Synthetic tools to study ubiquitin biology

Shahul Hameed, D.A.

Chapter 7

Summary and outlook

Hameed, D.S., Ovaa, H.

The scope of this thesis concerns the synthesis of novel tools based on ubiquitin (Ub) using Fmoc-based solid-phase peptide synthesis (SPPS) to study Ub signalling pathways. The chemical synthesis of large polypeptides and proteins has relied on combining fragments of peptide sequences using chemical ligation techniques [1]. These ligation techniques depend on the sequence of the polypeptide or protein and the ability of the final product to refold post-synthesis. The thiol group found in cysteine residues is frequently used as a chemical ligation handle [2]. For polypeptides and proteins that lack a cysteine residue in their sequence, cysteine can be introduced to replace an alanine residue to allow ligation to another peptide segment, followed by desulphurization to reinstate alanine. Ligation procedures have been refined over the years to improve ligation yield and efficiency. However, the intermediate purifications of peptide fragments make this approach less favourable for generating many polypeptides in a fully automated procedure and in parallel. This can be avoided by synthesizing polypeptides in a conceivably linear fashion. This approach has been helpful in the chemical synthesis of Ub [3]. Since Ub lacks a cysteine residue in its sequence, the most convenient approach is to synthesize it in a linear fashion. Furthermore, the short sequence of 76 amino acids and the excellent refolding capacity of Ub gives the edge to synthesize Ub in a linear fashion with a relatively short duration and high yields. This also helps us in making virtually any mutant Ub with both natural and unnatural amino acids at any position of our choice. SPSS also allows the introduction of fluorescent dyes, crosslinkers and other chemical handles conveniently at will.

In **Chapter 1**, I provide an overview of the various Ub conjugates, assay reagents and probes that have been generated using Fmoc-based SPPS [4]. The Ovaa lab has established an automatic procedure to synthesize full-length Ub by SPPS in parallel. This allows easy N-terminal modification of Ub with dyes such as rhodamine or tetramethylrhodamine (TAMRA), while the C-terminal carboxylate of Ub can also be easily modified to create, for example, activity-based probes (ABPs) or fluorogenic assay substrates. The internal residues of Ub can also be mutated at will (Figure 1). For example, the synthesis of a mutant Ub containing a ligation handle such as a protected δ- or γ-thiolysine residue at the location of a lysine residue has enabled the synthesis of Ub dimers of different linkage topologies. The resulting diubiquitin (diUb) molecules have allowed studies of the substrate specificity of deubiquitinases (DUBs) to better understand their biochemical properties [5]. Moreover, assay reagents containing Ub conjugated to synthetic fluorescently-labelled peptides containing enzyme-specific sequences have also been generated using thiolysine-mediated chemical Ub conjugation which has allowed studies of DUBs kinetics [6]. Modification of the C-terminus of Ub with an active-site cysteine-reactive element has enabled the synthesis of activity-based Ub probes specific for DUBs. The use of SPPS has also facilitated the incorporation of photo-crosslinkers in the sequence of Ub at positions deemed to interact with Ub interacting proteins. Such Ub reagents were generated and incorporated into polyUb chains that were generated enzymatically and the resulting polyUb chains containing photo-crosslinkers were used to trap linkage-specific interacting proteins in the Ub-proteasome system [7]. The procedure to synthesize Ub variants of virtually any sequence has accelerated the study of Ub biology in cells. The same procedure has also been applied to synthesize many Ub-like (Ubl) proteins in a linear fashion using Fmoc-SPPS.

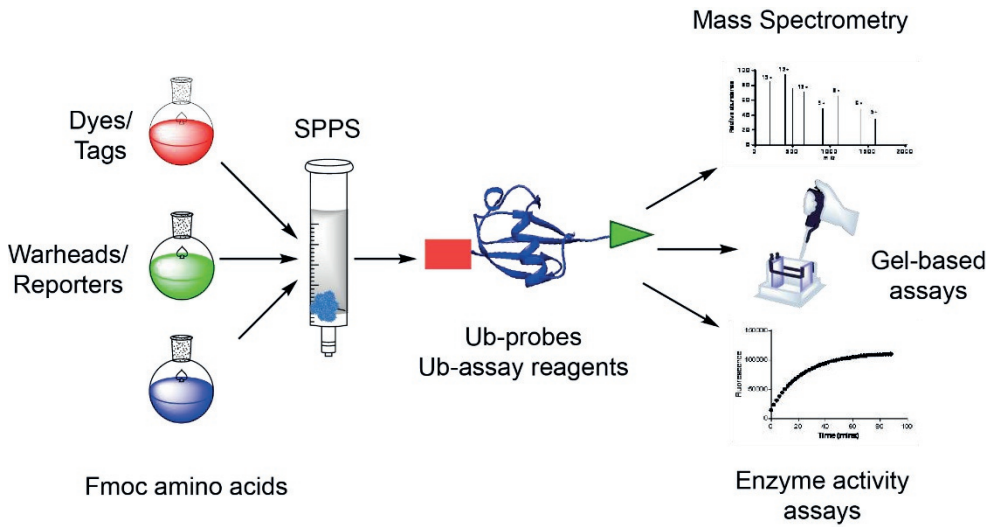

Figure 1: Overview of Fmoc-based SPPS of Ub conjugates. *Synthetic Ub can be generated in a linear fashion using SPPS. Modification of the C-terminus, N-terminus or internal residues of Ub is made possible during or after synthesis. Such reagents are used to study Ub biology.*

Synthesis of ubiquitin dimers linked by an isopeptide bond using enzymatic procedures is often difficult due to two main reasons: first, lack of knowledge of enzymes or enzyme combinations that can generate these specific linkages efficiently *in-vitro*; and secondly, the lack of control of the enzymatic reaction to make conjugates of well-defined lengths without the generation of undesired linkage types as a by-product of these enzymatic reactions [8-10]. In order to address these issues, an alternative procedure was developed to make Ub chains by chemical synthesis of Ub molecules using a native chemical ligation technique. In **Chapter 2**, I explain how the synthesis of a Ub molecule containing a native chemical ligation handle called δ-thiolysine at positions of lysine residues has enabled the synthesis of diubiquitins (diUbs) of specific linkages via chemical ligation [3, 11]. The Ub module whose C-terminal glycine residue participates in the isopeptide bond is called the distal Ub whereas the lysine-contributing Ub moiety is called the proximal Ub. I utilized together with my colleagues the biochemical activation of wild type Ub by Ub activating (E1) enzyme and isolated stable (distal) Ub-thioesters. Using SPPS, the proximal Ub containing δ-thiolysine at specific lysine sites was synthesized and conjugated to the activated Ub-thioesters to make isopeptide linked diubiquitins. After radical-mediated desulfurization, these diUbs were isolated and purified in high yields. These conjugates have allowed studies of the specificity of USP and OTU families of deubiquitinating enzymes [5, 12]. This procedure to make diUb topoisomers has also laid the groundwork for the synthesis of isopeptide-linked dimers of SUMO conjugates [13].

The ability to make Ub dimers of different lysine linkages has opened the possibility to study their structural properties. Different Ub chains are known to be involved in eliciting distinct cellular responses through binding ubiquitin-specific proteins [14]. The recognition of different Ub topoisomers by linkage-specific Ub receptor proteins proceeds through interactions with ubiquitin-binding domains (UBDs) [15]. The mechanism of interaction of UBDs and Ub chains can be studied using various biophysical techniques. Among them, NMR has been instrumental in providing insight into the solution-dynamics and Ub-UBD

interactions. In **Chapter 3**, I describe the generation of Ub dimers where only the distal Ub module is isotopically labelled with ^{15}N atoms. I used these diUbs in NMR experiments to provide information on the binding of the distal Ub moiety with respect to the proximal Ub moiety in different isopeptide-linked diUb molecules. I expressed ^{15}N-isotopically labelled Ub in E. Coli and a ^{15}N-Ub-thioester was then generated enzymatically. Similar to the procedure reported in Chapter 2, I made all seven isopeptide-linked diUbs but this time ^{15}N-distally labelled. Upon measuring the ^{1}H-^{15}N HSQC spectra of these reagents by NMR, I observed that the interactions between the two Ub modules in a diUb molecule are different for each lysine linkage-type. Next to obtaining information on the structural orientation of Ub modules in a diUb molecule, I also studied how the UBD UBXN1 interacts specifically with K6 diUb. UBDs function by interacting with Ub and Ub chains thereby forming the basis for ubiquitin signalling in cells [16]. One of the UBDs is the UBA domain that is found in many ubiquitin-interacting enzymes. These UBA domains are about 45 amino acids long containing three alpha-helices in their structure. A UBA domain found in the UBXN1 protein is specific for K6 diUb chains and UBXN1 is involved in DNA damage repair. Even though this domain was found to interact with K6 polyUb chains, [17] I now show with my colleagues how only the C-terminally extended version of the UBA domain of UBXN1 interacts with K6 diUb chains. Using a fluorescence polarization binding assay and microscale thermophoresis it was found by Gabrielle van Tilburg that this extended domain is needed for K6 specificity. I investigated this interaction further by NMR using a distally labelled K6 diUb molecule in solution. Moreover, studies using bioinformatics revealed that this C-terminal extension was conserved among commonly appearing UBA domains (data from Kay Hoffman, University of Cologne). This opens the possibility that the C-terminal extensions of UBA domains contain sequences that contribute to Ub-linkage selectivity, which remains to be investigated. Thus, I demonstrated the potential of using labelled diUb molecules in studying the interactions of Ub chains with UBDs.

In this study, I conveniently labelled distal Ub elements in Ub chains and studied their interactions with proximal Ub moieties and the specific interaction of distally labelled K6 di Ub with an extended UBD found in UBXN1. Since the proximal Ub that was used in the native chemical ligation technique contains a thiolysine residue, it is difficult to express it in bacteria. However, using an evolved $tRNA^{CUA}$/tRNA synthetase pair, this may potentially be achieved (Figure 2) [18]. An evolved $tRNA^{CUA}$/tRNA synthetase pair can be developed to efficiently incorporate δ- or γ- thiolysine in Ub in a bacterial expression. By using this technique, it is possible to express ^{15}N-labelled proximal Ub which can then be used to make proximally ^{15}N-labelled diUb molecules of all lysine-linkage types. This way, a complete diUb toolkit containing segmentally isotope-labelled ^{15}N-diUb molecules can be synthesized and used in NMR experiments to study the solution-dynamics of diUbs and their mode of interaction with specific UBDs.

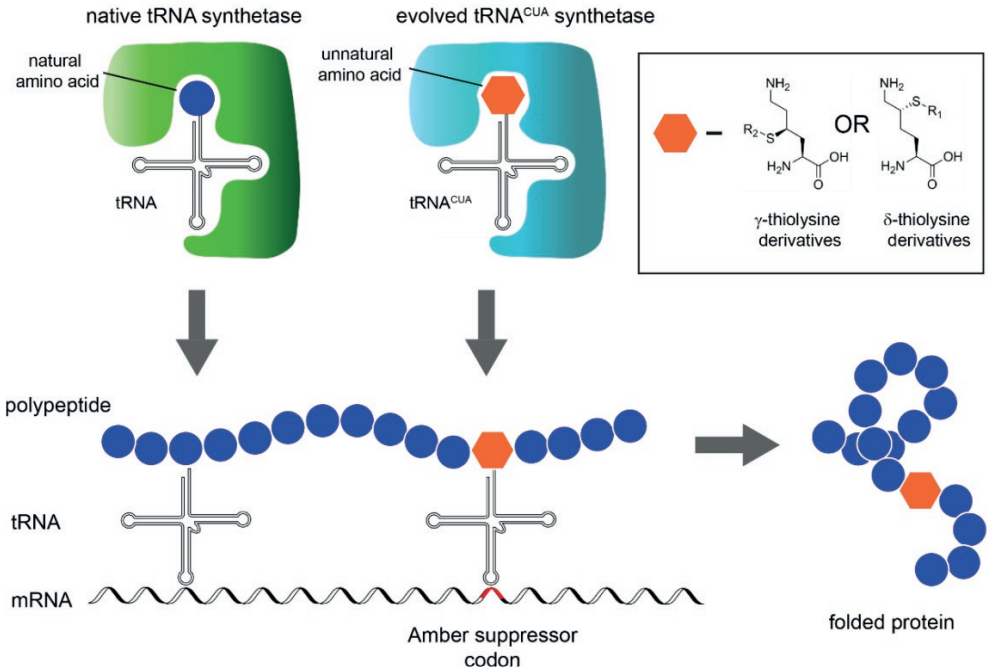

Figure 2: Incorporation of thiolysine derivatives into Ub or another protein by using an evolved tRNA^{CUA}/tRNA synthetase pair. *The tRNA^{CUA} recognizes a stop codon and incorporates the unnatural amino acid in that position, which can be translated into a folded protein and purified from the bacterial lysate. [19]. By evolving an efficient tRNA^{CUA}/tRNA synthetase pair, a γ- or δ-thiolysine (Inset) containing a protected thiol (R_1 and R_2) can be used as the unnatural amino acid and incorporated into full-length Ub in positions of lysine residues. This provides a convenient method to isotopically label the proximal Ub of a diUb molecule for studying them in NMR experiments.*

The feasibility of Ub synthesis using SPPS has opened the possibility to make Ub-based ABPs against enzymes involved in ubiquitination or deubiquitination. Among the different families of DUBs, the cysteine-protease DUBs have been extensively studied so far mainly because of the availability of Ub-probes and assay reagents [20, 21]. A Ub probe mimics upon reaction the covalent enzyme-substrate intermediate formed between the active site cysteine residue of the enzyme and the carbonyl group of the isopeptide bond at the C-terminus of Ub. However, these covalent intermediates are absent in catalysis by the metalloprotease family of DUBs (metalloDUBs) because these enzymes use Zn^{2+} that is chelated by two histidine residues and an aspartate residue in the active site of the enzyme. It is, therefore, a challenge to generate Ub-based ABPs for metalloDUBs [22]. In **Chapter 4**, I report the synthesis of a chelating Ub-based activity probe that targets metalloDUBs [23]. For this purpose, a chemically synthesized zinc-binding group (ZBG) was conjugated with the C-terminus of a truncated synthetic Ub obtained by SPPS. A metalloDUB called Rpn11 was used to validate our Ub-ZBG probe because of its vital role in the 26S proteasome [24, 25]. First, the mode of interaction in an Rpn11/Rpn8-Ub complex was analyzed and molecular docking was used to design the Ub-ZBG probe. Based on this information, Ub (1-74) was synthesized and conjugated with 8-mercaptoquinoline (8MQ), a Zn^{2+} chelating

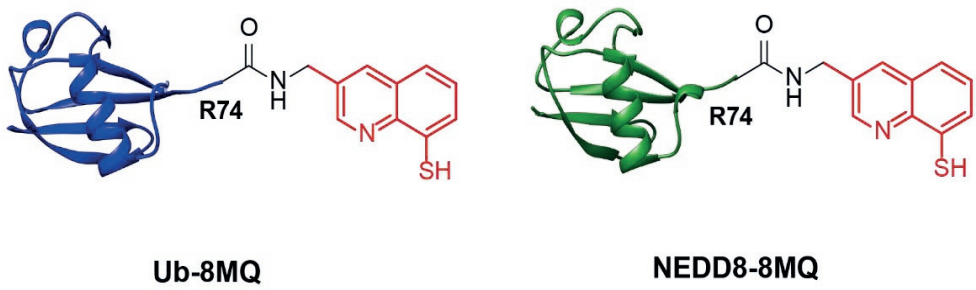

Figure 3: *Comparison of the structure of Ub-8MQ reagent that was validated against Rpn11/Rpn8 complex and a similar reagent made with NEDD8 that can be used to study the metalloprotease called CSN5 of the COP9 signalosome complex in cells.*

moiety, at the C-terminus of Arg74 of Ub [26]. Furthermore, Ub-8MQ was also labelled with a fluorescent dye or a biotin tag on the N-terminus, which facilitated studying their interaction with Rpn11/Rpn8 heterodimer complex and carrying out pull-down experiments of over-expressed metalloDUBs from cell lysate.

This technique can be easily expanded to the synthesis of probes that target metalloproteases specific for ubiquitin-like proteins (Ubl). One of the interesting Ubl-metalloproteases is the CSN5, a metalloprotease in the COP9 signalosome complex that de-NEDDylates Cullin proteins [27]. Since NEDD8 and Ub are similar in size, they can be synthesized in a similar fashion (Figure 3) [28]. This technique can be further extended to other Ub-like proteins like SUMO and ISG15, potentially leading to the discovery of putative metalloproteases.

So far, I described Ub reagents or probes that can be used mainly for *in-vitro* experiments. However, studying the ubiquitin machinery *in-vivo* is essential to understand the ubiquitin signalling pathway in the greatest detail. Such studies require the delivery of synthetic Ub reagents and conjugates into live cells so that the ubiquitination or deubiquitination machinery can be studied in cells and preferably in real-time [29]. One of the widely used biophysical techniques to deliver Ub into live cells is electroporation. For example, this technique was used to deliver Ub-dehydroalanine probes into mammalian cells to study the E1-E2-E3 enzyme cascade [30]. One of the drawbacks of electroporation is the harsh nature of this technique [31]. Therefore, I investigated other less-invasive methods to deliver synthetic Ub into live cells. In **Chapter 5**, I explain how I made a Ub molecule containing a synthetic cell-penetrating delivery vehicle [32]. The TAT peptide derived from the HIV virus has been extensively used to deliver large proteins into live cells by simple co-incubation [33]. However, the uptake efficiency is generally low and therefore TAT-fusion proteins (TFPs) were developed to circumvent this issue. TFPs containing a single copy of the TAT peptide are generally trapped at the endosomal uptake step without further delivery into the cytoplasm unless used at elevated concentrations which generally leads to cell lysis. However, surprisingly a dimeric disulfide-linked TAT peptide was reported to be able to escape from endosomes into the cytoplasm of cells [34] and this proved indeed the case in my hands. Based on this, a cell-permeable Ub conjugate was designed and synthesized using Fmoc-SPPS by making a dimeric disulfide-linked TAT fusion peptide at the C-terminus of a rhodamine-labelled Ub molecule. This delivery reagent efficiently traversed the cell membrane via the endosomal pathway and escaped from the endosomes into the cytoplasm

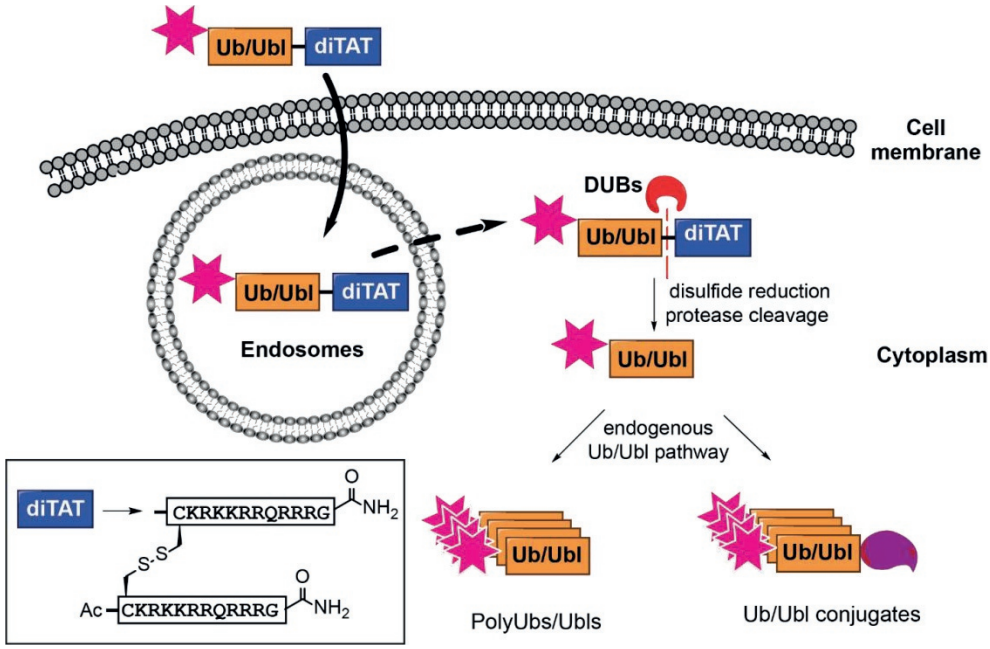

Figure 4: *The mechanism of delivery of labelled synthetic Ub or Ubl by using a dimeric disulfide-linked TAT peptide conjugated at the C-terminus of Ub. This reagent enters cells via the endosomal pathway and escapes from endosomes due to the dimeric nature of the TAT peptide used here. The delivery vehicle is then cleaved by cytosolic DUBs or Ubl-specific proteases. Once freed from the TAT peptide, the synthetic Ub or Ubl can be conveniently incorporated into the endogenous Ub or Ubl pathways.*

(Figure 4). Once in the cytoplasm, the dimeric disulfide-linked TAT peptide was reduced and removed from the C-terminus of rhodamine-Ub by the action of endogenous DUBs. Furthermore, these rhodamine-Ub reagents were incorporated into the intrinsic Ub machinery effectively. This study shows the potential to deliver virtually any synthetic Ub or Ubl reagent mildly into live cells.

Direct structural interactions between a Ub protein and DUBs are essential for deubiquitination. This interaction has been utilized to make Ub-based ABPs for DUBs and the X-ray crystal structures of different covalently trapped DUB-Ub complex have been determined [35]. One very interesting DUB that is associated with the 26S proteasome is the UCHL5 enzyme. An X-ray crystal structure of Ub-UCHL5 has been determined by trapping a UCHL5/Rpn13 complex with a Ub-PRG probe [36, 37]. From this structure, the residues Leu8 and Thr9 at the N-terminus of the Ub molecule were found to directly interact with a hydrophobic pocket in UCHL5. Therefore, a peptide sequence in Ub containing residues from 1 to 17 (that evidently includes the Leu8 and Thr9 residues) can potentially be used as an inhibitor of UCHL5 activity. **Chapter 6** deals with the synthesis of a stable cyclic β sheet peptide containing residues 1 to 17 of Ub, which was validated using the UCHL5/Rpn13 complex. I observed that this peptide inhibits UCHL5/Rpn13 both in isolation and in complex with the 26S proteasome. Moreover, the activity of the 26S proteasome was found to be increased upon inhibiting UCHL5, likely since UCHL5 has been implicated in Ub-chain

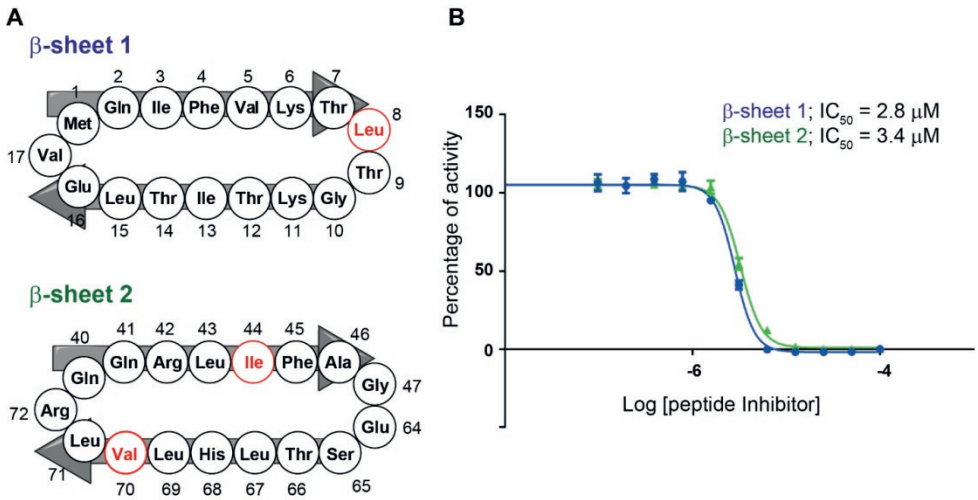

Figure 5: A: *Sequences of two β-sheet containing peptides derived from the Ub sequence. β-sheet peptide 1 contains residues 1 to 17 of Ub while β-sheet peptide 2 contains residues 40 to 47 and 64 to 72 of Ub which are combined and cyclized.* ***B:*** *IC_{50} values of both the β-sheet peptides show similar values when tested against UCHL5/Rpn13.*

trimming activity prior to substrate degradation by the 26S proteasome [38]. This inherent function of UCHL5 is hypothesized to save the substrate protein from being degraded by the 26S proteasome. Therefore, inhibition of UCHL5 with this cyclic peptide inhibitor renders substrate proteins potentially more prone to degradation. Further experiments enhance the properties of this UCHL5 inhibitor are ongoing. The use of SPPS offers more options to mutate the peptide with natural or unnatural amino acids and make them more selective to UCHL5.

Ubiquitin is known to contain four beta-sheets in its structure. This forms the core of the hydrophobic patch encompassing the residues of Leu8, Ile36, Ile44 and Val70. The UCHL5 inhibiting peptide contains the Leu8 residue. Other β-sheet peptides of Ub containing residues such as Ile36, Ile44 and Val70 can also be synthesized as a stable beta-sheet peptide inhibitor for DUBs. One such a peptide containing residues 40-47 and 64 to 72 of Ub were combined and cyclized. I observed that this peptide also inhibited UCHL5 with a similar IC_{50} value (Figure 5). The inhibitory potential of such Ub-derived β-sheet containing peptides can form the basis for future DUB inhibitors active in cells. Recent advances in generating large cyclic peptide libraries containing unnatural aminoacids have opened new possibilities in the discovery of highly efficient peptide inhibitors. One such method called RaPID screening uses modified ribozymes capable of incorporating unnatural aminoacids into peptide sequence generating a highly diverse peptide library, thereby increasing the chances of finding high affinity peptide inhibitors for DUBs [39]. Such a library can be developed using a Ub-derived peptide sequence as the basis and potentially identify better peptide inhibitors for UCHL5.

To summarize the thesis, I have investigated how the chemical synthesis of Ub conjugates and peptides based on Ub can be used to study and modulate the ubiquitination system both *in-vitro* and in live cells. With an expanding set of reagents to study the Ub system, it will become easier to develop future drugs that act on this system for therapeutic purposes.

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