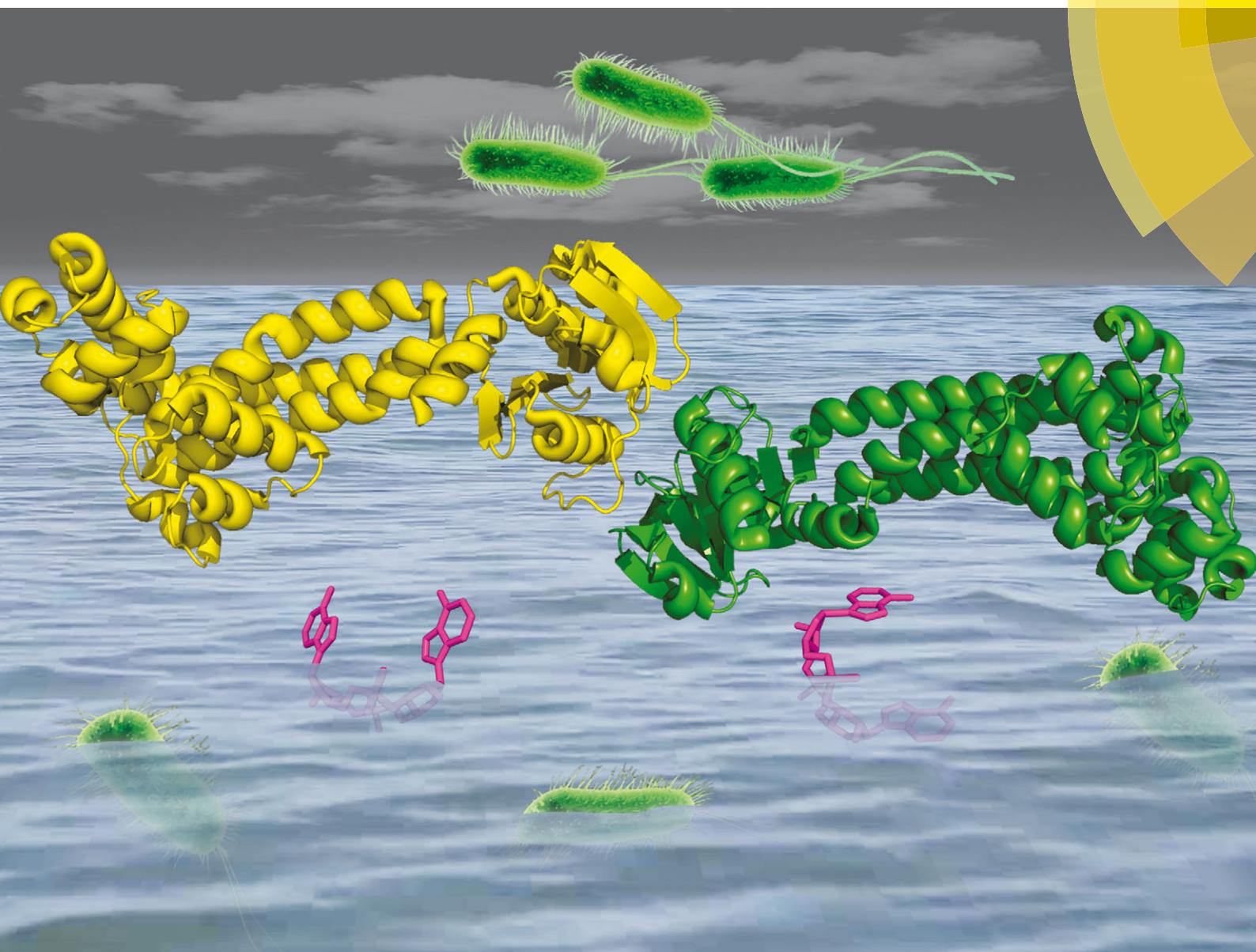


# ChemComm

Chemical Communications

[www.rsc.org/chemcomm](http://www.rsc.org/chemcomm)



ISSN 1359-7345



## COMMUNICATION

Clement Opoku-Temeng and Herman O. Sintim

Potent inhibition of cyclic diadenylate monophosphate cyclase by the antiparasitic drug, suramin

**175** YEARS



Cite this: *Chem. Commun.*, 2016, 52, 3754

Received 21st December 2015,  
Accepted 21st January 2016

DOI: 10.1039/c5cc10446g

[www.rsc.org/chemcomm](http://www.rsc.org/chemcomm)

C-di-AMP synthases are essential in several bacteria, including human pathogens; hence these enzymes are potential antibiotic targets. However, there is a dearth of small molecule inhibitors of c-di-AMP metabolism enzymes. Screening of 2000 known drugs against DisA has led to the identification of suramin, an antiparasitic drug as potent inhibitor of c-di-AMP synthase.

Cyclic diadenylate monophosphate (c-di-AMP) is an important second messenger found in Gram-positive Firmicutes and Actinobacteria.<sup>1–3</sup> It is synthesized from two molecules of adenosine triphosphate (ATP) by diadenylate cyclases, DAC (see Fig. 1), and degraded by c-di-AMP-specific phosphodiesterases (PDE).<sup>4–7</sup> C-di-AMP has been shown to regulate important processes in bacteria, such as virulence,<sup>8</sup> cell wall formation,<sup>8,9</sup> cell size,<sup>10</sup> ion transport<sup>11</sup> among others. For some Gram-positive bacteria, such as *Bacillus subtilis* and *Listeria monocytogenes*, it has been demonstrated that low intracellular levels of c-di-AMP increased the sensitivity of the cells to  $\beta$ -lactam antibiotic treatment; an effect attributed to defective peptidoglycan synthesis.<sup>12,13</sup> Attempts to delete DAC from human pathogens *L. monocytogenes*<sup>13</sup> and *Streptococcus pneumoniae*,<sup>14</sup> failed and this was probably due to the essentiality of DAC for maintaining the integrity of the peptidoglycan. Considering that the majority of antibiotics target peptidoglycan synthesis, there is an obvious interest in discovering inhibitors of DAC enzymes.<sup>15–17</sup>

Motivated by the potential of DAC inhibitors as antibacterial targets we developed an interesting fluorescent assay for monitoring c-di-AMP synthesis, using readily available coraline fluorophore.<sup>18</sup> Using this assay, we discovered the first small molecule inhibitor of a DAC (DisA), bromophenol thiohydantoin (or bromophenol-TH).<sup>15</sup> Bromophenol-TH is however a weak inhibitor of DisA

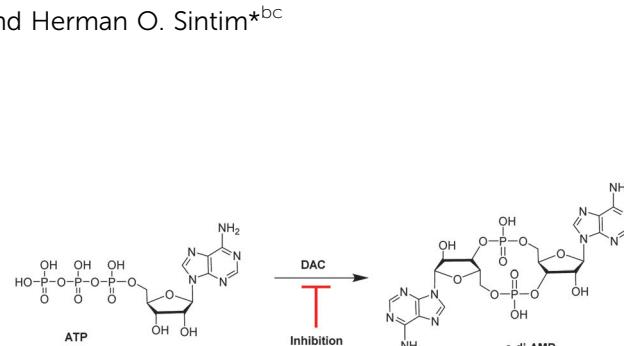


Fig. 1 Schematic of the synthesis of c-di-AMP by DisA from ATP. A DAC inhibitor will hinder the synthesis of c-di-AMP.<sup>15</sup>

(IC<sub>50</sub> of 56  $\mu$ M at 5  $\mu$ M DisA)<sup>15</sup> and attempts to improve its potency *via* modifications were unfruitful.<sup>17</sup> Recently, Müller *et al.* showed that cordycepin triphosphate (3'-deoxyATP) was an inhibitor of *Thermotoga maritima* DisA (TmaDisA) with an IC<sub>50</sub> of 3  $\mu$ M at 26 nM TmaDisA.<sup>16</sup>

In an effort to identify more potent and non-nucleotide-based inhibitors of c-di-AMP synthesis, we employed the coraline assay to screen a library of 2000 known drugs against DisA. This effort led to the identification of suramin (see Fig. 2), a drug used to treat parasitic infections, as a potent inhibitor of DisA.

Suramin is a symmetrical polysulfonated urea derivative that has long been used as an anti-parasitic drug for the treatment of African trypanosomiasis as well as onchocerciasis.<sup>19,20</sup> To delineate which moieties on suramin are responsible for DisA inhibition, we tested the enzyme against five structurally related compounds (see Fig. 2) using the coraline assay. To eliminate the possibility that a positive hit from the coraline assay was not due to the quenching of coraline's fluorescence by the tested compounds, we also used HPLC to monitor the DisA reaction in presence of the compounds. 8-Aminopyrene-1,3,6-trisulfonic acid (APTS), 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS), Ponceau S and benzothiazole-2,5-disulfonic (compounds that contain two or more sulfonic acid groups) did not inhibit DisA (see Fig. 3 and Fig. S1, ESI<sup>†</sup>), indicating that the presence of sulfonic acid alone was not sufficient to cause DisA inhibition. Both trypan blue and suramin inhibited DisA (see Fig. 3) but suramin was a better inhibitor. Using HPLC to monitor the DisA reaction (see Fig. S1, ESI<sup>†</sup>), we noted that that at 20  $\mu$ M, suramin

<sup>a</sup> Graduate Program in Biochemistry, University of Maryland, College Park, Maryland 20742, USA

<sup>b</sup> Department of Chemistry, Purdue University, West Lafayette, IN 47907, USA

<sup>c</sup> Center for Drug Discovery, Purdue University, West Lafayette, IN 47907, USA.

E-mail: hsintim@purdue.edu; Tel: +1 (765) 496-6078

<sup>†</sup> Electronic supplementary information (ESI) available: HPLC analysis of DisA and YbT with select compounds, Experimental section. See DOI: 10.1039/c5cc10446g



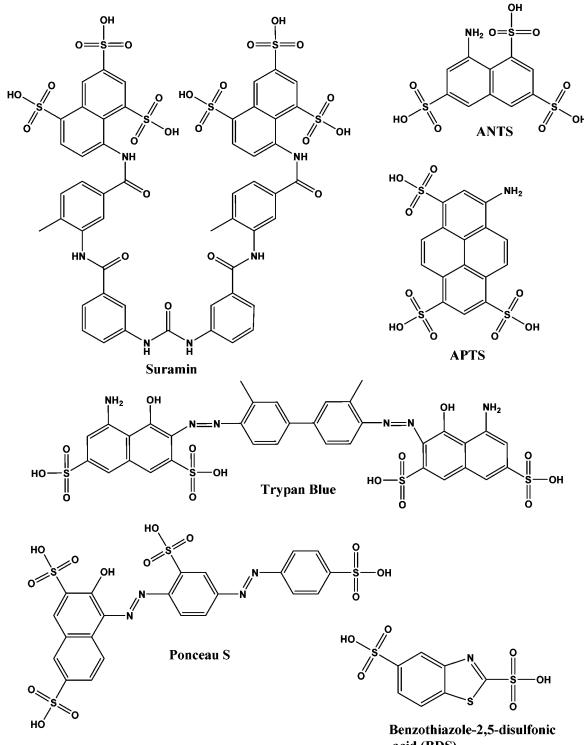


Fig. 2 Structures of suramin and other sulfonated molecules tested against DisA. Abbreviations: ANTS is 8-aminonaphthalene-1,3,6-trisulfonic acid; APTS is 8-aminopyrene-1,3,6-trisulfonic acid.

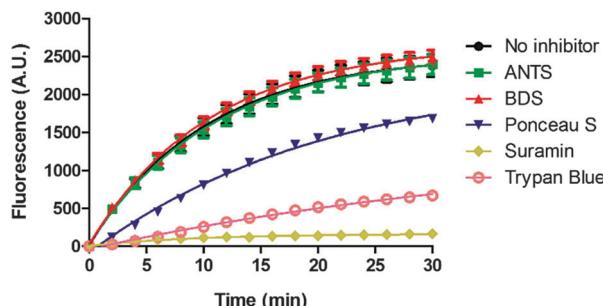


Fig. 3 Coralyne assay of suramin and suramin-related compounds. For the coralyne assay, emission fluorescence intensity of coralyne (at 475 nm) increases as the concentration of c-di-AMP increases.<sup>18</sup> BDS stands for benzothiazole-2,5-disulfonic acid. 8-Aminopyrene-1,3,6-trisulfonic acid (APTS) is highly fluorescent so could not be tested using the coralyne assay. Each experiment was done in triplicate.

inhibited DisA activity by 90% after 30 min while trypan blue only showed 20% inhibition. The coralyne assay indicated about 80% inhibition with trypan blue; the discrepancy between the coralyne and HPLC results for trypan blue inhibition is probably due to partial fluorescence quenching by trypan blue, which contains an azo moiety. Therefore, although the coralyne assay is more convenient than HPLC analysis for high throughput screening to discover c-di-AMP synthase inhibitors, it is crucial to use counter screens, such as HPLC analysis, to confirm “hits”.

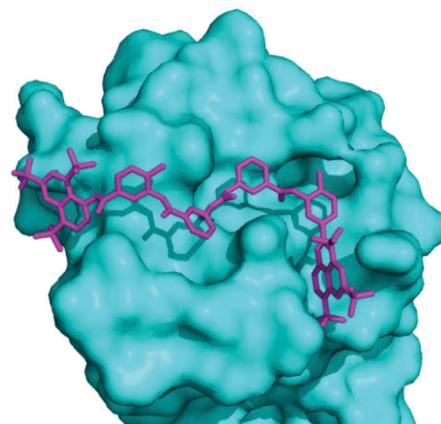


Fig. 4 Molecular docking used to obtain a suramin/TmaDisA complex. Suramin (magenta) binds in the nucleotide-binding pocket of TmaDisA. Image was generated with PyMOL.

We wondered if suramin was inhibiting DisA *via* ATP-competitive mechanism. Previous report by Avliyakulov *et al.* indicated that binding of suramin to L3, a ribosomal protein from *Trypanoplasma borreli*, completely abolished ATP binding.<sup>21</sup> Also, Morgan *et al.* crystallized suramin bound to the ATP binding site of *Leishmania mexicana* pyruvate kinase.<sup>22</sup> These precedents provide evidence of suramin binding to the nucleotide binding pockets of proteins.

Docking of suramin with a monomer of TmaDisA(PDB:3C1Z)<sup>3</sup> revealed that suramin bound to the ATP binding site of TmaDisA (Fig. 4) with binding affinity of  $-9.4 \text{ kcal mol}^{-1}$ . Future crystallography studies, beyond the scope of this work, should reveal if the docked structure is accurate and could provide more insight into how to design potent DisA inhibitors. *B. subtilis* DisA is similar to TmaDisA; computationally modelled 3D structure<sup>23</sup> of *B. subtilis* DisA aligned well with TmaDisA (see Fig. S2, ESI<sup>†</sup>).

To provide some experimental confirmation that suramin and ATP compete for the same binding site in DisA, we determined the  $IC_{50}$  values at various ATP concentrations (100  $\mu\text{M}$ , 500  $\mu\text{M}$  and 1 mM, see Fig. 5). The formation of  $\alpha^{32}\text{P}$ -c-di-AMP/c-di-AMP from  $\alpha^{32}\text{P}$ -ATP/ATP by 1  $\mu\text{M}$  DisA in the presence of increasing concentrations of suramin at the various ATP concentrations was monitored *via* TLC.  $IC_{50}$  values of 1.1  $\mu\text{M}$ , 3.1  $\mu\text{M}$  and 5.4  $\mu\text{M}$  were obtained at the ATP concentrations of 100  $\mu\text{M}$ , 500  $\mu\text{M}$  and 1 mM respectively. The observed increase in  $IC_{50}$  values upon increasing ATP concentration is consistent with both molecules competing for similar binding site.

We also compared the potency of suramin's inhibition with that of 3'-deoxyATP and bromophenol-TH. Here,  $IC_{50}$  values of 2.3  $\mu\text{M}$ , 3.8  $\mu\text{M}$  and 67.2  $\mu\text{M}$  (DisA concentration was 1  $\mu\text{M}$ ) were obtained for suramin, 3'-deoxyATP and bromophenol-TH respectively. This indicates that suramin is more potent than either of the two previously identified inhibitors of DisA (see Fig. S3, ESI<sup>†</sup>).

The addition of suramin to DisA resulted in a decrease in the protein intrinsic fluorescence (see Fig. S4, ESI<sup>†</sup>), probably due to changes in the microenvironment(s) of the tyrosine residues in the protein. Therefore we were able to determine the apparent  $K_d$  of suramin binding to DisA *via* fluorescence titration of the



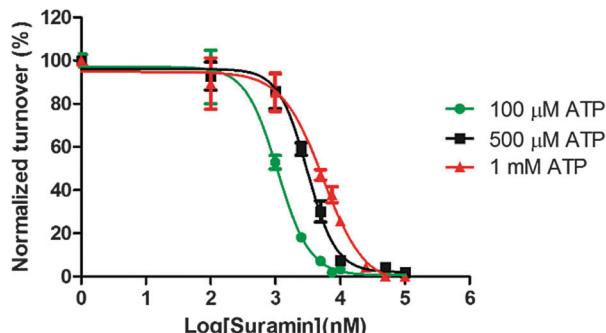


Fig. 5 Inhibition constant of suramin determination. The  $IC_{50}$  of suramin was determined by incubating DisA with either 100  $\mu$ M, 500  $\mu$ M or 1 mM  $\alpha$ -<sup>32</sup>P-ATP/ATP in reaction buffer. The  $IC_{50}$  values at 100  $\mu$ M, 500  $\mu$ M and 1 mM ATP were 1.1  $\mu$ M, 3.1  $\mu$ M and 5.4  $\mu$ M. Experiments were done in triplicate.

inhibitor with 5  $\mu$ M DisA. The apparent  $K_d$  was determined to be 5.4  $\mu$ M (Fig. 6A), assuming a 1:1 binding. Bromophenol-TH bound to DisA with an apparent  $K_d$  of 21  $\mu$ M,<sup>15</sup> so it appears that suramin binds tighter to DisA, compared to bromophenol-TH. We also analysed the fluorescence quenching data using the Stern–Volmer equation<sup>24,25</sup> (see Fig. S4, ESI†) and by the modified form of the Stern–Volmer equation (eqn (1) and Fig. 6B),<sup>26</sup>

$$\log \frac{(F_0 - F)}{F} = \log K + n \log [Q] \quad (1)$$

where  $F$  and  $F_0$  are respectively the fluorescence intensities (at 340 nm) in the presence and absence of the quencher (suramin) at concentration  $Q$ , the binding constant  $K$  (reciprocal of which gives the dissociation constant,  $K_d$ ) is the  $y$ -intercept of the line and the slope gives the number of binding sites,  $n$  on the

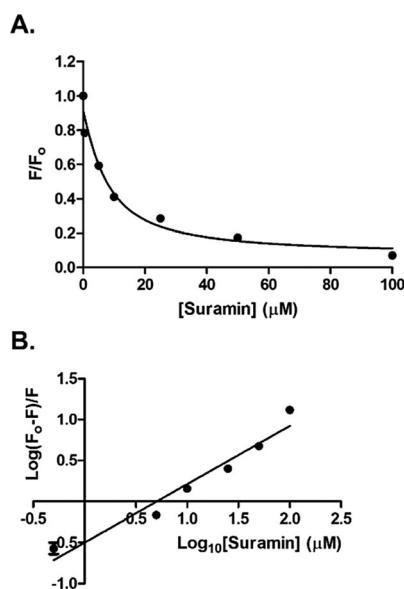


Fig. 6 (A) Plot of relative fluorescence of DisA (5  $\mu$ M) at 340 nm as a function of suramin concentration. The data was fitted to the non-linear regression, eqn (S3) (see ESI† Experimental section). (B) Stern–Volmer plot generated by fitting the fluorescence emission at 340 nm to eqn (1). GraphPad Prism was used to obtain both plots from triplicate measurements.

protein (DisA). From eqn (1), an apparent  $K_d$  of 3.2  $\mu$ M was determined and the number of binding sites ( $n$ ) was found to be  $\sim 1$ , implying a 1:1 binding between DisA and suramin.

Suramin did not inhibit the activity of *B. subtilis* YybT, a c-di-AMP specific phosphodiesterase (see Fig. S5, ESI†), indicating that the observed DisA inhibition is somehow specific.

In conclusion, we have identified suramin as a potent inhibitor of DisA. Suramin is already used in the clinic to treat parasitic infection and has also been shown to have anti-cancer<sup>27</sup> and anti-viral<sup>28</sup> properties. Recently Nautiyal *et al.* showed that suramin inhibited *Mycobacterium tuberculosis* RecA protein with submicromolar  $IC_{50}$ .<sup>29</sup> They also showed that suramin potentiated the activity of ciprofloxacin against *M. smegmatis*.<sup>29</sup> Here, we show that suramin also inhibits cyclic diadenylate cyclase enzyme and represents an interesting scaffold, which could be used to develop cyclic dinucleotide signalling inhibitors. The advances made in the identification of c-di-AMP related enzymes, receptor proteins and RNA far outpace the number of small molecule inhibitors of these proteins. This paper sets the tone for the discovery of such small molecules, which could find use in unravelling the intricacies of cyclic dinucleotide signalling as well as being used as antibacterial agents.

This work was supported by funding from National Science Foundation (CHE1307218) and Camille Dreyfus Foundation. We thank Dr Karl-Peter Hopfner and Dr Angelika Gründling for plasmids encoding DisA and also Dr Zhao-Xun Liang for YybT plasmid.

## Notes and references

- 1 R. M. Corrigan and A. Gründling, *Nat. Rev. Microbiol.*, 2013, **11**, 513–524.
- 2 (a) U. Römling, *Sci. Signaling*, 2008, **1**, pe39; (b) D. Kalia, G. Merey, S. Nakayama, Y. Zheng, J. Zhou, Y. Luo, M. Guo, B. T. Roembke and H. O. Sintim, *Chem. Soc. Rev.*, 2013, **42**, 305–341.
- 3 G. Witte, S. Hartung, K. Buettner and K.-P. Hopfner, *Mol. Cell*, 2008, **30**, 167–178.
- 4 Y. Bai, J. Yang, X. Zhou, X. Ding, L. E. Eisele and G. Bai, *PLoS One*, 2012, **7**, e35206.
- 5 T. Kamegaya, K. Kuroda and Y. Hayakawa, *Nagoya J. Med. Sci.*, 2011, **73**, 49–57.
- 6 T. N. Huynh, S. K. Luo, D. Pensinger, J. D. Sauer, L. Tong and J. J. Woodward, *Proc. Natl. Acad. Sci. U. S. A.*, 2015, **112**, E747–E756.
- 7 Y. Bai, J. Yang, L. E. Eisele, A. J. Underwood, B. J. Koestler, C. M. Waters, D. W. Metzger and G. Bai, *J. Bacteriol.*, 2013, **195**, 5123–5132.
- 8 C. E. Witte, A. T. Whiteley, T. P. Burke, J.-D. Sauer, D. A. Portnoy and J. J. Woodward, *mBio*, 2013, **4**, e00282–13.
- 9 M. Kaplan Zeevi, N. S. Shafir, S. Shaham, S. Friedman, N. Sigal, R. Nir Paz, I. G. Boneca and A. A. Herskovits, *J. Bacteriol.*, 2013, **195**, 5250–5261.
- 10 R. M. Corrigan, J. C. Abbott, H. Burhenne, V. Kaever and A. Gründling, *PLoS Pathog.*, 2011, **7**, e1002217.
- 11 Y. Bai, J. Yang, T. M. Zarrella, Y. Zhang, D. W. Metzger and G. Bai, *J. Bacteriol.*, 2014, **196**, 614–623.
- 12 Y. Luo and J. D. Helmann, *Mol. Microbiol.*, 2012, **83**, 623–639.
- 13 J. J. Woodward, A. T. Iavarone and D. A. Portnoy, *Science*, 2010, **328**, 1703–1705.
- 14 J. H. Song, K. S. Ko, J. Y. Lee, J. Y. Baek, W. S. Oh, H. S. Yoon, J. Y. Jeong and J. Chun, *Mol. Cells*, 2005, **19**, 365–374.
- 15 Y. Zheng, J. Zhou, D. A. Sayre and H. O. Sintim, *Chem. Commun.*, 2014, **50**, 11234–11237.
- 16 M. Müller, T. Deimling, K. P. Hopfner and G. Witte, *Biochem. J.*, 2015, **469**, 367–374.
- 17 Y. Zheng, J. Zhou, S. M. Cooper Jr, C. Opoku-Temeng, A. M. De Brito and H. O. Sintim, *Tetrahedron*, 2016, DOI: 10.1016/j.tet.2015.10.073.

18 J. Zhou, D. A. Sayre, Y. Zheng, H. Szmacinski and H. O. Sintim, *Anal. Chem.*, 2014, **86**, 2412–2420.

19 F. Hawking, *Adv. Pharmacol. Chemother.*, 1978, **15**, 289–322.

20 C. C. Wang, *Annu. Rev. Pharmacol. Toxicol.*, 1995, **35**, 93–127.

21 N. K. Avliyakulov, J. Lukes, A. V. Kajava, B. Liedberg, I. Lundstrom and S. P. S. Svensson, *Eur. J. Biochem.*, 2000, **267**, 1723–1731.

22 H. P. Morgan, I. W. McNae, M. W. Nowicki, W. Zhong, P. A. M. Michels, D. S. Auld, L. A. Fothergill-Gilmore and M. D. Walkinshaw, *J. Biol. Chem.*, 2011, **286**, 31232–31240.

23 L. A. Kelley, S. Mezulis, C. M. Yates, M. N. Wass and M. J. E. Sternberg, *Nat. Protoc.*, 2015, **10**, 845–858.

24 J. R. Lakowicz and G. Weber, *Biochemistry*, 1973, **12**, 4161–4170.

25 J. R. Lakowicz and G. Weber, *Biochemistry*, 1973, **12**, 4171–4179.

26 O. K. Abou-Zied and O. I. K. Al-Shihi, *J. Am. Chem. Soc.*, 2008, **130**, 10793–10801.

27 R. V. La Rocca, C. A. Stein, R. Danesi and C. E. Myers, *J. Steroid Biochem. Mol. Biol.*, 1990, **37**, 893–898.

28 I. C. Albulescu, M. van Hoolwerff, L. A. Wolters, E. Bottaro, C. Nastruzzi, S. C. Yang, S.-C. Tsay, J. R. Hwu, E. J. Snijder and M. J. van Hemert, *Antiviral Res.*, 2015, **121**, 39–46.

29 A. Nautiyal, K. N. Patil and K. Muniyappa, *J. Antimicrob. Chemother.*, 2014, **69**, 1834–1843.