Protein Chemistry

DOI: 10.1002/ange.201306118

Modifying the Vicinity of the Isopeptide Bond To Reveal Differential Behavior of Ubiquitin Chains with Interacting Proteins: Organic Chemistry Applied to Synthetic Proteins**

*Najat Haj-Yahya, Mahmood Haj-Yahya, Carlos A. Castañeda, Liat Spasser, Hosahalli P. Hemantha, Muhammad Jbara, Marlin Penner, Aaron Ciechanover, David Fushman, and Ashraf Brik**

Ubiquitination (the attachment of a ubiquitin (Ub) monomer, composed of 76 amino acids, or of a polyubiquitin (polyUb) chain to a protein target) is involved in a wide range of cellular processes, including protein degradation, trafficking, transcription, and the DNA damage response.[1] Polyubiquitination produces molecular signals that depend on which of the seven lysines in Ub is linked by an isopeptide bond to the C-terminal Gly76 of the consecutive Ub molecules.[2] Furthermore, Met1 of Ub can also be used to link the consecutive Ub molecules assembling the so-called linear chains.[2] Ubiquitination is a reversible posttranslational modification, and the reverse reaction, namely deubiquitination, is controlled by a family of enzymes known as deubiquitinases (DUBs), which hydrolyze isopeptide bonds within Ub bioconjugates.[3]

DUBs are involved in a variety of regulatory processes, such as cell-cycle progression and differentiation, and several DUBs have been implicated in various neurological infectious and neoplastic diseases,[3b] leading to the emergence of DUBs as potential therapeutic targets. Biochemical analyses of Ub chains revealed different specificities of DUBs towards these chains. Still unclear, however, is how most of these enzymes recognize the different Ub chains in their free or anchored form and what are the molecular bases for their specificities.[4,5] It appears that various factors are influencing recognition and specificity: among them are the structural topology and dynamics of these chains as well as the peptide sequences near the isopeptide bond that links two Ubs. For example, a single-molecule fluorescence study showed that conformational equilibria of these chains provide another level of regulation of the recognition and specificity of the chains with various DUBs and Ub binding domains (UBDs) and that by interfering with conformational dynamics one could affect chain hydrolysis by DUBs.[6]

Recent breakthroughs in the chemical and semisynthesis of Ub chains in their native forms offer exquisite control over the atomic structure of any chain[7] and have opened unprecedented opportunities to shed light on the modes of recognition and processing by the different ubiquitin-interacting proteins such as DUBs. For example, the role of the isopeptide bond in the recognition of Ub chains by the different ubiquitin-interacting proteins could be investigated. Along these lines, we have recently shown that *N*-methylation[8] of the isopeptide bond in H2B, ubiquitinated at Lys120, renders it unrecognizable by its specific monoclonal antibody.[9] This may indicate that the recognition event is mainly mediated by the isopeptide bond and its vicinity. Such understanding of the consequences of manipulating the isopeptide bond and its nearby residues on chain recognition and processing could also lead to the design of unique probes based on these chains (for example, highly labile or stable di-Ub[10]). Herein we present the chemical synthesis and characterization of 14 di-Ub analogues that are site-specifically modified at the nitrogen of the isopeptide bond, Gly76, or at the δ-carbon of the isopeptide Lys residue (Figure 1) and

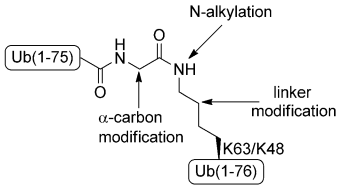


Figure 1. Points of interest within the Ub chains to be modified and tested.

[*] N. Haj-Yahya, M. Haj-Yahya, L. Spasser, Dr. H. P. Hemantha, M. Jbara, Prof. A. Brik
Department of Chemistry, Ben Gurion University of the Negev
Beer Sheva 84105 (Israel)
E-mail: abrik@bgu.ac.il
Homepage: http://www.bgu.ac.il/~abrik
Dr. C. A. Castañeda, Prof. D. Fushman
Department of Chemistry and Biochemistry, University of Maryland
College Park, MD 20742-3360 (USA)
Dr. M. Penner, Prof. A. Ciechanover
The Polak Cancer and Vascular Biology Research Center
The Rappaport Faculty of Medicine and Research Institute
Technion-Israel Institute of Technology, Haifa 31096 (Israel)
Prof. A. Ciechanover, Prof. A. Brik
The Institute of Chemistry & BioMedical Sciences
Nanjing University, Nanjing 210093 (P. R. China)

[**] This work was supported by the Israel Science foundation (A.B.) and the NIH grant GM065334 (D.F.). M.P. gratefully acknowledges the Azrieli Foundation for financial support. Research in the laboratory of A.C. is supported by grants from the Dr. Miriam and Sheldon G. Adelson Medical Research Foundation (AMRF), the I-CORE Program of the Planning and Budgeting Committee, the Israel Science Foundation (ISF) (grant no. 1775/12), The TreatPolyQ Network funded by the EU, and the Deutsch-Israelische Projektkooperation (DIP). A.C. is an Israel Cancer Research Fund (ICRF) USA Professor.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201306118.

their behavior with ubiquitin-interacting proteins. Our study revealed differential stability and affinities of these analogues with various DUBs and UBDs.

To investigate how small changes near the isopeptide bond could affect the recognition of ubiquitin-interacting proteins, we envisioned the chemical preparation of Ub chains with specific modifications at the nitrogen of the isopeptide bond, α-carbon of Gly76 of the distal Ub, or the δ-carbon of the isopeptide Lys residue (Figure 1). In pursuing the preparation of these analogues, three different strategies were adopted that enabled their highly efficient synthesis for biochemical and structural analyses with DUBs and UBDs. To prepare the modified analogues at the δ-carbon, we utilized our previously developed synthesis of di-Ub chains[7c] mediated by δ-mercaptolysine[11] to enable isopeptide chemical ligation (ICL) and generated the thiol-modified K48- or K63-linked di-Ub, **1**, **2**, respectively. These analogues were either desulfurized to obtain the native di-Ub (**3**, **4**) or alkylated at the thiol handle with iodoacetamide, methyl vinyl sulfone, or maleimide to give the analogues **5**–**10** (Scheme 1). Product isolation and refolding allowed us to study the effect of these chemical modifications on the activity of the DUBs.

Initially, we chose two enzymes from the USP family: Isopeptidase T (IsoT)[12] and Ubiquitin Specific Protease 2 (USP2),[13] which are known to cleave free ubiquitin chains with preference towards the K48 and K63-linked chains. To determine the hydrolysis percentage, our di-Ub analogues were incubated separately with each of the two enzymes and analyzed by HPLC and ESI-MS (see the Supporting Information). Surprisingly, despite the use of various alkyl groups of different sizes, all the analogues were cleaved in a similar percentage as the unmodified chains (Figure 2). These results may reflect the conformational flexibility of the isopeptide linker in the di-Ub chains. This could also be a result of the fact that the two DUBs examined here, IsoT and USP2, are not linkage specific, hence they are less sensitive to changes in the nearby residues of the isopeptide bond. Our results also indicate that modifying the thiol handle after ICL does not necessarily generate di-Ub chains that are highly stable against all DUBs. However, one may consider modifying the thiol with various probes to follow di-Ub cleavage, chains' dynamics and for other studies. Such strategy is currently under investigation.

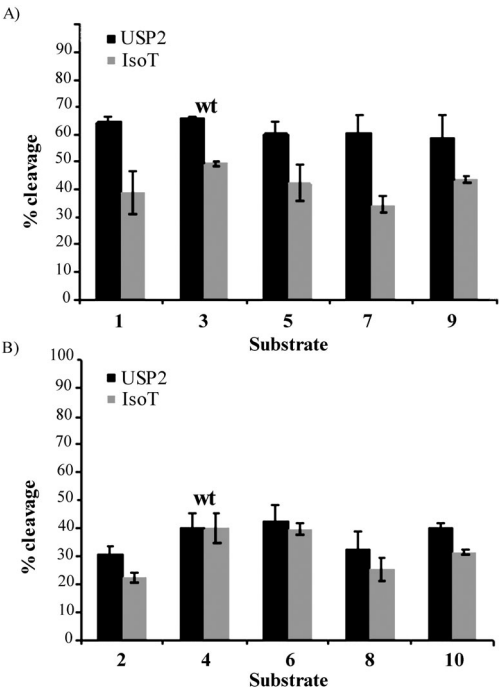
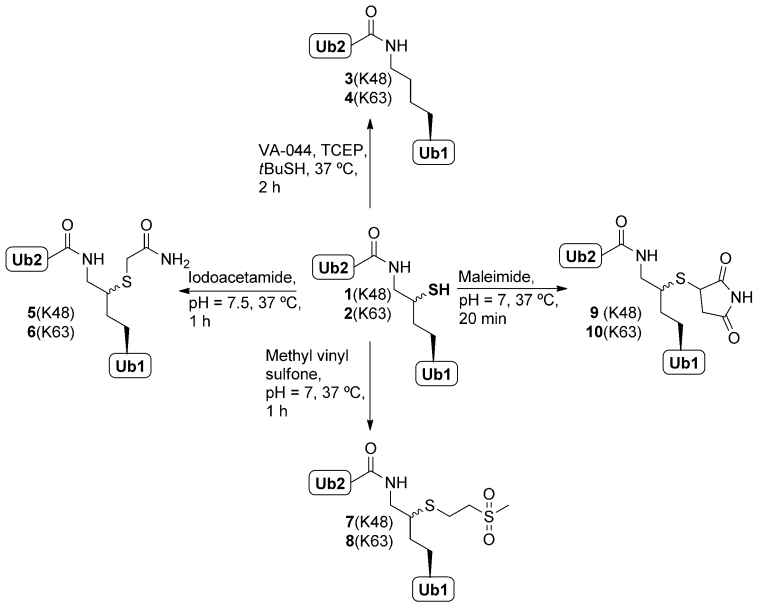


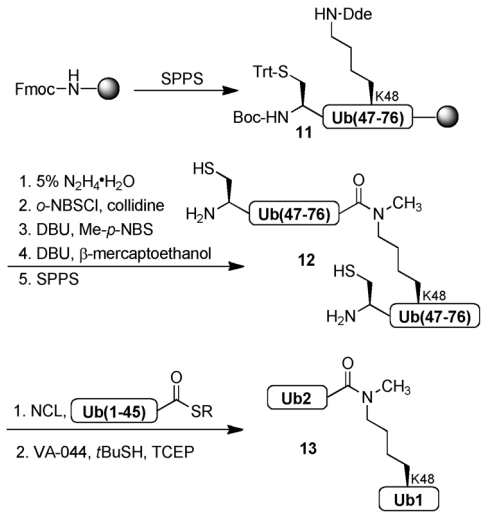
Figure 2. Enzymatic hydrolysis of di-Ub analogues (1–10) with IsoT and USP2: A) Lys48 linked di-Ub; B) Lys63 linked di-Ub. The percent of hydrolysis was determined from the HPLC data for each reaction using identical reaction conditions (30 min). Error bars correspond to the standard deviation of three measurements.



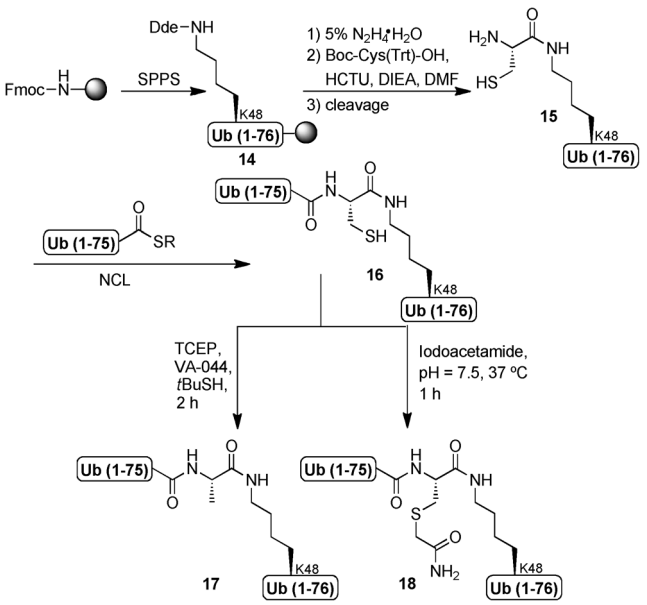
Scheme 1. Synthesis of native (**3**, **4**) and thiol alkylated (**1**, **2**, **5**–**10**) di-Ub chains. TCEP = tris(2-carboxyethyl)phosphine hydrochloride.

Next, we focused on studying the effect of *N*-methylation of the isopeptide on the Ub chain conformation, stability to DUBs, and binding to a UBD. We had recently reported that *N*-methylation of the isopeptide bond in ubiquitinated peptides and proteins makes these conjugates stable against DUBs.[8] However, this had not been examined with Ub chains. The strategy for the synthesis of *N*-methylated di-Ub (Scheme 2) relies on two-fragments that will be linked via native chemical ligation (NCL) followed by desulfurization.[14] Accordingly, we succeeded in synthesizing building block **12**, containing the C-terminal part of the distal and proximal Ub linked via an *N*-methylated isopeptide bond and each presenting N-terminal Cys for NCL with Ub(1-45)-thioester.

After cleavage and purification by HPLC, this peptide was ligated with the complementary part of Ub fragment, Ub(1-45)-thioester. The subsequent desulfurization of the two Cys residues to their parent Ala gave the desired product **13**, in 24% yield (for the two steps). Notably, this strategy has



Scheme 2. Synthesis of *N*-methylated K48-linked di-Ub chain. DBU = 1,8-diazabicyclo[5.4.0]undec-7-ene, *o*-NBSCl = 2-nitrobenzenesulfonyl chloride, Me-*p*-NBS = methyl 4-nitrobenzenesulfonate.



Scheme 3. Synthesis of the di-Ub chain with Gly76 modifications.

never been applied to the synthesis of Ub chains and can also be used in future studies related to the synthesis and studies of other Ub chains with the advantage of relying only on peptide synthesis and NCL, without the need for the special amino acid δ-mercaptolysine.

Interestingly, when examining analogue **13** with both IsoT and USP2, although it exhibited higher stability in the presence of IsoT or USP2 than the unmodified chain, **3**, (see SI), DUBs were still able to cleave it. These results are in sharp contrast to the *N*-methylated H2B-Ub, which exhibited remarkable stability even in cell lysate, and to the known behavior of *N*-methylation of backbone amide bond in peptides.[15] This provides another example of the complexity of recognition and processing by DUBs of their substrates. It also indicates that *N*-methylation of the isopeptide bond in Ub bioconjugates cannot be used as a general strategy for resisting DUB activities as it depends on both the type of substrate and its specific DUB.

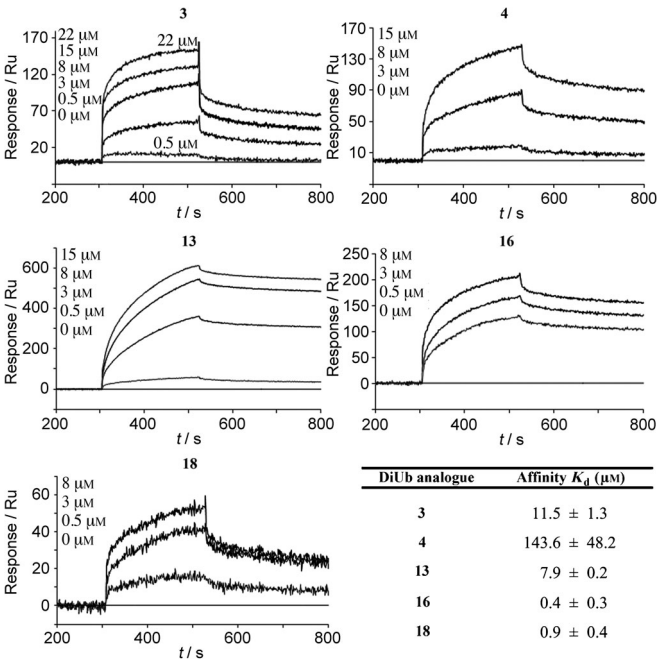
To complete the scanning of the isopeptide bond's vicinity and to study the effect of the modification at the C-terminus of the distal Ub, we modified Gly76 with other amino acids such as Ala, Cys, and Cys-(acetamide) (Scheme 3). The syntheses of these analogues were carried out using SPPS and NCL.[16,17] Here, Fmoc-Lys-(Dde)-OH was used as the orthogonally protected Lys to enable selective removal of the Lys protecting group for the formation of the isopeptide bond. After the synthesis of Ub with the Lys-(Dde) using Fmoc-SPPS, the ε-amine of the Lys residue was unmasked using hydrazine hydrate. Subsequently, the free amine was coupled with Boc-Cys(Trt)-OH to enable ligation with the distal Ub(1-75)-thioester. After peptide cleavage and purification, this fragment was ligated with the Ub(1-75)-thioester to afford di-Ub **16** in 40% yield. This di-Ub was either desulfurized to afford di-Ub **17** with the G76A mutation, or alkylated with iodoacetamide to furnish di-Ub **18**.

To determine the effect of these modifications on DUB activity, we tested the di-Ub analogues with IsoT and USP2 as described above. While di-Ub **16** and **18** were completely stable, di-Ub **17** exhibited some degree of cleavage (ca. 20%) after 30 min incubation. Previous studies by Hodgins et al.[18] showed that the Ub Gly76Ala mutation results in irreversible conjugation to proteins or to itself when forming Ub chains, causing a change in the Ub conjugate equilibrium. On the other hand, Muir's group has reported that H2B-Ub(G76A) was cleaved by UCH-L3 in a similar manner to the native substrate.[17] Our results show that K48-linked di-Ub chain, with the G76A mutation, also is not completely stable with IsoT (20% vs 49% after 30 min) and USP2 (13% vs 66% after 30 min), however it was cleaved to a lesser extent compared to the unmodified chains (Supporting Information, Figures S38, S39).

It is important to note that the two DUBs that we have examined so far are not linkage specific. It is likely that the recognition of the Ub surfaces is the driving force for DUB recognition and cleavage of the chains. Next, we questioned whether the linkage-specific DUB, OTUB1,[19] which is known to cleave only K48-linked chains, would recognize some of the modified analogues. For this, we examined three representative analogues, **5**, **13**, and **16** that were compared to enzymatically-assembled wild-type K48-linked di-Ub.[20] Interestingly, in all of the modified cases OTUB1 failed to cleave any of these chains (Supporting Information, Figure S42), which indicates the importance of the linkage and its nearby residues in the recognition and cleavage of these chains.

To examine the effect of modifying the isopeptide bond on the recognition of ubiquitin-interacting proteins, we decided to measure the binding affinity of representative analogues, **13**, **16**, and **18** as well as unmodified K48- and K63-linked di-Ub **3** and **4**. Specifically we were interested in studying the di-Ub analogues' affinities for Ubiquitin-Associated 2 domain (UBA2) of the human homologue of protein Rad23 (hHR23a), which binds preferentially to K48-linked chains

over K63-linked chains.[21] For this, we synthesized UBA2 that is biotinylated at its N-terminus in order to immobilize it onto a NeutrAvidin coated-chip. Binding affinity was measured by surface plasmon resonance (SPR). First, the biotin-UBA2 was bound to the NeutrAvidin coated-chip, followed by flowing each of the stable di-Ub analogues (**13**, **16**, and **18**) as well as the native analogues (**3** and **4**) as controls at various concentrations (0.5–22 μM), (Figure 3). The binding affinities



DiUb analogue	Affinity K_d (μM)
3	11.5 ± 1.3
4	143.6 ± 48.2
13	7.9 ± 0.2
16	0.4 ± 0.3
18	0.9 ± 0.4

Figure 3. Surface plasmon resonance (SPR) study of the native diUb-K48 (**3**), native diUb-K63 (**4**), analogues (**13**, **16**, and **18**), and their calculated K_d values.

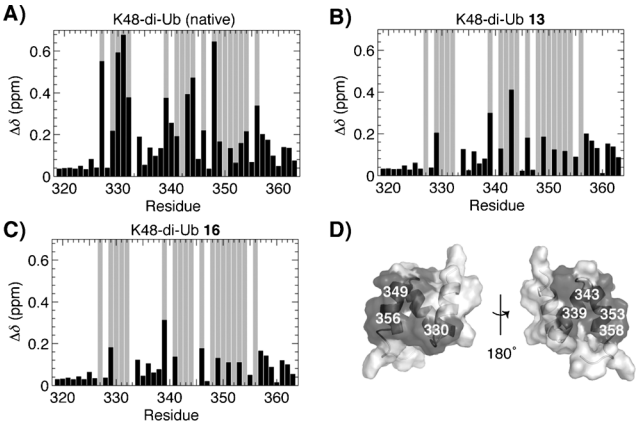


Figure 4. NMR mapping of the binding interactions of K48-linked di-Ub analogues with the UBA2 domain of hHR23a. A)–C) Amide chemical shift perturbations (CSPs) in backbone amides of the UBA2 domain at the titration endpoints with enzymatically-assembled K48-di-Ub (native) (A), K48-di-Ub **13** (B), and K48-di-Ub **16** (C). CSPs were calculated as $\Delta\delta = (\Delta\delta_H^2 + \Delta\delta_N^2/25)^{1/2}$, where $\Delta\delta_H$ and $\Delta\delta_N$ are the changes in the chemical shifts of ^{1}H and ^{15}N, respectively. Those residues that experienced significant attenuation during the course of the titration are marked by gray bars. A number of resonances remained attenuated at the titration endpoint (residues missing black bars in parts B)–C) as a consequence of not having attained full saturation with ligand, in contrast to part (A). D) Residues that experienced CSP > 0.2 ppm and/or strong signal attenuation are shaded dark gray on the surface of the UBA2 domain. The UBA2 domain coordinates are from the UBA2:K48-di-Ub complex (PDB ID: 1ZO6): the front-side of UBA2 (left) contacts the distal Ub (including the C-terminal linker) in K48-linked di-Ub, whereas the back-side of UBA2 (right) interacts with the proximal Ub.

of the native analogues matched very well with the reported values that were determined by NMR titrations.[21a] Furthermore, the N-methylated di-Ub **13** exhibited similar binding affinity, which indicated that the N-methylation had not interfered with the binding of UBA2. Surprisingly, the stable analogues **16** and **18** exhibited 10-fold higher affinities when compared to the native analogue. It is not clear yet why analogues **16** and **18** exhibited higher affinities towards UBA2. One possibility might be that restricting the conformation of the isopeptide-surrounding area by modifying Gly76 could induce favorable binding compared to the unmodified isopeptide.

To shed further light on the binding of analogues **13** and **16** to UBA2, we used NMR spectroscopy to examine and compare the residue-specific patterns of interactions of these analogues and enzymatically assembled wild-type K48-linked di-Ub[20] with ^{15}N-labelled UBA2. Changes in the backbone amide chemical shifts of UBA2 upon titration with di-Ub were monitored by ^{1}H–^{15}N SOFAST-HMQC experiments, quantitated as chemical shift perturbations (CSPs), and mapped onto the protein surface of UBA2 (Figure 4). With all three di-Ub analogues, a significant number of signal attenuations were observed in UBA2 during the course of the titration experiment, indicating slow exchange and thus tight binding. The overall signal attenuation profiles and signal trajectories for individual residues were similar across all three titrations, suggesting that UBA2 interacts with each di-Ub analogue in a similar manner, despite the fact that ligand saturation was not reached with analogues **13** and **16** by the titration endpoint. The similarity of NMR signal trajectories indicates that UBA2 makes similar physical and chemical contacts with these di-Ub analogues as with the wild-type K48-linked di-Ub, thus suggesting identical binding modes (Supporting Information, Figure S45). Importantly, the UBA2 residues that exhibited significant CSPs or signal attenuations map to the same UBA2 surfaces that were previously identified to interact specifically with K48-linked di-Ub.[21] In fact, UBA2 was shown to bind K48-linked di-Ub in a sandwich-like manner, where each side of UBA2 forms a specific interface with the corresponding Ub unit and the isopeptide linker (Figure 4D). Thus, our results suggest that despite the isopeptide bond modifications, both K48-linked di-Ub analogues (**13** and **16**) bind to UBA2 specifically and in a similar binding mode as native K48-linked di-Ub. As UBA2 is known to bind across the Ub-Ub linker, these findings suggest that UBA2 is able to recognize the K48 linkage in each of the di-Ub analogues.

In summary, we have presented three different approaches for the preparation of 14 different analogues of Ub chains modified in the vicinity of the isopeptide bond.

Examination of these analogues revealed differential behavior with DUBs and UBDs. In particular, DUBs that are linkage-specific exhibited greater sensitivity to these modifications while DUBs that are active against various chains were tolerant to many of these modifications. Notably, modifications of Gly76 generated the most stable analogues with increased binding affinity towards UBA2. Our results lay the groundwork for creating a new generation of probes based on Ub chains for various studies and should enable further understanding on the mode of recognition and processing of their native substrates.

Received: July 14, 2013
Published online: September 4, 2013

Keywords: deubiquitinases · peptide ligation · protein synthesis · ubiquitin chains · ubiquitination

[1] a) M. H. Glickman, A. Ciechanover, *Physiol. Rev.* **2002**, *82*, 373; b) Z. J. Chen, L. J. Sun, *Mol. Cell* **2009**, *33*, 275.
[2] M. J. Eddins, R. Varadan, D. Fushman, C. M. Pickart, C. Wolberger, *J. Mol. Biol.* **2007**, *367*, 204.
[3] a) F. E. Reyes-Turcu, K. D. Wilkinson, *Chem. Rev.* **2009**, *109*, 1495; b) K. R. Love, A. Catic, C. Schlieker, H. L. Ploegh, *Nat. Chem. Biol.* **2007**, *3*, 697.
[4] D. Komander, M. J. Clague, S. Urbe, *Nat. Rev. Mol. Cell Biol.* **2009**, *10*, 550.
[5] The only known structure of DUB with its substrate is the AMSH enzyme complexed with K63-linked di-Ub; for more details, see: Y. Sato, A. Yoshikawa, A. Yamagata, H. Mimura, M. Yamashita, K. Ookata, O. Nureki, K. Iwai, M. Komada, S. Fukai, *Nature* **2008**, *455*, 358.
[6] Y. Ye, G. Blaser, M. H. Horrocks, M. J. Ruedas-Rama, S. Ibrahim, A. A. Zhukov, A. Orte, D. Klenerman, S. E. Jackson, D. Komander, *Nature* **2012**, *492*, 266.
[7] a) L. Spasser, A. Brik, *Angew. Chem.* **2012**, *124*, 6946; *Angew. Chem. Int. Ed.* **2012**, *51*, 6840; b) E. R. Strieter, D. A. Korasick, *ACS Chem. Biol.* **2012**, *7*, 52; c) K. S. Kumar, L. Spasser, L. A. Erlich, S. N. Bavikar, A. Brik, *Angew. Chem.* **2010**, *122*, 9312; *Angew. Chem. Int. Ed.* **2010**, *49*, 9126; d) K. S. Kumar, S. N. Bavikar, L. Spasser, T. Moyal, S. Ohayon, A. Brik, *Angew. Chem.* **2011**, *123*, 6261; *Angew. Chem. Int. Ed. A* **2011**, *50*, 6137; e) F. El Oualid, R. Merkx, R. Ekkebus, D. S. Hameed, J. J. Smit, A. de Jong, H. Hilkmann, T. K. Sixma, H. Ovaa, *Angew. Chem.* **2010**, *122*, 10347; *Angew. Chem. Int. Ed.* **2010**, *49*, 10149; f) C. Castaneda, J. Liu, A. Chaturvedi, U. Nowicka, T. A. Cropp, D. Fushman, *J. Am. Chem. Soc.* **2011**, *133*, 17855; g) S. Virdee, Y. Ye, D. P. Nguyen, D. Komander, J. W. Chin, *Nat. Chem. Biol.* **2010**, *6*, 750; h) S. N. Bavikar, L. Spasser, M. Haj-Yahya, S. V. Karthikeyan, T. Moyal, K. S. Kumar, A. Brik, *Angew. Chem.* **2012**, *124*, 782; *Angew. Chem. Int. Ed.* **2012**, *51*, 758.
[8] M. Haj-Yahya, N. Eltarteer, S. Ohayon, E. Shema, E. Kotler, M. Oren, A. Brik, *Angew. Chem.* **2012**, *124*, 11703; *Angew. Chem. Int. Ed.* **2012**, *51*, 11535.
[9] N. Minsky, E. Shema, Y. Field, M. Schuster, E. Segal, M. Oren, *Nat. Cell Biol.* **2008**, *10*, 483.
[10] a) S. Eger, M. Scheffner, A. Marx, M. Rubini, *J. Am. Chem. Soc.* **2010**, *132*, 16337; b) N. D. Weikart, S. Sommer, H. D. Mootz, *Chem. Commun.* **2012**, *48*, 296.
[11] K. S. Ajish Kumar, M. Haj-Yahya, D. Olschewski, H. A. Lashuel, A. Brik, *Angew. Chem.* **2009**, *121*, 8234; *Angew. Chem. Int. Ed.* **2009**, *48*, 8090.
[12] K. D. Wilkinson, V. L. Tashayev, L. B. O'Connor, C. N. Larsen, E. Kasperek, C. M. Pickart, *Biochemistry* **1995**, *34*, 14535.
[13] M. Renatus, S. G. Parrado, A. D'Arcy, U. Eidhoff, B. Gerhartz, U. Hassiepen, B. Pierrat, R. Riedl, D. Vinzenz, S. Worpenberg, M. Kroemer, *Structure* **2006**, *14*, 1293.
[14] P. E. Dawson, *Isr. J. Chem.* **2011**, *51*, 862.
[15] J. Chatterjee, C. Gilon, A. Hoffman, H. Kessler, *Acc. Chem. Res.* **2008**, *41*, 1331.
[16] K. S. Kumar, L. Spasser, S. Ohayon, L. A. Erlich, A. Brik, *Bioconjugate Chem.* **2011**, *22*, 137.
[17] A similar approach was previously reported for the synthesis of H2B-Ub; for more details, see: R. K. McGinty, M. Kohn, C. Chatterjee, K. P. Chiang, M. R. Pratt, T. W. Muir, *ACS Chem. Biol.* **2009**, *4*, 958.
[18] R. R. Hodgins, K. S. Ellison, M. J. Ellison, *J. Biol. Chem.* **1992**, *267*, 8807.
[19] a) T. Wang, L. Yin, E. M. Cooper, M. Y. Lai, S. Dickey, C. M. Pickart, D. Fushman, K. D. Wilkinson, R. E. Cohen, C. Wolberger, *J. Mol. Biol.* **2009**, *386*, 1011; b) M. J. Edelmann, A. Iphofer, M. Akutsu, M. Altun, K. di Gleria, H. B. Kramer, E. Fiebiger, S. Dhe-Paganon, B. M. Kessler, *Biochem. J.* **2009**, *418*, 379.
[20] C. A. Castañeda, J. Liu, T. R. Kashyap, R. K. Singh, D. Fushman, T. A. Cropp, *Chem. Commun.* **2011**, *47*, 2026.
[21] a) R. Varadan, M. Assfalg, S. Raasi, C. Pickart, D. Fushman, *Mol. Cell* **2005**, *18*, 687; b) S. Raasi, R. Varadan, D. Fushman, C. M. Pickart, *Nat. Struct. Mol. Biol.* **2005**, *12*, 708.

