

# An Unexpected Example of Protein-Templated Click Chemistry\*\*

Takayoshi Suzuki,\* Yosuke Ota, Yuki Kasuya, Motoh Mutsuga, Yoko Kawamura, Hiroki Tsumoto, Hidehiko Nakagawa, M. G. Finn,\* and Naoki Miyata\*

Click chemistry is a popular approach to the synthesis of functionalized molecules, and emphasizes the use of practical and reliable reactions.<sup>[1]</sup> Copper(I)-catalyzed azide–alkyne cycloaddition<sup>[2]</sup> (CuAAC), which selectively produces *anti*-(1,4)-triazoles in preference to the *syn* isomer (1,5-triazole), is regarded as a superlative example of click chemistry. The CuAAC reaction can be accelerated by Cu<sup>I</sup>-stabilizing ligands, such as tris[(1-substituted-1*H*-1,2,3-triazol-4-yl)methyl]amines<sup>[3]</sup> and tris(2-benzimidazolylmethyl)amines.<sup>[4]</sup> The catalytic system has received a great deal of use in various fields such as chemical biology and materials science.<sup>[1,5]</sup> The 1,3-dipolar cycloaddition of azides with unactivated alkynes occurs much more slowly but is highly chemoselective. This property stimulated the development of “in situ click chemistry” for the field of drug discovery, in which target enzymes are allowed to assemble new inhibitors by linking azides and alkynes that bind to adjacent sites on the protein surface.<sup>[6]</sup> The linkage reaction does not employ Cu catalysis, but instead relies on acceleration of the otherwise sluggish [3+2] cycloaddition reaction when the reaction partners are

held in proximity to each other, often in or near the enzyme active site. In the course of an in situ click chemistry study on histone deacetylase (HDAC), we unexpectedly observed acceleration of the AAC reaction by trace copper associated with the protein in a structurally sensitive manner. Herein we report these findings, which constitute the first example of a Cu-protein complex catalyzing the AAC reaction.

HDAC inhibitors are attractive drug candidates for cancer, inflammation, and neurodegenerative disorders.<sup>[7]</sup> As shown in Figure 1, most HDAC inhibitors consist of a

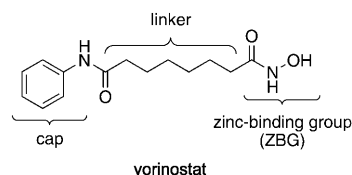
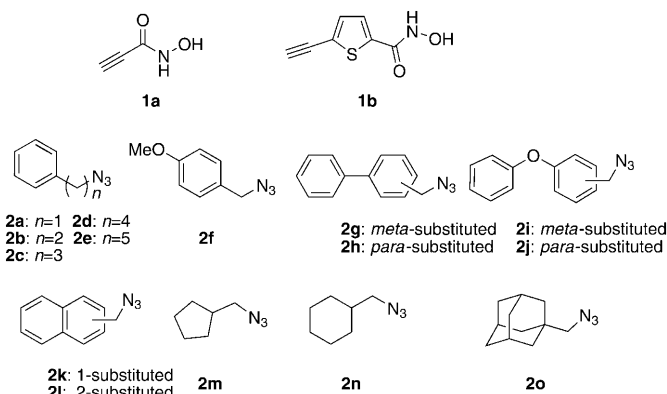


Figure 1. Structural characteristics of HDAC inhibitors.

Zn-binding group (ZBG) that coordinates with the Zn ion in the active site, a capping region that interacts with residues on the rim of the active site, and a linker that connects the cap and ZBG at an appropriate distance. For example, the clinically used HDAC inhibitor vorinostat (Figure 1) consists of a hydroxamic acid (ZBG), an anilide (cap), and an alkyl chain (linker). This general linked motif resembles that of acetylcholinesterase, the first target of in situ click chemistry.<sup>[6a]</sup> Accordingly, we prepared two alkynes with hydroxamic acid (**1a** and **1b**) and 15 alkyl azides (**2a–o**) as building blocks for in situ assembly screening (Scheme 1).

In conventional in situ click chemistry, a mixture of an alkyne and an azide is incubated in the presence of the target



Scheme 1. Structures of alkynes **1a** and **1b** and azides **2a–o**.

[\*] Dr. T. Suzuki, Y. Ota, Y. Kasuya, Dr. H. Tsumoto, Dr. H. Nakagawa, Prof. N. Miyata  
Graduate School of Pharmaceutical Sciences  
Nagoya City University  
3-1 Tanabe-dori, Mizuho-ku, Nagoya, Aichi 467-8603 (Japan)  
Fax: (+81) 52-836-3407  
E-mail: suzuki@phar.nagoya-cu.ac.jp  
miyata-n@phar.nagoya-cu.ac.jp

Dr. T. Suzuki  
PRESTO (Japan) Science and Technology Agency (JST)  
4-1-8 Honcho Kawaguchi, Saitama 332-0012 (Japan)

Prof. N. Miyata  
Division of Organic Chemistry  
National Institute of Health Sciences  
1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501 (Japan)

Dr. M. Mutsuga, Dr. Y. Kawamura  
Division of Food Additives  
National Institute of Health Sciences  
1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501 (Japan)

Prof. M. G. Finn  
Department of Chemistry  
The Scripps Research Institute  
10550 North Torrey Pines Road, La Jolla, CA 92037 (USA)  
Fax: (+1) 858-784-8850  
E-mail: mgfinn@scripps.edu

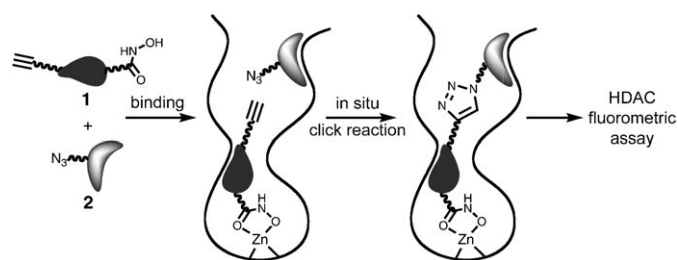
[\*\*] This work was supported in part by the JST PRESTO program (T.S.) and grants from Taisho Pharmaceutical Co., Ltd. (T.S.), the Uehara Memorial Foundation (T.S.), and the Skaggs Institute for Chemical Biology (M.G.F.).

Supporting information for this article is available on the WWW under <http://dx.doi.org/10.1002/anie.201002205>.

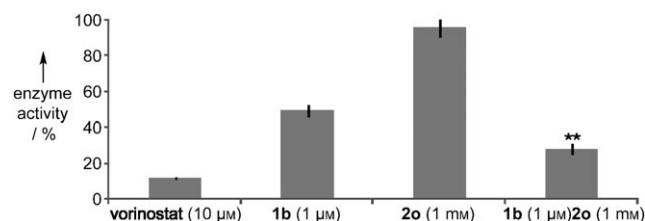
protein and the formation of the product is monitored by LC/MS analysis. The alkyne fragment is not activated by electron-withdrawing substituents, so as to make the uncatalyzed cycloaddition with azide very slow. At concentrations typically used for in situ inhibitor discovery, the background reaction has been estimated to have a half life of many years,<sup>[6a]</sup> making false positives very rare. It also makes even the target-templated reaction slow in absolute terms, such that only a percent or two of inhibitor is formed and detected by mass spectrometry. Independent synthesis is then necessary to confirm that the assembled triazole is indeed a good inhibitor.

Since we had a convenient assay of HDAC activity available,<sup>[8]</sup> we chose to see if enough of an inhibitor could be generated by in situ assembly to measurably affect enzyme function. We therefore incubated a mixture of each known alkyne ligand (at a concentration approximately equivalent to its  $IC_{50}$ ) with each candidate azide (in large excess) in the presence of HDAC and subsequently carried out a fluorometric assay for HDAC activity directly on the reaction mixture (Scheme 2). These experiments were conducted in parallel in 96-well microtiter plates using human recombinant HDAC8, which had been shown separately to be stable under the incubation and assay conditions. As shown in Figure 2, a significant decrease in fluorescence was observed only for the combination of alkyne **1b** and azide **2o** relative to the effect of **1b** and **2o** alone. Other alkyne/azide combinations resulted in no statistically significant change in fluorescence signal in the presence of both the azide and alkyne, relative to alkyne alone (data not shown).

The in situ and regiospecific formation of a triazole from **1b** and **2o** was also observed by HPLC (Figure 3). Incubation of **1b** and **2o** gave rise to a significant peak corresponding to *anti*-**3** in the presence of HDAC8 (Figure 3c), but not in its

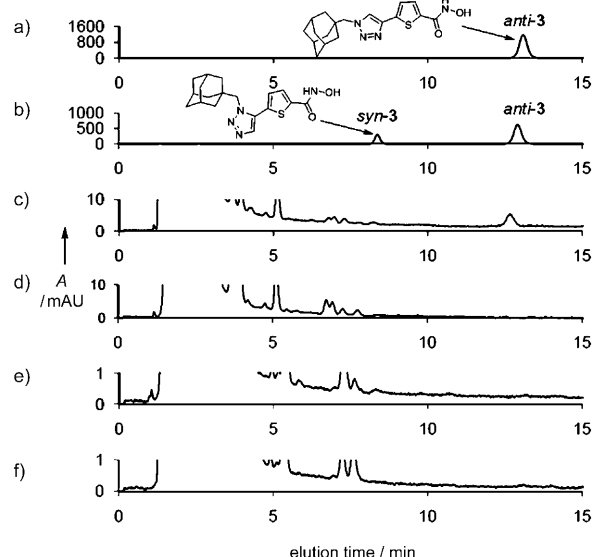


**Scheme 2.** In situ click chemistry screening using a fluorometric assay for HDAC activity.



**Figure 2.** In situ click chemistry screening using fluorometric assay for HDAC activity. \*\* $P < 0.01$ ; ANOVA and Bonferroni-type multiple  $t$  test results indicated differences between **1b** or **2o** alone and a combination of **1b** with **2o**.

absence (Figure 3d). The triazole was not formed when bovine serum albumin was substituted for HDAC8 (Figure 3e), nor when the HDAC8 reaction was performed in the presence of an excess of vorinostat, an active site-binding HDAC inhibitor (Figure 3f). These results show that the triazole formation takes place in the active site. Compound *anti*-**3** was synthesized independently and found to be a competent HDAC inhibitor (Table S1 in the Supporting Information).



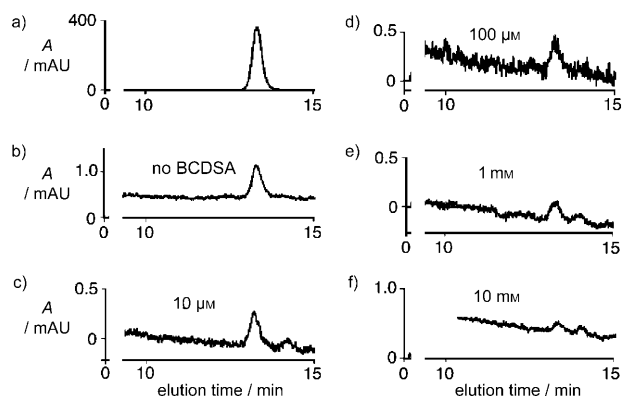
**Figure 3.** Identification of in situ click chemistry product generated from **1b** (1 mM) and **2o** (1 mM) by HPLC analysis: a) authentic sample *anti*-**3** (13.1 min); b) a mixture of *syn*-**3** (8.37 min) and *anti*-**3** (12.9 min); c) reaction in the presence of HDAC8 (0.6  $\mu$ M), in situ product (12.7 min); d) reaction without enzyme; e) reaction in the presence of BSA (0.6  $\mu$ M); f) reaction in the presence of HDAC8 (0.6  $\mu$ M) and vorinostat (10 mM). mAU = milli absorbance units.

Several aspects of these apparently successful in situ click chemistry results were unusual. First, while ordinary in situ reactions produce only very small amounts of triazole per enzyme, the yield of the triazole in this study was as much as 50%. Second, no *syn*-**3** was found in the in situ process, even though the *syn*-triazole isomer proved to be a better HDAC8 inhibitor ( $IC_{50} = 0.51 \mu$ M) than *anti*-**3** ( $IC_{50} = 4.0 \mu$ M). These factors suggested that the rapid and regioselective formation of *anti*-**3** in the presence of HDAC8 is consistent with acceleration of the reaction by a small amount of enzyme containing  $Cu^I$  instead of  $Zn^{II}$ .

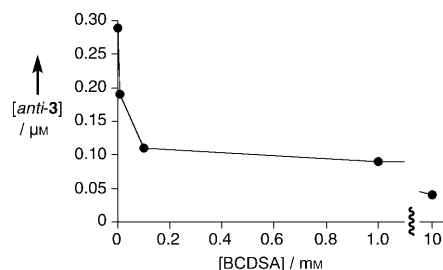
Indeed, ICP-MS analysis of a mixture of alkyne **1b** (1 mM), azide **2o** (1 mM), and HDAC8 (0.6  $\mu$ M) revealed the presence of both 0.95  $\mu$ M of Zn and 0.10  $\mu$ M of Cu. ICP-MS analysis of each component independently showed that alkyne **1b** contained 0.01 mol% Cu, which probably came from the Sonogashira reaction used for its synthesis (Scheme S2 in the Supporting Information).

To examine whether  $Cu^I$  is required for the cycloaddition, we repeated the in situ experiment in the presence of various concentrations of bathocuproine disulfonic acid (BCDSA),<sup>[9]</sup>

a Cu<sup>I</sup>-specific chelator and inhibitor of the CuAAC reaction. As shown in Figures 4 and 5, BCDSA inhibited the triazole formation in dose-dependent fashion. In addition, Zn(OAc)<sub>2</sub> was added to the mixture of alkyne **1b**, azide **2o**, and HDAC8 to displace Cu from the protein. This treatment completely suppressed the formation of *anti*-**3** (Figure S2 in the Supporting Information), whereas in solution Zn<sup>II</sup> has no effect on the CuAAC reaction.<sup>[10]</sup> Conversely, kinetic measurements of HDAC8 catalytic activity showed that added Cu<sup>I</sup> increased the *K<sub>m</sub>* value for substrate relative to the value observed in the absence of added cuprous ion (Lineweaver–Burk analysis, Figure S3 in the Supporting Information).



**Figure 4.** HPLC analysis of triazole formation from **1b** (1 mM) and **2o** (1 mM) in the presence of HDAC8 (0.6 μM): a) authentic sample of *anti*-**3**; b) reaction in the absence of the Cu<sup>I</sup>-specific chelator BCDSA; c–f) reaction in the presence of the indicated concentration of BCDSA.



**Figure 5.** Inhibition of triazole *anti*-**3** formation by the Cu<sup>I</sup>-specific chelator BCDSA.

These data strongly suggest that Cu<sup>I</sup>, acting in the active site of HDAC8, accelerates the rate of cycloaddition between alkyne **1b** and azide **2o**. Indeed, CuAAC reactions attempted under analogous conditions but without HDAC8 (CuBr at 0.1 or 0.6 μM; CuSO<sub>4</sub> at 0.1 or 0.6 μM and sodium ascorbate at 0.5 or 3.0 μM) resulted in no triazole formation (Figure S1 in the Supporting Information). This study is important for four reasons:

- 1) It shows for the first time that a protein–Cu complex can be a far better catalyst than Cu alone. HDAC8–Cu complex at 0.1 μM catalyzes triazole formation, whereas Cu<sup>I</sup> at submicromolar concentration provides very little reaction (Figure S1 f).<sup>[3b,4]</sup> In order to be effective, most

Cu–ligand catalysts of the CuAAC reaction have to be used at 10 μM or higher concentrations.<sup>[3b,4]</sup> These results suggest that protein–Cu complexes can be developed as highly active CuAAC catalysts.

- 2) It shows that a single Cu center may be enough to catalyze the reaction,<sup>[11]</sup> in contrast to most of our kinetics experiments, which indicate that two Cu atoms are required.<sup>[12]</sup> However, many possibilities exist for coordination of Cu in the HDAC binding pocket. In addition to the two Asp (178 and 267) and one His (180) residues that are shown to bind Zn in the X-ray crystal structures of HDAC8, four other potential metal-binding side chains are in the immediate vicinity (His142, His143, Met274, and Tyr306).<sup>[13]</sup> Thus, it is conceivable for two metal centers (Zn/Cu or Cu/Cu) to occupy the active site together, or for a single Cu center to do so in different ways. Further investigation is needed to determine the CuAAC active structure.
- 3) It shows that HDAC8 preserves Cu in the +1 oxidation state, even though the solution is not protected from air. The histidine and methionine residues in the active site of HDAC8 may contribute to the stabilization of Cu<sup>I</sup> in analogy to natural Cu<sup>I</sup> environments in copper-containing enzymes.<sup>[14]</sup>
- 4) It shows that the HDAC8–Cu complex, while being a fast catalyst, is not a general one, since it provides triazole only for the **1b**+**2a** combination among the possibilities tested, and therefore that the reaction is guided by the protein structure. The X-ray crystal structure of HDAC8 shows a large hydrophobic pocket next to the active site.<sup>[15]</sup> The adamantyl group of **2o** could be located in this pocket in an orientation that allows reaction with a Cu–acetylide generated from alkyne **1b** and Cu<sup>I</sup> in the active site.

If the protein holds the reaction components in such a way as to allow Cu in the Zn binding site to selectively assemble a triazole in situ, the metal center would interact with the alkyne portion of **1b** rather than the hydroxamate, essentially inverting the orientation shown schematically in Scheme 2. We would then expect the molecule so formed to be less likely than most hydroxamates to bind to the Zn atom, and therefore to be a less potent inhibitor of the enzyme. In addition to *syn*-**3**, preliminary experiments showed that other triazoles available from the azide–alkyne library, such as the **1b**/**2f** combination, were also more potent inhibitors than *anti*-**3**, but were not formed in the enzyme. These compounds can presumably benefit from hydroxamate–Zn binding to a greater degree than *anti*-**3**. While helpful and used by most HDAC inhibitors, such binding is not a requirement for HDAC8 inhibition (Figure S4 in the Supporting Information).<sup>[16]</sup> The fact that *anti*-**3** is only a moderate inhibitor is probably responsible for the catalytic nature of its production (more triazole is formed than there is Cu in the sample); with a binding affinity of 4 μM, most of the protein is not bound by *anti*-**3** during the course of the reaction, and the off-rate is likely to be fast, allowing for catalytic turnover.

In conclusion, we have established that a Cu<sup>I</sup> complex of HDAC8 accelerates a selective reaction between an azide and an alkyne, thereby forming a compound with greater inhib-

itory power than either of the pieces alone. In this case, the unwavering regioselectivity of the CuAAC process overrides the tendency of the Cu-free in situ click reaction to form the more potent of the two triazole isomers. We regard this as an important precedent in the search for highly active protein catalysts of the CuAAC reaction, as well as an interesting and potentially useful example of in situ inhibitor formation.

Received: April 14, 2010

Revised: June 13, 2010

Published online: August 16, 2010

**Keywords:** alkynes · azides · click chemistry · copper · proteins

- [1] a) H. C. Kolb, M. G. Finn, K. B. Sharpless, *Angew. Chem.* **2001**, *113*, 2056–2075; *Angew. Chem. Int. Ed.* **2001**, *40*, 2004–2021; b) M. G. Finn, V. V. Fokin, *Chem. Soc. Rev.* **2010**, *39*, 1231–1232.
- [2] a) C. W. Tornøe, C. Christensen, M. Meldal, *J. Org. Chem.* **2002**, *67*, 3057–3062; b) V. V. Rostovtsev, L. G. Green, V. V. Fokin, K. B. Sharpless, *Angew. Chem.* **2002**, *114*, 2708–2711; *Angew. Chem. Int. Ed.* **2002**, *41*, 2596–2599.
- [3] a) T. R. Chan, R. Hilgraf, K. B. Sharpless, V. V. Fokin, *Org. Lett.* **2004**, *6*, 2853–2855; b) V. Hong, S. I. Presolski, C. Ma, M. G. Finn, *Angew. Chem.* **2009**, *121*, 10063–10067; *Angew. Chem. Int. Ed.* **2009**, *48*, 9879–9883.
- [4] V. O. Rodionov, S. I. Presolski, S. Gardinier, Y. H. Lim, M. G. Finn, *J. Am. Chem. Soc.* **2007**, *129*, 12696–12704.
- [5] For recent reviews and original papers, see a) J. F. Lutz, *Angew. Chem.* **2007**, *119*, 1036–1043; *Angew. Chem. Int. Ed.* **2007**, *46*, 1018–1025; b) C. M. Salisbury, B. F. Cravatt, *J. Am. Chem. Soc.* **2008**, *130*, 2184–2194; c) C. Xu, E. Soragni, C. J. Chou, D. Herman, H. L. Plasterer, J. R. Rusche, J. M. Gottesfeld, *Chem. Biol.* **2009**, *16*, 980–989; d) A. Carlmark, C. Hawker, A. Hult, M. Malkoch, *Chem. Soc. Rev.* **2009**, *38*, 352–362.
- [6] a) W. G. Lewis, L. G. Green, F. Grynszpan, Z. Radić, P. R. Carlier, P. Taylor, M. G. Finn, K. B. Sharpless, *Angew. Chem.* **2002**, *114*, 1095–1099; *Angew. Chem. Int. Ed.* **2002**, *41*, 1053–1057; b) H. C. Kolb, K. B. Sharpless, *Drug Discovery Today* **2003**, *8*, 1128–1137; c) S. K. Mamidyalala, M. G. Finn, *Chem. Soc. Rev.* **2010**, *39*, 1252–1261; d) X. Hu, R. Manetsch, *Chem. Soc. Rev.* **2010**, *39*, 1316–1324.
- [7] a) T. Suzuki, *Chem. Pharm. Bull.* **2009**, *57*, 897–906; b) M. Paris, M. Porcelloni, M. Binaschi, D. Fattori, *J. Med. Chem.* **2008**, *51*, 1505–1529.
- [8] a) T. Suzuki, Y. Nagano, A. Kouketsu, A. Matsuura, S. Maruyama, M. Kurotaki, H. Nakagawa, N. Miyata, *J. Med. Chem.* **2005**, *48*, 1019–1032; b) T. Suzuki, A. Kouketsu, Y. Itoh, S. Hisakawa, S. Maeda, M. Yoshida, H. Nakagawa, N. Miyata, *J. Med. Chem.* **2006**, *49*, 4809–4812; c) Y. Itoh, T. Suzuki, A. Kouketsu, N. Suzuki, S. Maeda, M. Yoshida, H. Nakagawa, N. Miyata, *J. Med. Chem.* **2007**, *50*, 5425–5438; d) N. Suzuki, T. Suzuki, Y. Ota, T. Nakano, M. Kurihara, H. Okuda, T. Yamori, H. Tsumoto, H. Nakagawa, N. Miyata, *J. Med. Chem.* **2009**, *52*, 2902–2922; e) T. Asaba, T. Suzuki, R. Ueda, H. Tsumoto, H. Nakagawa, N. Miyata, *J. Am. Chem. Soc.* **2009**, *131*, 6989–6996.
- [9] A. T. Faizullah, A. Townshend, *Anal. Chim. Acta* **1985**, *172*, 291–296.
- [10] See section G in the Supporting Information of reference [3b].
- [11] C. Nolte, P. Mayer, B. F. Straub, *Angew. Chem.* **2007**, *119*, 2147–2149; *Angew. Chem. Int. Ed.* **2007**, *46*, 2101–2103.
- [12] a) V. O. Rodionov, V. V. Fokin, M. G. Finn, *Angew. Chem.* **2005**, *117*, 2250–2255; *Angew. Chem. Int. Ed.* **2005**, *44*, 2210–2215; b) V. O. Rodionov, S. I. Presolski, D. D. Díaz, V. V. Fokin, M. G. Finn, *J. Am. Chem. Soc.* **2007**, *129*, 12705–12712.
- [13] A. Vannini, C. Volpari, G. Filocamo, E. C. Casavola, M. Brunetti, D. Renzoni, P. Chakravarty, C. Paolini, R. De Francesco, P. Gallinari, C. Steinkühler, S. Di Marco, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 15064–15069.
- [14] a) Y. Rondelez, G. Bertho, O. Reinaud, *Angew. Chem.* **2002**, *114*, 1086–1088; *Angew. Chem. Int. Ed.* **2002**, *41*, 1044–1046; b) S. T. Prigge, B. A. Eipper, R. E. Mains, L. M. Amzel, *Science* **2004**, *304*, 864–867.
- [15] a) J. R. Somoza, R. J. Skene, B. A. Katz, C. Mol, J. D. Ho, A. J. Jennings, C. Luong, A. Arvai, J. J. Buggy, E. Chi, J. Tang, B. C. Sang, E. Verner, R. Wynands, E. M. Leahy, D. R. Dougan, G. Snell, M. Navre, M. W. Knuth, R. V. Swanson, D. E. McRee, L. W. Tari, *Structure* **2004**, *12*, 1325–1334; b) K. KrennHrubec, B. L. Marshall, M. Hedglin, E. Verdin, S. M. Ulrich, *Bioorg. Med. Chem. Lett.* **2007**, *17*, 2874–2878.
- [16] G. Estiu, N. West, R. Mazitschek, E. Greenberg, J. E. Bradner, O. Wiest, *Bioorg. Med. Chem.* **2010**, *18*, 4103–4110.