Stable Isopeptide Bond

DOI: 10.1002/anie.201205771

N-Methylation of Isopeptide Bond as a Strategy to Resist Deubiquitinases**

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The reversibility of the amide bond in a cellular environment, achieved by the action of proteases, has for many years engaged chemists to develop strategies to stabilize this bond within biomolecules for various goals. The introduction of an amide bond isostere into peptide sequences is a particularly useful approach for enhancing the stability of therapeutic peptides for prolonged activity.[1] In this regard, nature has evolved several ways to stabilize highly rich peptidic scaffolds, for example, backbone N-methylation, to carry out their function while resisting proteases.[2] Not surprisingly chemists have been using N-methylation as a tool to change the pharmacological properties of peptides by enhancing their stability to enzymatic degradation and their cellular permeability.[2] As a result, several synthetic strategies, in solution and on solid phase have already been developed to achieve backbone N-methylation.[3]

In the context described above the hydrolysis of the isopeptide bond by the action of proteases is not an exception. Such a bond is widely used in nature to link, for example, the lysine side-chain with either glutamic/aspartic acids or the C-terminus of various proteins.[4] In the C-terminus case, this linkage is introduced by a well-coordinated enzymatic process that involves attachment of the Lys side chain to the C-terminus of ubiquitin (Ub) protein (i.e. ubiquitination) or of ubiquitin-like modifiers (e.g. SUMO) to initiate and regulate a multitude of signaling pathways that are highly important in numerous cellular processes (e.g. protein degradation).[5] In the Ub system, there are as many as 100 deubiquitinases (DUBs) encoded in the human genome and cleave the isopeptide bond by mechanisms similar to the ones employed by proteases that hydrolyze the backbone amide bond (e.g. Cys proteases).[6] Not surprisingly, DUBs are involved in a variety of regulatory processes and are linked to numerous diseases (e.g. cancer).[6] Therefore, studying the activity, selectivity, and inhibition of DUBs at the molecular level, and identifying their protein partners are crucial steps towards understanding their roles in health and disease and ultimately targeting them for drug development.

Towards these goals, several groups have pioneered the development of a stable isopeptide bond of Ub conjugates using advanced chemical methods.[7] These efforts include, for example, the use of stable di-Ub analogues that were cross-linked through dichloroacetone,[8] the 1,4-triazole bond,[9] the oxime bond.[10,11] Motivated by these advances and the potential use of such strategies for understanding and targeting DUBs, we reasoned that expanding the toolbox to allow the introduction of a stable isopeptide bond using a straightforward method with minimal perturbation of the isopeptide bond and its nearby sequence would be highly beneficial to the field. Herein we report on the development of a highly efficient method for the synthesis of stable ubiquitinated peptides and proteins based on N-methylation of the isopeptide bond. We then describe the application of this strategy to shed light on the behavior of several Ub conjugates with different DUBs in vitro and within a cellular environment.

To achieve the goals of our strategy we had first to develop a straightforward synthesis of N-methylated isopeptide bond and its incorporation into different ubiquitin bioconjugates. For this, we chose a model peptide GLS**K**AKE derived from α-synuclein wherein the orthogonally protected Lys was used to introduce the native and stable isopeptide in a site-specific manner. We have recently reported a novel method for the expeditious synthesis of ubiquitinated peptides with native isopeptide bond,[12] relying only on solid-phase peptide synthesis (SPPS) and Cys based native chemical ligation (NCL). In this method, Fmoc-Lys-(ivDde)-OH was used to enable selective unmasking of the Lys residue for the following formation of the isopeptide bond with Gly76 and the elongation of the remaining Ub peptide. To prepare the stable isopeptide bond we modified this strategy, where we first synthesized the GLS**K**AKE peptide **1** using Fmoc-SPPS with the Lys-(ivDde) followed by unmasking the ε-amine of the Lys residue. Subsequently, the free amine was coupled with *o*-nitrobenzenesulfonyl chloride (*o*-NBS) to facilitate N-methylation by selective deprotonation of the sulfonamide with DBU and alkylation with methyl *p*-nitrobenzenesulfonate (Scheme 1).[3d] Selective removal of the *o*-NBS was achieved by using mercaptoethanol and DBU to generate the secondary amine ready for peptide elongation of the Ub(46–76) to afford fragment **2**. After cleavage and purification, this peptide was ligated with Ub fragment **3**, which after a desulfurization step gave the desired product, **5**, in 40% yield (for the two steps). One limitation of such a strategy is

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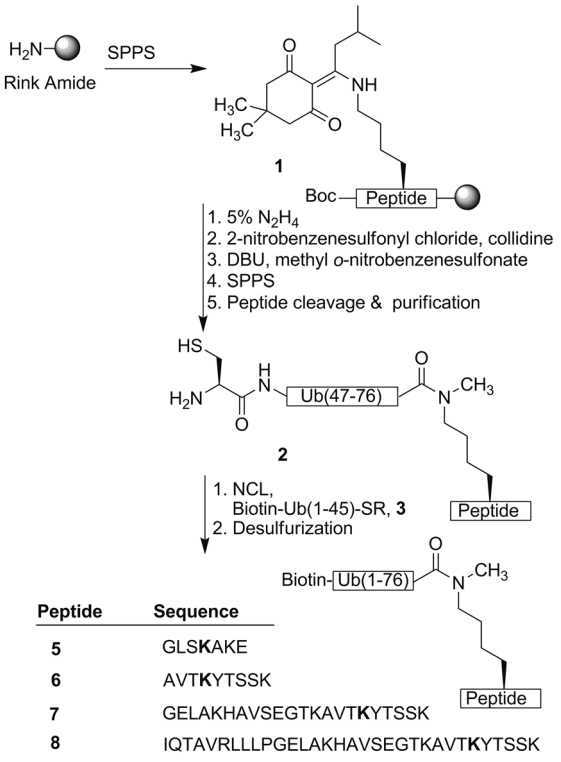
[**] This work was supported by the Israel Science foundation (A.B.), the Israel Ministry of Science and Technology (M.H.-Y.), and the European Research Council grant 293438 (RUBICAN) (M.O.).

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201205771.



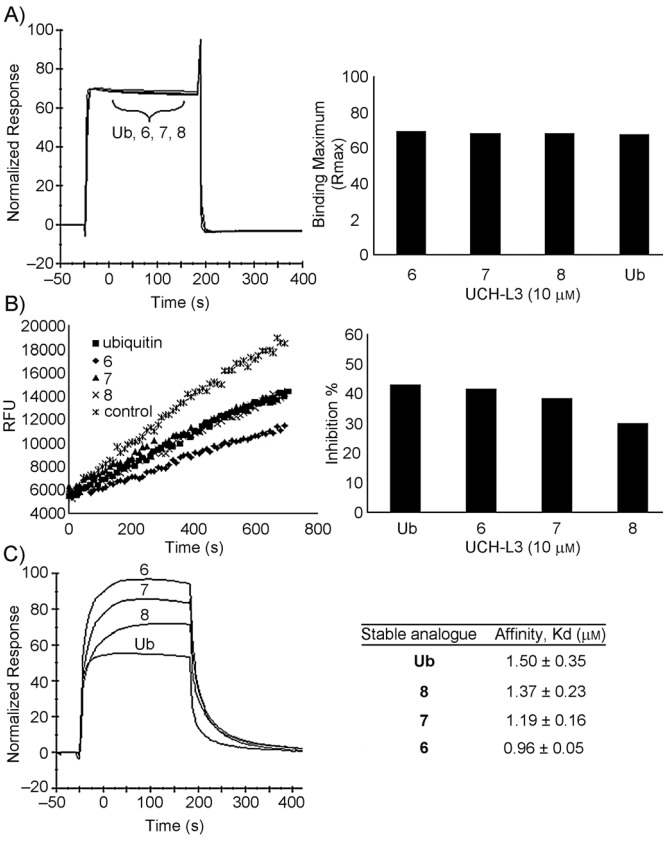
Peptide	Sequence
5	GLS**K**AKE
6	AVT**K**YTSSK
7	GELAKHAVSEGTKAVT**K**YTSSK
8	IQTAVRLLLPGELAKHAVSEGTKAVT**K**YTSSK

Scheme 1. The synthesis of ubiquitinated peptides with N-methylated isopeptide bond, (R = -CH_2-CH_2-COOMe). The peptides that are used in this study derived from the C-terminal region of H2B.

the presence of Cys residues in the peptide fragments that are obtained by expression, which cannot be protected in the desulfurization step.

To check the stability of ubiquitinated peptide **5** bearing the N-methylated isopeptide bond, we tested it with Ub C-terminal hydrolase (UCH-L3), a cysteine protease that cleaves small adducts from the C-terminal of Ub.[13] We have also compared it to ubiquitinated peptide **4** (see Supporting Information) with the same sequence and bearing the native isopeptide bond, prepared as previously described.[12] When both ubiquitinated peptides were folded and incubated under similar conditions with UCH-L3, we found that over 50 % of substrate **4** was cleaved within 30 min, while the N-methylated ubiquitinated peptide **5** was completely stable even after 24 h (Figure S8, Supporting Information). These results confirmed our design and the stability of the N-methylated isopeptide bond against the action of DUBs.

Using the above strategy, three other stable ubiquitinated peptides **6**–**8** having 9, 22, 32-mer peptides, respectively, derived from the C-terminal of histone H2B were also assembled (Scheme 1). In these analogues, we have also attached a biotin molecule to the N-terminus of Ub for immobilization onto streptavidin coated-chips to enable binding affinity measurements by surface plasmon resonance (SPR). We next focused on studying their affinities to UCH-L3. We have previously reported that the labile version of these peptides are cleaved by this DUB with preferences to peptides with lengths of less than 20 amino acids.[12] We used SPR to examine their binding affinity to UCH-L3, where the enzyme was used at various concentrations (1–35 μM) as the flowing solution over the streptavidin-coated biacore sensor chip bound to **6**–**8** and biotin-Ub as a control. This study revealed that the three stable ubiquitinated peptides and Ub have very similar binding affinities to the UCH-L3 (Figure 1 A), despite the lower activity observed previously with



Stable analogue	Affinity, Kd (μM)
Ub	1.50 ± 0.35
8	1.37 ± 0.23
7	1.19 ± 0.16
6	0.96 ± 0.05

Figure 1. SPR and inhibition studies of substrates **6**–**8** and Ub with UCH-L3 (A,B) and USP7 (C) showing a representative concentration measurement of each substrate in the SPR studies and their calculated Kd values (see Supporting Information).

the longer peptide having the native isopeptide bond. The similar binding affinities of these substrates and Ub with UCH-L3 may indicate that most of the interaction is due to Ub itself while very little contribution is coming from the peptidic component. This is also consistent with the high promiscuity of UCH-L3 towards short peptides linked to Ub. Hence, the peptide's ability to pass the crossover loop of UCH-L3 and reach the catalytic Cys could determine its ability to be cleaved rapidly.[14]

When these ubiquitinated peptides were tested in a FRET-based assay that we have recently developed to report on UCH-L3 activity and inhibition,[15] they exhibited similar inhibition (30–40 %) at 10 μM, further supporting the SPR studies (Figure 1 B). Finally, when the Ub sequence in conjugate **6** was truncated from the N-terminal side, leaving Ub(50–76) or Ub(55–76), a complete loss of inhibition was observed (see Supporting Information).

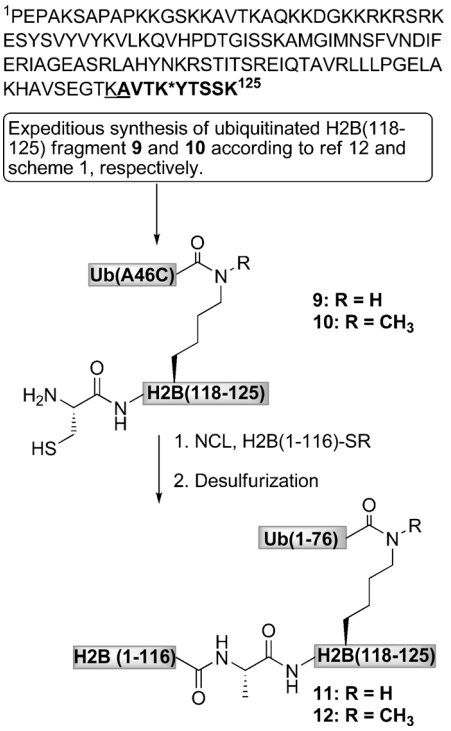
In a similar study to that performed with the UCH-L3, we employed SPR to examine the binding of these substrates to the Ub-specific protease 7 (USP7) DUB, at various concentrations (0.045–0.45 μM). This cysteine isopeptidase removes Ub from several substrates, such as p53[16] and H2B.[17] Regulation of USP7 expression and activity is necessary for maintaining an appropriate cell survival, proliferation, and apoptosis and hence is of high relevance to cancer studies. Previous studies with the stable ubiquitinated peptides based on oxime linkage showed that the affinity of USP7 to ubiquitinated peptides with different sequences but with similar lengths could vary significantly.[10] On the other hand, our study with our stable ubiquitinated peptides, **6**–**8**, derived from H2B, shows that their affinities to USP7 are comparable (Figure 1 C). Our results indicate that both DUBs investigated in our study, recognize mainly the Ub fold rather than the linked peptides.

Having established the utility of the N-methylated isopeptide bond in peptides, we then aimed to extend this method to ubiquitinated proteins and generate stable analogues that resist DUBs for various applications. We chose the human histone H2B protein since its monoubiquitination by Lys120 (H2Bub1) is a highly important modification, affecting chromatin dynamics, gene expression and DNA repair.[18] Removal of Ub from H2Bub1 was proposed to occur by several DUBs, including USP22 and USP44, which have been linked to disease development and in particular to cancer.[19] Hence generating stable analogues of ubiquitinated H2B can have important implications for understanding DUBs that act on H2Bub1, and could be used for identifying H2Bub1-interacting proteins and protein complexes.

The synthesis of labile and stable H2Bub1 is described in Scheme 2.[20] A key aspect of this synthesis features the preparation of fragments **9** and **10** for the synthesis of the labile ubiquitinated H2B **11** and the stable analogue **12**, respectively. In the present study, we completed the preparation of the H2Bub1 by ligating **9**[12] with H2B(1–116)-thioester[20] followed by desulfurization to generate **11** in 20 % yield (for four steps). The stable analogue **12** was prepared using a similar strategy and by applying our N-methylation approach of the isopeptide bond to prepare the key fragment **10**. Both analogues were prepared in milligram quantities allowing further characterization and studies (Figure 2). An additional two analogues of **11** and **12** each bearing a His-tag at the N-terminal of the H2B were prepared for detection with anti-His antibodies.

To examine the behavior of the semi-synthetic stable **12** and labile **11** H2Bub1 within a cellular context we transfected these proteins, as well as unmodified H2B, into human HeLa cells. Cells were harvested after either 6 h or 24 h of transfection, and cell extracts were analyzed by Western blot. As seen in Figure 3, while the labile H2Bub1 was still present after 6 h of transfection, it became completely undetectable after 24 h, presumably owing to its deubiquitination; this is supported by the appearance of a band migrating at the position of non-ubiquitinated H2B (24 h, lane 2). In contrast, the stable H2Bub1 persisted also after 24 h, confirming its resistance to ubiquitination in a cellular context; this is supported by the fact that there was no detectable conversion to non-ubiquitinated H2B (lane 3).

Interestingly, the N-methylated version of H2Bub1 was inefficiently recognized by the monoclonal H2Bub1 antibody,



Scheme 2. Synthetic strategy for the stable and labile H2Bub1. The sequence of H2B is shown, highlighting the ligation site.

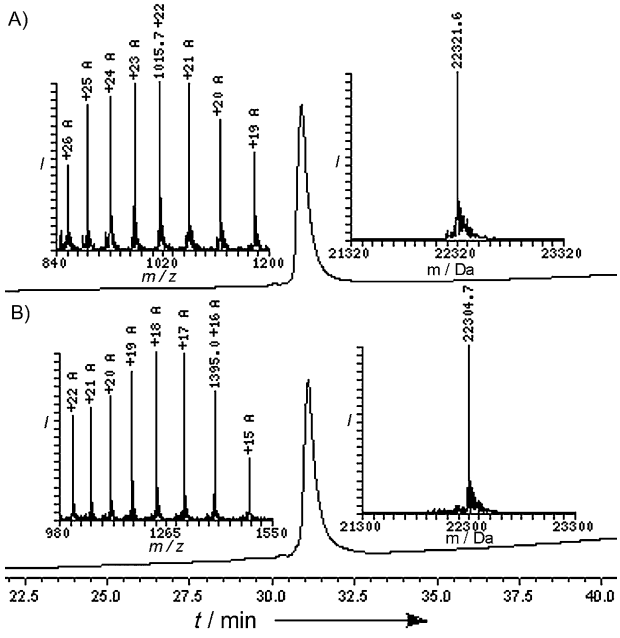


Figure 2. Characterization of semi-synthetic stable and labile H2Bub1. A) Analytical HPLC of the purified stable H2Bub1, **12**, containing the N-methylated isopeptide bond; observed mass of 22321.3 Da (calcd 22317.8 Da). B) Analytical HPLC of the purified labile H2Bub1, **11**; observed mass of 22304.7 Da (calcd 22302.8 Da).

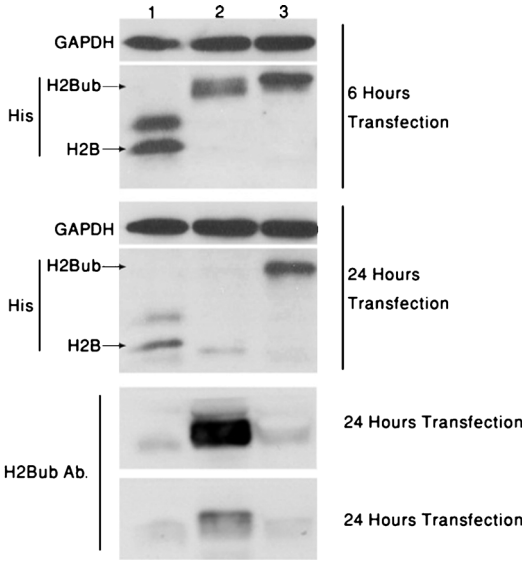


Figure 3. Behavior of the semi-synthetic stable, **12**, and labile, **11**, H2Bub1 in a cellular context. HeLa cells were transfected with unmodified H2B protein (1), labile H2Bub1 (2), or stable H2Bub1 (3). After 6 or 24 h of transfection cell lysates were subjected to Western blot analysis with anti-His antibodies (upper panels) or anti-H2Bub1 monoclonal antibody (lower panels). Blotting with antibodies against endogenous GAPDH served as a loading to confirm equal loading of all extracts.

despite the fact that its secondary structure, as was measured by circular dichroism (CD), was indistinguishable from the labile H2Bub1 (see Supporting Information). This antibody was generated against a 10-mer peptide that is branched by an isopeptide bond and linked to a LRGG peptide derived from the C-terminal of Ub.[21] Thus, our results show clearly that even small modifications of the isopeptide bond interfere strongly with the binding of this antibody to H2Bub1. These results shed light on the nature of the interactions of this antibody with H2Bub1, which appear to involve mainly the isopeptide bond and its surrounding sequence.[22] On the other hand, this observation indicates that the use of the various stable analogues should be approached carefully, as protein partners that interact mainly with the isopeptide bond could be affected once this bond is modified. In our case, although the methyl group can be viewed as a relatively small change to the isopeptide bond, such a group could also affect *cis–trans* isomerization of the isopeptide bond and further perturb the interactions of DUBs, Ub binding domains and the specific antibodies that recognize mainly the isopeptide region.

In summary, we present herein a highly efficient synthetic method to prepare ubiquitinated peptides and proteins bearing an N-methylated isopeptide bond, which is resistant to the activity of DUBs. Several stable ubiquitinated peptides were prepared and their binding affinities were examined with two known DUBs. Importantly, we have shown that our method is not only amenable to the preparation of stable ubiquitinated peptides but also of stable ubiquitinated proteins, as we demonstrated with the synthesis of stable H2Bub1, which exhibited high stability both in vitro and in a cellular context. This stable H2Bub1 might be highly useful to study the dynamics and various roles of H2Bub1 in mammalian cells since it can be transfected into cells, where it resists hydrolysis by deubiquitinases. Our chemical synthetic strategy opens new opportunities for studying various aspects of DUBs including their role in transcription and other chromatine-associated processes.[23]

Received: July 19, 2012
Revised: August 23, 2012
Published online: October 12, 2012

Keywords: deubiquitination · histone synthesis · N-methylation · stable isopeptide bond · ubiquitination

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