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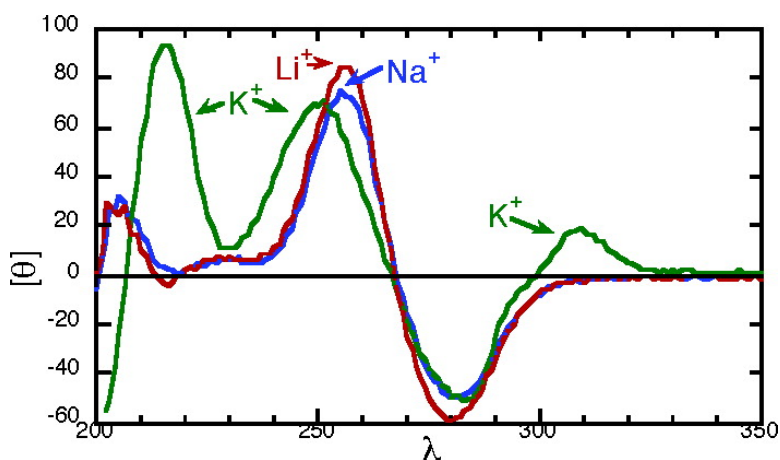
c-di-GMP Displays A Monovalent Metal Ion-Dependent Polymorphism

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c-di-GMP Displays A Monovalent Metal Ion-Dependent Polymorphism

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Naturally occurring bis-(3′→5′)-cyclic guanosine monophosphate (c-di-GMP, **6**) is an essential but poorly understood bacterial signaling molecule[1,2] that regulates a variety of functions involved in the transition to the stationary state found in biofilms.[3] Crystal structures of c-di-GMP showed that the 12-membered cyclic sugar–phosphate backbone forms a rigid structure with two dimers self-intercalated,[4,5] perhaps leading to an assumption that the molecule was severely limited in the range of structures available to it. An arrangement of four c-di-GMP molecules aligned so as to form two parallel guanine quartets with a central cavity was proposed by Wang in 1990, although it was not observed in the crystal structure.[5] To our knowledge, no circular dichroism spectra of this or other cyclic dinucleotides have been reported.

Elucidation of the mechanisms by which c-di-GMP functions in vivo will benefit significantly from a better understanding of its properties and potential interactions. As part of a program to further explore cyclic dinucleotides, we sought first to develop an improved synthesis of these compounds. Our original strategy for synthesizing cyclic dinucleotides[6] used the phosphotriester method, as have most of the subsequent routes for both ribo and deoxyribo cyclic dinucleotides.[1,7] Subsequently, H-phosphonate[8,9] and combinations of amidite and triester methods[10] were reported. As the H-phosphonate cyclization had worked particularly well in our hands,[9] we chose to develop an approach in which a standard phosphoramidite coupling to prepare the linear dimer is combined with an H-phosphonate cyclization (Scheme 1).

In this approach, the H-phosphonate group also serves as a 3′ protecting group during the coupling step, thus obviating the need for additional protection/deprotection steps and significantly simplifying the overall procedure. We have used both the standard cyanoethyl-protected phosphoramidite monomer **2b** and the methyl phosphoramidite **2a** to give the linear dimers (**4b** and **4a**, respectively) in excellent yields. An advantage of the latter is that after the cyclization reaction, the H-phosphonate diester is conveniently oxidized to the methyl triester (**5a**), so that both triesters have the same protecting group. Although **5a** is still a mixture of three diastereomers, it is reasonably stable and can be purified on silica gel (73% from **4a**). In either path, the final purification of **6** is done by RP HPLC (79% from **5a**). Details of the synthetic procedures starting from **1** via both **2a** and **2b** are provided as Supporting Information.

We converted c-di-GMP (**6**) to the Li+, Na+, and K+ salt forms by cation exchange chromatography to study the effect of metals on its properties by a combination of UV, CD, and NMR spectroscopies. We find that c-di-GMP adopts one or more structured forms in solution, depending on the counterion present. UV melting curves for the Li+ and Na+ forms show the upper portion of an apparent conformational transition, while the K+ form shows at least two at higher concentrations (Supporting Information). All of them are concentration-dependent, indicating intermolecular interactions. Circular dichroism spectra (Figure 1) at

Scheme 1[a]
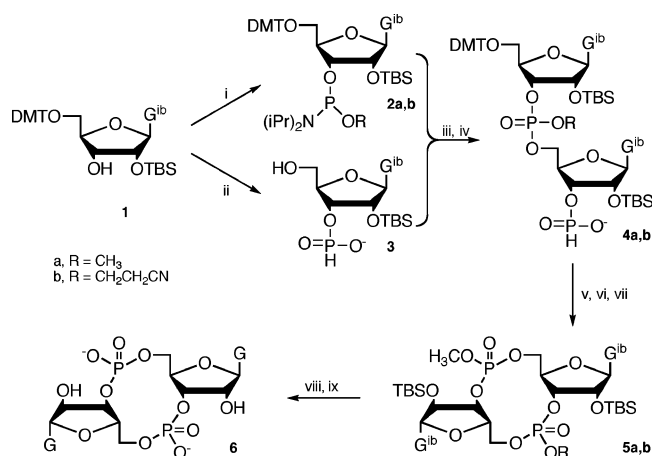
[a] (i) Bis(diisopropylamino)methyl or bis(diisopropylamino)cyanoethyl phosphoramidite and pyridinium trifluoroacetate; (ii) 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one; (iii) pyridinium trifluoroacetate; (iv) *tert*-butylhydroperoxide; (v) sulfonic acid resin; (vi) adamantoylcarbonyl chloride; (vii) methanol/NBS; (viii) pyridine/aq NH₃ (1:1); (ix) TEA/HF.
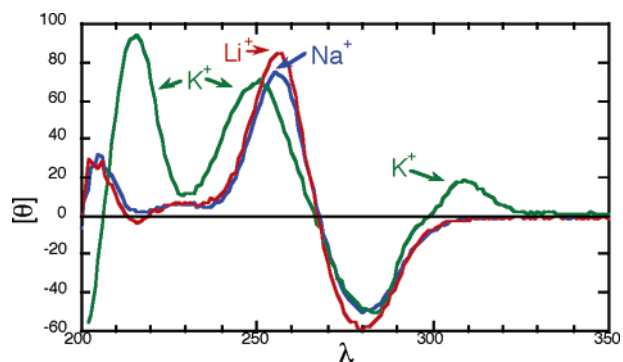

Figure 1. CD spectra at 5 °C of the Li+, Na+, and K+ forms of **6** in 0.1 M LiCl, NaCl, or KCl and 0.01 M LiOAc, Na+ phosphate, or K+ phosphate, respectively (pH 7.0). Wavelength, λ, is in nm and molar ellipticity, $[\theta]$, is in [deg M^{-1} cm^{-1}] × 10^{-3}.

5 °C for the Li+, Na+, and K+ forms all have very strong molar ellipticities with patterns (negative at 280 nm and positive near 250 nm) that are inverted relative to those of right-handed DNA but similar to those of 5′-GMP and 3′-GMP in 0.1 M K+,[11] left-handed 5′-5′ GpG,[11] and left-handed Z DNA.[12] However, CD spectra obtained under the same conditions for the linear dimer, 3′-5′ GpG, in all three salt forms show typical right-handed patterns (Supporting Information). For the linear dimer, the K+ > Na+ ≫ Li+ order of molar ellipticity for the positive band near 260 nm is associated with the known formation of guanine quartet structures.[13] For **6**, on the other hand, the molar ellipticities of the positive band near 250 nm for all salt forms are approximately similar to each other and are 20 times more intense than the Li+

form of the linear dimer, which does not self-associate. At 75 °C, all forms of **6** retain some residual ellipticity, while the linear dimers do not. Thus, the strong, inverted pattern shown by all salt forms of **6** is likely a consequence of the constrained structure in which the guanine rings are held in a fixed, left-handed orientation, presumably with additional intermolecular interactions, including intercalation and/or end-to-end stacking.

In addition to the band at 250 nm, the K^+ form of **6** displays positive bands at 215 (very strong) and 309 nm (moderate) that are weak or absent in the Li^+ and Na^+ forms. Monitoring of the 215, 250, and 309 nm bands while changing the sample temperature reveals that upon cooling, the 250 nm band appears immediately in all salt forms, while the 215 and 309 nm bands present in the K^+ form are slow to appear (Supporting Information). When the samples of the Na^+ and Li^+ forms are warmed, the 250 nm band disappears immediately. In contrast, with the K^+ form, all of the bands disappear only slowly at the same apparent rate. At equilibrium at 40 °C, the 250 nm band is substantially diminished, while the 215 and 309 nm bands are nearly absent (Supporting Information). These results are consistent with the presence of at least two types of structural alignments, one that is generated quickly and is the dominant form present with Na^+ or Li^+ (reflected in the 250 nm band), and another that is formed by a slow association and is the dominant form present with K^+ (reflected in the 215 and 309 nm bands). Upon heating, disruption of this second alignment is rate-determining.
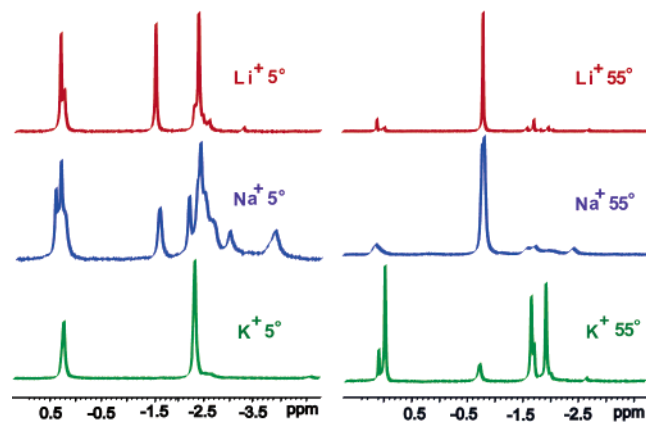

Figure 2. ^{31}P NMR spectra of the Li^+ (33 mM), Na^+ (37 mM), and K^+ (37 mM) forms of **6** in 0.1 M LiCl, NaCl, or KCl, respectively, in 90/10 H_2O/D_2O (pH 7).

^{31}P NMR spectra (Figure 2) at 55 °C for the Li^+ and Na^+ forms of **6** show a dominant signal at −0.8 ppm. Upon cooling to 5 °C, this signal quickly diminishes and moves to −1.5 ppm. Simultaneously, new families of signals appear slightly upfield and downfield of the original peak. For the K^+ form, similar families of peaks are dominant even at 55 °C, with only a very small central signal at −0.7 ppm. ^1H NMR spectra of **6** at 5 °C in all salt forms display multiple peaks for the guanine H8 atoms near 8 ppm as well as for the N1H atoms near 11 ppm, indicating that the latter are protected from exchange (Figure 3). This protection greatly diminishes at 55 °C for the Li^+ and Na^+ forms, but not the K^+ form, which is again consistent with two different alignments.

The UV, CD, and NMR data presented here demonstrate that this small molecule displays a surprising polymorphism that is exquisitely sensitive to monovalent metal ions. In Li^+ and Na^+, the data are consistent with a dominant form that involves primarily aromatic stacking, probably intercalation, and/or end-to-end stack-
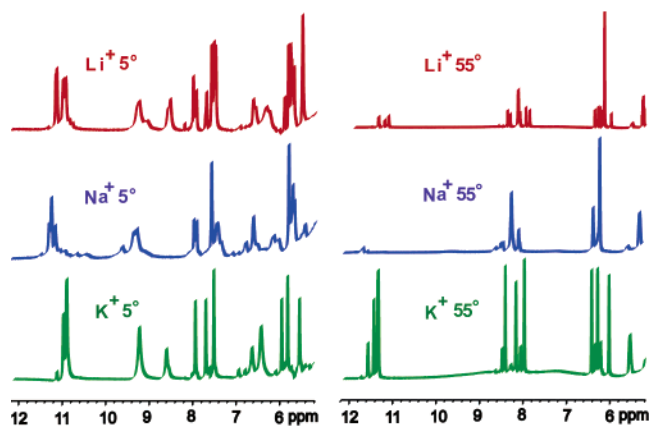

Figure 3. ^1H NMR spectra of the Li^+ (33 mM), Na^+ (37 mM), and K^+ (37 mM) forms of **6** in 0.1 M LiCl, NaCl, or KCl, respectively, in 90/10 H_2O/D_2O (pH 7).

ing. In contrast in K^+, the data support a guanine quartet structure that is increasingly favored as the concentration of the dimer increases. Further structural and energetic studies will be necessary to clarify the kinds of interactions that c-di-GMP can accommodate.

Supporting Information Available: Synthetic procedures for **6**, UV melting curves of **6**, CD spectra of **6** and the linear dimer, and plots of CD of **6** after increasing or decreasing the temperature. This material is available free of charge via the Internet at http://pubs.acs.org.

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