Electronic Supplementary Material (ESI) for RSC Advances


# 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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## Supporting information

**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 Sigma Life Science. C-di-GMP was made using the Jones protocol.[1]

**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.[2] 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 $^{o}$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 for a few hours without disaggregating into monomeric form.[2]

For the preparation of monomeric c-di-GMP, 20 or 40 μM of c-di-GMP was heated to 95 $^{o}$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 analog in buffers (50 mM Tris-HCl (pH = 7.5) containing no extra salt) 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:

$$K_{cat} = V_{max}/[E]$$

Where [E] = 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.

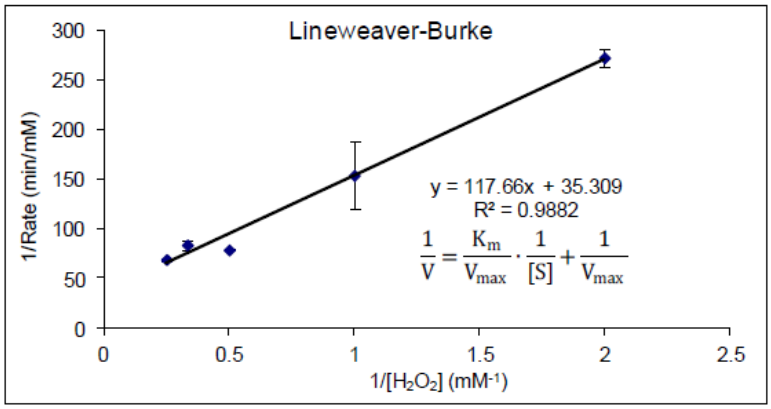


Figure S1. calculation of $K_m$, $V_{max}$ and $k_{cat}$ with PS2.M-hemin catalyst. This value was calculated with 10 µM PS2.M, 0.1 µM Hemin, 2 mM ABTS, 0-4 mM $H_2O_2$, 50 mM Tris-HCl (pH = 7.9) 250 mM $NH_4Cl$.

**Calculation of Dissociation constants ($K_d$)**

Dissociation constant ($K_d$) was determined by the method described by Wang et al.[3] The absorbance change in the Soret band was plotted as a function of DNA concentration and fitted by the equation:

$$[\mathrm{DNA}]_0 = \mathrm{K_d}\frac{\mathrm{A}-\mathrm{A}_0}{\mathrm{A}_\infty-\mathrm{A}} + [\mathrm{P}_0]\frac{\mathrm{A}-\mathrm{A}_\infty}{\mathrm{A}_\infty-\mathrm{A}_0}$$

Where $[DNA]_0$ is the initial concentration of DNA/c-di-GMP, $[P]_0$ is the initial concentration of hemin/hemin analog, $A_\infty$ 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.

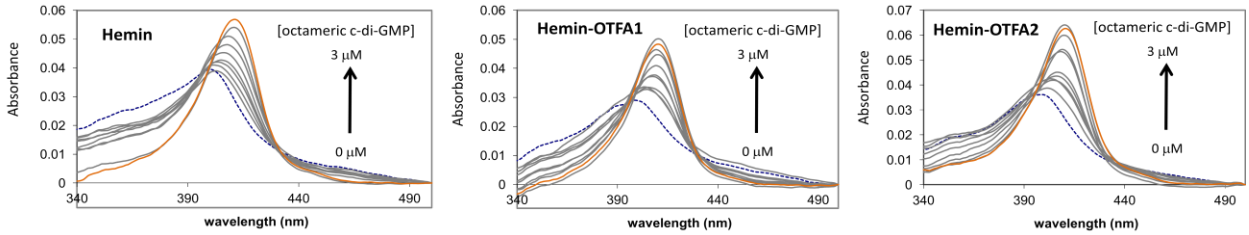


Figure S2. Dissociation constant determination of hemin and analogs to octameric c-di-GMP. Conditions: [octameric c-di-GMP] = 0, 0.1, 0.15, 0.2, 0.25, 0.3, 0.4, 0.5, 1.0, 1.5 and 3 µM, [Hemin/Hemin analog] = 1 µM (contained 2% DMSO), buffer: 50 mM Tris-HCl (pH = 7.9), [$NH_4Cl$] = 250 mM, [KCl] = 3 mM, Triton = 0.05%. Dotted navy blue line = no added c-di-GMP; Orange line = 3 µM c-di-GMP.

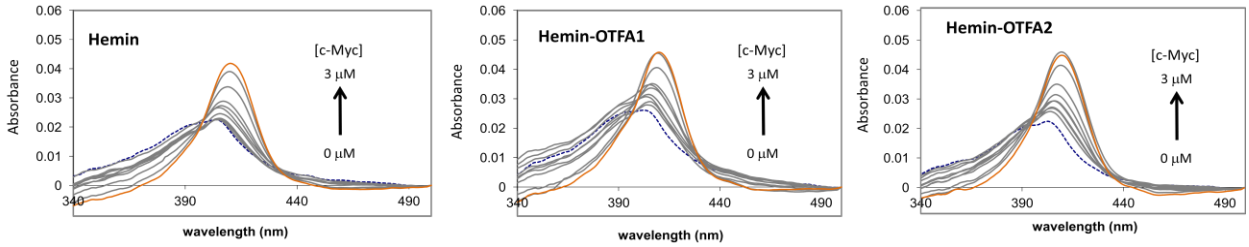


Figure S3. Dissociation constant determination of hemin and analogs to c-Myc. Conditions: [c-Myc] = 0, 0.1, 0.15, 0.2, 0.25, 0.3, 0.4, 0.5, 1.0, 1.5 and 3 µM, [Hemin/Hemin analog] = 1 µM (contained 2% DMSO) buffer: 50 mM Tris-HCl (pH = 7.9), [$NH_4Cl$] = 250 mM, [KCl] = 3 mM, Triton = 0.05%. Dotted navy blue line = no added c-Myc; Orange line = 3 µM c-Myc.

**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, 1.02 mmol), N-hydroxysuccinimide (0.15 g, 1.30 mmol) and (2-aminoethyl)trimethylammonium chloride hydrochloride (0.17 g, 0.99 mmol). The reaction was allowed to stir for 6 hours. 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. The method was as follows:

| Time (min.) | Temperature (°C) | Flow Rate (ml/min.) | % Composition 0.1 % TFA in water | % Composition 0.1 % TFA in 9.9% water and 90% Acetonitrile |
|---|---|---|---|---|
| 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 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 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)

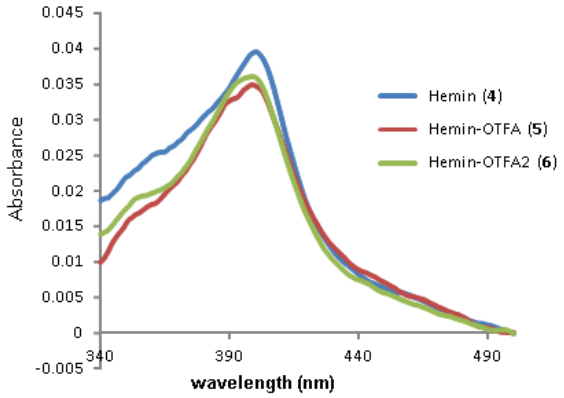


Figure S4. UV-visible spectra of hemin and hemin analogs in 50 mM Tris-HCl (pH = 7.9), 0.05 % triton and 2% DMSO. Concentrations of hemin, hemin-(OTFA) and hemin-$(OTFA)_2$ was 1 µM.

## Monomeric and Octameric c-di-GMP peroxidation

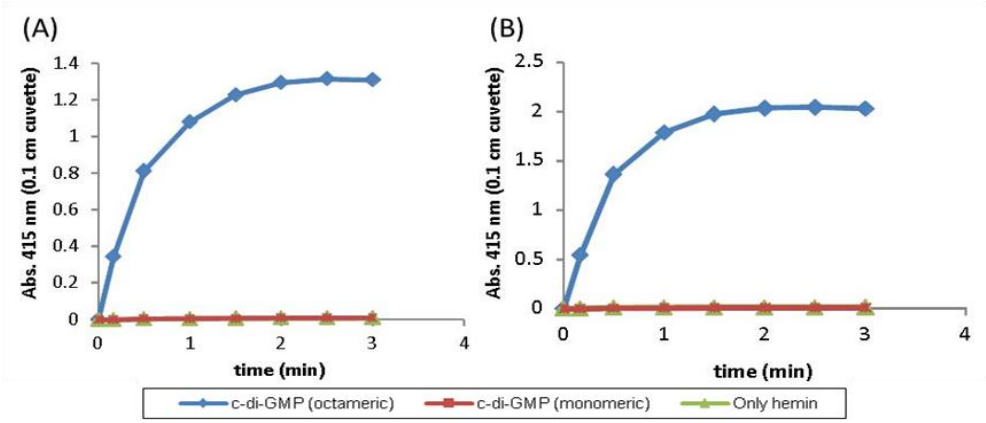


Figure S5. Comparison of monomeric and octameric c-di-GMP peroxidation activity. Conditions: [c-di-GMP] = 20 µM, [Hemin] = 0.5 µM, [ABTS] = 2 mM, [$H_2O_2$] = 2 mM, Buffer: Tris-Cl (pH = 7.9). A) No additional cation B) 250 mM $NH_4Cl$ was added. Octameric peroxidation: Peroxidation was initiated immediately after addition of hemin to octameric c-di-GMP. Monomeric c-di-GMP: 2 mM stock solution of c-di-GMP was heated for 5 min at 95 ºC and cooled down to room temperature for 15 min then incubated at 4 °C for 12 hr. Peroxidation was initiated immediately after addition of hemin.

## DNA sequences

Table S1. Sequences of hemin binding DNAs.

| DNA | Sequence |
|---|---|
| TBA | GGTTGGTGTGGTTG |
| c-Myc | TGAGGGTGGGGAGGGTGGGGAA |
| PS2.M | GTGGGTAGGGCGGGTTGG |

## References

1 B. L. Gaffney, E. Veliath, J. W. Zhao and R. A. Jones, *Org. Lett.*, 2010, **12**, 3269-3271.
2 M. Gentner, M. G. Allan, F. Zaehringer, T. Schirmer and S. Grzesiek, *J. Am. Chem. Soc.*, 2012, **134**, 1019-1029.
3 Y. Wang, K. Hamasaki and R. R. Rando, *Biochemistry*, 1997, **36**, 768-779.