ChemPubSoc Europe

DOI: 10.1002/cbic.201400013

Phosphoramidates as Novel Activity-Based Probes for Serine Proteases

Ute R. Haedke,[a] Sandra C. Frommel,[a] Fabian Hansen,[a] Hannes Hahne,[b] Bernhard Kuster,[b] Matthew Bogyo,[c] and Steven H. L. Verhelst*[a]

Activity-based probes (ABPs) are small molecules that exclusively form covalent bonds with catalytically active enzymes. In the last decade, they have especially been used in functional proteomics studies of proteases. Here, we present phosphoramidate peptides as a novel type of ABP for serine proteases. These molecules can be made in a straightforward manner by standard Fmoc-based solid-phase peptide synthesis, allowing rapid diversification. The resulting ABPs covalently bind different serine proteases, depending on the amino acid recognition element adjacent to the reactive group. A reporter tag enables downstream gel-based analysis or LC-MS/MS-mediated identification of the targeted proteases. Overall, we believe that these readily accessible probes will provide new avenues for the functional study of serine proteases in complex proteomes.

Functional analysis of the proteome has been enormously enhanced by the use of activity-based probes (ABPs, Figure 1 A).[1] ABPs specifically label a targeted fraction of active enzymes, often by using a mechanism-based reaction.[2] Distinguishing between active and inactive enzymes is important because most enzymes are expressed as inactive zymogens and are, upon activation, dynamically regulated by a variety of post-translational mechanisms. More importantly, up- or down-regulation of enzyme activities underlies many pathologies. Due to the expanding applicability of ABPs in profiling, imaging and inhibitor screening methods,[3] quick synthetic access to ABPs is crucial. Furthermore, some applications require the ability to tune the ABP selectivity towards a specific subset of enzymes or even a single enzyme.[4] To achieve both rapid synthesis and probe optimization, solid-phase synthesis has been successfully applied in the construction of ABPs for cysteine proteases,[5–7] the proteasome,[8] and tyrosine phosphatases,[9] for example. In the past, we and others have generated ABPs for serine proteases (Figure 1 B), including fluorophosphonates,[10] which label virtually all serine hydrolases,[11] diphenyl phosphonates,[12] 4-chloro-isocoumarins,[13,14] and sulfonyl fluorides,[15] but all of these are only accessible by solution-phase synthesis. Here, we report phosphoramidates as a novel type of serine protease ABP. The main benefit lies in their ability to be exclusively constructed on solid support, using only commercially available reagents and standard amino acid building blocks. We show that we can control the selectivity of these probes by varying the recognition element adjacent to the reactive phosphoramidate group. In addition, we demonstrate that they can be used for labeling and identification of serine proteases in complex proteomes.

In solid-phase synthesis of ABPs, the reactive electrophilic "warhead" is ideally introduced during the last step in order to prevent inactivation during the elongation procedure. Such a strategy can be achieved by a safety-catch approach[8] or by simply capping the N terminus of a peptide chain with a reactive electrophile.[5,7,16,17] Although the latter strategy results in reversed backbone polarity compared to a protease substrate, it has successfully resulted in ABPs for cysteine cathepsins based on electrophiles such as epoxysuccinates[5] and O-acylhydroxyureas.[7] For the design of serine protease ABPs, we decided to cap the N terminus of a growing peptide chain on resin with a phosphor-containing electrophile, analogous to diphenyl phosphonates. This resulted in the formation of phosphoramidate peptides (PAPs). Compared to an α-aminoalkyl diphenyl phosphonate (Figure 1 B), the diphenyl phosphoramidate might be less electronegative and therefore less reactive, due to donation of the free pair of electrons on the nitrogen to an empty 3d orbital on the phosphor atom. PAPs were synthesized on a Rink amide resin from Fmoc-protected building blocks by using standard solid-phase peptide synthesis (SPPS) elongation conditions (Scheme 1). Fmoc deprotection was performed with 20% piperidine in dimethylformamide, and each amino acid coupling was achieved by using diisopropyl carbodiimide (DIC) and hydroxybenzotriazole (HOBt). As a first building block, the reporter tag (either a propargylglycine or an ε-biotinylated lysine) was coupled to the resin. Next, the peptide was elongated with a spacer and a recognition element, and the reactive electrophile was coupled by using diphenyl chlorophosphate (DCP) and triethylamine (TEA). The probes were cleaved from the resin with 95% trifluoroacetic acid and purified by HPLC, giving the desired PAP probes in 2–39% yield, which is comparable to previously reported SPPS for cysteine protease probes.[18] With this procedure, we synthesized two sets of ABPs carrying either an alkyne moiety (set a) or a biotin (set b) as a reporter tag (Scheme 2). The residues adjacent to

[a] Dr. U. R. Haedke, S. C. Frommel, F. Hansen, Dr. S. H. L. Verhelst
Lehrstuhl für Chemie der Biopolymere, Technische Universität München
Weihenstephaner Berg 3, 85354 Freising (Germany)
E-mail: verhelst@wzw.tum.de

[b] Dr. H. Hahne, Prof. Dr. B. Kuster
Chair of Proteomics and Bioanalytics, Technische Universität München
Emil Erlenmeyer Forum 5, 85354 Freising (Germany)

[c] Prof. Dr. M. Bogyo
Departments of Pathology and Microbiology & Immunology
Stanford University, School of Medicine
300 Pasteur Drive, CA 94305 Stanford (USA)

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cbic.201400013.

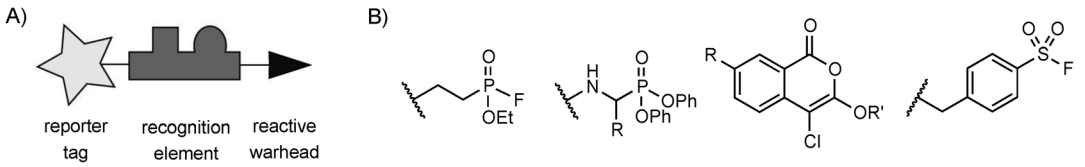
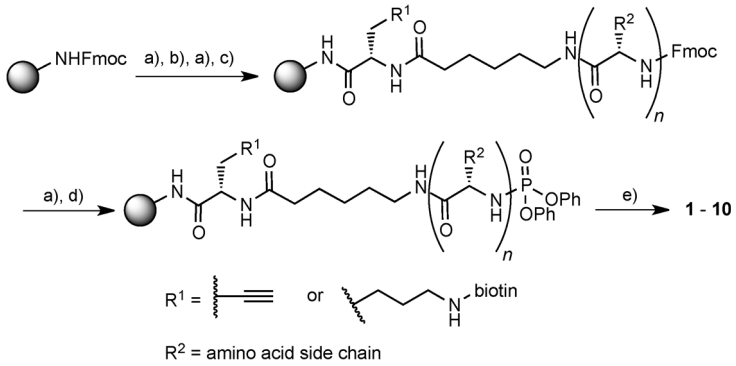
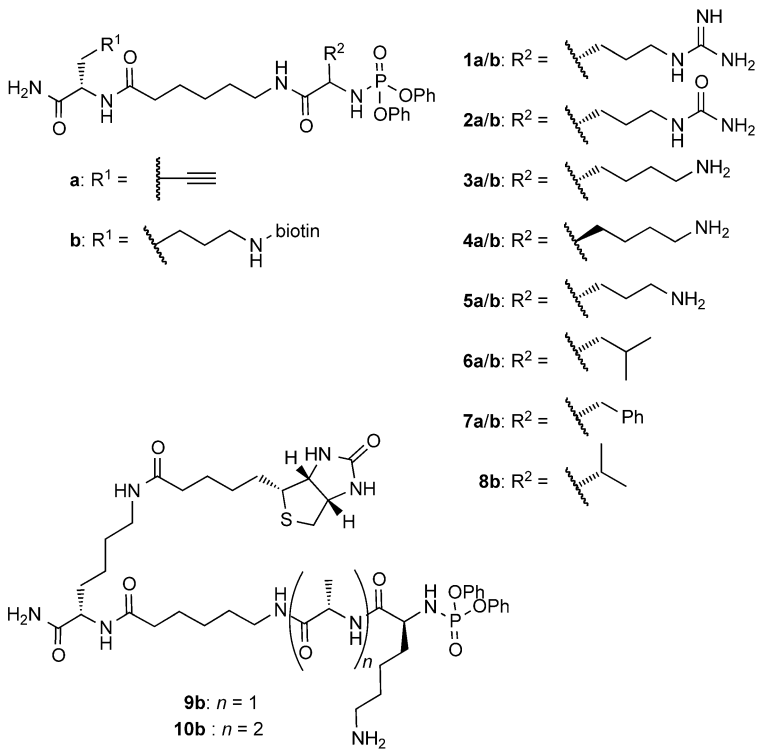


Figure 1. A) Generally, ABPs consist of three elements: 1) an electrophilic warhead that forms a covalent bond to an active site residue, 2) a recognition element that induces selectivity for certain members of the targeted enzyme class, and 3) a reporter tag enabling the analysis of the enzyme–ABP complex. B) Existing ABP warheads for serine hydrolases and serine proteases: fluorophosphonates, α-amino-alkyl diphenyl phosphonates, 4-chloro-isocoumarins, and sulfonyl fluorides.



Scheme 1. Fmoc-based solid-phase synthesis of PAPs. a) 20% piperidine in DMF, 15 min; b) Fmoc-Lys(Biotin)-OH (1.5 equiv), HBTU (1.5 equiv), DIEA (3 equiv), NMP, overnight; or Fmoc-Pra-OH (2 equiv), DIC (2 equiv), HOBt (2 equiv), DMF, 2 h. c) Fmoc-aa-OH (3 equiv), HOBt (3 equiv), DIC (3 equiv), DMF, 2 h; d) diphenylchlorophosphate (5 equiv), TEA (5 equiv), DCM, 1 h; e) TFA/TIS/H_2O (95:2.5:2.5), 1 h, 2–39% yield after HPLC purification.



Scheme 2. The set of synthesized phosphoramidate peptide probes. Compounds **1a**–**7a** and **1b**–**8b** carry a single amino acid constituting the recognition element; compound **9b** has one and **10b** two additional alanines.

the electrophile included hydrophobic, basic, and polar amino acids. For the set of biotinylated probes, two extended peptides (**9b** and **10b**) were synthesized to increase both the length and peptide character of the probes.

We tested the stability of the phosphoramidate electrophile by incubating ABP **3b** at physiological pH and found no substantial degradation under our assay conditions (Supporting Information, Figure S1). We also found that the relatively hydrophobic alkyne probes, such as ABP **6a**, displayed enough water solubility for use in aqueous environment (Figure S2). At this point, we used the alkyne-containing ABPs **1a**–**7a** to label three purified serine proteases that display different substrate specificities: bovine trypsin, bovine chymotrypsin, and human neutrophil elastase (Figure 2A). Trypsin was labeled by probes **1a** and **3a**, containing an arginine or lysine, respectively, as an amino acid residue next to the reactive electrophile. This is in accordance with the preference of trypsin for basic residues in the P1 position. Interestingly, probes with D-lysine or ornithine did not result in labeling, indicating that probe binding to the active site is highly selective. Chymotrypsin and neutrophil elastase (NE) reacted with probes having a bulky hydrophobic or small hydrophobic residue next to the phosphoramidate electrophile, consistent with their established substrate specificity. The biotinylated probes **3b**, **7b**, and **8b**, incorporating a lysine, phenylalanine, and valine residue, respectively, also show selective labeling of the protease target with matching substrate selectivity (Figure 2B). For all proteases, preincubation with a known active site inhibitor resulted in abolishment of the labeling, indicating the need for an intact active site (Figure 2A). To further confirm that the labeling is activity-dependent, we incubated both trypsin and trypsinogen with ABP **3b**. Trypsin yields robust labeling upon incubation with 10 μM of this probe, whereas its zymogen form is not labeled at all (Figure 2C). Overall, this indicates that PAPs function as genuine ABPs that require active enzyme for labeling.

To determine the different labeling sensitivities, we performed a dose–response experiment on trypsin and PAPs **1b**, **3b**, and **9b**, as shown in Figure 2D. All three probes result in saturated labeling of trypsin

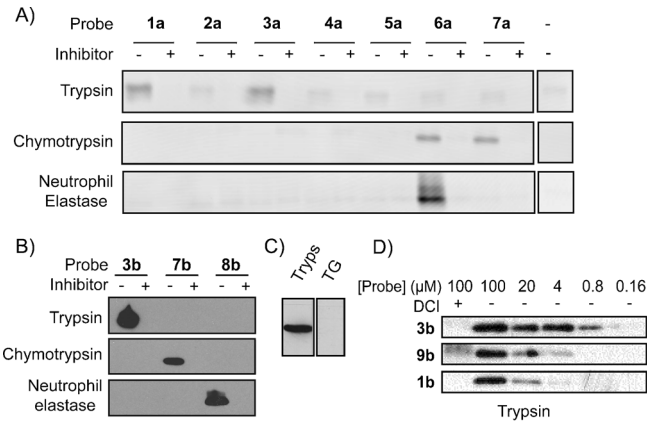


Figure 2. Labeling of purified serine proteases. A) Proteases were preincubated for 30 min with DMSO or inhibitor (TLCK for bovine trypsin, TPCK for bovine chymotrypsin, and DCI for human neutrophil elastase) and were subsequently reacted with the indicated probe (0.5 μM for trypsin, 2 μM for chymotrypsin, 10 μM for elastase). A tetramethylrhodamine fluorophore was attached by Cu^{I}-catalyzed click chemistry. Labeled proteins were resolved on SDS-PAGE and detected through fluorescent scanning. B) Proteases were preincubated with DMSO or an active site inhibitor for 30 min and subsequently reacted with the indicated probe (10 μM). Labeled proteins were detected by biotin–streptavidin blot. C) Probe **10b** (10 μM) was used to label either trypsin or trypsinogen. After SDS-PAGE, labeled proteins were detected by biotin–streptavidin blot. D) Trypsin was labeled with decreasing concentrations of the indicated probe and detected by biotin–streptavidin blot.

when used at 100 μM. Probe **3b** turned out to be the most potent probe, as it detects trypsin when used at concentrations as low as 0.8 μM. For the other two probes, higher concentrations were needed to saturate labeling. This result demonstrates that the structure next to the reactive electrophile has a strong influence on the affinity of a probe for its target enzyme. A direct comparison of PAP **3a** with a trypsin-targeting diphenyl phosphonate probe[12] shows that detection of both probes is possible at submicromolar concentrations (Figure S3), but that the phosphoramidate needs a concentration approximately five times higher than that of diphenyl phosphonate, which could be due to less optimal recognition by the S1 pocket or the expected lower electrophilicity of the PAP.

Overall, the above labeling experiments show that the inverse peptide backbone of the probes compared to a peptide substrate does not prevent the ABP from binding and productively reacting with the active site of a serine protease target. Similar to inverse backbone probes for cysteine cathepsins,[5,7] these probes have enough flexibility to interact with the protease specificity pockets and simultaneously display the reactive warhead to the active site residues. For PAPs, we hypothesized that covalent probe binding occurs by reaction at the phosphor atom and concomitant expulsion of a phenoxy leaving group, similar to the mechanism of peptidyl diphenyl phosphonates.[19] To confirm this, we treated bovine trypsin with probe **3a** and performed mass spectrometry experiments on the intact protein species. All multiple-charged species showed a clear mass shift compared to a dimethylsulfoxide (DMSO) control (Figure 3A). The mass difference between the unlabeled and the labeled trypsin, calculated after deconvolution (Figure 3B), indeed corresponded to a trypsin species that had reacted with the PAP, with elimination of one phenol. Bovine chymotrypsin and probe **7a** also showed a mass shift matching this mechanism (Figure S4).

We now set out to use PAPs as tools to label and identify endogenously expressed serine proteases. Rat pancreas lysate, activated with enteropeptidase, was incubated with 10 μM of each biotinylated probe, and labeled proteins were detected by streptavidin blot. Probes carrying an L-lysine afforded robust labeling of a band around 27 kDa (Figure 4). Pretreatment with an active site inhibitor resulted in disappearance of these bands, indicating activity-dependent labeling. ABP **3b** was used to enrich and identify the labeled targets. To this end, lysate treated with **3b** was subjected to a desalting column to remove unreacted probe, and the eluate was incubated with streptavidin-coated agarose beads. After extensive washing, the bound proteins were eluted with heat by boiling the beads with sample buffer for 10 min. The sample was then separated on SDS-PAGE. The 27 kDa protein was the only Coomassie-stainable band and was excised, reduced, alkylated, digested with GluC, and analyzed by LC-MS/MS. In addition to a few common laboratory contaminants (Table S1), the only identified proteins were three different trypsins (Table 1), which matches the selectivity pattern of probe **3b**.

In summary, we have designed PAPs as a new type of ABP for serine proteases. PAPs can be synthesized entirely on solid

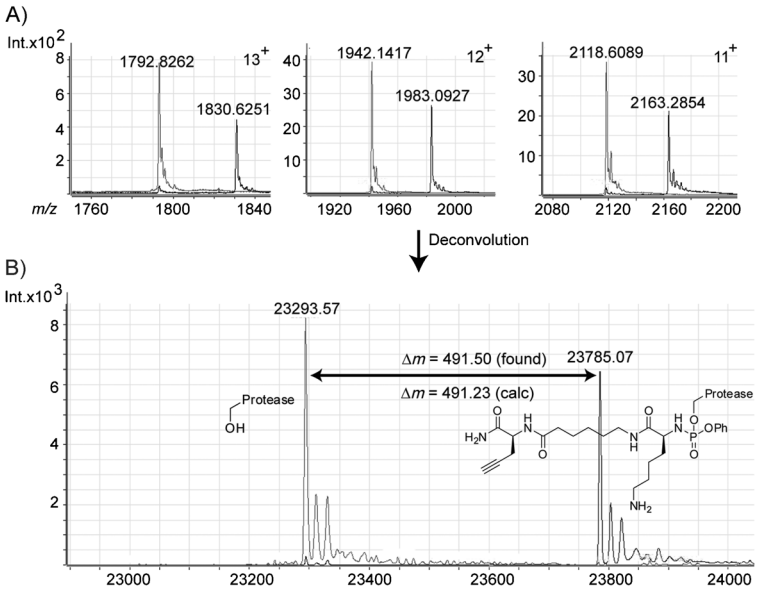


Figure 3. PAPs label their target protease by substitution of one phenol on the phosphoramidate warhead. Bovine trypsin was incubated with **3a** (100 μM), excess probe was removed by dialysis, and the sample was analyzed by ESI-MS. An untreated sample of trypsin served as a reference. A) Overlays of the spectra of the 13^{+}-, 12^{+}-, and 11^{+}-charged species, indicating a clear mass shift (trace in black) compared to the unreacted sample (blue). B) After deconvolution, the mass shift corresponds to covalent modification of the PAP, minus a phenol leaving group.

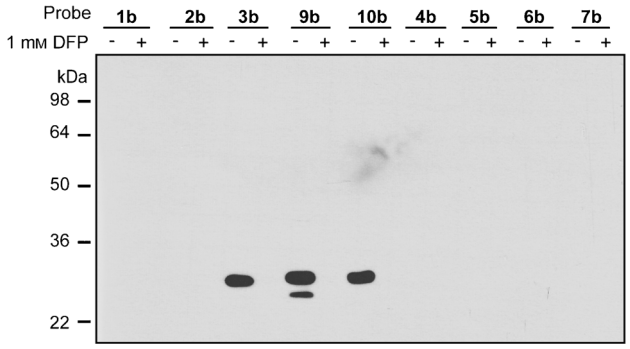


Figure 4. Labeling of endogenous serine proteases. Rat pancreas lysate was treated with enteropeptidase and labeled with the indicated probes (10 μM). Subsequently, the samples were separated by SDS-PAGE and analyzed by biotin–streptavidin blot.

support, which is a clear advantage over previously reported serine protease ABPs, as it represents an easy synthesis and allows for rapid generation of probe libraries. We have demonstrated that PAPs are functional ABPs that covalently label active serine proteases by release of a phenoxy leaving group. The selectivity of this class of ABPs is influenced by the amino acid residue located directly proximal to the phosphoramidate warhead, and matches the P1 substrate specificity of the targeted proteases. Furthermore, we showed that the probes can be utilized for MS-based target identification. Taken together, PAPs are a valuable addition to the ABP toolbox directed against serine proteases.

Table 1. Enriched and identified peptides from activated rat pancreas lysate.

Protein name	Molecular weight [kDa]	Accession number	Unique peptides (sequence coverage [%])	ID probability [%]
anionic trypsin-1	26	TRY1_RAT	5 (27)	100
anionic trypsin-2	26	TRY2_RAT	3 (15)	100
trypsin V-A	27	TRYA_RAT	2 (13)	100

Experimental Section

Solid-phase peptide synthesis: PAPs were synthesized on Rink amide resin (Creosalus, USA). Completion of each coupling was confirmed by the Kaiser test. Fmoc groups were deprotected with piperidine/DMF (1:4) for 15 min. The first amino acids, containing the detection tag, were coupled as follows: Fmoc-Lys(Biotin)-OH (1.5 equiv), HBTU (1.5 equiv), and DIEA (3 equiv) in NMP, overnight. Fmoc-Pra-OH (2 equiv), HOBt (2 equiv), and DIC (2 equiv) in DMF, 2 h. Other Fmoc amino acids (3 equiv) were coupled by using HOBt/DIC (3 equiv) in DMF. For Fmoc-Ahx-OH, a double coupling was performed to ensure complete reaction. To introduce the phosphoramidate warhead, diphenyl chlorophosphate (5 equiv) and TEA (5 equiv) in dichloromethane (DCM) were incubated with the resin for 1 h. Cleavage with 95% TFA, 2.5% triisopropylsilane (TIS), and 2.5% H_2O yielded the crude product, which was concentrated under reduced pressure or triturated in cold ether followed by drying under nitrogen flow. Crude products were purified by reversed-phase HPLC.

Preparation of rat pancreas lysate: Unless otherwise stated, all following procedures were performed on ice. A piece of rat pancreas tissue was forced through a 70 μm cell strainer and suspended in cold lysis buffer (50 mM NaH_2PO_4, 150 mM NaCl, 0.5% Nonidet P-40 substitute, pH 7.4). Cell debris was removed by using a tabletop centrifuge at 15000 rpm; the supernatant was snap-frozen and stored at −80 °C. For enzyme labeling, the lysate was diluted with phosphate-buffered saline (PBS) to 1 mg mL^{-1} protein content after thawing. The lysate was then incubated for 2 h on ice with 1 U enteropeptidase per mg total protein. Subsequently, the standard procedure for enzyme labeling was performed.

Target enrichment: For pull-down assays, pancreas lysate (19 mg mL^{-1}) was used undiluted. Following labeling with PAP **3b** (10 μM), unreacted probe was removed by using a Zeba spin gel filtration column (M_w cutoff 7 kDa, Thermo Fisher Scientific). The eluate was shaken with streptavidin-coated agarose beads (Merck/Calbiochem) for 2 h at room temperature in the presence of EDTA and EDTA-free protease inhibitor cocktail (50 μM, Roche). Beads were washed three times (50 mM Tris·HCl, 4 M urea, 150 mM NaCl, 1 mM EDTA, and 0.1% Triton-X 100 *v/v*), and bound proteins were eluted by heating the sample for 10 min at 95 °C in elution buffer (50 mM Tris·HCl, 4 mM biotin, 150 mM NaCl, 1 mM EDTA, and 0.1% Triton-X 100 *v/v*). Eluted proteins were separated by SDS-PAGE. Gel bands were cut and analyzed as described in the Supporting Information.

Acknowledgements

We acknowledge financial support from the DFG (Emmy Noether program), the Netherlands Organisation for Scientific Research (NWO), and the Center for Integrated Protein Science Munich. We thank Dr. Dieter Edbauer for providing rat pancreas tissue and Prof. Dr. Dieter Langosch for general support.

Keywords: activity-based probes · proteases · proteomics · solid-phase synthesis · target identification

[1] a) W. P. Heal, T. H. T. Dang, E. W. Tate, *Chem. Soc. Rev.* **2011**, *40*, 246–257; b) N. Li, H. S. Overkleeft, B. I. Florea, *Curr. Opin. Chem. Biol.* **2012**, *16*, 227–233.
[2] a) B. F. Cravatt, A. T. Wright, J. W. Kozarich, *Annu. Rev. Biochem.* **2008**, *77*, 383–414; b) S. H. L. Verhelst, M. Bogyo, *QSAR Comb. Sci.* **2005**, *24*, 261–269.
[3] S. Serim, U. Haedke, S. H. L. Verhelst, *ChemMedChem* **2012**, *7*, 1146–1159.
[4] U. Haedke, E. V. Kuttler, O. Vosyka, Y. Yang, S. H. L. Verhelst, *Curr. Opin. Chem. Biol.* **2013**, *17*, 102–109.
[5] a) D. Greenbaum, K. F. Medzihradszky, A. Burlingame, M. Bogyo, *Chem. Biol.* **2000**, *7*, 569–581; b) D. Greenbaum, A. Baruch, L. Hayrapetian, Z. Darula, A. Burlingame, K. F. Medzihradszky, M. Bogyo, *Mol. Cell. Proteomics* **2002**, *1*, 60–68.
[6] a) G. Wang, U. Mahesh, G. Y. Chen, S. Q. Yao, *Org. Lett.* **2003**, *5*, 737–740; b) S. H. L. Verhelst, M. Bogyo, *ChemBioChem* **2005**, *6*, 824–827.
[7] S. H. L. Verhelst, M. D. Witte, S. Arastu-Kapur, M. Fonovic, M. Bogyo, *ChemBioChem* **2006**, *7*, 943–950.
[8] H. S. Overkleeft, P. R. Bos, B. G. Hekking, E. J. Gordon, H. L. Ploegh, B. M. Kessler, *Tetrahedron Lett.* **2000**, *41*, 6005–6009.
[9] K. A. Kalesh, L. P. Tan, K. Lu, L. Gao, J. Wang, S. Q. Yao, *Chem. Commun.* **2010**, *46*, 589–591.

[10] a) Y. S. Liu, M. P. Patricelli, B. F. Cravatt, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 14694 – 14699; b) D. Kidd, Y. S. Liu, B. F. Cravatt, *Biochemistry* **2001**, *40*, 4005 – 4015.
[11] D. A. Bachovchin, T. Ji, W. Li, G. M. Simon, J. L. Blankman, A. Adibekian, H. Hoover, S. Niessen, B. F. Cravatt, *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 20941 – 20946.
[12] a) S. Mahrus, C. S. Craik, *Chem. Biol.* **2005**, *12*, 567 – 577; b) Z. Pan, D. A. Jeffery, K. Chehade, J. Beltman, J. M. Clark, P. Grothaus, M. Bogyo, A. Baruch, *Bioorg. Med. Chem. Lett.* **2006**, *16*, 2882 – 2885; c) A. S. Abuelyaman, D. Hudig, S. L. Woodard, J. C. Powers, *Bioconjugate Chem.* **1994**, *5*, 400 – 405; d) S. Serim, S. V. Mayer, S. H. L. Verhelst, *Org. Biomol. Chem.* **2013**, *11*, 5714 – 5721.
[13] a) C. M. Kam, A. S. Abuelyaman, Z. Z. Li, D. Hudig, J. C. Powers, *Bioconjugate Chem.* **1993**, *4*, 560 – 567; b) S. Arastu-Kapur, E. L. Ponder, U. P. Fonovic, S. Yeoh, F. Yuan, M. Fonovic, M. Grainger, C. I. Phillips, J. C. Powers, M. Bogyo, *Nat. Chem. Biol.* **2008**, *4*, 203 – 213.
[14] U. Haedke, M. Gotz, P. Baer, S. H. L. Verhelst, *Bioorg. Med. Chem.* **2012**, *20*, 633 – 640.
[15] D. A. Shannon, C. Gu, C. J. McLaughlin, M. Kaiser, R. A. van der Hoorn, E. Weerapana, *ChemBioChem* **2012**, *13*, 2327 – 2330.
[16] K. T. Barglow, B. F. Cravatt, *Chem. Biol.* **2004**, *11*, 1523 – 1531.
[17] N. J. Pace, D. R. Pimental, E. Weerapana, *Angew. Chem. Int. Ed.* **2012**, *51*, 8365 – 8368; *Angew. Chem.* **2012**, *124*, 8490 – 8493.
[18] D. Kato, K. M. Boatright, A. B. Berger, T. Nazif, G. Blum, C. Ryan, K. A. H. Chehade, G. S. Salvesen, M. Bogyo, *Nat. Chem. Biol.* **2005**, *1*, 33 – 38.
[19] a) J. Oleksyszyn, J. C. Powers, *Biochemistry* **1991**, *30*, 485 – 493; b) J. A. Bertrand, J. Oleksyszyn, C. M. Kam, B. Boduszek, S. Presnell, R. R. Plaskon, F. L. Suddath, J. C. Powers, L. D. Williams, *Biochemistry* **1996**, *35*, 3147 – 3155; c) J. C. Powers, J. L. Asgian, O. D. Ekici, K. E. James, *Chem. Rev.* **2002**, *102*, 4639 – 4750.

Received: January 6, 2014
Published online on May 9, 2014