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 Probing the *hras*-1^Y i-motif with small molecules†

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Non-B DNA structures represent intriguing and challenging targets for small molecules. For example, the promoter of the *HRAS* oncogene contains multiple G-quadruplex and i-motif structures, atypical globular folds that serve as molecular switches for gene expression. Of the two, i-motif structures are far less studied. Here, we report the first example of small organic compounds that directly interact with the *hras*-1^Y i-motif. We use a small molecule microarray screen to identify drug-like small molecules that bind to the *hras*-1^Y i-motif but not to several other DNA or RNA secondary structures. Two different lead compounds, **1** and **2**, were discovered to have $7.4 \pm 5.3 \mu\text{M}$ and $5.9 \pm 3.7 \mu\text{M}$ binding affinity by surface plasmon resonance and similar affinity by fluorescence titration. A structure–activity relationship (SAR) was developed and two improved analogues of **2** demonstrated submicromolar binding affinities. Both compounds display pH-dependent binding, indicating that they interact with the DNA only when the i-motif is properly folded. Chemical shift perturbation shows that **1** alters the structure of the i-motif, while **2** has no effect on the i-motif conformation, indicating different modes of interaction.

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Introduction

Genomic research has demonstrated that noncanonical DNA structures occur throughout the mammalian genome.¹ In addition to the classical B-DNA structure, DNA can adopt diverse secondary and tertiary structures including hairpins, triplexes, G-quadruplexes (G4s), i-motifs (also known as C-quadruplexes), three-way junctions, four-way junctions (exemplified by Holliday Junctions), and repeat expansions, among other structures.² Alternatively folded DNA is often associated with changes in gene regulation and is therefore associated with many diseases, including cancer. Many DNA structures have roles in regulating the expression of specific genes by forming in promoter regions. One prominent example of this is G4 structures, which are globular, folded nucleic acid structures that occur in G-rich regions of gene promoters.³ G4s have been broadly demonstrated to regulate the expression of many oncogenes and are well-studied as targets for small molecules. A less studied, but likely equally important structure is the i-motif, or C-quadruplex.⁴

Folded i-motif structures occur in C-rich regions of the genome, typically on the complementary strand to a G4-forming sequence.^{4a} In contrast to G4s, which are stabilized

by monovalent cations such as potassium, i-motifs are stabilized by multiple $[\text{C}\cdot\text{CH}]^+$ base pairing events. The $[\text{C}\cdot\text{CH}]^+$ base pair depends on one of the cytosine bases being protonated; as a consequence, the formation of an i-motif is highly pH dependent. While the specific pH at which unfolding occurs is sequence dependent, folded i-motifs generally exist below pH ~ 7 while unfolded structures predominate at higher pH. From a thermodynamic perspective, i-motifs are less stable than the corresponding G4 structures and typically

Fig. 1 (A) Structure and pH dependence of the HRAS i-motif. (B) Illustration of the $[\text{C}\cdot\text{C}]^+$ basepair. (C) Sequence of the *HRAS* i-motif and fluorescently labeled screening construct used in this manuscript. A647 = AlexaFluor 647.

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unfold at lower temperatures, even at low pH. A second factor that governs i-motif folding is molecular crowding,⁵ and biochemical conditions that mimic crowding cause increased folding of i-motif sequences, even in cases of higher pH. A third important contributing factor is torsional stress. Under negative superhelicity (induced by transcription), both G- and C-quadruplexes form to eliminate disfavored single-stranded sequences (Fig. 1).

There is considerable evidence that i-motifs serve as regulatory structures within cells.⁶ A recent study revealed that i-motifs are folded and can be visualized in living cells.⁷ Proteins have also been characterized that bind and unfold i-motifs, as is the case with hnRNP LL and the *BCI2* i-motif.⁸ Furthermore, while G4 structures typically suppress gene expression, i-motif-forming sequences are often associated with increased expression of the associated gene. To further support this observation, several small molecules that bind to i-motifs have been shown to control gene expression as well.^{4a,c} One example of an oncogene containing an i-motif is the *HRAS* gene.⁹ *HRAS* encodes for a small GTPase in the RAS family of oncoproteins that stimulate proliferation by signaling to the nucleus. Driver mutations that render the *HRAS* enzyme constitutively active are found in many cancers, for example in 29% of young adult patients with bladder cancer.¹⁰ However, it has proven difficult to develop small molecules that target the *HRAS* protein due to the high affinity of the protein for its GTP cofactor (coupled with millimolar concentrations of GTP in the cell) as well as limited sites for small molecule ligands to bind on the enzyme.

To overcome the difficulties associated with targeting *HRAS* at the protein level, controlling *HRAS* expression with small molecules that bind to the i-motif is an attractive strategy. For example, a small molecule might either stabilize or destabilize the i-motif structure, or interfere with protein/DNA interactions, any of which might have an impact on gene expression.¹¹ However, there are relatively few examples of small molecules that bind to i-motifs in general. A major challenge in establishing these important structures as potential drug targets is to characterize druglike compounds that specifically bind to the i-motif fold. More broadly, the discovery of small molecules that specifically recognize folded nucleic acid structures is of significant interest to both the chemical biology and drug discovery communities. For the specific case of the *hras-1*^Y i-motif, to date there have been no small molecule ligands reported.

In this study, we report the discovery of new classes of small molecules that bind to the *hras-1*^Y i-motif. We use a small molecule microarray (SMM) screening strategy to rapidly identify small organic compounds that interact with the *hras-1*^Y i-motif but do not bind to other folded oligonucleotides. Multiple orthogonal affinity measurement techniques reveal that compounds from these series have affinities ranging from the high nanomolar to low micromolar range and are readily chemically synthesized. Furthermore, circular dichroism, NMR, and fluorescence resonant energy transfer (FRET) assays reveal ligand-induced effects on i-motif struc-

ture. Thus, multiple distinct chemotypes bind to the *HRAS* i-motif with varying affinity and effects on the structure and are likely suitable for further development into chemical probes.

Results and discussion

To identify small molecules that bind to the *HRAS* i-motif, we used an SMM screening approach.¹² This approach has been used previously in our laboratory to identify small molecules that bind to other folded nucleic acid structures such as RNA hairpins and DNA G4s.^{12c,13} We designed a 5'-AlexaFluor 647-labeled *hras-1*^Y i-motif construct for use in the SMM screen (Fig. 2).¹⁴ Briefly, SMM slides containing 7042 compounds were incubated for one hour at room temperature with 150 nM labeled i-motif sequence in a Tris buffer. Higher concentrations of DNA resulted in high background and did not provide usable data. After washing, slides were dried and imaged. A composite Z-score was generated for each compound on the array, and compounds with a Z-score greater than three were selected as hits. 61 compounds were identified for further study after visual inspection of hit spots and comparison to 15 other structured oligonucleotides screened using the SMM platform.^{12a} We selected 13 compounds to purchase from this list for further study on the basis of availability and representation from different structural classes. Of the 13 compounds that were purchased, many were found to be impure by LC/MS and ¹H NMR analysis upon receipt, and we obtained pure samples of eight by HPLC purification.

Next, each of the eight compounds was qualitatively analyzed for binding to the *hras-1*^Y i-motif by surface plasmon resonance (SPR). We designed a 5'-biotin-labeled i-motif sequence and immobilized the oligonucleotide to a streptavidin surface. Each compound was injected at a concentration of 100 μ M to assess binding to the folded DNA. Of the eight compounds, six displayed binding to the DNA. We next used SPR to measure equilibrium dissociation constants (K_D) for each of the six compounds. Two compounds, 1 and 2, were found to have K_D values in the single digit micromolar range (7.4 ± 5.3 μ M and 5.9 ± 3.7 μ M, respectively). Other compounds showed weaker binding or data that was difficult to accurately fit to binding models (Table 1).

To independently measure K_D values using an orthogonal biophysical technique, we developed a fluorescence titration assay.^{13a} In this assay, we used the 5'-AlexaFluor 647 construct designed for screening. K_D values were derived by measuring ligand-induced changes in the fluorescence of the tagged oligonucleotide construct. In this assay, both compounds were validated to bind to the i-motif. Compound 1 was found to have a K_D of 1.6 ± 0.3 μ M, in excellent agreement with SPR experiments. In contrast, 2 had a K_D of 36.6 ± 13.7 μ M, higher than the value recorded by SPR. As compound 1 was the tightest binding compound, we chemically synthesized this compound and demonstrated that it had equivalent binding by both SPR and fluorescence titration and was used for all future experiments (Fig. 3).

ence of DNA, compounds that bind are protected from magnetization transfer and ligand peaks exhibit positive phasing (indicated as up in Fig. 4). Importantly, compounds that aggregate in aqueous solution (a common source of false positives in biochemical assays) can also be easily identified in this experiment by positive phasing in the waterLOGSY spectrum in the absence of DNA. Gratifyingly, **1** did not aggregate

Table 1 Chemical structures and affinity measurements of compounds to the *HRAS* i-motif

Entry	Compound structure	SPR K_D^a (μ M)	FIA K_D^b (μ M)
1		7.4 ± 5.3	1.6 ± 0.3
2		5.9 ± 3.7	35.6 ± 13.7
3		0.2 ± 0.2	0.6 ± 0.5
4		0.3 ± 0.3	3.0 ± 2.3
5		11.6 ± 4.8	16.0 ± 18.1
6		51.0 ± 3.0	254.5 ± 124.1
7		78.0 ± 99.0	30.8 ± 3.4
8		149.2 ± 15.3	90.7 ± 12.6
9		242.6 ± 97.5	>209
10		>300	106.3 ± 35.9
11		>300	50.4 ± 15.1
12		>300	>209
13		>300	>209

^a K_D values represent the mean of two experiments performed with at least four concentrations. ^b K_D values represent the mean of three replicates.

in solution as observed by negatively phased peaks in waterLOGSY spectrum in the absence of DNA. Furthermore, upon addition of an unlabeled *hras*-1^Y i-motif sequence, the protons from **1** were phased positively, confirming that the compound directly interacts with the DNA in the solution phase (Fig. 4). Next, we used waterLOGSY NMR to evaluate the pH-dependence of this binding interaction. Direct binding to the DNA was observed at pH 6.02, where the i-motif is

folded. In contrast, when the pH was raised to 8.02, where the DNA exists predominantly as a hairpin, no binding was observed. Thus, **1** binds to DNA in a fold-dependent manner. Similar effects were observed with **2**, which only bound to the *HRAS* DNA in the i-motif form.

Next, we evaluated the effects of compound binding on the chemical shifts of the i-motif protons in NMR (Fig. 5). Although there is no structure of the *hras*-1^Y i-motif available, characteristic peaks for i-motif imino protons are clearly visible at $\delta_{\text{ppm}} \sim 15$ –16.^{16,17} In the presence of increasing concentrations of **1**, the characteristic i-motif protons broaden considerably (Fig. 5A and B). However, no significant changes are observed elsewhere in the spectrum. Furthermore, no corresponding increase of Watson–Crick imino protons was observed at $\delta_{\text{ppm}} \sim 12.5$ –14.

To further probe the effects of ligands on the conformation of the i-motif, we used circular dichroism (CD) spectroscopy. An unlabeled *hras*-1^Y i-motif sequence displayed a peak minimum at 248 nm and a maximum at 289 nm, characteristic of a properly folded i-motif.^{4a,9} In the presence of **1**, this peak height decreased and shifted to a shorter wavelength, indicating that binding of **1** has a small but measurable effect on the conformation of the i-motif (Fig. 5). In contrast, **2** had minimal effects on the structure of the i-motif. We then evaluated the effects of ligand binding on the thermal denaturation of the i-motif. Neither **1** nor **2** had any statistically significant effect on the melting temperature of the DNA.

Next, we performed a FRET assay.⁹ In this assay, as previously described, an i-motif construct was purchased containing FRET donor/acceptor pairs on the 3' and 5' ends. Compounds that cause a large change in the i-motif conformation (for example unfolding) cause changes in FRET efficiency and can readily be monitored. Compound **1** had minimal effects in this assay, in contrast with a previously reported protein that unfolds the structure and causes a large change in FRET signal (see Fig. S56†).⁹ Combined with observations in the ¹H NMR, these data together suggest that the compound directly interacts with the i-motif core, but potentially not with other regions of the structure such as the loops. In contrast to **1**, evaluation of the effects of **2** on the i-motif NMR showed no changes in the i-motif imino protons with increasing concentrations of compound **2**. However, some changes could be observed in other regions of the spectrum, suggesting that the compound interacts with other regions of the DNA, potentially the loop regions. Taken together, these experiments suggest that small molecules may interact with i-motif structures by multiple distinct modes of binding (e.g. groove or loop binding).

To investigate the structure–activity relationship (SAR) of these compounds, we evaluated a collection of commercially available analogs (Table 1). After purchasing, each compound was assessed for purity by LC/MS analysis and purified by HPLC if necessary. The binding affinity (K_D) of each compound was measured by both fluorescence intensity titration and SPR to give orthogonal measures of affinity (Table 1).

Fig. 3 Synthesis of compound 1.

With respect to **1**, replacement of the hydroxyethyl amino sidechain with a methoxy substituent abolished binding, as did replacement of this group with other, bulky substituents. Similarly, replacement of the amino group on the pyrimidine ring with a dimethylamino group also caused a substantial decrease in binding. The compound tolerated other heterocyclic rings on the right-hand side chain, though binding was generally weaker. Finally, an amide analog exhibited no binding.

For **2**, a strong relationship was observed between compound affinity and chemical structure. Compound **2** also contains an amino-substituted pyrimidine group. Replacement of this group with a methyl or methoxy substituent led to a marked increase in binding affinity, with K_D values in the nanomolar range. However, a dimethylamino-substituted analog had no binding activity, pointing to a role for this group in binding. Furthermore, two analogs lacking the amine embedded in the sidechain also had no observable binding, again indicating a critical role for this functionality (Fig. 2). Because **3** and **4** had improved binding relative to **2**, we validated its binding in other assays. In comparison to **2**, **3** and **4** behaved similarly in circular dichroism and chemical shift perturbation experiments, indicating that it is likely binding by a similar mechanism (see ESI†).

In an attempt to further investigate potential binding modes for these compounds, we used computational docking. Because no structure of the *hras*-1^Y i-motif is available, we considered generating a homology model based on another reported intramolecular i-motif (PDB: 1ELN). However, modeling the loop regions proved extremely challenging due to the complex energy landscape governing loop pairing and stacking interactions. Given that experiments suggested that **1** binds directly to the i-motif core we generated a minimal i-motif model instead (lacking the loops) from the crystal structure of the d(ACCCT) i-motif (PDB: 1CN0).¹⁸ We docked **1** and **2** to the major groove of this structure (see modeling methods in the ESI† for details). Compound **1** docked in two nearly equivalent poses (Fig. 6A and B), with the pyrimidine and benzyl groups flat across the bottom of the groove, forming amino-aromatic interactions with the projecting cytosine N4 amines. The amino substituent on the pyridine, the hydroxyl group, and the pyrrolidine (protonated at pH 6) can all hydrogen bond to the phosphate backbone. The amino-pyrimidine group in **2** can dock similarly to that in **1** (Fig. S59†), but the rest of the structure does not interact strongly with the i-motif core, suggesting that the methoxy-containing ring may bind to the loops.

In conclusion, we have identified a series of small chemical ligands that directly bind to the *hras*-1^Y i-motif. This structure, an important regulatory element of the *HRAS* oncogene promoter, is an unexplored target for small molecules. Here we demonstrate through SMM screening that diverse small molecule ligands can bind to the *hras*-1^Y i-motif by different mechanisms. Furthermore, these compounds have

Fig. 4 WaterLOGSY experiments demonstrate **1** exhibits pH-dependent binding to i-motif DNA. (A) ¹H NMR and WaterLOGSY NMR in the (B) absence and (C) presence of i-motif DNA at pH 6. (D) ¹H NMR and WaterLOGSY NMR in the (E) absence and (F) presence of i-motif DNA at pH 8. Methyl peaks for *N*-methyl-L-valine (used as an internal negative control) can be observed at ~0.8 ppm. Other peaks in the spectrum correspond to protons from **1**.

Fig. 5 ^1H NMR chemical shift perturbations of i-motif DNA in the presence of increasing concentrations of (A) compound 1 and (B) compound 2 in deuterated Tris-acetate buffer at pH 6. Circular dichroism spectra of i-motif DNA in the presence of increasing concentrations of (C) compound 1 and (D) compound 2 in Tris-acetate buffer at pH 6 (except the purple spectrum, which was collected at pH 8).

strong SAR and can achieve submicromolar affinity to the i-motif. In-depth biophysical characterization demonstrates that small changes in the chemical structure can lead to large changes in affinity, indicating highly specific interactions. Finally, modeling studies provide a compelling plausible mode of interaction for 1. The observation that the aminopyrimidine group in both 1 and 2 adopts a similar interaction with the cytosine and phosphates suggests that this fragment may be a general scaffold that is suitable for further development for ligands that bind to the *hras-1^Y* or

other i-motif structures. A related observation is that proteins bearing unnatural amino acids containing a similar functionality exhibit improved binding to i-motifs.¹⁹ The chemical structure of several of these ligands is somewhat reminiscent of the pyrimidine core of a cytosine residue; this may play a role in the affinity of these compounds for the DNA, though further experimentation will be required to validate this observation. Future work will focus on establishing the structural basis by which such compounds can achieve high affinity with the i-motif structure, and to

Fig. 6 Representative docked pose of compound **1** with the i-motif core (PDB: 1CN0). A) Top view looking down into the major groove. The surface of the i-motif core is colored according to electrostatic potential. B) Side view with transparent surface. Hydrogen bonds to the phosphate groups are indicated with dashed orange lines and the amino-aromatic interaction between the pyrimidine group and the cytosine N4 amine is indicated with a dashed green line.

evaluate their role in modulating gene expression in cancer cells.

Conflicts of interest

There are no conflicts to declare.

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References

- (a) F. Kouzine, D. Wojtowicz, L. Baranello, A. Yamane, S. Nelson, W. Resch, K. R. Kieffer-Kwon, C. J. Benham, R. Casellas, T. M. Przytycka and D. Levens, *Cell Syst.*, 2017, **4**, 344–356; (b) A. Bacolla and R. D. Wells, *J. Biol. Chem.*, 2004, **279**, 47411–47414.
- (a) M. Kaushik, S. Kaushik, K. Roy, A. Singh, S. Mahendru, M. Kumar, S. Chaudhary, S. Ahmed and S. Kukreti, *Biochem. Biophys. Rep.*, 2016, **5**, 388–395; (b) T. A. Brooks, S. Kendrick and L. Hurley, *Rev. Geophys.*, 2010, **277**, 3459–3469.
- (a) S. A. Ohnmacht and S. Neidle, *Bioorg. Med. Chem. Lett.*, 2014, **24**, 2602–2612; (b) J. L. Huppert and S. Balasubramanian, *Nucleic Acids Res.*, 2007, **35**, 406–413.
- (a) H. A. Day, P. Pavlou and Z. A. Waller, *Bioorg. Med. Chem.*, 2014, **22**, 4407–4418; (b) C. E. Kaiser, N. A. Van Ert, P. Agrawal, R. Chawla, D. Yang and L. H. Hurley, *J. Am. Chem. Soc.*, 2017, **139**, 8522–8536; (c) M. Gueron and J. L. Leroy, *Curr. Opin. Struct. Biol.*, 2000, **10**, 326–331.
- T. Nguyen, C. Fraire and R. D. Sheardy, *J. Phys. Chem. B*, 2017, **121**, 7872–7877.
- S. Jonchhe, P. Shrestha, K. Ascencio and H. Mao, *Anal. Chem.*, 2018, **90**, 3205–3210.
- M. Zeraati, D. B. Langley, P. Schofield, A. L. Moye, R. Rouet, W. E. Hughes, T. M. Bryan, M. E. Dinger and D. Christ, *Nat. Chem.*, 2018, **10**, 631–637.
- (a) B. Roy, P. Talukder, H. J. Kang, S. S. Tsuen, M. P. Alam, L. H. Hurley and S. M. Hecht, *J. Am. Chem. Soc.*, 2016, **138**, 10950–10962; (b) H. J. Kang, S. Kendrick, S. M. Hecht and L. H. Hurley, *J. Am. Chem. Soc.*, 2014, **136**, 4172–4185; (c) M. Debnath, S. Ghosh, A. Chauhan, R. Paul, K. Bhattacharyya and J. Dash, *Chem. Sci.*, 2017, **8**, 7448–7456; (d) S. Kendrick, Y. Akiyama, S. M. Hecht and L. H. Hurley, *J. Am. Chem. Soc.*, 2009, **131**, 17667–17676.
- G. Miglietta, S. Cogoi, E. B. Pedersen and L. E. Xodo, *Sci. Rep.*, 2015, **5**, 18097.
- W. Beukers, A. Hercegovic and E. C. Zwarthoff, *Eur. J. Hum. Genet.*, 2014, **22**, 837–839.
- S. Kendrick, A. Muranyi, V. Gokhale, L. H. Hurley and L. M. Rimsza, *J. Med. Chem.*, 2017, **60**, 6587–6597.
- (a) C. M. Connelly, F. A. Abulwerdi and J. S. Schneekloth Jr., *Methods Mol. Biol.*, 2017, **1518**, 157–175; (b) P. J. Hergenrother, K. M. Depew and S. L. Schreiber, *J. Am. Chem. Soc.*, 2000, **122**, 7849–7850; (c) J. E. Bradner, O. M. McPherson and A. N. Koehler, *Nat. Protoc.*, 2006, **1**, 2344–2352; (d) M. D. Disney, S. Magnet, J. S. Blanchard and P. H. Seeberger, *Angew. Chem., Int. Ed.*, 2004, **43**, 1591–1594; (e) J. W. Puckett, K. A. Muzikar, J. Tietjen, C. L. Warren, A. Z. Ansari and P. B. Dervan, *J. Am. Chem. Soc.*, 2007, **129**, 12310–12319; (f) S. P. Velagapudi, A. Pushechnikov, L. P. Labuda, J. M. French and M. D. Disney, *ACS Chem. Biol.*, 2012, **7**, 1902–1909; (g) M. D. Disney, L. P. Labuda, D. J. Paul, S. G. Poplawski, A. Pushechnikov, T. Tran, S. P.

- Velagapudi, M. Wu and J. L. Childs-Disney, *J. Am. Chem. Soc.*, 2008, **130**, 11185–11194; (h) J. L. Childs-Disney, M. L. Wu, A. Pushechnikov, O. Aminova and M. D. Disney, *ACS Chem. Biol.*, 2007, **2**, 745–754.
- 13 (a) C. M. Connelly, R. E. Boer, M. H. Moon, P. Gareiss and J. S. Schneekloth Jr., *ACS Chem. Biol.*, 2017, **12**, 435–443; (b) K. M. Felsenstein, L. B. Saunders, J. K. Simmons, E. Leon, D. R. Calabrese, S. Zhang, A. Michalowski, P. Gareiss, B. A. Mock and J. S. Schneekloth Jr., *ACS Chem. Biol.*, 2016, **11**, 139–148; (c) J. Sztuba-Solinska, S. R. Shenoy, P. Gareiss, L. R. Krumpe, S. F. Le Grice, B. R. O'Keefe and J. S. Schneekloth Jr., *J. Am. Chem. Soc.*, 2014, **136**, 8402–8410; (d) D. R. Calabrese, K. Zlotkowski, S. Alden, W. M. Hewitt, C. M. Connelly, R. M. Wilson, S. Gaikwad, L. Chen, R. Guha, C. J. Thomas, B. A. Mock and J. S. Schneekloth Jr., *Nucleic Acids Res.*, 2018, **46**, 2722–2732; (e) J. A. Hong, D. V. Neel, D. Wassaf, F. Caballero and A. N. Koehler, *Curr. Opin. Chem. Biol.*, 2014, **18**, 21–28; (f) J. L. Duffner, P. A. Clemons and A. N. Koehler, *Curr. Opin. Chem. Biol.*, 2007, **11**, 74–82.
- 14 A. M. Fleming, Y. Ding, R. A. Rogers, J. Zhu, J. Zhu, A. D. Burton, C. B. Carlisle and C. J. Burrows, *J. Am. Chem. Soc.*, 2017, **139**, 4682–4689.
- 15 (a) C. Dalvit, P. Pevarello, M. Tato, M. Veronesi, A. Vulpetti and M. Sundstrom, *J. Biomol. NMR*, 2000, **18**, 65–68; (b) M. Pellecchia, B. Becattini, K. J. Crowell, R. Fattorusso, M. Forino, M. Fragai, D. Jung, T. Mustelin and L. Tautz, *Expert Opin. Ther. Targets*, 2004, **8**, 597–611.
- 16 I. Bertini, K. S. McGreevy and G. Parigi, *NMR of biomolecules : towards mechanistic systems biology*, Wiley-VCH, Weinheim, Germany, 2012.
- 17 V. V. Jolad, F. K. Murad, J. R. Arnold and J. Fisher, *Org. Biomol. Chem.*, 2005, **3**, 2234–2236.
- 18 J. Weil, T. Min, C. Yang, S. Wang, C. Sutherland, N. Sinha and C. Kang, *Acta Crystallogr., Sect. D: Biol. Crystallogr.*, 1999, **55**, 422–429.
- 19 X. Bai, P. Talukder, S. M. Daskalova, B. Roy, S. Chen, Z. Li, L. M. Dedkova and S. M. Hecht, *J. Am. Chem. Soc.*, 2017, **139**, 4611–4614.