependence of the fluorescence of 3',3'-cG(d2AP)MP (Figure 3.9) suggested to us that 3',3'-cG(d2AP)MP could be used to monitor the synthesis of c-di-GMP by WspR (Figure 3.10). For the detection of enzymatically synthesized c-di-GMP, we first performed the reaction for 30 min. and then added the fluorescent probe and Mn^{2+} to detect the synthesized cyclic dinucleotide. The Mn^{2+} and the fluorescent probe were added after the c-di-GMP was made because WspR requires Mg^{2+} and not Mn^{2+} for the synthesis of c-di-GMP. This new assay for the detection of enzymatically produced c-di-GMP is complementary to the thiazole orange assay, previously reported by us and it does not require long incubation time, in contrast to the thiazole orange assay.[16] We also hypothesized that upon cleavage of 3',3'-cG(d2AP)MP by phosphodiesterases, the microenvironment of the 2AP moiety would change and would result in a change in fluorescence, hence this probe could be used to monitor the activity of PDE's, such as SVPD. Its inhibition could also be monitored by the addition of EDTA.[33] SVPD could cleave the probe into the linear dinucleotide and this could be monitored in real-time, see Figure 3.11 and Figure 3.12 for the HPLC analysis of the cleavage. For c-di-AMP phosphodiesterase, YybT, cleavage also occurred but this time the reaction was slower than the cleavage by SVPD. Nonetheless the fluorescence of the probe, subjected to YybT, was 81% of the uncleaved 3',3'-cG(d2AP)MP after 140 min (data not shown) so the probe can also be used to discover inhibitors of c-di-AMP-specific PDE's. Pleasingly, this new fluorescent dinucleotide probe, 3',3'-cG(d2AP)MP, could be cleaved by both enzymes and hence one could monitor the inhibition of these enzymes with PDE inhibitors (Figure 3.11).[33, 34]
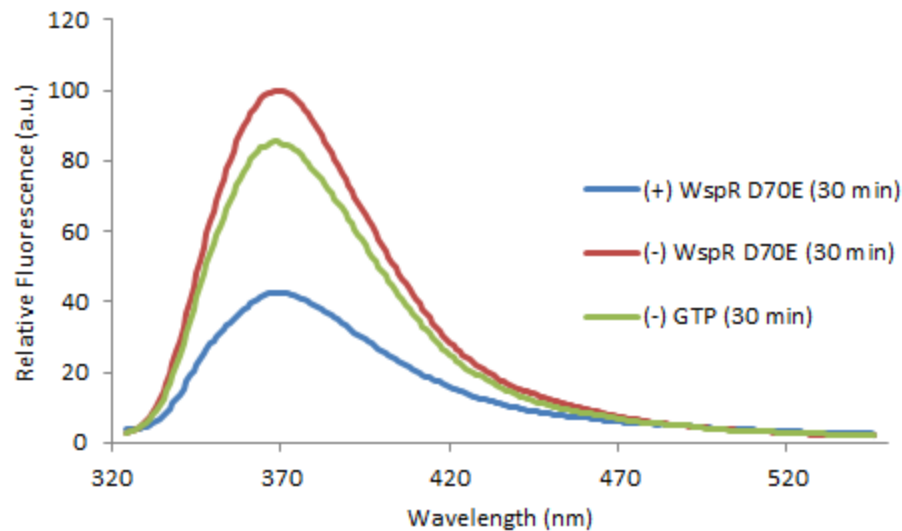
Figure 3.29. The 3',3'-cG(d2AP)MP probe was added to the DGC reaction after 30 min, when c-di-GMP was produced. (+) WspR D70E: contains enzyme and GTP; (-) WspR D70E contains no WsprD70E; (-) GTP contains no GTP. Enzymatic conditions: 10 μM WspR D70E, 100 μM GTP, 100 mM NaCl, 5 mM MgCl$_2$ in 10 mM Tris (pH = 8.0). Sample was allowed to react for 30 min and then heated to 95 °C to stop reaction. Fluorescence conditions: 1 μM 3',3'-cG(d2AP)MP, 15 mM MnCl$_2$, 69/100 dilution of the enzymatic reaction solution in 50 mM Tris-HCl (pH = 7.5). Sample allowed to incubate with 3',3'-cG(d2AP)MP and Mn^{2+} for at least 30 min prior to fluorescence measurement. Spectra are averages of triplicate measurements.
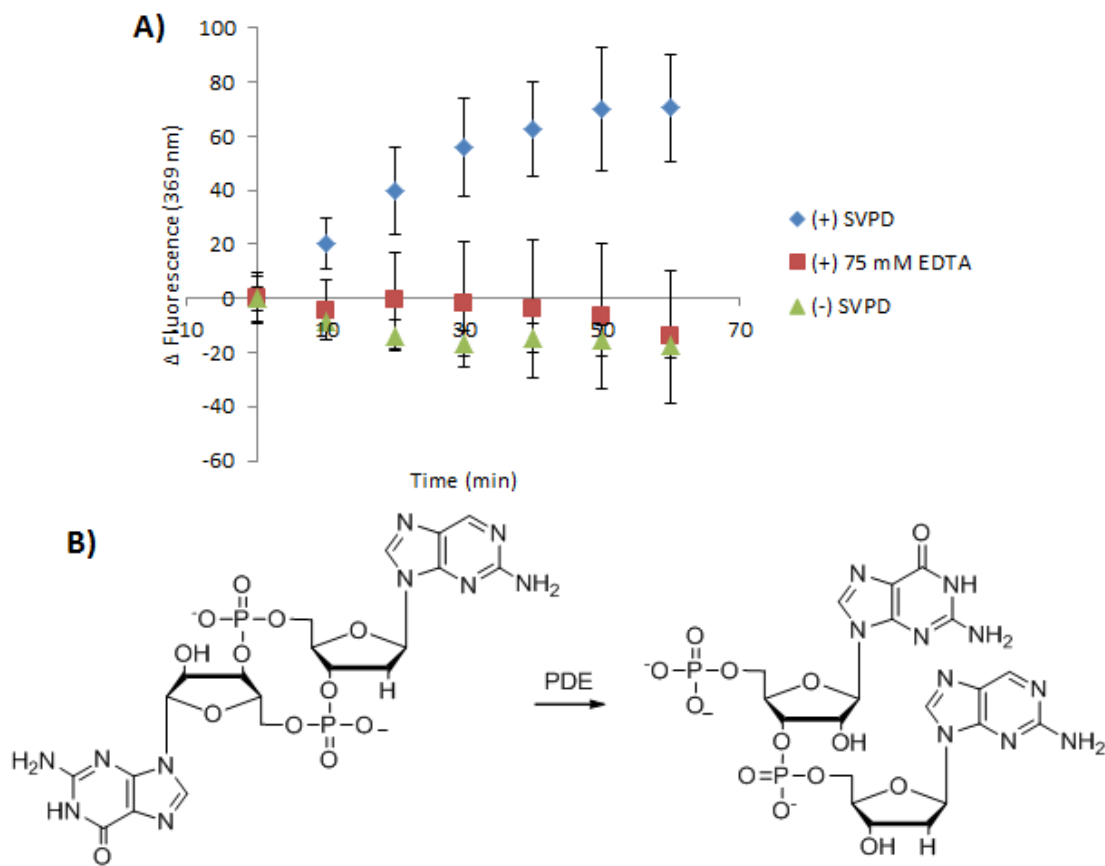
Figure 3.30. A) Cleavage of 3',3'-cG(d2AP)MP by the PDE SVPD indicated by the fluorescence emission of 3',3'-cG(d2AP)MP at 369 nm (Δ Fluorescence = F – F_0; where F_0 is initial fluorescence emission at time and F is fluorescence emission at time = t min). Blue diamonds: enzymatic reaction; Red squares: enzymatic reaction plus the EDTA, an inhibitor; Green triangles: reaction without enzyme. B) Scheme depicting 3',3'-cG(d2AP)MP cleavage by a PDE. Conditions: A) 1 µM 3',3'-cG(d2AP)MP, 15 mM MgCl$_2$, 0.001 units/mL SVPD in 50 mM Tris-HCl (pH = 8.5). Measurements were performed in triplicate.
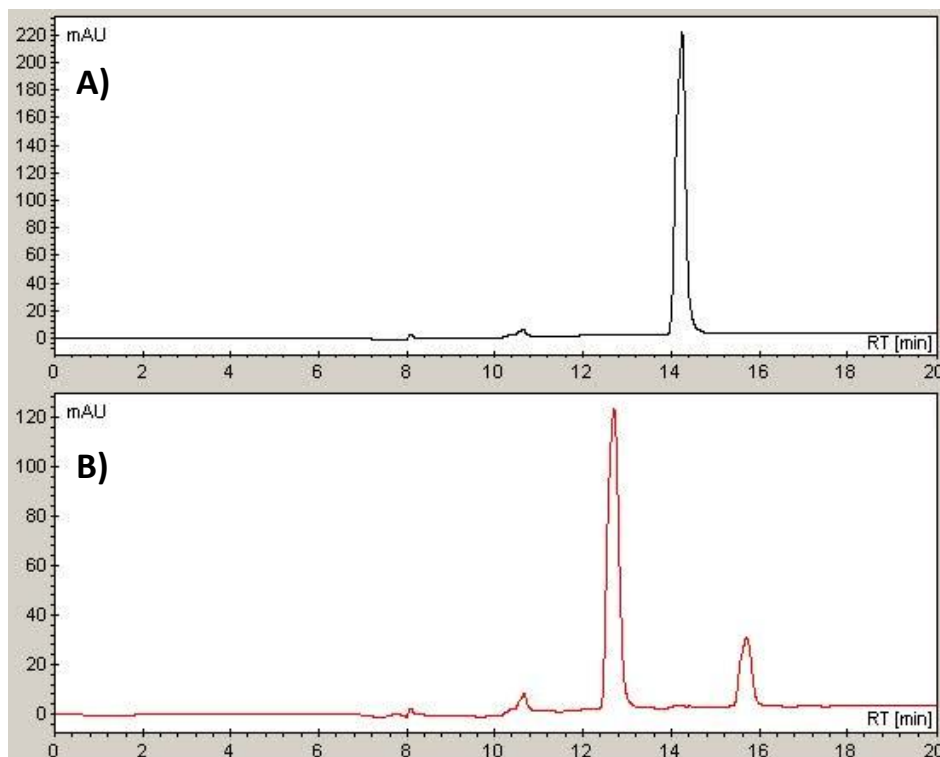
Figure 3.31. HPLC enzymatic cleavage assays. A) 3',3'-cG(d2AP)MP alone and B) 3',3'-cG(d2AP)MP with PDE SVPD. Conditions: A)100 μM 3',3'-cG(d2AP)MP, 15 mM $MgCl_2$ in 50 mM Tris-HCl (pH = 8,5). B) 100 μM 3',3'-cG(d2AP)MP, 15 mM $MgCl_2$ and 0.001 units/mL SVPD in 50 mM Tris-HCl (pH = 8.5).

3.3. Conclusion

We have demonstrated that cyclic dinucleotide analog containing 2-aminoprurine is a general probe that can be used to monitor the enzymatic activities of PDE and DGC and could be a useful tool for high-throughput assays that seek to discover new inhibitors of c-di-GMP metabolism enzymes.[35] Both cGAMP and 2',3'-cGAMP have been observed in nature and the distinction between the two is non-trivial and requires careful HPLC separation followed by MS-MS analysis. 3',3'-cG(d2AP)MP

can discriminate between the 2',3'- and 3',3'-forms of cGAMP and could be used as a confirmatory probe, whenever the phosphodiester linkage of an isolated cGAMP molecule comes into question.

Cyclic dinucleotides are known to aggregate into different polymorphs and it has been shown that both the sugar backbone and nature of nucleobase affect polymorphism.[36] Recently, it has also been demonstrated that both c-di-GMP and c-di-AMP interact with diverse π-systems to form supramolecular aggregates.[16-18, 27, 37] Herein, we use the fluorescent analog to show that under appropriate conditions, c-di-NMPs can also associate with each other to form heterodimers with K_d's that are similar to cytoplasmic concentrations (low single digit micromolar). Considering that some bacteria (such as *V. Cholerae*) are known to make both c-di-GMP and cGAMP,[3, 7] could it be possible that beyond binding of these nucleotides to their respective receptors, different cyclic dinucleotides also directly associate (or "communicate") with each other and intracellular aromatic molecules to affect signaling? Or bacteria may possess a keen ability to spatially separate these signaling molecules in the cell. Cyclic dinucleotide signaling might have another complex layer of interactions that is not yet fully appreciated.

3.4. Experimental

Materials

All reagents were purchased from industrial chemical sources and used without further purification. Pyridine and CH_3CN were distilled over CaH_2 to remove water.

Synthesis of 3',''3-cG(d2AP)MP

0.45 g 5'-Dimethoxytrityl-N-isobutyryl-Guanosine,2'-O-TBDMS-3'-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite **8** was placed in a 100 mL round bottom flask and dissolved in CH_3CN (10 mL). To this flask was added several 4 Å molecular sieves, and then the flask was capped and placed under an Argon atmosphere. To another 250 mL round bottom flask was added 0.25 g 5'-Dimethoxytrityl-N2-(dimethylaminomethylidene)-2'-deoxypurine riboside,3'-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite (**7**) which was dissolved in CH_3CN (10 mL). To this flask, 0.65 g pyridinium trifluoroacetate and H_2O (90 μL) were added. This was allowed to stir for 1 minute. Then 10 mL t-$BuNH_2$ was added and allowed to stir for 10 minutes. This reaction mixture was concentrated to a foam. After concentration, CH_3CN (10 mL) was added and then the mixture was concentrated again. This was repeated one more time. The concentrate was then dissolved in CH_2Cl_2 (25 mL) and H_2O (180 μL). 28 mL of 6% (v/v) DCA in CH_2Cl_2 was then added to this solution. After 10 min of stirring the acid was quenched with pyridine (20 mL). The solution was the concentrated under vacuum and then redissolved in CH_3CN (20 mL). This was repeated three more times. After the last addition of CH_3CN, the flask was capped with a septum and put under an Argon atmosphere. The dry solution of **8** was then transferred by cannula into reaction flask. The flask containing **8** was rinsed twice with CH_3CN (1 mL) which was also transferred into the flask by cannula. The linear coupling was allowed to react for 2 min. Then 1.3 mL anhydrous 5.5 M t-BuOOH was added and allowed to stir for 30 min. The reaction was then quenched by addition of 0.6 g $Na_2S_2O_3$ dissolved in H_2O (1.2 mL). This was allowed to stir for

2 min after which it was concentrated. The reaction mixture was then dissolved in

CH_2Cl_2 (30 mL) and H_2O (500 μL) and then 30 mL 6% (v/v) DCA in CH_2Cl_2 was

added. After 10 min the reaction was quenched by addition of pyridine (20 mL) and

then concentrated. This addition and concentration was repeated two more times.

After the last concentration the flask was capped with a septum and put under an

Argon atmosphere. This concentrate was dissolved in pyridine (10 mL) and 1.6 g 2-

chloro-5,5-dimethyl-1,3,2-dioxaphosphorinane 2-oxide was added. After 10 min H_2O

(1.5 mL) was added which was immediately followed by 0.8 g I_2. This was stirred for

5 min. The reaction mixture was then poured into and an Erlenmeyer flask containing

0.5 g $Na_2S_2O_3$ in H_2O (350 mL). This was allowed to stir for 5 min. To neutralize the

aqueous solution, 9.8 g $NaHCO_3$ was slowly added. The aqueous solution was then

extracted with 1:1 EtOAC:Et_2O (400 mL), EtOAC (400 mL) and 10:1 EtOAC:i-PrOH

(400 mL). Each organic layer was kept separate, concentrated and allowed to dry in a

vacuum desiccator overnight. Each organic extract was then independently subjected

to the following deprotection protocol. To remove excess pyridine, EtOAC (30 mL)

was added to the concentrated organic extract and subsequently concentrated under

vacuum. This was repeated three times in total. The concentrate was then dissolved in

CH_3CN (10 mL) and to which was added 10 mL t-BuNH$_2$. This was stirred for 10

minutes and then concentrated. To the resulting solid was then added CH_3CN (10

mL) and the suspension was sonicated. The suspension was then concentrated, and

this process was repeated two more times. The solid was then dissolved in a minimal

amount of CH_3OH and filtered to remove and remaining solids. The filtrate was

collected and concentrated. The resulting concentrate was then subjected to 20 mL

CH_3NH_2. This was allowed to stir for 90 min. The CH_3NH_2 was then removed under vacuum being careful to prevent bumping of the volatile amine. The concentrate was then dissolved in a 2:1 mixture of pyridine:TEA (6 mL) and concentrated. This was repeated two more times. The concentrate was then dissolved in 2 mL pyridine. The flask was capped with a septum and placed in a preheated 50 °C oil bath. Then, by syringe, TEA (3 mL) and 2 mL TEA·3HF was slowly added over a period of about 1 minute (Note: anything that came in contact with TEA·3HF was washed with a saturated aqueous solution of K_2CO_3). After 90 minutes of stirring the product was precipitated by addition of copious amounts of acetone. The solid was collected by centrifugation and dried in a vacuum desiccator overnight. The solid was then subjected to purification by standard RP-HPLC (see below). ^1H NMR (400 MHz, D_2O) δ 8.48 (s, 1H), 8.33 (s, 1H), 8.07 (s, 1H), 6.36 (d, J = 6.1 Hz, 1H), 5.95 (s, 1H), 5.08 (p, J = 7.5 Hz, 1H), 4.92 (td, J = 8.3, 4.9 Hz, 1H), 4.79 –4.73 (m, 1H), 4.41 (d, J = 8.7 Hz, 1H), 4.34 (d, J = 12.1 Hz, 1H), 4.28 (d, J = 6.0 Hz, 1H), 4.21 (d, J = 11.9 Hz, 1H), 4.11 – 4.03 (m, 2H), 3.08-3.02 (m, 1H), 2.78-2.71 (m, 1H); ^{13}C-NMR (126 MHz, D_2O) δ 160.21, 159.25, 154.62, 153.08, 151.37, 149.14, 143.35, 137.81, 127.80, 116.86, 90.16, 84.17, 83.36, 80.81, 74.18, 72.08, 71.40, 63.25, 62.97, 38.69; ^{31}P-NMR (202 MHz, D_2O) δ -19.14, -19.42; ESI-MS: Negative mode, predicted: 657.0978, observed: 657.0990.
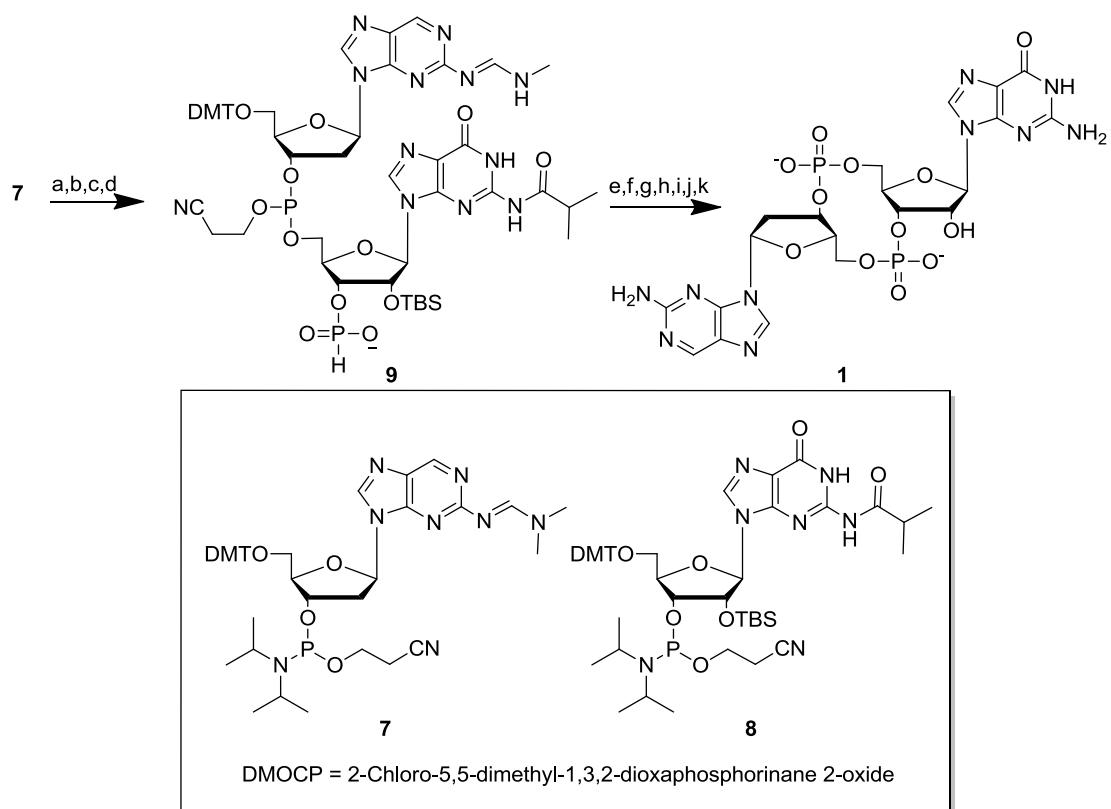
Scheme 3.3. Synthesis of compound **1** a) **7**, pyridinium-TFA, H_2O, CH_3CN b) *t*-BuNH$_2$ c) *i*) 6% DCA in CH_2Cl_2, H_2O, CH_2Cl_2 *ii*) pyridine d) **8** e) *i*) 5.5 M *t*-BuOOH *ii*) $Na_2S_2O_3$, H_2O f) *i*) 6% DCA in CH_2Cl_2, H_2O, CH_2Cl_2 *ii*) pyridine g) 2-chloro-5,5-dimethyl-1,3,2-dioxaphosphorinane 2-oxide h) *i*) H_2O, I_2 *ii*) $Na_2S_2O_3$ i) *t*-BuNH$_2$ j) CH_3NH_2 k) TEA·3HF, TEA, pyridine.

Purification of 3',3'-cG(d2AP)MP

RP-HPLC was performed on a Varian model 325 equipped with a Varian model 210 binary solvent delivery system. A Merck Purospher STAR RP-18 (250 mm x 10 mm; 5 μm) HPLC column was used. The method utilized 0.1 M TEAA buffer (solvent A) and CH_3CN (solvent B). The solvent gradient was as follows:

Table 3.6. RP-HPLC method for purification of 3',''3-cG(d2AP)MP

Time	Flow (ml/min)	% A	% B
0	2	95	5
30	2	90	10
35	2	80	20
40	2	0	100

The peak corresponding to an elution time of 20 minutes was collected and characterized. See Figure 3.13 for HPLC trace.
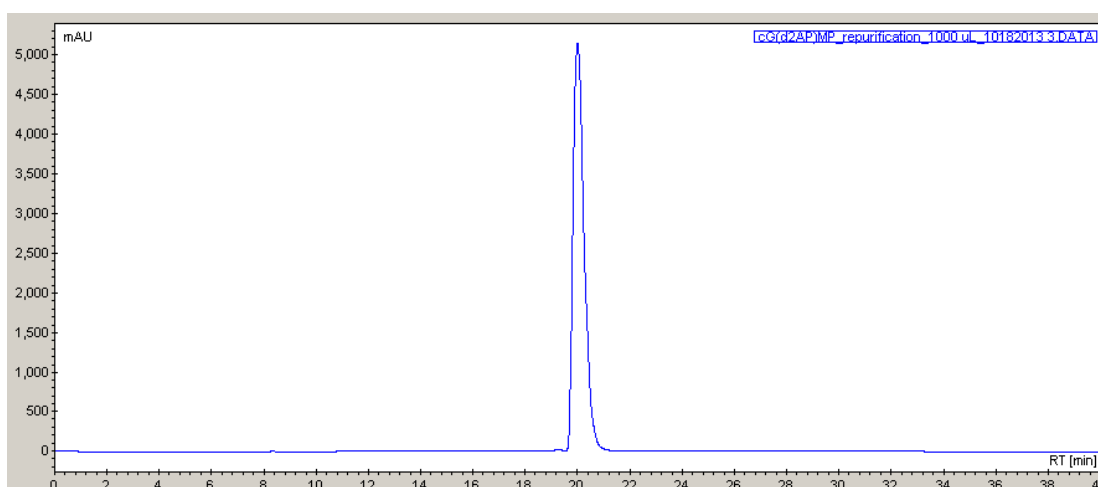
Figure 3.32. RP-HPLC trace of 3',3'-cG(d2AP)MP **1**.

HPLC Methodology for HPLC Cleavage Assay

Total sample volume was 100 μL. Column was a Merck Purospher STAR RP-18 (250 mm x 10 mm; 5 μm). 0.1 M TEAA buffer (solvent A) and CH_3CN (solvent B) were used as the mobile phase with a flow rate of 2 mL/min. Samples containing PDE's were filtered through a 5000 kDa centrifugal filter to remove enzymes. The solvent gradient was as follows:

Table 3.7. Solvent gradient for HPLC cleavage assay

Time	Flow (ml/min)	% A	% B
0	2	95	5
16	2	80	20
18.5	2	10	90
20	2	10	90

Protein overexpresson and purification

WspR, WspR D70E and RocR overexpression were induced by adding 1 mM IPTG at an OD_{600} of 0.6. After 6-hour induction at 30 °C, bacteria cells were collected by centrifugation and resuspended in lysis buffer (10 mM Tris-HCl, pH = 8.0, 100 mM NaCl). Cells were lysed by sonication and lysate was centrifuged at 22,000 rpm for 25 min. Supernatant was collected and passed through a Nickel affinity column (GE HisTrap HP). The purified proteins were then dialyzed in 10 mM Tris-HCl (pH = 8.0) with 100 mM NaCl overnight.

Fluorescence measurements

All measurements were done on either a Varian Cary Eclipse spectrophotometer or a Molecular Devices SpectraMax M5e plate reader. Before measurement all samples were annealed, with the exception of enzymatic samples, at 95 °C for 5 min and then allowed to slowly cool to room temperature for 15 min. All samples were then incubated at 4 °C for at least 2 h.

Derivation of Eq. 3
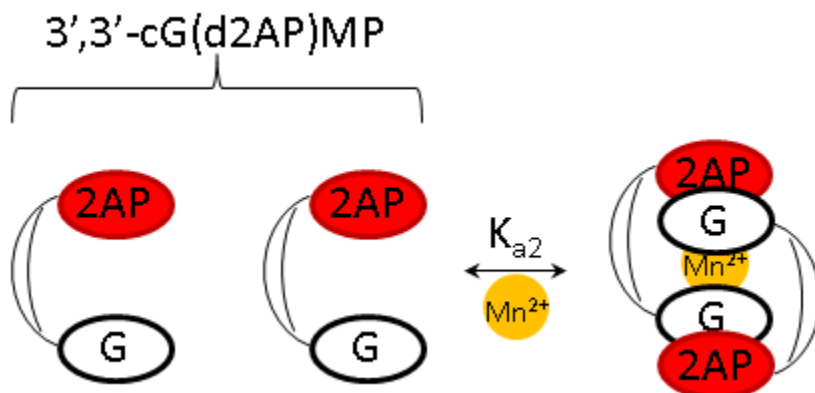
Figure 3.33. Schematic representation of homodimerization of 3',3'-cG(d2AP)MP

Because of mass balance,

$$[2AP] + 2[2AP_2] = [2AP]_T \qquad \text{Eq. 5}$$

Then we can describe [2AP] as a fraction of $[2AP]_T$.

$$\alpha = \frac{[2AP]}{[2AP]_T} \qquad \text{Eq. 6}$$

Substitution of Eq. 6 into Eq. 5 gives,

$$1 - \alpha = \frac{2[2AP_2]}{[2AP]_T} \qquad \text{Eq. 7}$$

Total fluorescence is given by Eq. 2,

$$F_T = f_1[2AP] + f_2[2AP_2] \qquad \text{Eq. 2}$$

Substituting in Eq. 6 and 7 into Eq. 2 and rearranging gives,

$$\alpha = \frac{F_T - \dfrac{[2AP]_T f_2}{2}}{[2AP]_T f_1 - \dfrac{[2AP]_T f_2}{2}} \qquad \text{Eq. 8}$$

The homodimerization constant, K_{a2} is given by,

$$K_{a2} = \frac{[2AP_2]}{[2AP]^2} \qquad \text{Eq. 1}$$

Substituting Eq. 5 into Eq. 1 gives,

$$K_{a2} = \frac{[2AP]_T - [2AP]}{2[2AP]^2} \qquad \text{Eq. 9}$$

Expanding this equation and solving using the quadratic equation gives,

$$[2AP] = \frac{-1 + \sqrt{1 + 8K_{a2}[2AP]_T}}{4K_{a2}} \qquad \text{Eq. 10}$$

Substituting Eq. 6 into Eq. 10 gives,

$$\alpha = \frac{-1 + \sqrt{1 + 8K_{a2}[2AP]_T}}{4K_{a2}[2AP]_T} \qquad \text{Eq. 11}$$

Setting Eq. 11 equal to Eq. 8 and rearranging yields Eq. 3.

$$F_T = \left(\frac{-1 + \sqrt{1 + 8K_{a2}[2AP]_T}}{4K_{a2}[2AP]_T}\right)\left([2AP]_T f_1 - \frac{[2AP]_T f_2}{2}\right) + \frac{[2AP]_T f_2}{2} \qquad \text{Eq. 3}$$

Determination of IC$_{50}$'s of cyclic dinucleotides with 3',3'-cG(d2AP)MP
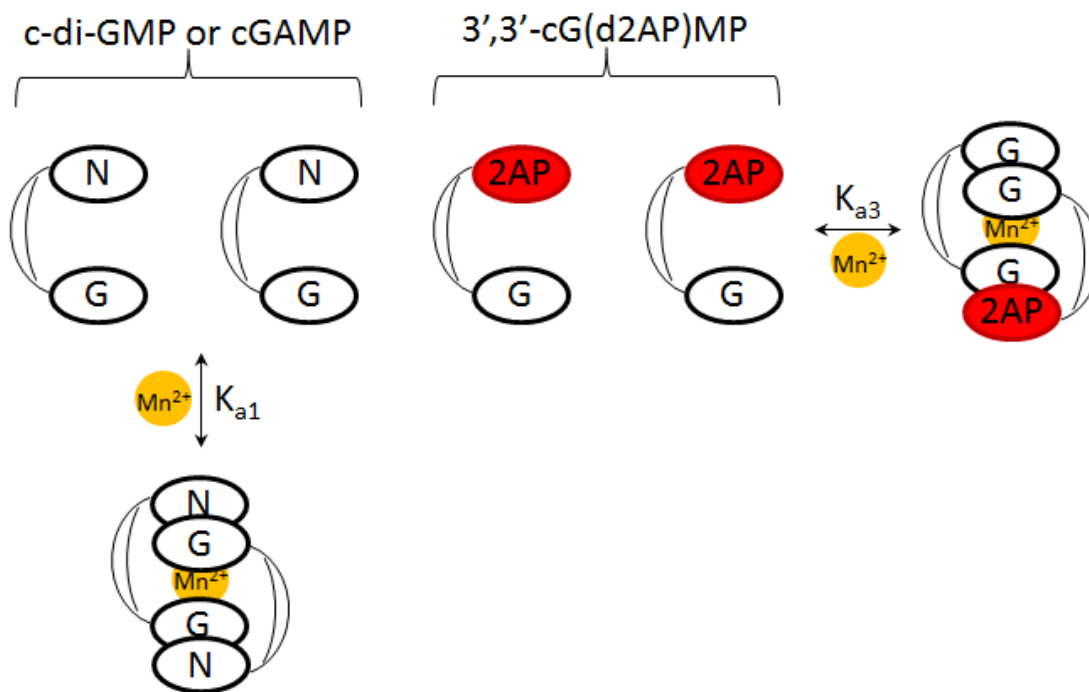
Figure 3.34. Schematic representation of c-di-GMP or cGAMP homodimerizing and binding to 3',3'-cG(d2AP)MP. A low concentration of cG(d2AP)MP is used so that the homodimerization of this nucleotide can be ignored.

To fit titration data, the Wang method was used.[38]

$$[cNMP]_T = IC_{50} \frac{f - f_0}{f_\infty - f} + [2AP]_T \frac{f - f_0}{f_\infty - f_0}$$

Eq. 12

Where $[cNMP]_T$ is the total concentration of added c-di-GMP or cGAMP, IC_{50} is the IC_{50} of binding of c-di-GMP or cGAMP to 3',3'-cG(d2AP)MP, f is the fluorescence of a sample with n concentration of cyclic dinucleotide, f_0 is the fluorescence of 3',3'-cG(d2AP)MP with no additional cyclic dinucleotides, f_∞ is the fluorescence when 100% of 3',3'-cG(d2AP)MP is bound and $[2AP]_T$ is the total concentration of 3',3'-cG(d2AP)MP.

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Chapter 4: Detection of c-di-GMP with the Smart Ligands PyroTASQ and NaphthoTASQ

4.1. Introduction

Several detection methodologies for cyclic dinucleotides have been discussed herein. Some of the most appealing have been those that utilize off-the-shelf chemicals to aggregate c-di-GMP G-quadruplexes.[1-3] In turn, the complex has some measureable signal by which it can be quantified. There are however several issues with this technique. The first of which is that the aggregative process is extremely slow.[1, 4] This limits the applicability of the assay to endpoint techniques and eliminates the possibility of real-time detection. Additionally, tetramer formation is an incomplete process, resulting in a small fraction of the concentration of c-di-GMP forming tetramer. This prevents detection of nM concentration of c-di-GMP because the concentration of tetramer is inherently low. It is therefore of interest, to discover new ways of forming c-di-GMP tetramer with new and innovative ligands. Presumably this would allow, faster and more complete formation of the c-di-GMP tetramer, resulting in a more sensitive, real-time detection of c-di-GMP.

Monchaud and coworkers have created several G-quadruplex forming compounds which contain, four guanine monomers attached to a central cyclic structure via flexible linkers known as template-assembled synthetic G-quartets (TASQ) and herein referred to as smart ligands.[5-7] These G-Quadruplex forming ligands have provided tremendous insight into G-quadruplex structures as well as provide a new platform for the ligation and detection of G-quadruplex structures.[7, 8]
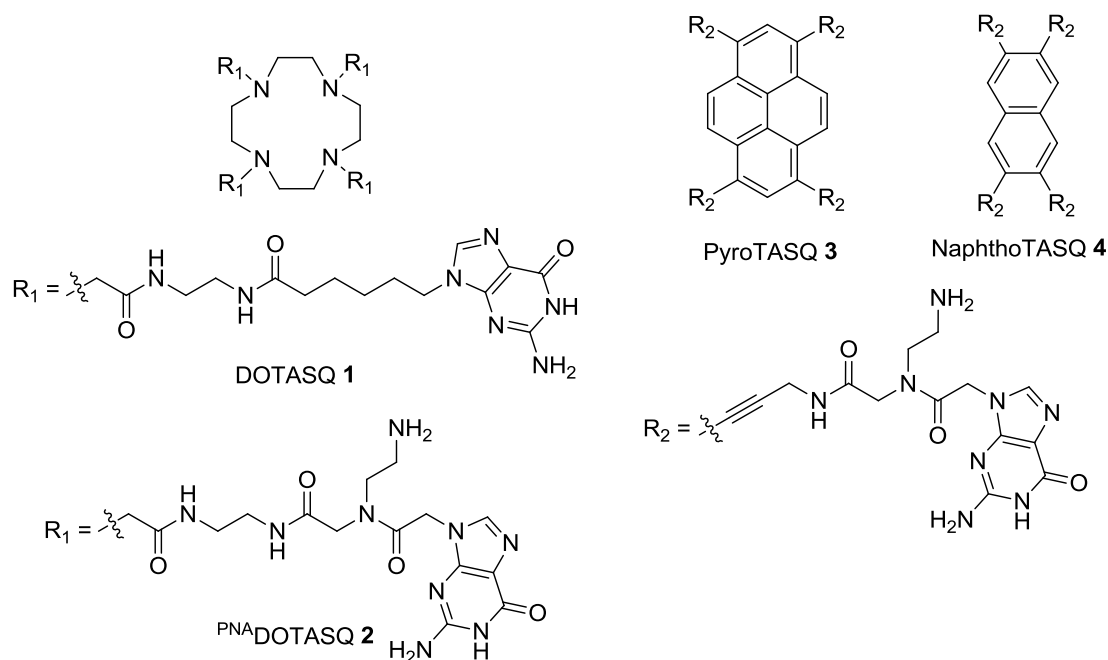
Figure 4.35. Template-assisted synthetic G-qaurtet type ligands described by Monchaud and coworkers.

The first ligand described by Monchaud and coworkers utilized 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) as a central template to attach four guanines capable of forming a TASQ (see Figure 4.1, **1**).[5] It was shown that these compounds were capable of forming G-quadruplexes when the DOTA template was ligated to a terbium cation.[5] The terbium cation presumably forces the DOTA template into a planar structure as well as forces the linker arms connected to each of the guanines onto one side of the macrocycle via ligation of the oxygen atoms in the linker.[5] The terbium bound complex was shown to stabilize G-quadruplex DNA even in the presence of excess duplex DNA.[5] The DOTASQ compounds, without the presence of Terbium (Terbium affects peroxidation reaction), were even

capable of catalyzing the peroxidation reaction in the presence of the iron cofactor, hemin, albeit to a lesser degree than natural G-quadruplexes such as 22AG.[9]

Later generations of the TASQ type compounds utilized other central templating structures such as porphyrin,[6] pyrene (see Figure 4.1, **3**),[7] and naphthalene (see Figure 4.1, **4**). These systems provide planar aromatic templates that would π-stack with the formed G-quadruplex.[6, 7] The pyrene-TASQ and naphthalene-TASQ so named PyroTASQ and NaphthoTASQ respectively, provide a detection modality when bound to G-quadruplex structures.[7] When bound to G-quadruplex, PyroTASQ and NaphthoTASQ become fluorescent. The fluorescence is a result of an interruption of an intramolecular photoinduced electron transfer (iPET, see Figure 4.2).[7, 10] The energy of the highest occupied molecular orbital (HOMO) of guanine is highly dependent on its coordination state (i.e. unbound or involved in a G-tetrad).[11] When unbound, the guanine HOMO sits in between the HOMO and lowest unoccupied molecular orbital (LUMO) of the templating molecule (pyrene or naphthalene) which results in a fluorescence-quenching iPET when the pyrene is excited with its absorbance wavelength (see Figure 4.2).[7] However, when the guanines of the smart ligand become involved in a G-terad (e.g. when bound to a G-quadruplex forming structure) the HOMO of coordinated guanine is reduced in energy.[7] This prevents the iPET from occurring and as a result the fluorescence of the pyrene/naphthalene template is enhanced.[7]
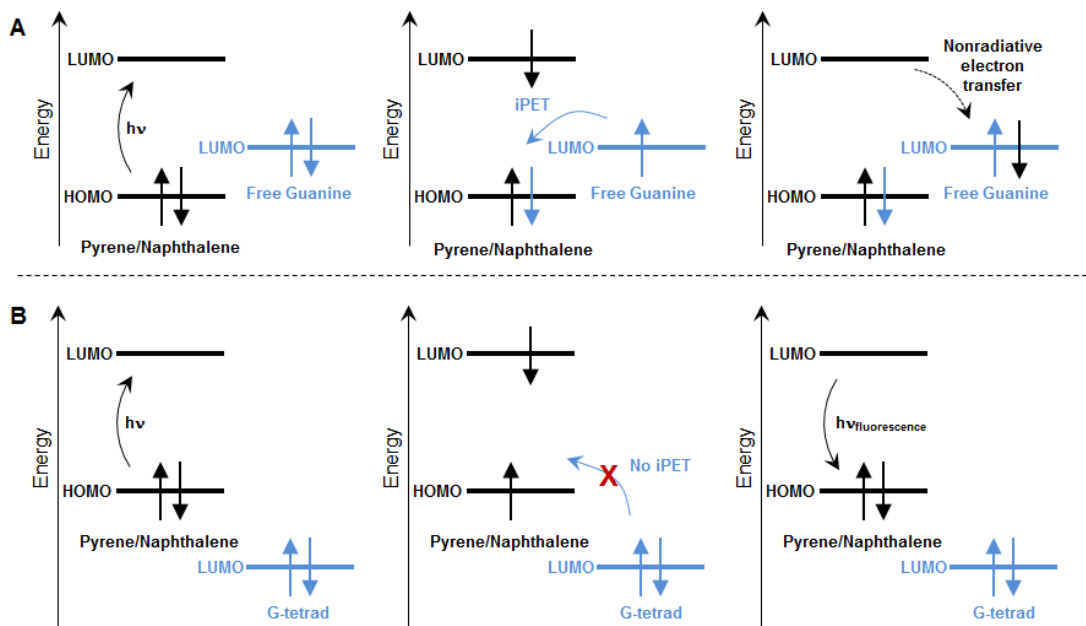
Figure 4.36. Fronteir molecular orbital diagram depicting iPET which leads to A) quenching of pyrene/naphthalene fluorescence in the presence of free guanine and B) enhancement of pyrene/naphthalene fluorescence in the presence of G-tetrads. Adapted with permission from Laguerre, A.; Stefan, L.; Larrouy, M.; Genest, D.; Novotna, J.; Pirrotta, M.; Monchaud, D. *J. Am. Chem. Soc.* **2014**, *136*, 12406-12414. Copyright

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These smart ligands show a particular propensity to detect parallel quadruplexes, presumably because the loops of a parallel G-quadruplex block the approach of the smart ligand.[7] This makes c-di-GMP an ideal candidate for detection using these smart ligands as G-qaudruplexes formed by c-di-GMP are devoid of any loops that may interfere with binding.

C-di-GMP is known to adopt G-quadruplex structures in the presence of monovalent cations[4, 12] and also in the presence of planar aromatic intercalators.[1-3] It is therefore of interest to investigate the interaction of c-di-GMP with PyroTASQ and NapthoTASQ. As PyroTASQ and NaphthoTASQ are capable of forming G-quadruplexes in the presence of high (100 mM) concentrations of monovalent cation (see Figure 4.3B),[7] it is possible that they will seed the formation of c-di-GMP G-quadruplex and speed up the formation of the c-di-GMP higher order aggregates (see Figure 4.3A) to give smart ligand c-di-GMP aggregates of several possible forms (see Figure 4.3C).
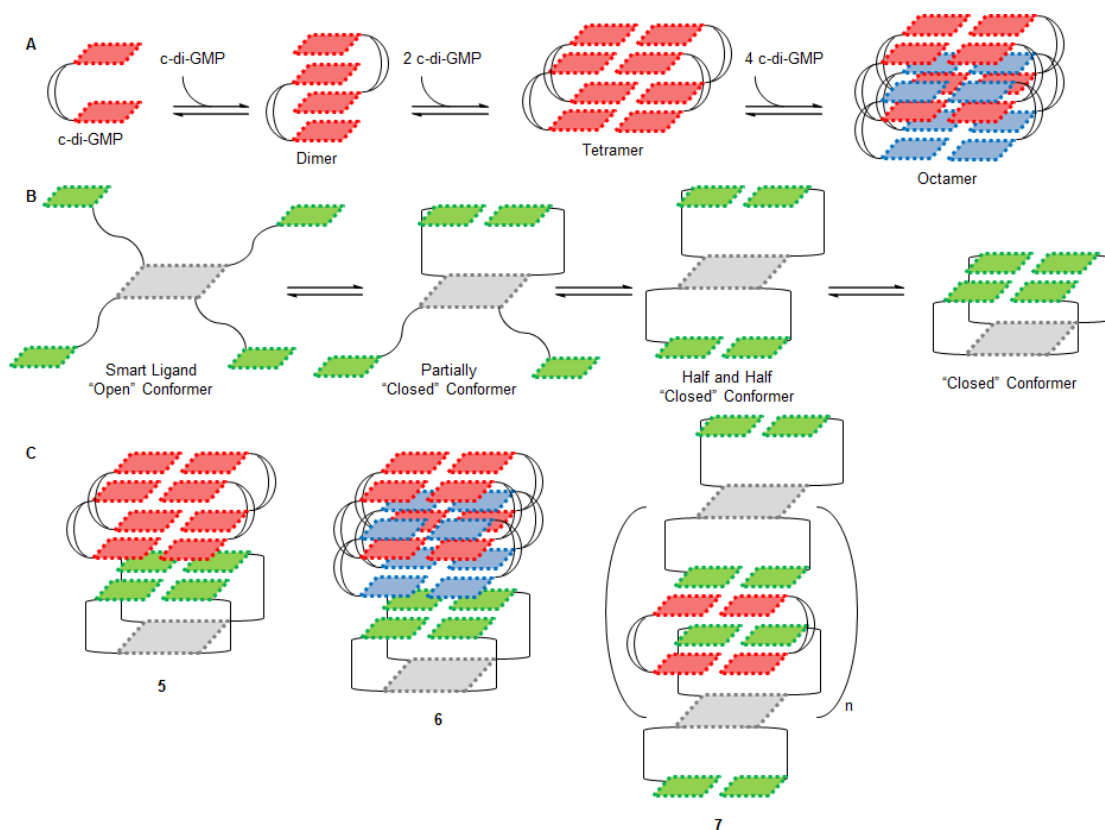
Figure 4.37. Cartoon representation of A) c-di-GMP aggregation equilibria B) smart ligand conformation equilibria and C) putative smart ligand c-di-GMP complexes.

4.2.   Results and Discussion

   With smart ligands in hand, we began to investigate the spectroscopic properties of PyroTASQ and NaphthoTASQ and if they were capable of forming a complex with the bacterial signaling molecule c-di-GMP (see Figure 4.4). As a point of comparison, fluorescence spectra of the smart ligands with the G-quadruplex forming oligonucleotide c-Myc were also collected (Figure 4.4) Polymorphism of c-di-GMP is dependent on the presence of cations, therefore Na^+ and K^+ were added to buffer at

different concentrations to promote c-di-GMP G-quadruplex structures and to optimize detection conditions (see Figure 4.5).[4, 12] Because NH_4^+ had a large effect on pH of the buffer system, it was omitted from the trial (data not shown).
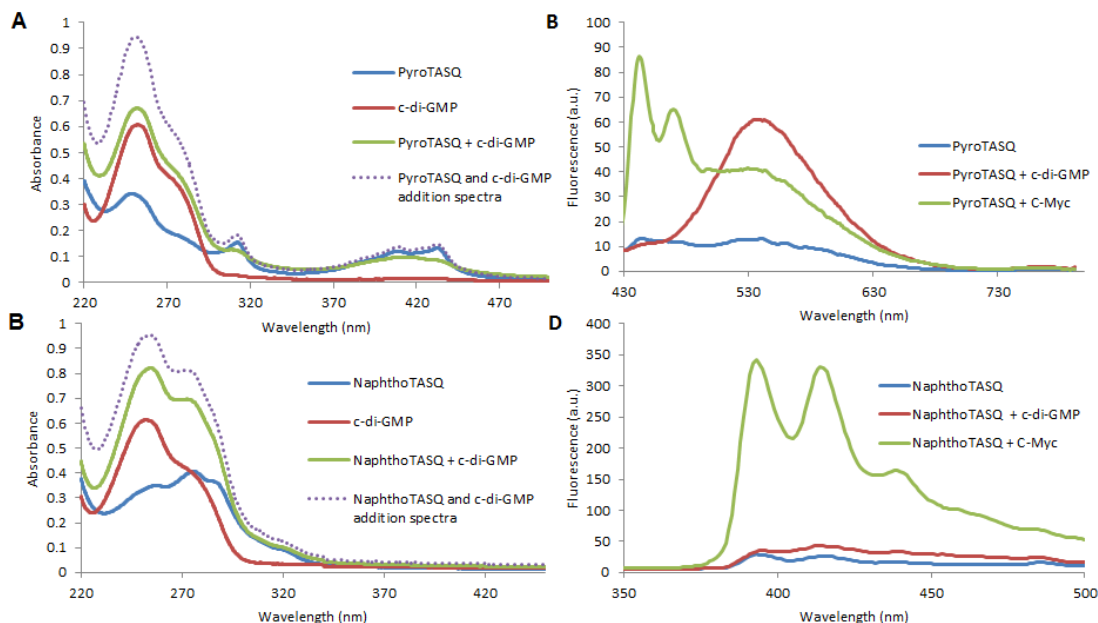
Figure 4.38. A) UV-visible and B) Fluorescence spectra of PyroTASQ alone and with G-quadruplex forming nucleotides. C) UV-visible and D) Fluorescence spectra of NaphthoTASQ alone and with G-quadruplex forming nucleotides. Spectra were run in duplicate and averaged. Conditions (A/C): 10 μM smart ligand, 20 μM c-di-GMP with 100 mM KCl 50 mM Tris-HCl buffer (pH = 7.5). PyroTASQ ex: 410 nm; em: 420-750 nm. NaphthoTASQ ex: 276 nm; em: 286-500 nm. Conditions (B/D): 10 μM smart ligand, 20 μM c-di-GMP or 2.5 μM c-Myc with 100 mM KCl 50 mM Tris-HCl buffer (pH = 7.5). PyroTASQ ex: 410 nm; em: 420-750 nm. NaphthoTASQ ex: 276 nm; em: 286-500 nm.
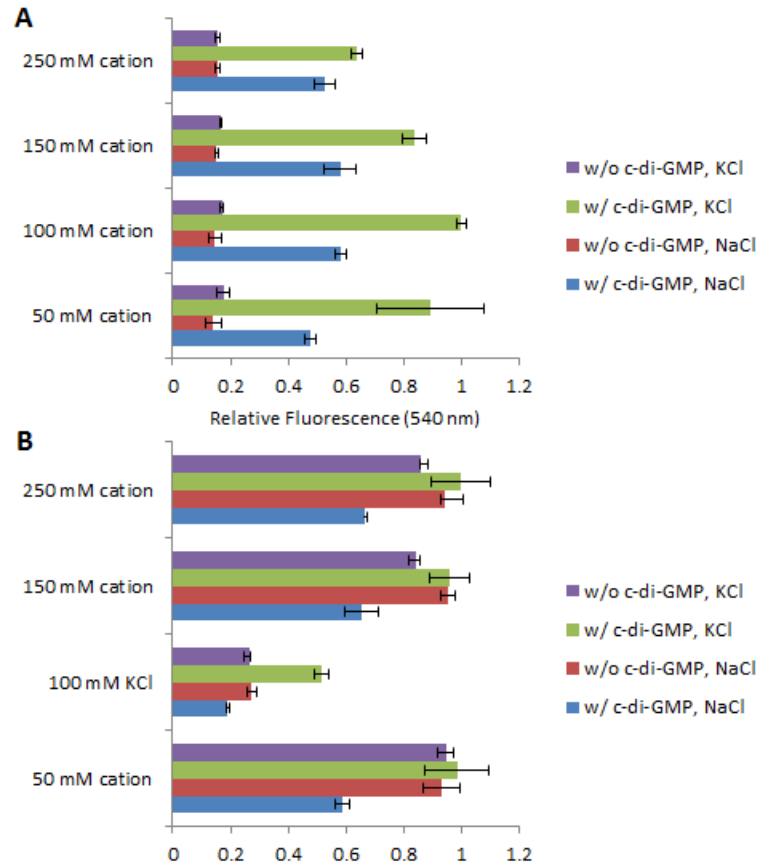
Figure 4.39. Relative fluorescence intensities of A) PyroTASQ or B) NaphthoTASQ and c-di-GMP in the presence of different monovalent cations at different concentrations. Samples were run in triplicate. Conditions: 10 µM smart ligand, 20 µM c-di-GMP with shown concentration of monovalent cation in 50 mM Tris-HCl buffer (pH = 7.5). PyroTASQ ex: 410 nm; em: 540 nm. NaphthoTASQ ex: 276 nm; em: 411 nm.

Interestingly, PyroTASQ but not NaphthoTASQ, showed increased fluorescence in the presence of c-di-GMP and KCl. Additionally c-di-GMP led to a red-shifted fluorescence emission (540 nm) as compared to that observed when c-Myc is bound to PyroTASQ. K^+ concentrations above 100 mM showed a decrease in fluorescence

intensity. This could be due to the fact that Cl⁻, a known quencher, concentration

increases with additional cation concentration. However, when K_2SO_4 is used in place

of KCl, little change in fluorescence intensity is observed (see Figure 4S.1). Without

a fluorescent readout for NaphthoTASQ other spectroscopic techniques had to be

utilized to study a potential binding event with c-di-GMP. Circular dichroism was

used to determine the presence of c-di-GMP G-quadruplex and to determine whether

the CD trace would change upon introduction of smart ligand (see Figure 4.6).
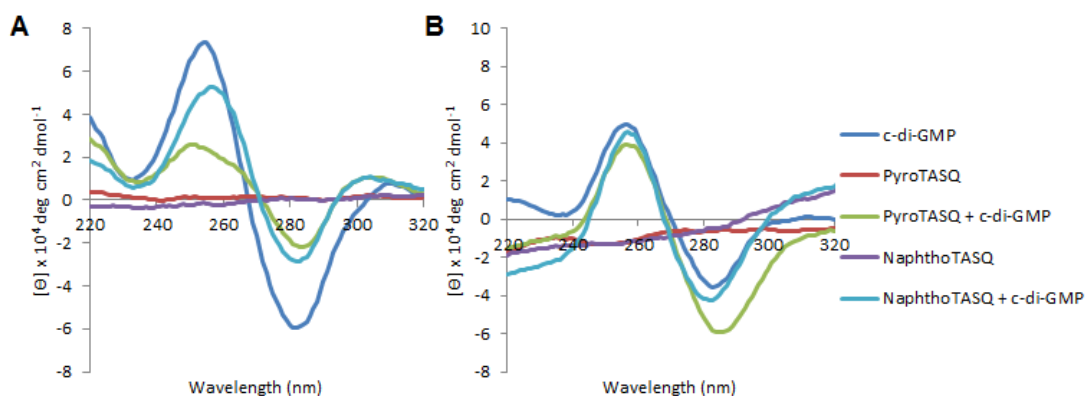
Figure 4.40. CD spectra of the smart ligands and c-di-GMP in the presence of A) 100

mM KCl and B) without presence of cation. Conditions: 100 μM smart ligand, 100

μM c-di-GMP with 100 mM KCl in 50 mM Tris-HCl buffer (pH = 7.5).

Due to the insensitivity of CD, high concentrations of c-di-GMP and smart ligand

had to be used. High concentrations of c-di-GMP shift its complex polymorphic

equilibria towards the formation of G-quadruplex structures,[4] therefore samples

without monovalent cation showed peaks indicative of G-quadruplexes (indicated by

a positive peak at ~260 nm). However, CD spectra showed modest changes when

smart ligands were introduced into solution with c-di-GMP suggesting a possible

interaction with the second messenger. We therefore, sought direct proof of a c-di-GMP-smart ligand aggregate. We employed a gel shift assay to see if high order aggregates are formed when smart ligands are present in solution with radioactive ^{32}P-c-di-GMP (see Figure 4.7). The gel showed a small band that migrated a short distance as compared to the bulk (monomeric) c-di-GMP when in the presence of smart ligands with a simultaneous attenuation of the monomeric c-di-GMP band seen at the bottom of the gel (see Figure 4.7). This is highly suggestive of a high molecular weight aggregate between c-di-GMP and the smart ligands.
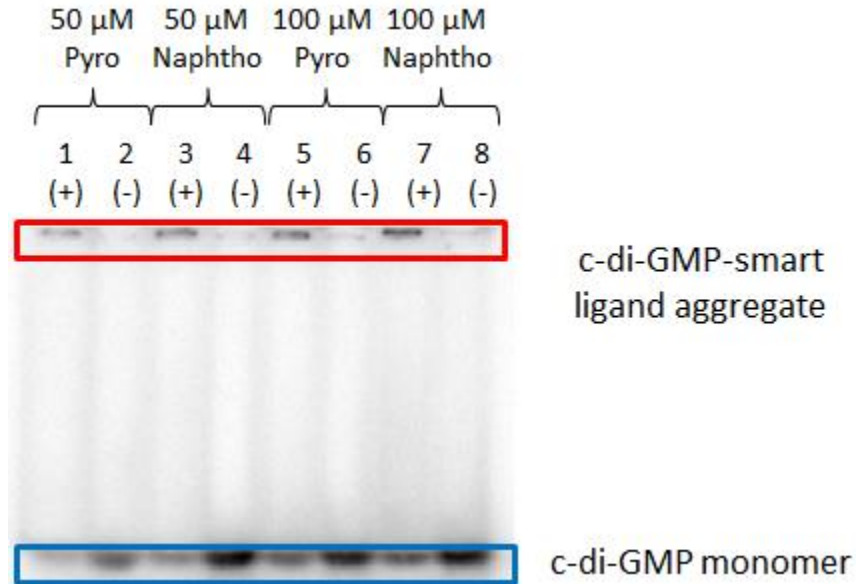
Figure 4.41. Gel shift assay of c-di-GMP with smart ligands. Lanes 1-4: c-di-GMP with and without PyroTASQ. Lanes 5-8: c-di-GMP with and without NaphthoTASQ. (+) indicates samples with smart ligand and (-) indicates those without. Conditions: Lanes 1, 2, 5 and 6: 50 μM smart ligand (where applicable), 50 μM c-di-GMP with 100 mM KCl in 50 mM Tris-HCl buffer (pH = 7.5). Lanes 3, 4, 7 and 8: 100 μM smart ligand (where applicable), 100 μM c-di-GMP with 100 mM KCl in 50 mM

Tris-HCl buffer (pH = 7.5). Run at 140 V for 20 min in 8% polyacrylamide gel, in 90 mM TBE buffer with 100 mM KCl.

Because of the fluorescent readout of PyroTASQ, its interaction with c-di-GMP was further characterized. In particular, the interaction of PyroTASQ and other nucleotides was studied to determine if the interaction with c-di-GMP was specific. Therefore we tested several cyclic and linear mono and di-nucleotides to see if it would give rise to a fluorescent signal from PyroTASQ (see Figure 4.8)
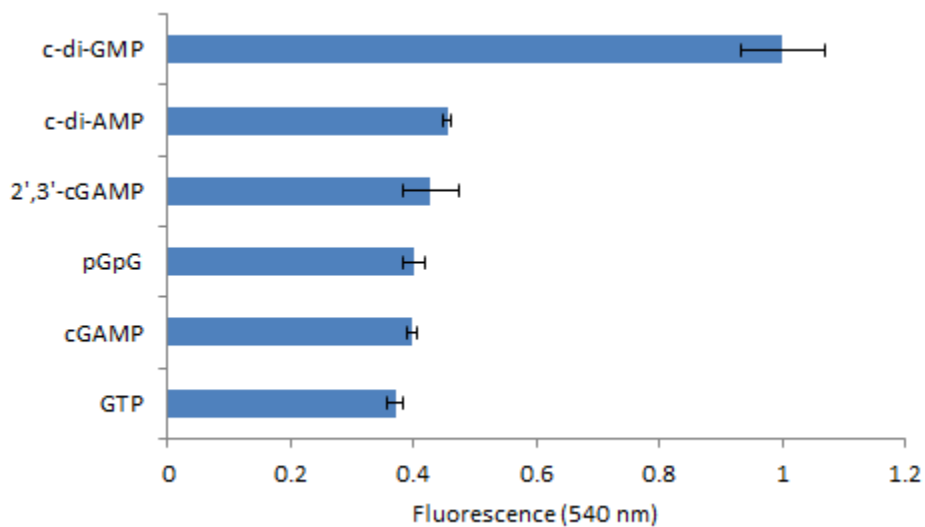
Figure 4.42. Specificity of PyroTASQ binding to small, biologically relevant nucleotides. Conditions: 5 µM PyroTASQ, 20 µM nucleotide (indicated in figure), 100 mM KCl in 50 mM Tris-HCl buffer (pH = 7.5). All fluorescence measurements were done in triplicate.

Pleasingly, among the nucleotides tested, c-di-GMP was the only nucleotide reported by PyroTASQ. This was not surprising however, because among the several nucleotides used, c-di-GMP is the only compound capable of forming G-quadruplex structures.[1] Because c-di-GMP G-quadruplex is a slow forming structure it was of interest to see if our initial hypothesis was correct, in that the presence of the PyroTASQ G-quadruplex would "seed" the formation of the c-di-GMP G-quadruplex and improve the kinetics of formation of the fluorescent aggregate. A time course study was undertaken to see how long maximal fluorescence would take to achieve (see Figure 4.9).
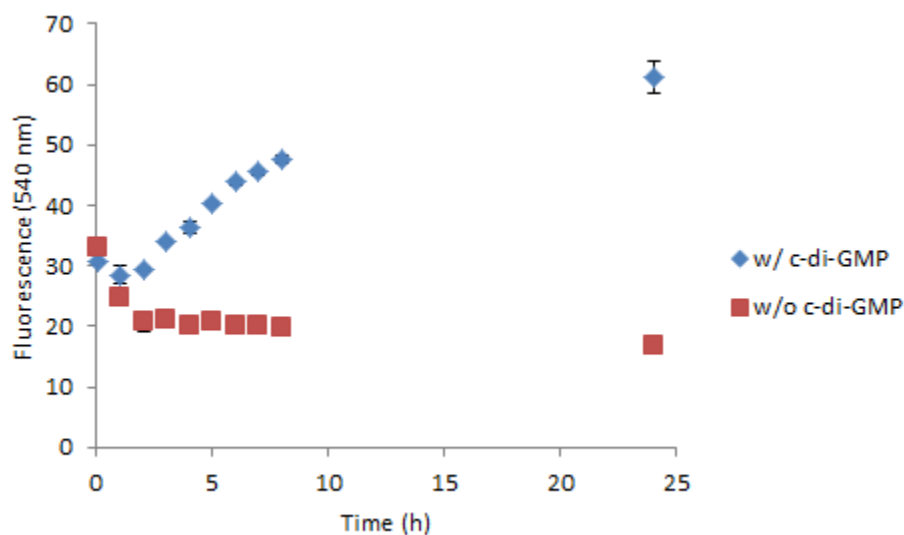
Figure 4.43. Time course study of PyroTASQ fluorescence over time after incubation with c-di-GMP. Conditions: 20 μM c-di-GMP, 5 μM PyroTASQ and 100 mM KCl in 50 mM Tris-HCl (pH = 7.5). Fluorescence measurements were done in triplicate.

Unfortunately, the kinetics of the formation of the putative c-di-GMP G-quadruplex-PyroTASQ aggregate was not fast. The formation of the complex still takes approximately 24 h to complete. Unfortunately, the detection method cannot be used for real-time detection of c-di-GMP. Despite this set back it was still of academic interest to evaluate the complex that is formed between c-di-GMP and PyroTASQ. To that end, binding stoichiometry was evaluated using a Job plot analysis (see Figure 4.10).
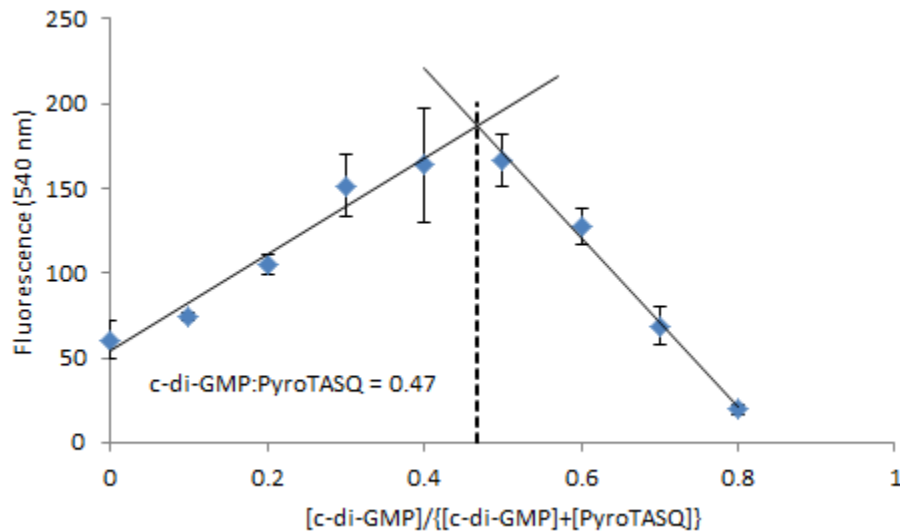
Figure 4.44. Job plot showing binding stoichiometry of c-di-GMP to PyroTASQ. Conditions: Concentration of c-di-GMP and PyroTASQ were collectively held at 20 μM. Samples each contained 100 mM KCl in 50 mM Tris-HCl (pH = 7.5). Note: The points corresponding to the c-di-GMP:PyroTASQ ratio of 0.9 and 1 were removed as it improved linear fit. This is because concentrations of c-di-GMP were below the limit of detection of the assay, *vide infra*. Measurements were performed in triplicate.

Job plot analysis revealed a binding stoichiometry of approxiamtely 1:1, an interesting result as a 4:1 or 8:1 stoichiometry would be expected for the binding of PyroTASQ to a c-di-GMP tetramer of octamer. However, it is possible that c-di-GMP and PyroTASQ form 1:1 quadruplexes similar to that aggregate **7** depicted in Figure 4.3.

With the binding stoichiometry in hand, it was of interest to determine the binding affinity of c-di-GMP with PyroTASQ as well as the limit of detection for the detection method being developed (see Figure 4.11).
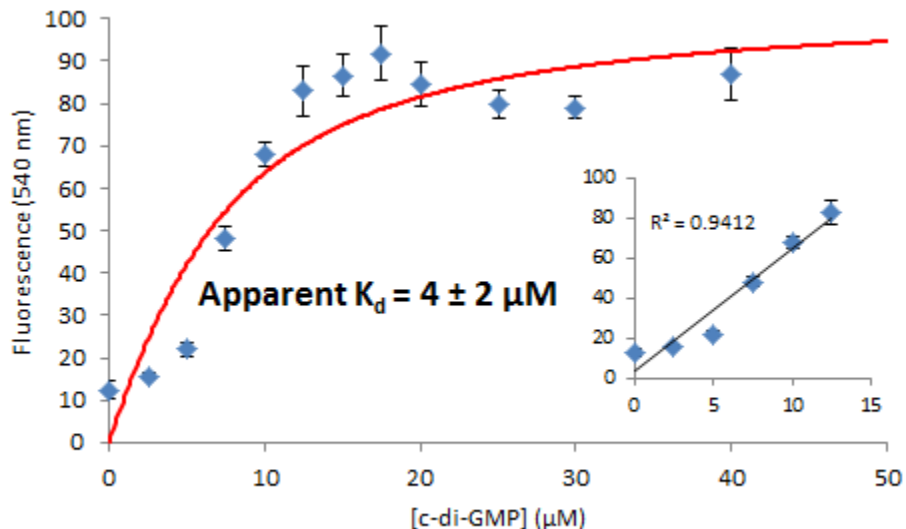
Figure 4.45. Apparent dissociation constant determination of c-di-GMP with PyroTASQ using the model described by Lin and coworkers.[13] Inset: A plot of [c-di-GMP] versus fluorescence at 540 nm shows a linear relationship until 12.5 µM c-di-GMP. Conditions: 5 µM PyroTASQ, various concentrations of c-di-GMP (0-40 µM) and 100 mM KCl in 50 mM Tris-HCl buffer (pH = 7.5). Measurements were performed in triplicate.

C-di-GMP showed a moderate binding affinity with PyroTASQ with an apparent

K_d of 4 ± 2 μM. It also proves to be a fairly sensitive detection method, with a limit of

detection of ~5 μM c-di-GMP similar to other intercalator based methods developed

by our group previously.[1, 3]

4.3.   *Conclusion*

Smart ligands, and more specifically PyroTASQ, provide a new method by which

to detect the presence of c-di-GMP *in vitro* although, limit of detection was not

improved. Pleasingly, PyroTASQ does provide a unique way of identifying c-di-GMP

as compared to other G-quadruplex forming oligonucleotides. The Stokes shift of

PyroTASQ when bound to c-di-GMP is significantly red-shifted as compared to the

Stokes shift observed when PyroTASQ is bound to the DNA based oligonucleotide c-

Myc. This may prove useful in more complex sample systems in which G-quadruplex

DNA may be present. This is a similar observation to thiazole orange detection of c-

di-GMP[1] which showed a red shift in the Stokes shift of thiazole orange when bound

to c-di-GMP as compared to emission spectra of thiazole orange in the presence of G-

quadruplex forming DNA.[14] This suggests a slightly different interaction of planar

aromatic intercalators with c-di-GMP G-quadruplex.

Another advantage of the PyroTASQ detection methodology described herein is

the fluorescent properties of the pyrene fluorophore. Pyrene is an ideal fluorophore

for the detection of biomolecules because of its long fluorescence lifetime.[15] This

allows experimenters to use time-resolved fluorescence to detect emission after the

decay of background fluorescence.[15] In turn this improves, signal to noise ratio particularly in those samples which may contain other molecules with emission wavelengths similar to those observed in the PyroTASQ-c-di-GMP aggregate. This would perhaps allow detection of c-di-GMP in more complex samples including cell lysates and live cells.

Despite the ability of smart ligands to form G-quadruplexes they are incapable of drastically speeding up formation of higher order c-di-GMP aggregates. The high entropic cost of the aggregation of c-di-GMP may be difficult to overcome. Other methods which improve the kinetics of this process would be advantageous for future detection methodologies utilizing c-di-GMP quadruplexes.

4.4.　*Experimental*

Materials

PyroTASQ was generously given to the Sintim lab from David Monchaud at Institut de Chimie Moléculaire, Université de Bourgogne. C-di-GMP was synthesized using previously described techniques and purified using RP-HPLC.[16] All other materials were obtained from commercial sources and used without further purification.

C-di-GMP Sample Preparation

All samples were prepared as follows: buffer, cation and c-di-GMP stock solutions were combined and mixed thoroughly. Solutions were then exposed to an annealing process in which the solution was placed on a heat block set to 95 °C for 5 min. The samples were then allowed to cool at room temperature for 15 minutes before adding

the smart ligand to the desired final concentration. Samples were then allowed to incubate at 4 °C for at least 18 h. PyroTASQ was excited at 410 nm and spectra were collected from 420-700 nm or emission endpoint was measured at 540 nm. NaphthoTASQ was excited at 276 nm and spectra were collected from 286-650 nm or emission endpoint was measured at 411 nm.

c-Myc Sample Preparation

C-Myc was first folded in 50 mM Tris-HCl buffer (pH = 7.5) containing 100 mM KCl. This was accomplished by dissolving 10 μM c-Myc in cation containing buffer and annealing the DNA by heating it at 95 °C for 5 min. The DNA solution was then cooled at room temperature for 15 min. The DNA solution was then incubated at 4 °C overnight. This stock solution was then used to prepare samples with smart ligands. To this stock solution was added freshly prepared smart ligand in 50 mM Tris-HCl (pH = 7.5) containing 100 mM KCl. The smart ligand was allowed to bind the DNA for 15 minutes prior to measurement. Samples containing PyroTASQ were excited at 410 nm and spectra were collected from 420-700 nm. Samples containing NaphthoTASQ were excited at 276 nm and spectra were collected from 286-650 nm. The DNA sequence used in this study was ordered from IDT and used without further purification. c-Myc: 5'-TGA GGG TGG GGA GGG TGG GG AA-3'. Concentration of the DNA was measured using the extinction coefficient provided by IDT, $\varepsilon_{260} =$ 229,900 M^{-1} cm^{-1}.

Fluorescence Measurements

All fluorescence measurements were made on either a Varian Cary Eclipse

spectrphotometer or a Molecular Devices SpectraMax M5e plate reader. All samples

were prepared using the general methodology described above.

CD Measurements

CD was carried out on an Applied Photophysics Chirascan CD spectrometer. Spectra

were collected in a 1 mm path length quartz cuvette from a 220-600 nm.

Gel Shift Assay

Radiolabeled ^{32}P-c-di-GMP was prepared as previously described using the mutated

DGC WspRD70E and α-^{32}P-GTP.[17] Radiolabeled c-di-GMP was prepared as a

mixture of "hot" and "cold" c-di-GMP (stock concentration of 500 µM). See general

sample preparation above for protocol for preparing c-di-GMP-smart ligand

complexes. The gel was run in 90 mM TBE buffer with 100 mM KCl at 140 V for 20

min, dried and then imaged using a phosphorimager.

K_d Determination

Dissociation constants were determined using a previously described method and fit

using OriginPro 8.0.[13] The model is described by the following equation:

$$F = F_0 + (F_0 - F_\infty)\frac{(K_d + [Pyro] + [cdG]) - \sqrt{(K_d + [Pyro] + [cdG])^2 - 4[cdG][Pyro]}}{2[cdG]} \qquad \text{Eq. 1}$$

Where F is the fluorescence of PyroTASQ in the presence of n µM c-di-GMP, F_0 is

the fluorescence when no c-di-GMP is present, F_∞ is the fluorescence when

PyroTASQ is saturated with c-di-GMP, K_d is the dissociation constant, [Pyro] is the concentration of PyroTASQ and [cdG] is the concentration of c-di-GMP.

4.5   Supporting Information
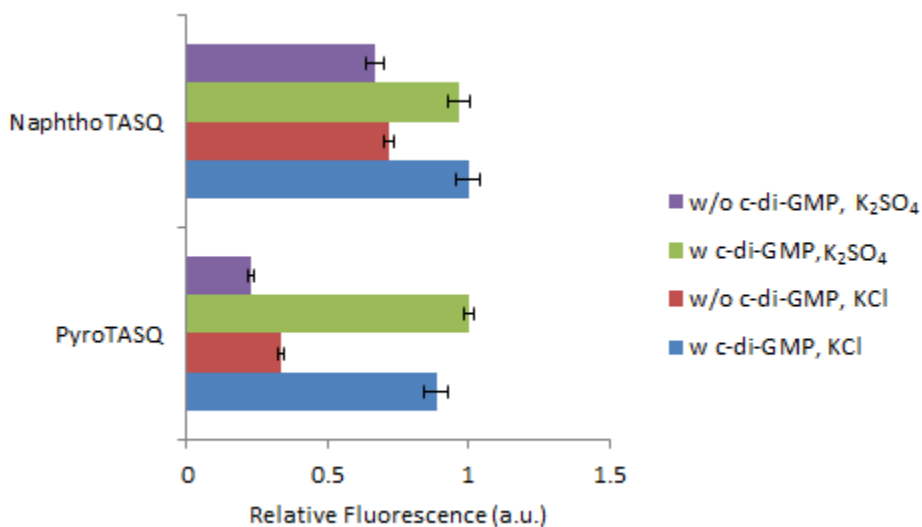
Figure 4S.1. Effect of anion on fluorescence of smart ligands bound to c-di-GMP. All samples were run in triplicate. Conditions: 10 µM smart ligand, 20 µM c-di-GMP with 100 mM KCl or 50 mM K_2SO_4 in 50 mM Tris-HCl buffer (pH = 7.5). PyroTASQ ex: 410 nm; em: 540 nm. NaphthoTASQ ex: 276 nm; em: 411 nm.

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Chapter 5: Conclusion and Future Perspectives

Conclusions

Herein we have reported the use of the two major classes of cyclic dinucleotide

polymorphs which include the intercalated dimer and the G-quadruplex (c-di-GMP is

the only cyclic dinucleotide capable of forming G-quadruplex; see Figure 5.1).[1, 2]

These two classes of polymorphs were able to be applied in to study nucleotide

peroxidases[3] as well as provide convenient methods to detect cyclic dinucleotides

utilizing fluorescent chemical probes.[4] Each of these methods has distinct advantages

and disadvantages which would allow judicious use of each detection methodology in

different scenarios.
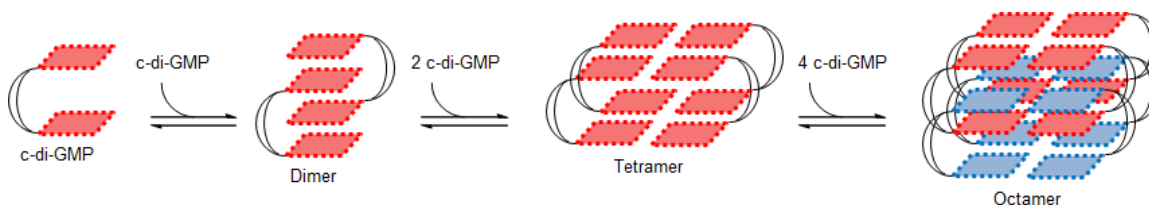
Figure 5.46. Polymorphs formed by c-di-GMP. Other cyclic dinculeotides are capable

of forming dimeric structures as wel.

In chapter 1, an octameric c-di-GMP peroxidase was introduced as a useful system

to study the effect of DNA loops in the peroxidation catalytic cycle. It was discovered

that the DNA loops are disposable structural features in the peroxidation reaction as

the c-di-GMP octameric peroxidase shows as good or even improved peroxidation

over the DNA sequences PS2.M and c-Myc. This is a significant result, as both of these DNA sequences are proficient peroxidases when bound by the hemin cofactor. Both PS2.M and c-Myc show a parallel structure in the presence of K^+ (see Figure 5.2).[5] The parallel structure of these two sequences allows good binding to hemin (hemin is even shown to promote parallel G-quadruplex structures when bound to anti-parallel structures) which in turn promotes proficient catalysis.[6]
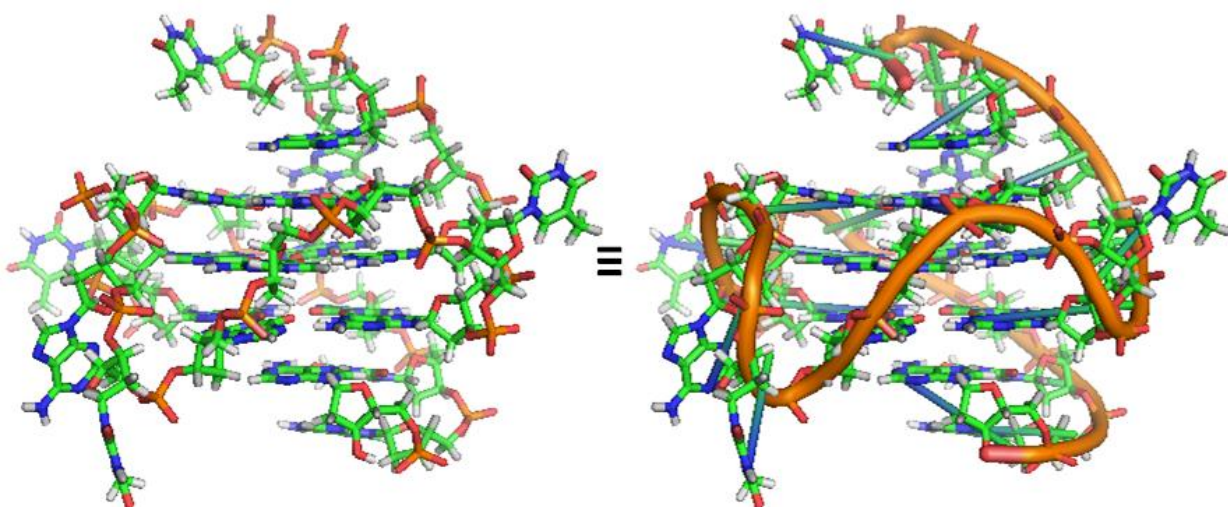
Figure 5.47. Solution structure of c-Myc showing parallel topology of c-Myc. Figure was created using PyMol.[7]

Future research may shed light on the possibility of these cyclic dinucleotide bases peroxidases to catalyze important chemical reactions such as Friedel-Crafts acylation[8] and epoxidation[9], in water to promote green chemistry initiatives. Potential downfalls of applications of the octameric c-di-GMP peroxidase may arise, however, due to the lack of loops in the active catalyst. Although this may improve rates of reaction as shown in the study discussed in chapter 1, the loops also may impart

enantioselectivity onto the organic reductant.[8, 9] In this particular application, this could be one distinct advantage of traditional DNA G-Quadruplex peroxidation catalysts.

In chapter 2, the chemical probe 3',3'-cG(d2AP)MP exploits the propensity of cyclic dinucleotides to form intercalated dimers to detect cyclic dinucleotides and revealed that cyclic dinucleotides have a great propensity to form dimers even at fairly low concentrations (8 µM). Once 3',3'-cG(d2AP)MP is incorporated into a dimer (either a heterodimer with c-di-GMP or cGAMP or a homodimer with another equivalent of 3',3'-cG(d2AP)MP) the fluorescence of the 2-aminopurine nucleobase is quenched, thereby giving rise to a facile fluorescent turn-off method to detect the cyclic dinucleotides, c-di-GMP or cGAMP.

As dimer formation is extremely fast (monomer and dimer H8 peaks cannot be resolved via ^{1}H-NMR[2]) this method allows for the real time detection of these molecules and subsequently, allows kinetic analysis of cyclic dinucleotide metabolic enzymes. Moreover, because 3',3'-cG(d2AP)MP is a cyclic dinucleotide analog itself it can be used as the substrate for metabolic enzymes which degrade c-di-GMP, cGAMP or other cyclic dinucleotides. Furthermore, the system itself is modular, allowing incorporation of 2-aminopurine into other cyclic dinucleotide analogs which may be used to study the kinetics of metabolic enzymes of these other classes of cyclic dinucleotides. This would allow high throughput screening for inhibitors of PDE enzymes which cleave the phosphodiester backbone of cyclic dinucleotides. Once the phosphodiester linkage is cleaved the fluorescence of the 2-aminopurine is increased. High throughput inhibitor screening assays such as the one described in

chapter 2 are invaluable in industrial laboratories where robotics can be used to discover new drugs from vast libraries of potential therapeutics.

Future research in this field should aim to improve the sensitivity of the 3',3'-cG(d2AP)MP enzymatic cleavage. Various cyclic dinucleotide PDE's cleave only one of the phosphodiester linkages in cyclic dinculeotides.[10, 11] For example the c-di-AMP specific PDE, YybT found in *B. subtilis* cleaves c-di-AMP to the linear dinucleotide 5'-pApA-3' (see Scheme 5.1).[11] Because one of the phosphodiester linkages remains, the fluorescence of the 2-aminpurine is not greatly enhanced. Additionally, because the analog studied herein (3',3'-cG(d2AP)MP) contains a guanine nucleobase, the kinetics of hydrolysis may be slower due to the greater rates observed for c-di-AMP hydrolysis rather than c-di-GMP.[11] To improve kinetics and sensitivity, analogs containing adenine and 2-aminopurine nucleobases (3',3'-cA(2AP)MP) could be used to better study enzymes which degrade c-di-AMP. Additionally, enzyme cascades could be used to further cleave 5'-pNpN-3' (where N is G or A) to the NMP products via an exonuclease (enzymes that cleave terminal nucleotides). The enzyme cascade would allow greater fluorescent enhancement of the 2-aminopurine to improve sensitivity of the enzyme kinetics assay using enzymes which only cleave to linear dinucleotide products rather than the NMP monomer products (see Scheme 5.1)
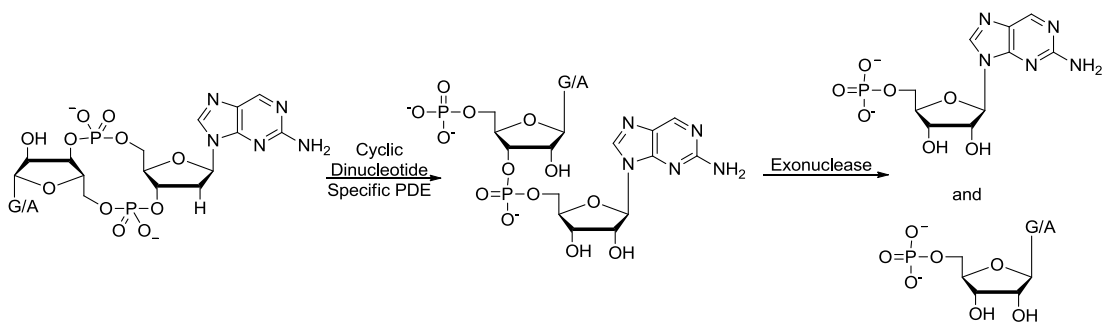
Scheme 5.4. Potential enzymatic cascade reaction to improve sensitivity of the enzymatic kinetics analysis using 2-aminopurine containing cyclic dinucleotides.

It would also be of interest to investigate the kinetics of cyclic dinucleotide synthases via the 2-aminopurine nucleobase. Cyclic dinucleotide synthases condense two equivalents of NTP (nucleotide triphosphate) to yield one equivalent of cyclic dinucleotide (see Schemes 1.1 and 1.2). If 2-aminpurine (commercially available from BioLog) was used in place of the canonical GTP or ATP, a cyclic dinucleotide containing 2-aminopurine could be enzymatically synthesized (see Scheme 5.2). In turn, fluorescence would be slowly quenched in the presence of Mn^{2+} as cyclic dinucleotide was synthesized and then subsequently dimerized. Again this assay could be used to screen inhibitors of the cyclic dinucleotide synthases, particularly DACs (c-di-AMP synthases) as 2-aminopurine most adequately mimics the adenosine nucleobase.
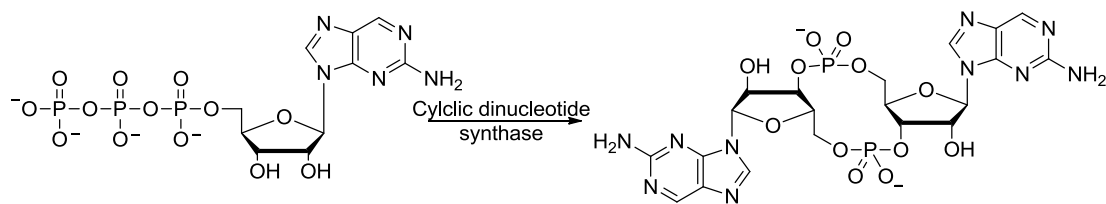
Scheme 5. Enzymatic synthesis of a 2-aminopurine cyclic dinucleotide.

The smart ligands, PyroTASQ and NaphthoTASQ, were investigated for their ability to bind c-di-GMP in chapter 4. They show promise in their ability to bind c-di-GMP G-quadruplex structures. Interestingly only PyroTASQ showed promise as a fluorescent chemical probe to detect the presence of c-di-GMP whereas NaphthoTASQ did not show fluorescent enhancement in the presence of c-di-GMP. However, both smart ligands showed the ability to aggregate c-di-GMP into higher order structures as shown by a gel shift assay. The smart ligands excel in their high solubility in aqueous solutions which can be an issue when developing new G-Quadruplex binding ligands as well as utilizing fluorescent probes that show large stokes shifts and long fluorescent lifetimes.

Future directions in this research may aim to utilize time resolved fluorescence measurements to detect biomolecules (both DNA based G-Quadruplex structures and c-di-GMP G-Quadruplex structures) to allow *in vivo* detection modalities.[12] This technique allows an experimenter to wait until background noise has faded to collect fluorescence of the pyrene core to improve signal to noise ratios thereby improving detection sensitivity.

Additionally, it would be of interest to show if these smart ligands promote formation of G-wires. Because the most likely binding mode of the smart ligands to c-di-GMP involves incorporation of guanines from c-di-GMP and the smart ligand

into each G-quadruplex this may lead to long polymeric strands of G-tetrads known as G-wires.[13] These have been previously observed by scanning probe microscopy and have various applications as nanomaterials.[13, 14]

In conclusion, cyclic dinucleotides are fascinating molecules due to their ability to control myriad processes in living cells as well as their proclivity to form various polymorphic structures. Future research in the detection of these important molecules will reveal new ways in which these molecules control important biological processes and form useful catalysts and nanomaterials.

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Appendix

3',3'-cG(d2AP)MP
1H-NMR

3',3'-cG(d2AP)MP
^{13}C-NMR

38.69

62.97
63.25

71.40
72.08
74.18

80.81
83.36
84.17

90.16

116.86

127.80

137.81

143.35

149.14
151.37
153.08
154.62
159.25
160.21

f1 (ppm)

113

-19.42
-19.14

3',3'-cG(d2AP)MP
^{31}P-NMR

f1 (ppm)

114
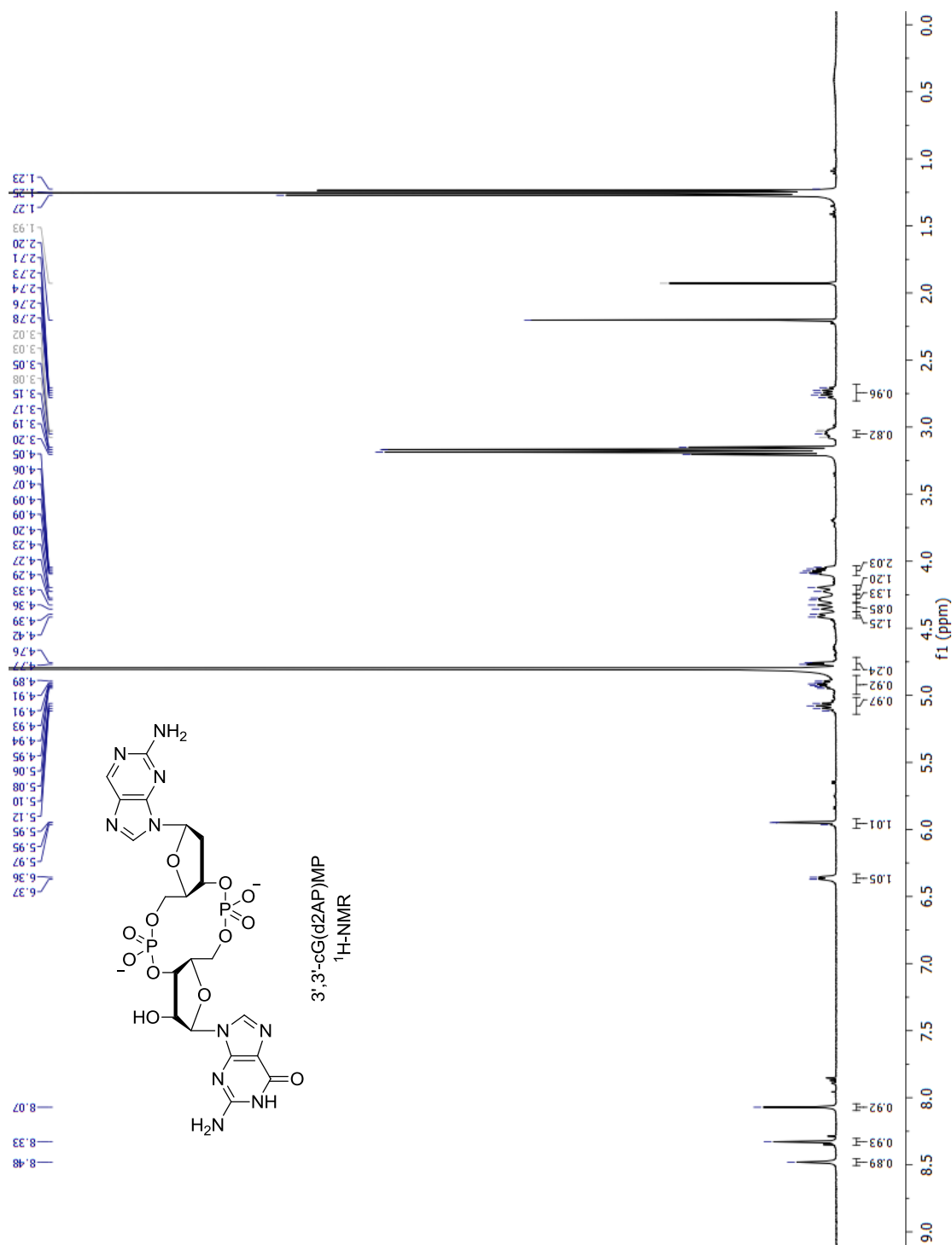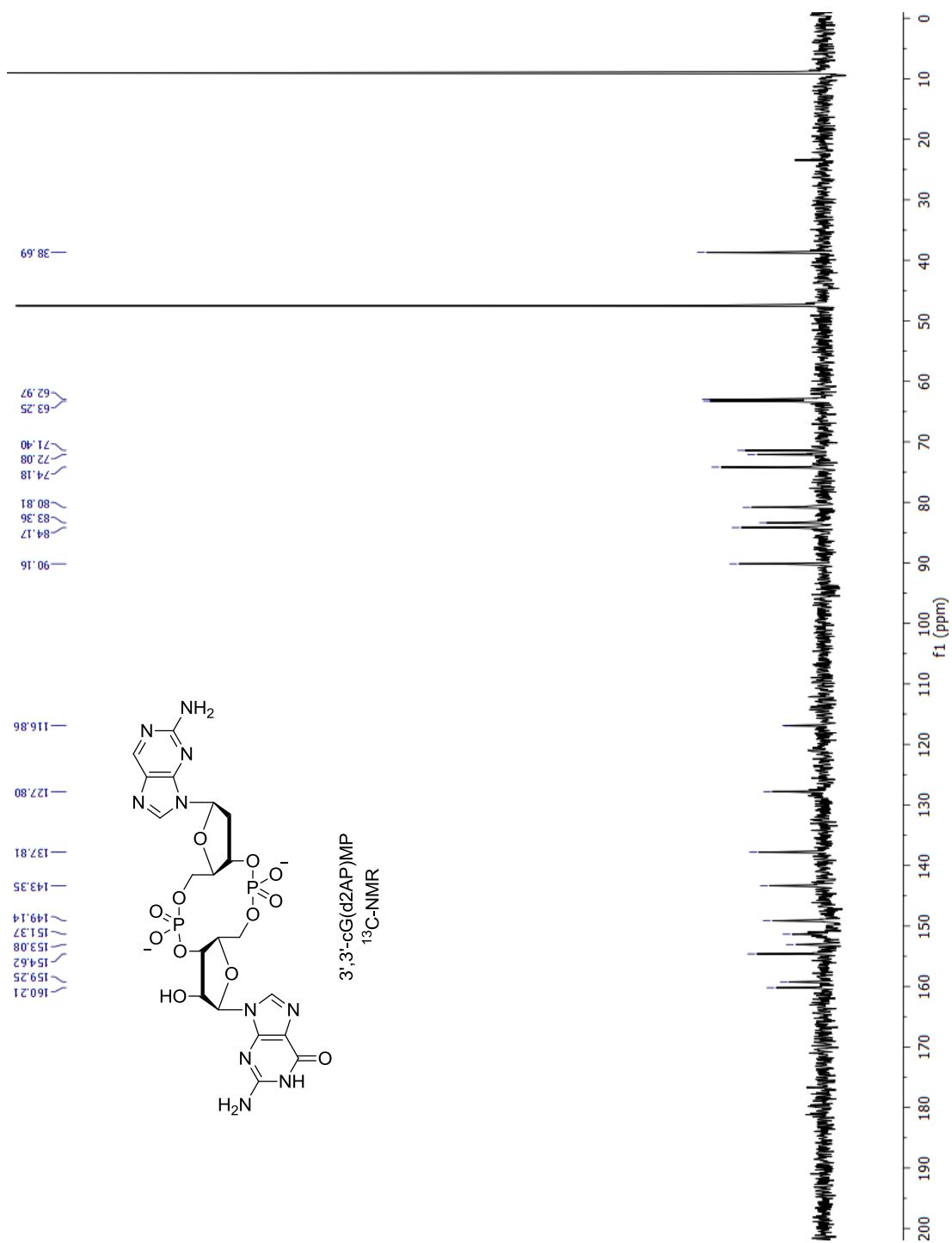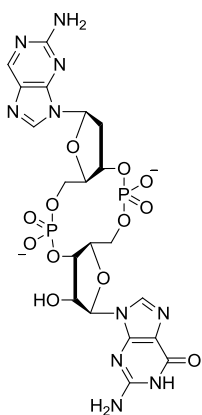
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Chapter 1

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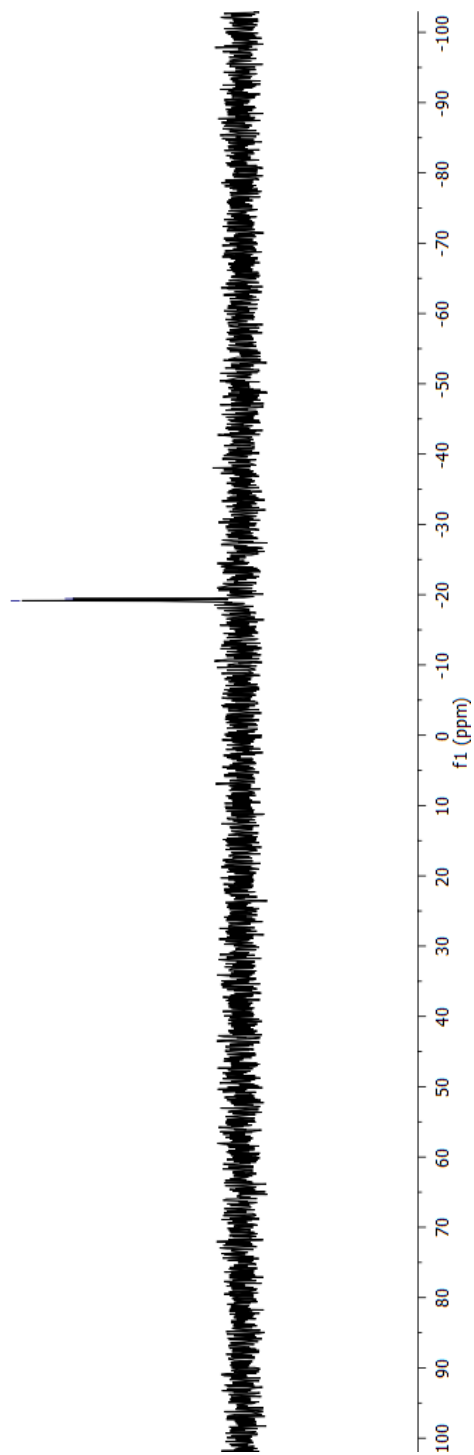
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Chapter 4

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