# Oligomer formation of the bacterial second messenger c-di-GMP: reaction rates and equilibrium constants indicate a monomeric state at physiological concentrations

## Supporting Information

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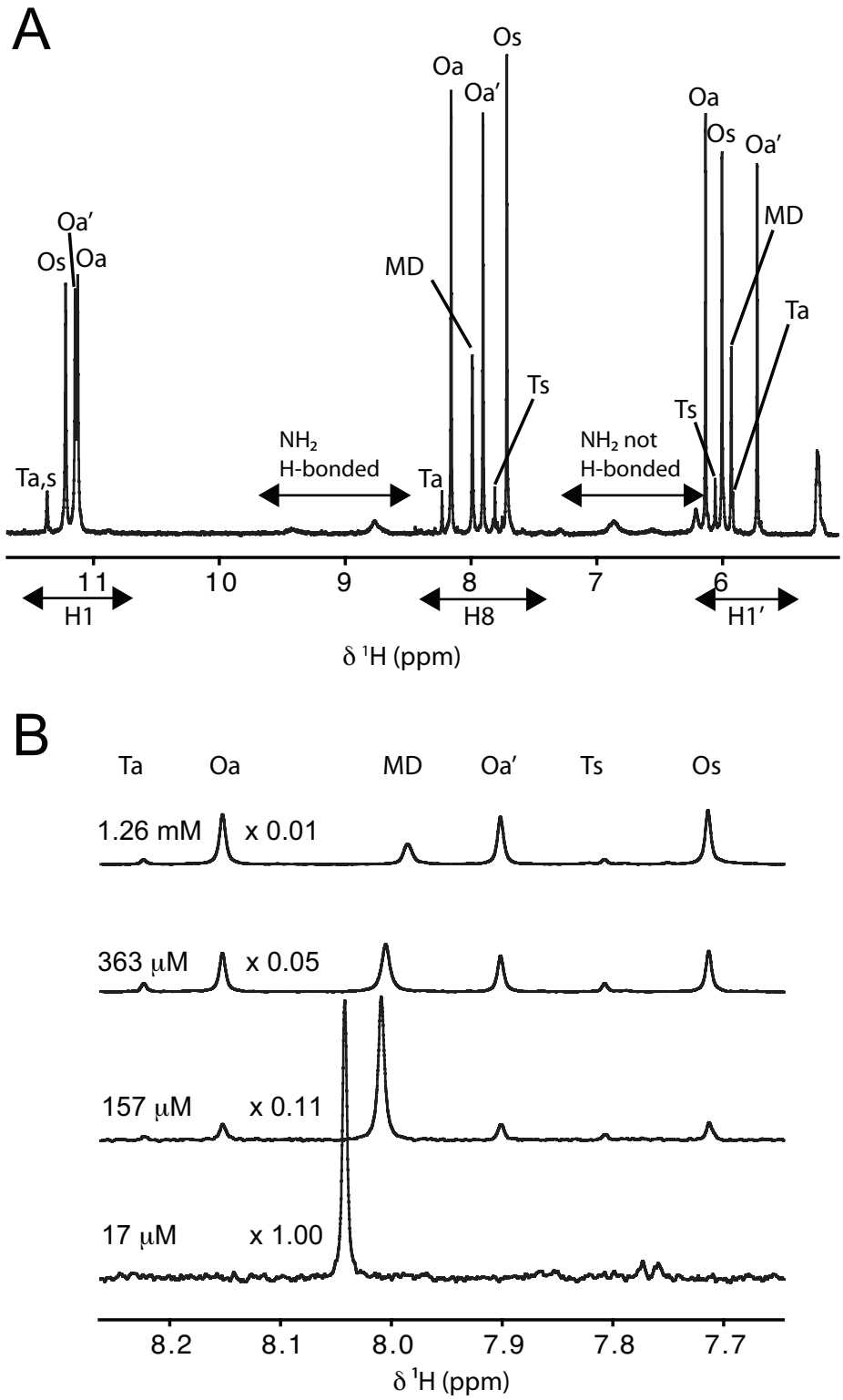



**Figure S1.** $^{1}$H NMR spectra of c-di-GMP dissolved in buffer B. (A) H1, H8 and H1' regions of the $^{1}$H NMR spectrum of 1.3 mM c-di-GMP in buffer B. Resonance assignments are identical as in Figure 2. (B) H8 region of $^{1}$H NMR spectra at various c-di-GMP concentrations in buffer B.

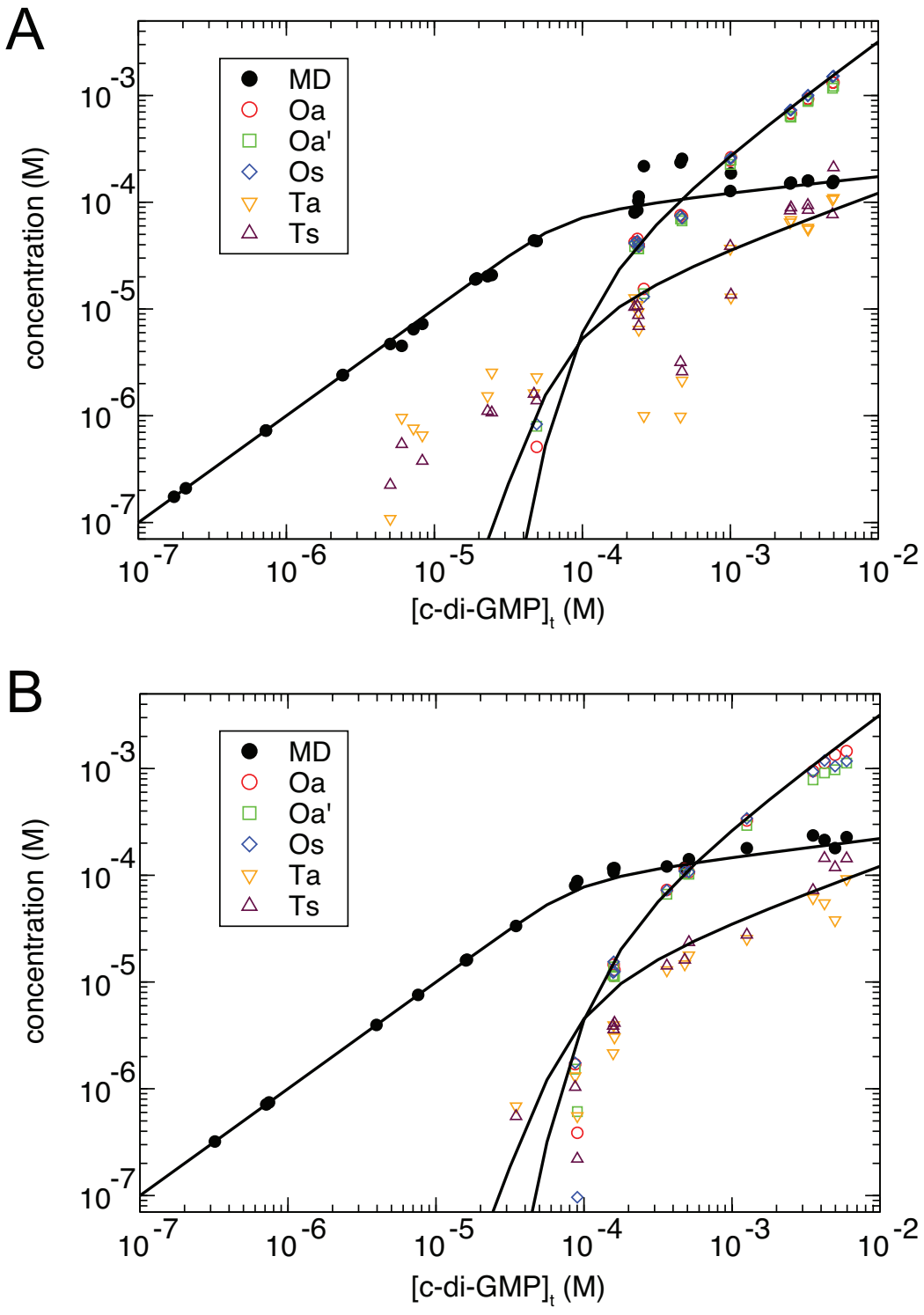



**Figure S2.** Monomeric concentrations of individual oligomer species as a function of total c-di-GMP concentration. Data points are according to experimental H8 peak intensities. Solid lines are the result of the fit to the kinetic model. The results for c-di-GMP in buffer A and B are shown in panels (A) and (B) respectively. Deviations from the fit can be explained by incomplete equilibration due to the slow oligomer formation/dissociation kinetics and imperfections of the model (unknown species).

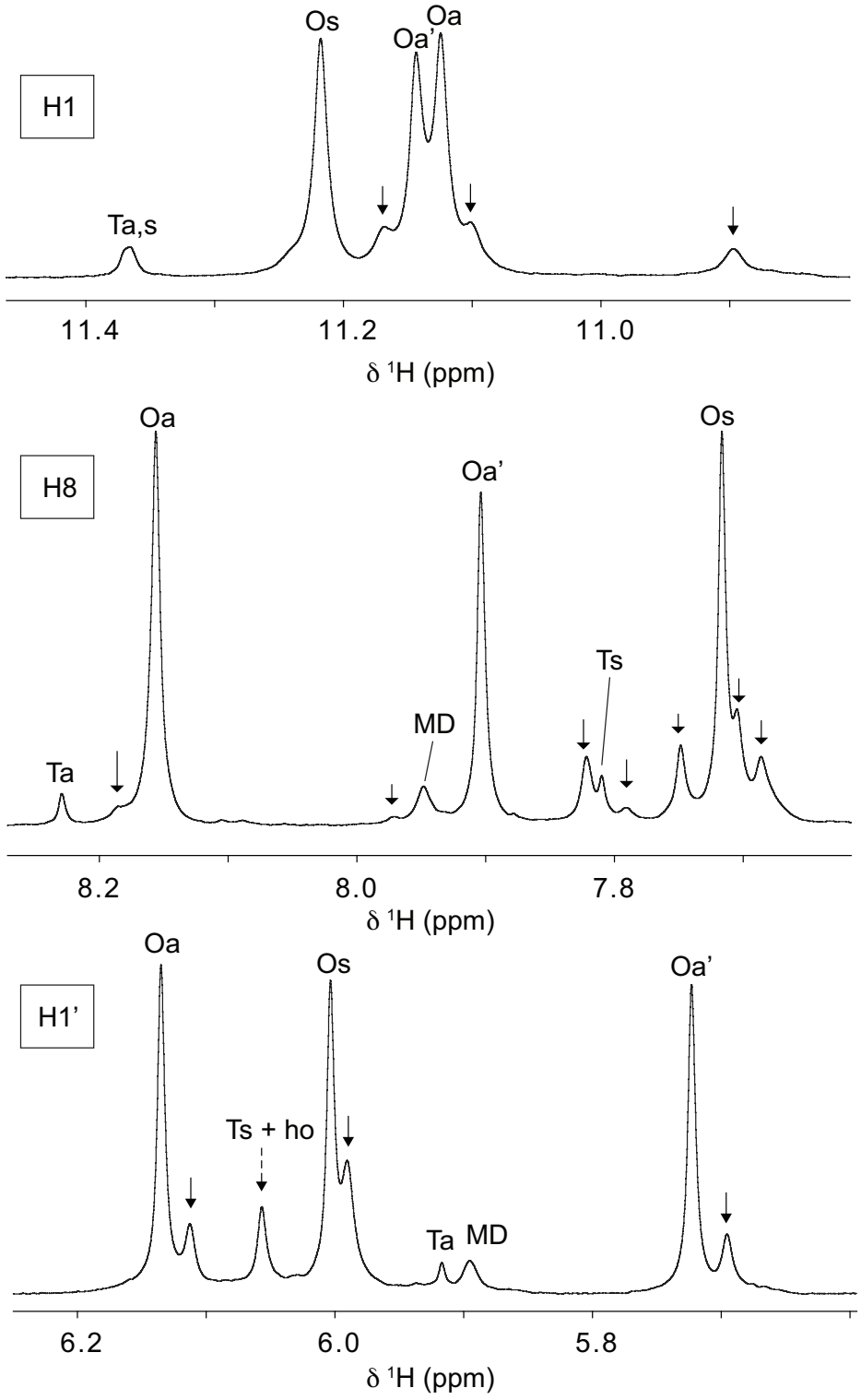



**Figure S3.** 1D $^{1}$H NMR spectrum of 5.9 mM c-di-GMP in buffer B. Spectral regions of H1 (top), H8 (middle) and H1' (bottom) are shown. Arrows indicate additional resonances from higher oligomers (ho), that start to emerge at c-di-GMP concentrations > 3 mM. An almost identical behavior was observed in buffer A.

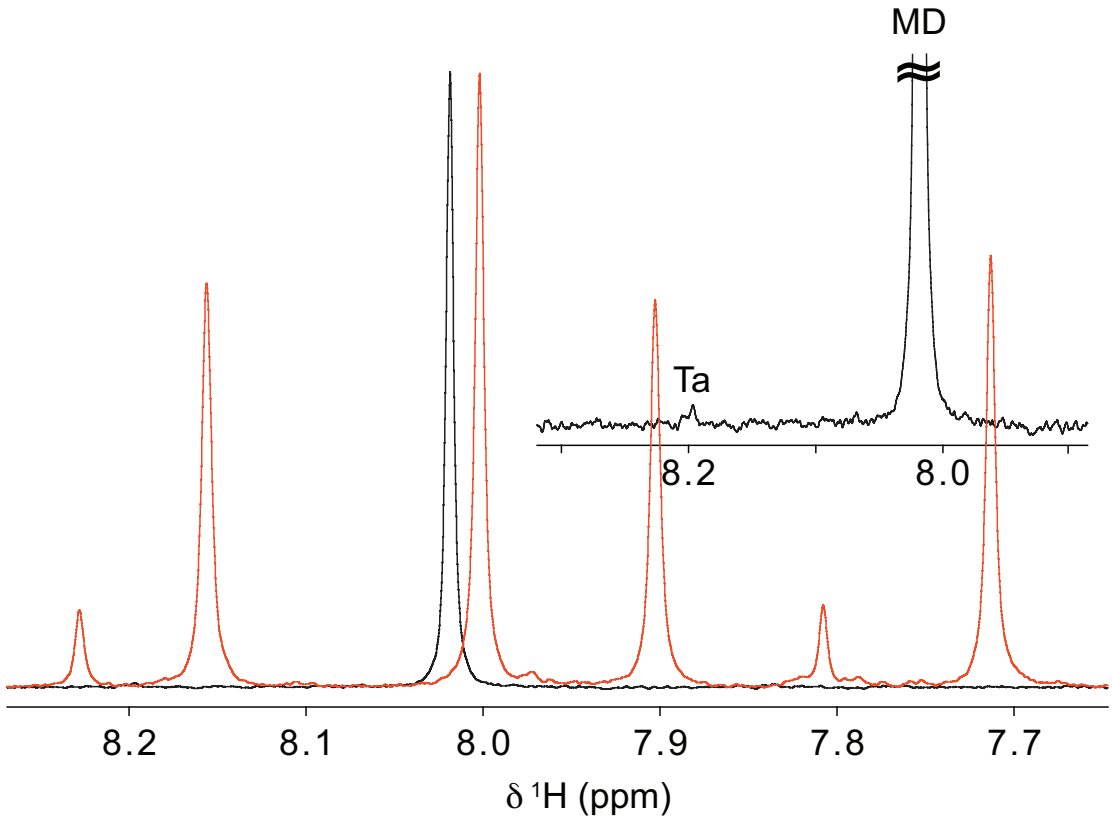



**Figure S4.** H8 region of the 1D $^{1}$H NMR spectrum of 512 μM c-di-GMP in buffer B at 60 °C (black) and at 24 °C (red). Spectra have been normalized with respect to the MD peak height. The inset shows enlarged the only detectable H8 resonance (Ta) apart from MD with an intensity of approximately 0.7% of the MD intensity indicating nearly complete dissociation of the oligomers.

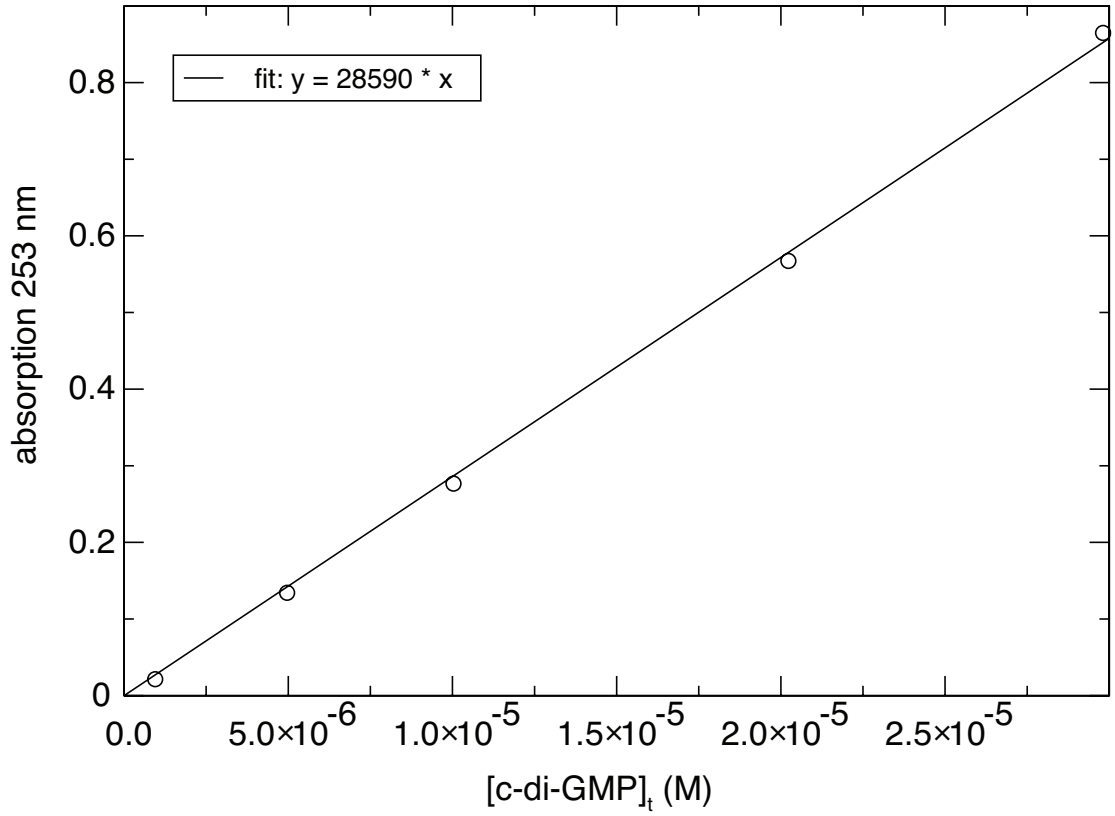



**Figure S5.** UV absorption (room temperature) at 253 nm as a function of c-di-GMP concentration in 5 mM TRIS buffer. A linear fit of the concentration-dependent UV absorption at 253 nm reveals a molar extinction coefficient of 28590 $M^{-1}$ $cm^{-1}$.

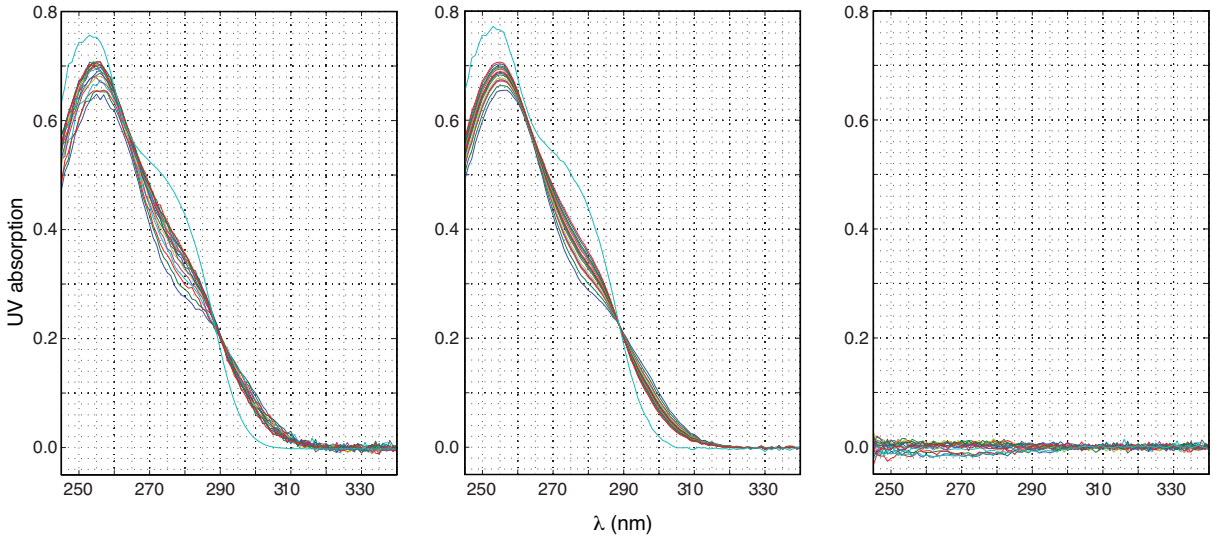
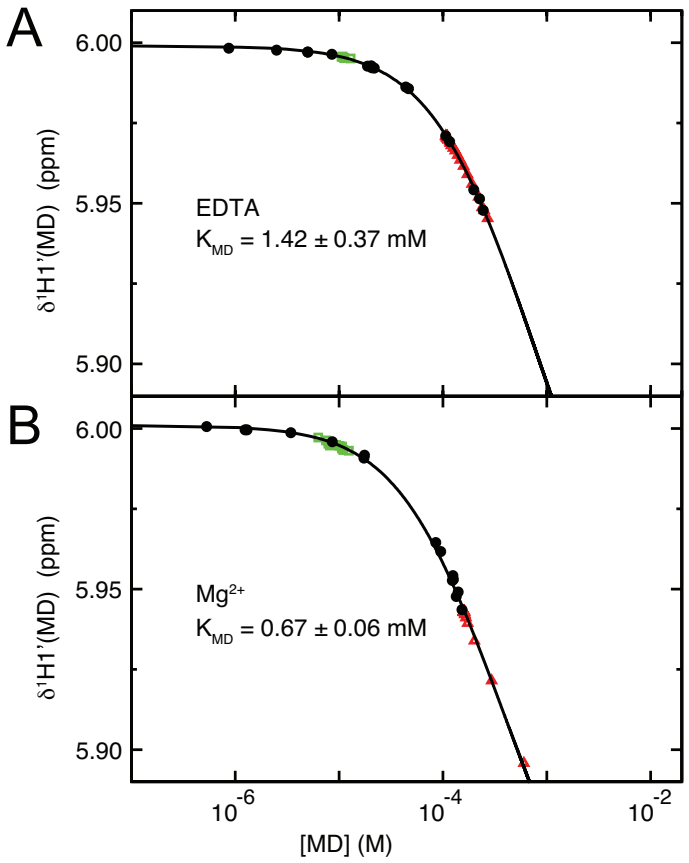



**Figure S6.** UV absorption spectra of 25.3 μM c-di-GMP at different time points after dilution from a 1.25 mM stock. Experimental (left), theoretical (middle) and the difference between the former (right) are shown. Theoretical curves were obtained by a two-state fit, assuming significantly different UV absorption spectra between unstacked monomers and stacked oligomers. From the theoretical data, an isosbestic point of 288.8 nm was determined. Even though the model assumes that all species other than monomers have identical absorption spectra, the agreement is very good.



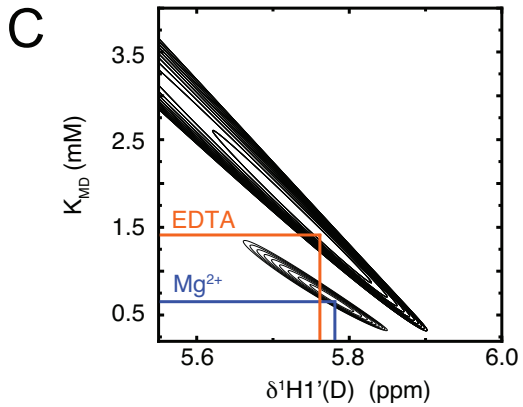




**Figure S7.** Concentration dependence of the c-di-GMP monomer/dimer equilibrium as evidenced from the chemical shift of the MD H1' resonance. (A) H1' chemical shift of c-di-GMP in buffer A as function of monomer/dimer concentration [MD] as determined from the H1' peak intensity. (B) same as (A) but spectra were recorded in buffer B. Filled black circles in (A) and (B) represent data from a titration experiment, while red triangles and green squares indicate data from oligomer reassociation and dilution experiments, respectively. The solid lines in (A) and (B) represent fits to a two-state model according to Eq. 1. (C) Mean square deviation $\chi^2$ of fitted and experimental shifts according to Eq. 4 as a function of the fit parameters $K_{MD}$, $\delta_D$(H1') for buffer A (EDTA) and B ($Mg^{2+}$).

**Table S8.** Rates and equilibrium constants of c-di-GMP oligomerization.

| | 2M ↔ D | 4M ↔ T | | | T + 4M ↔ O | | |
|---|---|---|---|---|---|---|---|
| buffer | $K_{MD}$ [M][a] | $k_{TM}$ [$M^{-3}s^{-1}$] | $k_{MT}$ [$s^{-1}$] | $K_{MT}$ [$M^{3}$] | $k_{OT}$ [$M^{-4}s^{-1}$] | $k_{TO}$ [$s^{-1}$] | $K_{TO}$ [$M^{4}$] |
| A (EDTA)[b] | $1.4 \pm 0.4 \cdot 10^{-3}$ | $5.4 \pm 0.8 \cdot 10^{5}$ | $6.5 \pm 0.5 \cdot 10^{-6}$ | $12.0 \pm 0.9 \cdot 10^{-12}$ | $1.8 \pm 0.2 \cdot 10^{12}$ | $4.9 \pm 0.3 \cdot 10^{-5}$ | $2.8 \pm 0.1 \cdot 10^{-17}$ |
| B ($Mg^{2+}$) | $6.7 \pm 0.6 \cdot 10^{-4}$ | | | | | | |

[a]In contrast to Table 1, equilibrium constants were determined from MD $^{1}$H1' chemical shift and peak intensities.
[b]M ↔ T and T ↔ O reaction parameters derived from the fit of the kinetic model to the oligomer reassociation experiment in buffer A.

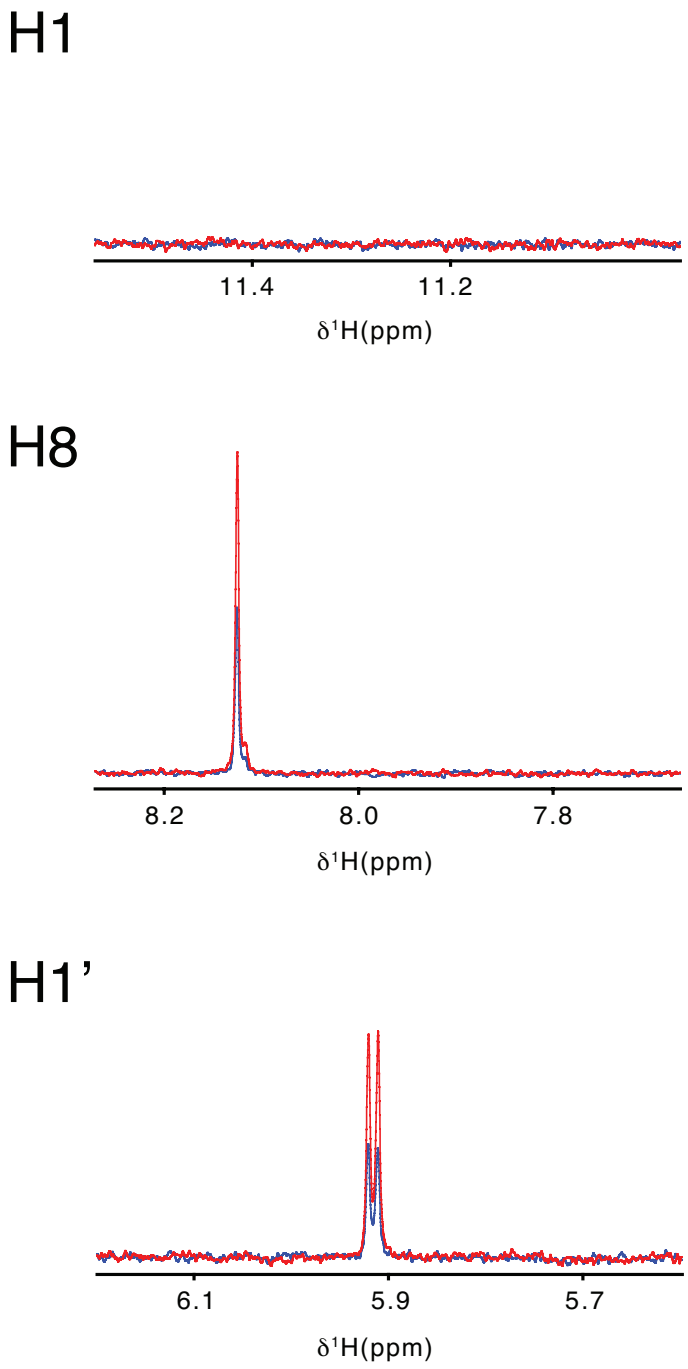



**Figure S9.** NMR spectra of 100 μM (red) and 50 μM (blue) GTP dissolved in buffer A. Samples were not annealed, but directly placed into the spectrometer at 24°C. No H1 resonances indicative of H-bonding are observable, no shift changes are observed for the other resonances, and the intensities in the 100 μM spectrum are twice those of the 50 μM spectrum. Thus formation of oligomers is not detectable.

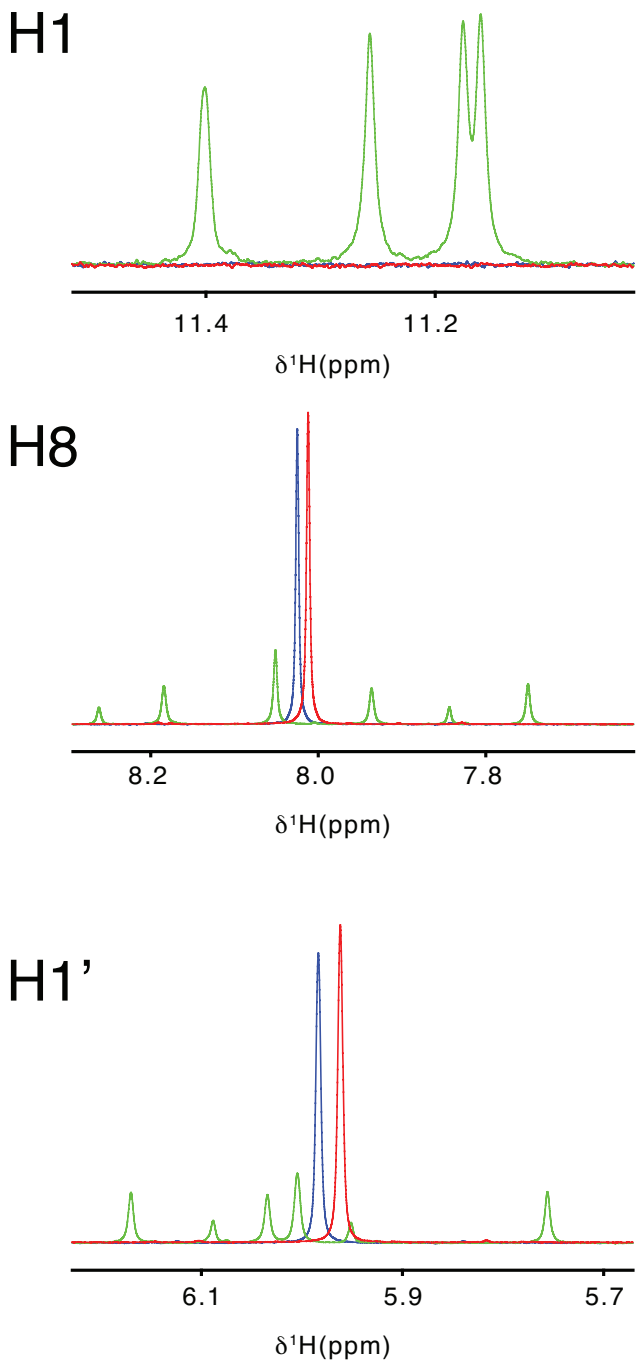



**Figure S10.** NMR spectra at 297 K of ~1 mM c-di-GMP dissolved in 5 mM TRIS/HCl, pH8, in presence of 150 mM KCl (green) or 250 mM NaCl (blue) or 250 mM LiCl (red). Neither for NaCl buffer nor for LiCl buffer H1 resonances indicative of H-bonding due to oligomer formation are observable. However the characteristic set of tetramer and octamer resonances appears in presence of KCl. This clearly indicates that $K^+$ is the major determinant for c-di-GMP oligomerization at low mM concentrations.

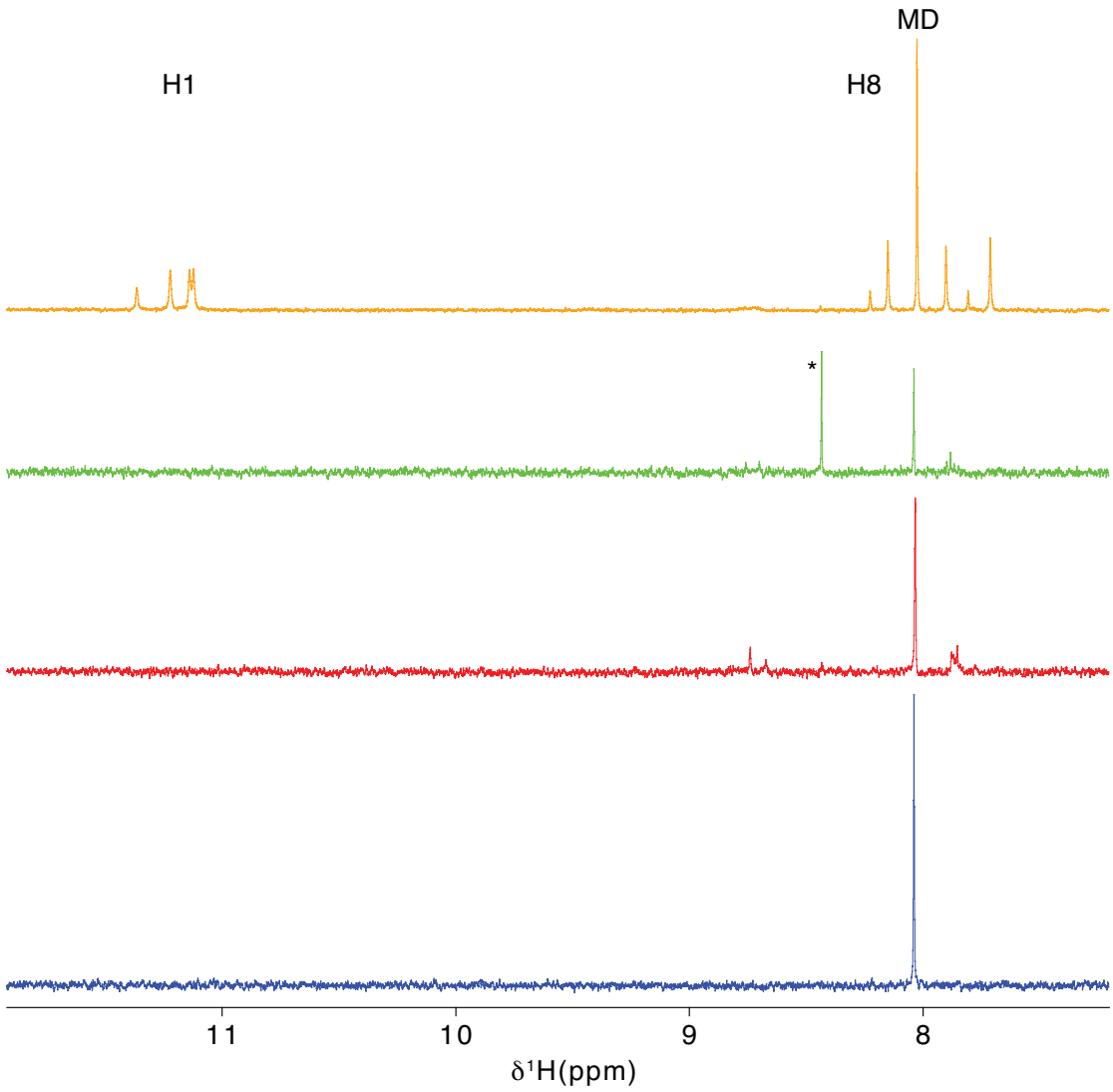



**Figure S11.** NMR spectroscopic test for interaction of c-di-GMP with aromatic dye. All spectra are recorded in c-di-GMP dissolved in buffer A at 297 K. Blue: 25 µM c-di-GMP sample in absence of dye. Red: same as blue directly after addition of 16 µM acriflavin/proflavin. Green: same as red after three days. Orange: 280 µM sample containing G-quartet-based oligomers (shown for comparison).

After addition of dye, a decrease in c-di-GMP and dye peak intensities is observed over a period of several days. No new signals can be detected that would match the dimer, tetramer or octamer resonances. In particular, also no H1 resonances are detected that would indicate H-bond formation. After several days, an additional resonance can be observed at 8.43 ppm (*), which does not match any of the previously identified species. It is possible that this resonance corresponds to an intercalated form, which, however, is not a canonical H-bonded dimer, tetramer or octamer.