Peptide Stapling

International Edition: DOI: 10.1002/anie.201508416
German Edition: DOI: 10.1002/ange.201508416

Double Strain-Promoted Macrocyclization for the Rapid Selection of Cell-Active Stapled Peptides

*Yu Heng Lau, Yuteng Wu, Maxim Rossmann, Ban Xiong Tan, Peterson de Andrade, Yaw Sing Tan, Chandra Verma, Grahame J. McKenzie, Ashok R. Venkitaraman, Marko Hyvönen, and David R. Spring**

Abstract: *Peptide stapling is a method for designing macrocyclic alpha-helical inhibitors of protein–protein interactions. However, obtaining a cell-active inhibitor can require significant optimization. We report a novel stapling technique based on a double strain-promoted azide–alkyne reaction, and exploit its biocompatibility to accelerate the discovery of cell-active stapled peptides. As a proof of concept, MDM2-binding peptides were stapled in parallel, directly in cell culture medium in 96-well plates, and simultaneously evaluated in a p53 reporter assay. This in situ stapling/screening process gave an optimal candidate that showed improved proteolytic stability and nanomolar binding to MDM2 in subsequent biophysical assays. α-Helicity was confirmed by a crystal structure of the MDM2-peptide complex. This work introduces in situ stapling as a versatile biocompatible technique with many other potential high-throughput biological applications.*

Macrocyclization is an effective strategy for reinforcing peptides in stable secondary structures.[1] Whilst short peptides derived from proteins can lack a well-defined conformation when used in isolation, cyclisation can restore the native bioactive conformation and hence function of a peptide. By mimicking native binding motifs, cyclized peptides can competitively inhibit protein–protein interactions of clinical relevance, and they have received substantial attention as potential therapeutics.[1,2] One of the most intensely studied targets is the oncogenic p53/MDM2 interaction, in which an N-terminal α-helix of the tumor suppressor p53 binds a hotspot on MDM2, an E3 ubiquitin ligase that downregulates p53 and is overexpressed in some cancers.[3,4] Numerous peptide therapeutics have been developed to target this interaction, with some reaching as far as early-phase clinical trials.[5–10]

Stapled peptides are an important subset of macrocyclic peptides specific to α-helices.[11–15] Stapling involves two amino acids with non-native side chains that can be covalently linked, producing a cyclized peptide that may display enhanced proteolytic stability, binding affinity, and cellular uptake. Whilst the term "stapling" was originally coined by Verdine and Walensky for work on all-hydrocarbon linkers generated by metathesis (after Grubbs),[11,12,16] there is now great interest in alternative chemistries for side-chain cross-linking to give peptides with novel structures and biological properties. Of particular note are modular two-component strategies, where the staple linkage and peptide are separate moieties prior to cyclisation (Figure 1).[14] We recently developed a two-component stapling technique that makes use of double Cu-catalyzed azide–alkyne cycloaddition (CuAAC),[7,17,18] following related work by Bong[19] and other click systems.[20–22] Combining a two-component approach with click chemistry enables easy access to peptides bearing different functional staples. Whilst we appended cell-permeabilizing motifs to the staple, others have used a two-component approach to create photoswitchable,[23] reversible,[24] and dynamic linkers.[25]

When screening for an optimal inhibitor, stapling reactions are typically carried out on many peptide variants. A practical bottleneck for two-component strategies is that, unlike hydrocarbon stapling, cyclisation is typically done in solution to avoid on-resin site isolation.[7,23,25] Hence, from the pure unstapled peptide, extra purification is needed after cyclisation to remove reagents/catalyst prior to assays (Figure 1).

We decided to develop a stapling technique that would be biocompatible and so simple to conduct that it could be done in parallel on a large peptide library, even directly in the culture medium of a primary cell-based 96-well assay (Figure 1). This in situ approach would be faster than setting up a dedicated stapling reaction for each library variant, which is required in all current methods, and eliminates the

[*] Dr. Y. H. Lau,[+] Y. Wu,[+] Dr. P. de Andrade, Prof. D. R. Spring
Department of Chemistry, University of Cambridge
Lensfield Rd, Cambridge CB2 1EW (UK)
E-mail: spring@ch.cam.ac.uk

Dr. M. Rossmann, Dr. M. Hyvönen
Department of Biochemistry, University of Cambridge
80 Tennis Court Rd, Cambridge CB2 1GA (UK)

Dr. B. X. Tan
p53Lab, A*STAR, 8A Biomedical Grove
#06-04/05 Neuros/Immunos, Singapore 138648 (Singapore)

Dr. Y. S. Tan, Dr. C. Verma
Bioinformatics Institute, A*STAR, 30 Biopolis St
#07-01 Matrix, Singapore 138671 (Singapore)

Dr. G. J. McKenzie, Prof. A. R. Venkitaraman
Hutchison/MRC Research Centre
Hills Rd, Cambridge CB2 0XZ (UK)

Dr. C. Verma
Department of Biological Sciences, National University of Singapore
14 Science Drive 4, Singapore 117543

Dr. C. Verma
School of Biological Sciences, Nanyang Technological University
50 Nanyang Drive, Singapore 637551

[+] co-first authors

Supporting information and ORCID(s) from the author(s) for this article are available on the WWW under http://dx.doi.org/10.1002/anie.201508416.

 © 2015 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

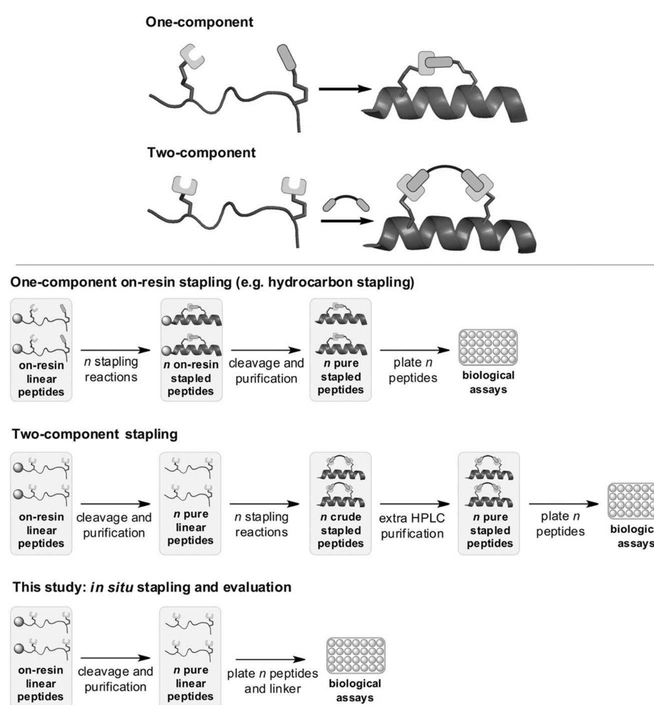


Figure 1. Top: One- and two-component macrocyclization approaches. Bottom: Unlike classical one-component stapling[15] and existing two-component strategies such as our CuAAC method,[7,18] the in situ strategy combines stapling and the primary biological assay in a single step.

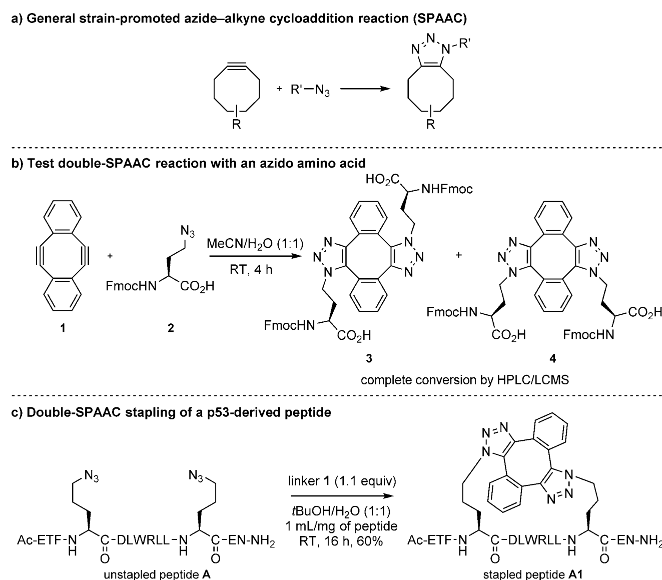


Figure 2. a) Strain-promoted azide–alkyne cycloaddition (SPAAC).[26] b) Test double SPAAC on Fmoc-Aha-OH. c) Double-SPAAC stapling of p53-derived diazidopeptide **A**.

extra purification step needed in other two-component strategies.

Inspired by the strain-promoted azide–alkyne cycloaddition reaction (Figure 2a),[26,27] we report a catalyst-free double-click technique for parallel stapling in cell culture. This in situ strategy is the first example of stapling in a live biological context, since most techniques are incompatible owing to metal catalysis,[7,19,20] inert atmosphere or protecting groups,[28,29] or thiols[30–33]/amines[34] that might cross-react in biological systems. Whilst the oxime/hydrazone staples by Horne,[25] photocycloaddition staples by Lin,[35] and macrocyclic organo–peptide hybrids by Fasan have potential for in situ use,[10] we favored a non-dynamic system without UV irradiation or recombinant precursors.

Strained diyne **1** was prepared according to the method of Orita et al.,[36] and a test reaction with Fmoc-Aha-OH **2** gave the expected bis(triazole) compounds **3** and **4** (Figure 2b and Figure S1.4.1 in the Supporting Information). Stapling of p53-derived diazidopeptide **A** (Figure 2c) with linker **1** in 1:1 H_2O/*t*BuOH gave stapled peptide **A1** in 60% yield (Figure 2c; assigned as the *anti* regioisomer, see later crystallography on an analogous peptide). Minor byproducts of the same mass were observed. These may be stable alternative conformations of the *syn* form, with MD simulations suggesting the possibility of at least two extra non-interchanging conformations (Figure S12.1.3). An excess of **1** did not affect the reaction, and comparable results were obtained when swapping *t*BuOH for MeOH, MeCN, or DMSO. Importantly, the reaction also proceeded in Dulbecco's Modified Eagle's medium (DMEM) with fetal calf serum and 1% DMSO.

The binding affinity of **A1** for MDM2 was 3.1 ± 0.4 nM by competitive fluorescence polarization (FP),[7] which is more potent than that of wild-type $p53_{17\text{-}29}$ (820 ± 60 nM) and unstapled **A** (16 ± 1 nM). Despite this encouraging in vitro result, up to 100 μM of **A1** did not induce activation of p53 in an established T22 cell reporter assay,[9] which is in line with previous studies showing poor uptake of this sequence.[6,7]

To test our in situ approach, we investigated sequence variants **B**–**E**, which are based on the phage-derived peptides PMI/PDI and were previously investigated for hydrocarbon stapling (Figure 3a).[5,8,9,37] To determine which variant would induce greatest p53 activation upon stapling with **1**, we directly treated p53 reporter cells for the cell-based assay[9] in 96-well format with 0.5 mM **1** and 50 μM unstapled peptides **A**–**E** in DMEM. All five peptides were stapled in situ with similar yields (54–58%). After 18 h incubation, p53 activation was observed for **B**–**E**, whilst no activation was observed for **A** (Figure 3a). Cells treated with **1** or **A**–**E** only showed no p53 activation. From this in situ procedure, we were rapidly able to rank peptide activity, finding that **E** stapled with **1** (**E** + **1**) was the most potent activator of p53.

We resynthesized and isolated stapled peptides **A1**–**E1** to verify that the activity ranking from in situ stapling was consistent with standard testing of pure peptides.[7] Stapling in H_2O/*t*BuOH proceeded with similar yield in each case, and the same activity ranking was observed for the purified peptides in the reporter assay, with **E1** again inducing the greatest response (Figure 3b). An F3A control for **E1** was found to have no cellular activity (Figure S11.2.1). The response using pure peptides was greater than in the in situ procedure, potentially due to gradual product formation over 18 h and losses due to *syn*/*anti* byproducts. Despite these possible confounding factors, the reactivity of linker **1** was still sufficiently robust to rank peptide activity.

 © 2015 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

a) Cellular activity of peptides using *in situ* stapling

Peptide + linker	*Sequence*	*Fold p53 activation (50 μM)*
A + 1	$Ac-ETFXDLWRLLXEN-NH_2$	1.03 ± 0.03
B + 1	$Ac-LTFXHYWAQLXS-NH_2$	1.5 ± 0.3
C + 1	$Ac-TSFXEYWALLX-NH_2$	1.5 ± 0.2
D + 1	$Ac-LTFXEYWAQLXSAA-NH_2$	2.3 ± 0.2
E + 1	$Ac-LTFXEYWAQLXS-NH_2$	2.9 ± 0.2

increasing cellular activity

b) Cellular activity of pre-stapled and purified peptides

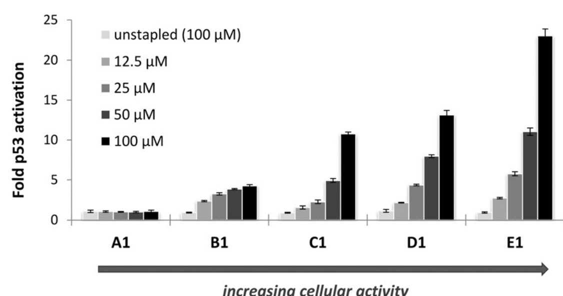


Figure 3. p53 activation in a cellular reporter assay for a) in situ stapling with peptides **A**–**E** (50 μM) and linker **1** (0.5 mM), and b) pre-stapled peptides **A1**–**E1**. Unstapled peptide controls **A**–**E** were tested at 100 μM (also see Figure S2.2.1). X = Orn(N_3), data reported as fold activation over 1% DMSO. **A** is a p53-derived peptide (K24R) we previously used,[7] **C** is based on phage peptide PMI, and[8,9] **B**, **D**, and **E** are based on phage peptide PDI.[5,37]

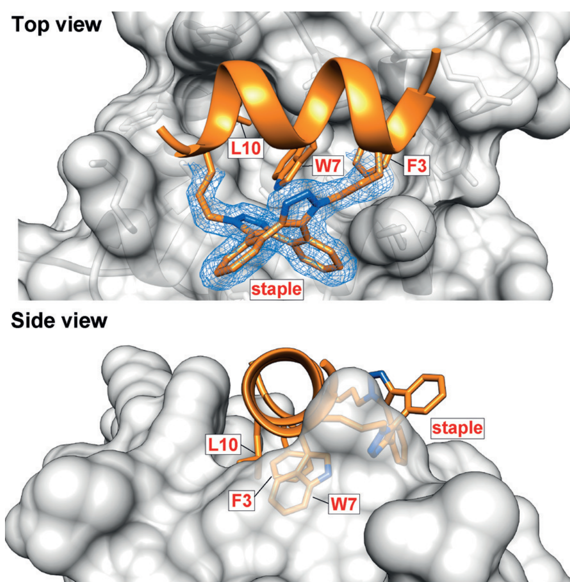


Figure 4. Crystal structure of **E1** bound to MDM2 at 1.9 Å resolution (PDB ID: 5afg), showing the α-helical conformation and the *anti* regioisomer of the staple. For clarity, only the staple and side chains of the three binding residues are shown. The $2F_o-F_c$ electron density map is contoured at 1σ.

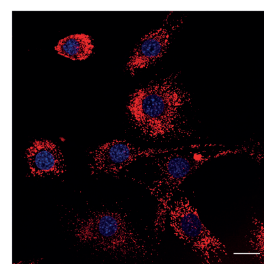
Taking **E1** forward, we obtained its crystal structure in complex with MDM2 (17–108, E69A/K70A;[38] Figure 4). The structure shows **E1** in α-helical conformation, placing the binding triad (F3, W7, L10) in the correct orientation for engaging the MDM2 hotspot. The bis(triazolyl) staple is found as the *anti* regioisomer and forms interactions with the protein, a phenomenon only previously seen with hydrocarbon-stapled peptides (Figure S5.3.2).[5,39–41]

By FP,[7,9] **A1**–**E1** all showed potent affinity for MDM2 (Figure 5a), thus exemplifying how in vitro binding does not always translate to cellular activity, owing to other factors such as uptake. A comparable binding affinity for **E1** was obtained by isothermal calorimetry (12 ± 3 nM, Figure S4.2.1).

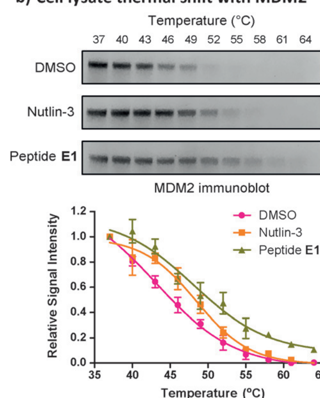
a) *In vitro* binding affinity for MDM2 (K_d, nM)

Peptide	*Unstapled*	*Stapled with linker **1***
$p53_{17-29}$	820 ± 60	n/a
A	16 ± 1	3.1 ± 0.4
B	36 ± 3	14 ± 1
C	9 ± 1	7.6 ± 0.7
D	6.0 ± 0.6	2.5 ± 0.3
E	6.5 ± 0.6	7.5 ± 0.7

c) Confocal microscopy of E1



b) Cell lysate thermal shift with MDM2



d) Chymotrypsin stability assay

Intact peptide (%)
stapled peptide E1
unstapled peptide E
Time (h)

Figure 5. a) Binding affinities by fluorescence polarization (FP). b) MDM2 is stabilized by **E1** as shown by cell lysate thermal shift. c) Confocal microscopy of TAMRA-**E1** (20 μM) in live T22 cells, scale bar: 25 μm. Peptide shown in red, nuclei in blue. d) **E1** shows increased stability to proteolysis by chymotrypsin.

Based on thermal shift in OCI/AML-2 lysate,[42,43] both **E1** and control Nutlin-3 show increased stabilization of MDM2 (Figure 5b). Uptake of **E1** was observed by confocal microscopy in T22 cells (Figure 5c). **E1** did not exhibit non-specific toxicity in an LDH leakage assay (Figure S9.1.1), and was not observed to aggregate in solution by gel filtration and UV/Vis spectroscopy (Figures S10.1.1, S10.1.2). Finally, **E1** showed significantly improved proteolytic stability in a chymotrypsin assay (Figure 5d).

In conclusion, this work introduces a new stapling technique with unique biocompatibility. Linker **1** was used for stapling in situ, leading to rapid selection of optimal candidate **E1**. Having established the chemistry, we now pave the way for applying in situ stapling to new biological targets. Only five peptides were screened in this study, since well-characterized sequences were already available. For new targets, more variants will be needed before finding a promising hit. A major advantage of our method is the potential to

 © 2015 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

staple these variants with different strained linkers, efficiently covering a wider area of chemical space. Finally, different high-throughput assays can enable rapid evaluation of other properties, for example, high-content analysis of peptide uptake/localization, whilst stapling biosynthetic diazidopeptides could lead to vast screening libraries.

Acknowledgements

This work was supported by the EPSRC, BBSRC, MRC, Wellcome Trust (090340/Z/09/Z) and ERC (FP7/2007-2013; 279337/DOS). We thank Dr Judith Reeks for providing the MDM2 expression plasmid, and the Department of Biochemistry biophysics and X-ray facilities for crystallization and data collection equipment.

Keywords: bioorthogonal chemistry · click chemistry · macrocycles · peptides · peptide stapling

How to cite: *Angew. Chem. Int. Ed.* **2015**, *54*, 15410–15413
Angew. Chem. **2015**, *127*, 15630–15633

[1] M. Pelay-Gimeno, A. Glas, O. Koch, T. N. Grossmann, *Angew. Chem. Int. Ed.* **2015**, *54*, 8896–8927; *Angew. Chem.* **2015**, *127*, 9022–9054.
[2] M. A. Klein, *ACS Med. Chem. Lett.* **2014**, *5*, 838–839.
[3] C. J. Brown, S. Lain, C. S. Verma, A. R. Fersht, D. P. Lane, *Nat. Rev. Cancer* **2009**, *9*, 862–873.
[4] P. H. Kussie, S. Gorina, V. Marechal, B. Elenbaas, J. Moreau, A. J. Levine, N. P. Pavletich, *Science* **1996**, *274*, 948–953.
[5] Y. S. Chang, B. Graves, V. Guerlavais, C. Tovar, K. Packman, K.-H. To, K. A. Olson, K. Kesavan, P. Gangurde, A. Mukherjee, et al., *Proc. Natl. Acad. Sci. USA* **2013**, *110*, E3445–E3454.
[6] F. Bernal, A. F. Tyler, S. J. Korsmeyer, L. D. Walensky, G. L. Verdine, *J. Am. Chem. Soc.* **2007**, *129*, 2456–2457.
[7] Y. H. Lau, P. de Andrade, S.-T. Quah, M. Rossmann, L. Laraia, N. Sköld, T. J. Sum, P. J. E. Rowling, T. L. Joseph, C. Verma, et al., *Chem. Sci.* **2014**, *5*, 1804–1809.
[8] M. Pazgier, M. Liu, G. Zou, W. Yuan, C. Li, C. Li, J. Li, J. Monbo, D. Zella, S. G. Tarasov, et al., *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 4665–4670.
[9] C. J. Brown, S. T. Quah, J. Jong, A. M. Goh, P. C. Chiam, K. H. Khoo, M. L. Choong, M. A. Lee, L. Yurlova, K. Zolghadr, et al., *ACS Chem. Biol.* **2013**, *8*, 506–512.
[10] J. M. Smith, J. R. Frost, R. Fasan, *Chem. Commun.* **2014**, *50*, 5027–5030.
[11] C. E. Schafmeister, J. Po, G. L. Verdine, *J. Am. Chem. Soc.* **2000**, *122*, 5891–5892.
[12] H. E. Blackwell, R. H. Grubbs, *Angew. Chem. Int. Ed.* **1998**, *37*, 3281–3284; *Angew. Chem.* **1998**, *110*, 3469–3472.
[13] V. Azzarito, K. Long, N. S. Murphy, A. J. Wilson, *Nat. Chem.* **2013**, *5*, 161–173.
[14] Y. H. Lau, P. de Andrade, Y. Wu, D. R. Spring, *Chem. Soc. Rev.* **2015**, *44*, 91–102.
[15] L. D. Walensky, G. H. Bird, *J. Med. Chem.* **2014**, *57*, 6275–6288.
[16] L. D. Walensky, A. L. Kung, I. Escher, T. J. Malia, S. Barbuto, R. D. Wright, G. Wagner, G. L. Verdine, S. J. Korsmeyer, *Science* **2004**, *305*, 1466–1470.
[17] Y. H. Lau, P. de Andrade, N. Skold, G. J. McKenzie, A. R. Venkitaraman, C. Verma, D. P. Lane, D. R. Spring, *Org. Biomol. Chem.* **2014**, *12*, 4074–4077.
[18] Y. H. Lau, Y. Wu, P. de Andrade, W. R. J. D. Galloway, D. R. Spring, *Nat. Protoc.* **2015**, *10*, 585–594.
[19] O. Torres, D. Yüksel, M. Bernardina, K. Kumar, D. Bong, *ChemBioChem* **2008**, *9*, 1701–1705.
[20] S. A. Kawamoto, A. Coleska, X. Ran, H. Yi, C.-Y. Yang, S. Wang, *J. Med. Chem.* **2012**, *55*, 1137–1146.
[21] S. Ingale, P. E. Dawson, *Org. Lett.* **2011**, *13*, 2822–2825.
[22] M. Scrima, A. Le Chevalier-Isaad, P. Rovero, A. M. Papini, M. Chorev, A. M. D'Ursi, *Eur. J. Org. Chem.* **2010**, *2010*, 446–457.
[23] L. Nevola, A. Martín-Quirós, K. Eckelt, N. Camarero, S. Tosi, A. Llobet, E. Giralt, P. Gorostiza, *Angew. Chem. Int. Ed.* **2013**, *52*, 7704–7708; *Angew. Chem.* **2013**, *125*, 7858–7862.
[24] S. P. Brown, A. B. Smith, *J. Am. Chem. Soc.* **2015**, *137*, 4034–4037.
[25] C. M. Haney, M. T. Loch, W. S. Horne, *Chem. Commun.* **2011**, *47*, 10915–10917.
[26] N. J. Agard, J. A. Prescher, C. R. Bertozzi, *J. Am. Chem. Soc.* **2004**, *126*, 15046–15047.
[27] I. Kii, A. Shiraishi, T. Hiramatsu, T. Matsushita, H. Uekusa, S. Yoshida, M. Yamamoto, A. Kudo, M. Hagiwara, T. Hosoya, *Org. Biomol. Chem.* **2010**, *8*, 4051–4055.
[28] Y.-W. Kim, T. N. Grossmann, G. L. Verdine, *Nat. Protoc.* **2011**, *6*, 761–771.
[29] N. E. Shepherd, H. N. Hoang, G. Abbenante, D. P. Fairlie, *J. Am. Chem. Soc.* **2005**, *127*, 2974–2983.
[30] A. M. Spokoyny, Y. Zou, J. J. Ling, H. Yu, Y.-S. Lin, B. L. Pentelute, *J. Am. Chem. Soc.* **2013**, *135*, 5946–5949.
[31] H. Jo, N. Meinhardt, Y. Wu, S. Kulkarni, X. Hu, K. E. Low, P. L. Davies, W. F. DeGrado, D. C. Greenbaum, *J. Am. Chem. Soc.* **2012**, *134*, 17704–17713.
[32] F. Zhang, O. Sadovski, S. J. Xin, G. A. Woolley, *J. Am. Chem. Soc.* **2007**, *129*, 14154–14155.
[33] A. Muppidi, K. Doi, S. Edwardraja, E. J. Drake, A. M. Gulick, H.-G. Wang, Q. Lin, *J. Am. Chem. Soc.* **2012**, *134*, 14734–14737.
[34] K. Fujimoto, M. Kajino, M. Inouye, *Chem. Eur. J.* **2008**, *14*, 857–863.
[35] M. M. Madden, C. I. Rivera Vera, W. Song, Q. Lin, *Chem. Commun.* **2009**, 5588–5590.
[36] A. Orita, D. Hasegawa, T. Nakano, J. Otera, *Chem. Eur. J.* **2002**, *8*, 2000–2004.
[37] J. Phan, Z. Li, A. Kasprzak, B. Li, S. Sebti, W. Guida, E. Schönbrunn, J. Chen, *J. Biol. Chem.* **2010**, *285*, 2174–2183.
[38] B. Anil, C. Riedinger, J. A. Endicott, M. E. M. Noble, *Acta Crystallogr. Sect. D* **2013**, *69*, 1358–1366.
[39] M. L. Stewart, E. Fire, A. E. Keating, L. D. Walensky, *Nat. Chem. Biol.* **2010**, *6*, 595–601.
[40] C. Phillips, L. R. Roberts, M. Schade, R. Bazin, A. Bent, N. L. Davies, R. Moore, A. D. Pannifer, A. R. Pickford, S. H. Prior, et al., *J. Am. Chem. Soc.* **2011**, *133*, 9696–9699.
[41] A. Glas, D. Bier, G. Hahne, C. Rademacher, C. Ottmann, T. N. Grossmann, *Angew. Chem. Int. Ed.* **2014**, *53*, 2489–2493; *Angew. Chem.* **2014**, *126*, 2522–2526.
[42] D. M. Molina, R. Jafari, M. Ignatushchenko, T. Seki, E. A. Larsson, C. Dan, L. Sreekumar, Y. Cao, P. Nordlund, *Science* **2013**, *341*, 84–87.
[43] B. X. Tan, C. J. Brown, F. J. Ferrer, T. Y. Yuen, S. T. Quah, B. H. Chan, A. E. Jansson, H. L. Teo, P. Nordlund, D. P. Lane, *Sci. Rep.* **2015**, *5*, 12116.

Received: September 8, 2015
Published online: November 2, 2015

© 2015 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim