DESIGN AND DEVELOPMENT OF NOVEL CHEMICAL BIOLOGY TOOLS FOR INVESTIGATING DEUBIQUITINATING ENZYMES

A Dissertation

by

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ABSTRACT

Ubiquitination, also known as ubiquitylation, is a major type of post-translational modification (PTM), which is closely associated with numerous cellular processes, such as protein degradation, intracellular trafficking, cell signaling, and DNA damage response. Substrate proteins can be monoubiquitinated at one or multiple lysine residues or further polyubiquitinated at one of the eight possible sites (seven lysines and N-terminal methionine) of ubiquitin (Ub) itself. Thus, various ubiquitin polymers (poly-Ub) with distinct linkage types will be produced and they possess different topological characteristics and cellular functions. Deubiquitinases (DUBs), also called deubiquitinating enzymes, can remove Ub from target proteins and regulate most Ub-dependent processes. The human genome encodes ~100 DUBs, which are essential for ubiquitin homeostasis, protein stability, and a wide range of signaling pathways. Consistent with this, their misregulations have been implicated in the pathogenesis of many human diseases, including chronic inflammatory diseases and various types of cancer. In contrast to the increasing interest in deubiquitinases study, ubiquitin conjugates that allow quantitative analyses of DUB activities and high-throughput screening (HTS) of DUB inhibitors, as well as poly-Ub with various linkage types for DUB structural and functional studies are not readily accessible.

A straightforward and readily adoptable approach that employed ubiquitin-activating enzyme (E1)-catalyzed amidation reaction to append ubiquitin C-terminus with multiple functionalities for further syntheses of various Ub-based conjugates, as

well as poly-Ub mimics with high efficiency was developed. With this facile method, Ub-based fluorescent substrates with distinct flexible linkages, which could mimic the native isopeptide bond in physiological DUB substrates, were produced and tested with one common DUB, UCH-L3 (ubiquitin carboxyl-terminal hydrolase isozyme L3). Moreover, desirable poly-Ub with diverse linkage types could also be readily generated, in combination with thiol-ene coupling (TEC) chemistry, thereby providing abundant versatile substrates for DUB specificity study. A novel luminescence-based experiment for high-throughput screening (HTS) was also developed and applied to the discovery of ubiquitin specific peptidase 2 (USP2) inhibitors for cancer therapy. Altogether, these novel chemical biology tools allow accelerated studies of DUBs that have pivotal roles in numerous cellular pathways, but whose investigations have been hampered by the difficulty of access to suitable substrates.

DEDICATION

To my parents-Mr. & Mrs. Wang, and my husband

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NOMENCLATURE

2×YT	Yeast extract tryptone
5-IAF	5-Iodoacetamidofluorescein
AMP	Adenosine monophosphate
Asn	Asparagine
Asp	Aspartic acid
Atg8	Autophagy-related protein 8
Atg12	Autophagy-related protein 12
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid assay
CAPS	*N*-Cyclohexyl-3-aminopropanesulfonic acid
Cys	Cysteine
DMF	*N*,*N*-Dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
DUB	Deubiquitinating enzyme or deubiquitinase
E1	Ubiquitin-activating enzyme
E2	Ubiquitin conjugating enzyme
E3	Ubiquitin ligase enzyme
EDTA	Ethylenediaminetetraacetic acid

EGFR	Epidermal growth factor receptor
ESI-MS	Electrospray ionization mass spectrometry
EtOAc	Ethyl acetate
FAS	Fatty acid synthase
FPLC	Fast protein liquid chromatography
GFP	Green fluorescent protein
Gln	Glutamine
Gly	Glycine
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
His	Histidine
HPLC	High performance liquid chromatography
HTS	High-throughput screening
HUB1	Homologous to ubiquitin 1
IPTG	Isopropyl β-D-1-thiogalactopyranoside
ISG15	Interferon-induced 15 kDa protein
JAMM	JAMM/MPN domain-associated metallopeptidases
LAP	Lithium acylphosphinate
Lys or K	Lysine
MCPIP	Monocyte chemotactic protein-induced protein
MDM2	Mouse double minute 2 homolog
Met	Methionine
MJD	Machado-Joseph disease

mRNA	Messenger ribonucleic acid
NCC	NIH Clinical Collection
NCL	Native chemical ligation
Nedd8	Neural precursor cell expressed, developmentally down-regulated 8
NEM	*N*-Ethylmaleimide
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NMR	Nuclear magnetic resonance
OTU	Ovarian-tumor proteases
PBS	Phosphate-buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulfonyl fluoride
PTM	Post-translational modification
RIPK1	Receptor-interacting serine-threonine kinase 1
RNAi	RNA interference
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SUMO	Small ubiquitin-like modifier
TBTA	Tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine
TCEP	Tris(2-carboxyethyl)phosphine
TEC	Thiol-ene coupling
THF	Tetrahydrofuran

TLC	Thin-layer chromatography
Ub	Ubiquitin
Ub-Al	Ubiquitin-allylamine
Ub-AMC	Ubiquitin-7-amido-4-methylcoumarin
Ub-D-Cys	Ub-D-cysteine
Ubl	Ubiquitin-like proteins
Ub-Lys-TAMRA	Ubiquitin-$^{\varepsilon}$N-($^{\alpha}$N-tetramethyl-rhodamine)-lysine
UbR110	Ubiquitin rhodamine110
UCH	Ubiquitin carboxyl-terminal hydrolase
UCH-L3	Ubiquitin carboxyl-terminal hydrolase isozyme L3
UFM1	Ubiquitin-fold modifier 1
UIM	Ubiquitin-interacting motif
URM1	Ubiquitin-related modifier-1
USP	Ubiquitin-specific proteases
YUH1	Yeast ubiquitin carboxyl-terminal hydrolyase

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CHAPTER I

INTRODUCTION

Protein Post-Translational Modifications

In eukaryotes, the diversity and complexity of the proteome (>1,000,000 molecular species of proteins) is achieved by two major routes that greatly expand the number of proteins predicted by DNA coding capacities (30,000 human genes) to two to three orders of magnitude, namely mRNA splicing at the transcriptional level and covalent post-translational modifications (PTMs) of proteins at one or more sites at the translational level.[1] PTM occurs after mRNA has been translated into proteins, especially after release of polypeptides from ribosome during the biosynthesis of proteins, and refers to a series of chemical reactions catalyzed by specific enzymes whereby the side chain or backbone of a ribosomally coded amino acid residue is converted into a non-standard amino acid residue.[2] For example, protein splicing, green fluorescent protein (GFP) maturation and proteasome autoactivations are representative PTM processes.[1] It is well known that 20 amino acids are the building blocks of proteins, and 15 of them contain side chains that can undergo covalent additions to constitute more than 100 different enzymatically modified amino acid residues found in various proteins.[3] Prominent posttranslational modifications include (a) phosphorylation of serine, threonine, tyrosine, histidine, aspartic acid, arginine, and lysine; (b) lysine acetylation; (c) tyrosine sulfation; (d) lysine, arginine and proline methylation; (e) lysine and proline hydroxylation; (f) lysine ubiquitination, *etc.* More than 300 different types of

PTMs have already been illustrated and discovery of new ones is still one of the most active fields of research currently.[1-4]

These major and minor categories of post-translational modifications lead to tremendous diversity, complexity and heterogeneity of gene products and generate millions of possible molecular variants of proteins in eukaryotic cells. Far from being "decorations", PTMs can grant proteins with expanded opportunities for catalysis, regulating signal transduction, controlling gene expression, integration of information at many metabolic intersections, and alternation of cellular locations. Thus, they are fundamental to cellular signaling, growth and transformation. For example, in signaling, kinase cascades are turned on and off by the reversible addition and removal of phosphate groups, and in the cell cycle ubiquitination marks cyclins for destruction at defined time points. Failure to control complex PTM processes is detrimental to the survival of the cell and a range of post-translationally modified proteins and their substrates are implicated in human diseases, including cancers, diabetes, and numerous neurological disorders.[5-7]

Protein Ubiquitination Process

The diversity and functional breadth of an organism's proteome are significantly more complex than genome and transcriptome due to extensive PTMs of proteins.[1,3] Hundreds of different modifications exist, including modifications by small molecules such as phosphate, methyl, or acetyl groups and by small proteins such as ubiquitin (Ub) and ubiquitin-like (Ubl) proteins that can be attached covalently to protein substrates.[2] The classic example of a protein that modifies other proteins is ubiquitin, an essential

76-residue polypeptide that is ubiquitously expressed and highly conserved among eukaryotic organisms but is absent from bacteria and archaea.[1,8,9]

Ubiquitin was first discovered in the mid-1970s, and its most widely understood function is to tag intracellular proteins for proteasomal degradation.[10] The 2004 Nobel Prize in Chemistry was awarded to Avram Hershko, Aaron Ciechanover and Irwin Rose for this finding.[11] Ubiquitin is a highly stable protein that adopts a compact β-grasp fold with a flexible six-residue C-terminal tail (**Figure 1A**).[10] With three conservative changes, ubiquitin is almost invariant from yeast to plants and mammals. This strong sequence conservation suggests high evolutionary pressure to conserve the structure of ubiquitin and that ubiquitin plays a fundamental role in maintaining cell function and in species evolution.[12,13] Actually, ubiquitin is involved in almost all aspects of cellular activities by regulating its extensively targeted proteins via ubiquitination (also called ubiquitylation), one of the most common and important protein post-translational modifications.[14,15]

Ubiquitination is an ATP-dependent biological process where a single Ub moiety is covalently tagged to target proteins via an amide bond between the Ub C-terminal di-glycine motif and ε-amino group in a lysine (K) residue, or less frequently, the free amino-terminus of target proteins.[10,11] The attachment of ubiquitin molecules to their targets is carried out by the sequential actions of a cascade of enzymes including ubiquitin-activating enzymes (E1s),[16,17] ubiquitin conjugating enzymes (E2s),[18] and ubiquitin ligase enzymes (E3s)[19] in a highly complex, temporally controlled and tightly

regulated manner. Thus, the ubiquitination process is also called as E1-E2-E3 cascade (**Figure 1B**).

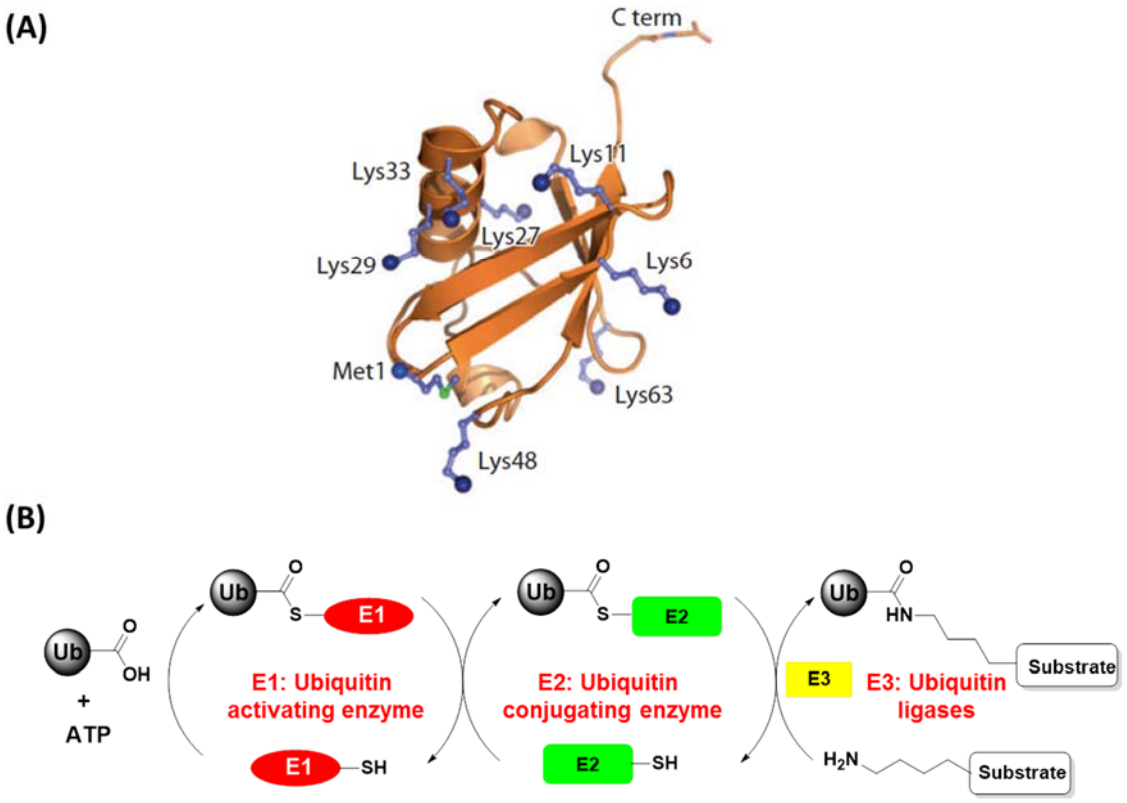

Figure 1. (A) The structure of ubiquitin. Seven lysines, the first methionine and the C-terminal carboxylate group are labeled.[10] (B) Scheme of protein ubiquitination process, the E1-E2-E3 cascade.

In the process of ubiquitination, initially, the C-terminal glycine (Gly76) of Ub is activated by the E1 enzyme using ATP as an energy source to adenylate Ub at its C terminus, forming a ubiquitin-adenylate intermediate. Subsequently, the high-energy mixed anhydride bond is quickly attacked by the sulfhydryl group of the active-site cysteine in E1 enzyme, forming a high-energy thioester bond between the E1 and the C terminal carboxyl group of ubiquitin.[20-22] Secondly, the activated ubiquitin is passed from E1 to the active-site cysteine residue of E2 enzyme via a trans(thio)esterification reaction, and a novel thioester is formed. Finally, Ub is conjugated to its substrate, creating an isopeptide/peptide bond between a carboxyl group at the C-terminus of ubiquitin and an amino group in the substrate with the aid of an E3 ligase enzyme, which identifies specific recognition modules in the target proteins and is capable of interactions with both E2 enzymes and target proteins.[23,24]

Theoretically, any lysine residues in a protein can be targeted by ubiquitination process, including ubiquitin itself. There are seven lysine residues (K6, K11, K27, K29, K33, K48, and K63) among the 76 amino acids of ubiquitin (**Figure 1A**), as well as the N-terminal amino group of methionine, which can themselves be targeted for ubiquitination to generate polyubiquitin (poly-Ub) chains of different linkage types depending on which amino group acts as the acceptor site for the incoming ubiquitin (**Figure 2A**).[25,26] In particular, the C terminus of the more distal ubiquitin molecule is attached to the previous ubiquitin molecule through an isopeptide bond with an ε-amino group of a Ub lysine. The last ubiquitin moiety in a poly-ubiquitin chain without a modified lysine is called distal ubiquitin.[10] In general, ubiquitination can be categorized

into three groups based on the number of tagged ubiquitins: i) monoubiquitination: proteins are modified by a single ubiquitin, such as Lys164 in proliferating cell nuclear antigen (PCNA);[27,28] ii) multi-monoubiquitination: proteins are tagged with several single ubiquitin molecules at multiple sites, with the epidermal growth factor receptor (EGFR) as an example; iii) polyubiquitination: proteins are attached with chains of ubiquitin molecules. These polyubiquitin chains can be as short as only two ubiquitin molecules or as long as more than ten moieties (up to 20).[1,10,27,29-33] Moreover, these chains can be homogenous if the same residue is modified during elongation, or mixed if different linkages alternate at succeeding positions of the chain, or branched when a single ubiquitin is modified at multiple lysine sites (**Figure 2**), and all possible linkages mentioned above have been detected in cells, but only Lys48- and Lys63-linked chains have been extensively studied.[30,34]

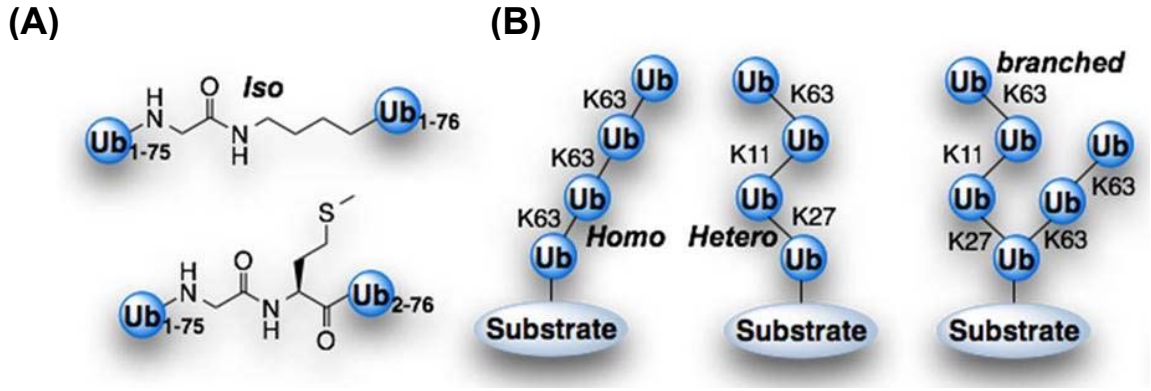

Figure 2 (A) The isopeptide and peptide linkages between two Ubs, (B) the homo- and hetero-typic linear and branched polyubiquitin chains.

Considering seven internal lysine residues and one N-terminus amino group in ubiquitin, there are eight possible positions for the second ubiquitin to attach, while for the third ubiquitin, there are 15 possible positions of attachment, thus, with chains of varying lengths and types, the structures of polyubiquitin chains in cells can be remarkably diverse, which has a profound impact on the fate of the modified proteins.[10,11,35] The syntheses of specific linked polyubiquitin chains are guaranteed in a sophisticated and economic way via the E1-E2-E3 cascade. In the human genome, there are two genes encoding different isoforms of E1 enzymes and each form has a distinct preference for E2s. There are at least 37 genes that encode distinctive E2s.[18] The number of the genes encoding E3s is over one thousand, which approximately equals the number of genes encoding kinases, underlining the universal significance of ubiquitination.[19] In this ubiquitination cascade, E1 can bind to dozens of E2s, which can bind to hundreds of E3s, and E3s specifically target thousands of substrate proteins. Numerous E2s and E3s and the combinatorial possibilities of various E2s and E3s provide the molecular basis for the stringent substrate specificity of polyubiquitination, thus, for any target substrate, there are two decisions for a specific E2/E3 pair to make: which substrate residue to modify and which Ub linkage to use for chain assembly. Therefore, this E1-E2-E3 cascade forms a hierarchical structure to control the whole ubiquitination process.[11,24]

Attachment of a Ub or Ub chain to a protein changes the protein surface topography in the conjugate substantially compared to that of the unmodified protein, resulting in either enhanced or inhibited binding capacity of the target protein to another macromolecule, thereby changing the location, conformation, stability, or activity of

tagged proteins.[24,25,29] Because myriad proteins can be ubiquitinated, ubiquitination has emerged as one of the most important PTMs in regulation of protein function. Various ubiquitin modifications adopt distinct conformations and provide an information-rich architectural scaffold with key roles in nearly all the important cell biological activities, including protein degradation, cell metabolism, signal transduction, membrane traffic, transcriptional regulation, endocytosis, DNA repair, chromatin remodeling, peroxisome biogenesis and viral budding.[2,31,33] The functional consequences of ubiquitination are gradually being deciphered, and the number of biological pathways known to be regulated by ubiquitination continues to grow. Not surprisingly, abnormalities in ubiquitin-mediated processes are causally linked to pathologies, including inflammation, arthritis, heart disease and cancers.[9,10,27,28]

The most well-established physiological role for ubiquitination is to target proteins for degradation by the proteasome.[28] For this purpose, ubiquitin is attached to the substrate in the form of a polyubiquitin chain that can be recognized by specific receptors within the proteasome, and subsequently removed hydrolytically during ATP-driven unfolding of the targeted protein.[11] The unfolded protein is then threaded into the chamber of the proteasome where the active sites of the protease subunits degrade it into small peptides.[36] The "classical" signal for protein degradation is the Lys48-linked polyubiquitin chain of at least four ubiquitin moieties to the target protein, which constitutes an architectural for efficiently binding to the proteasome and for degradation. Lys11-, Lys29-, Lys48-, and Lys63-linked chains might all have roles in proteasomal degradation, while Lys11- and Lys48-linked chains trigger degradation more frequently

than other modifications, which is possibly due to the fact that enzymes synthesizing Lys11- and Lys48-linked chains are less likely to introduce branches, which can impede degradation.[8,13,36-38]

Although mostly associated with proteasomal degradation, ubiquitination is also involved in regulatory events in a proteasome-independent manner, mostly resulting from monoubiquitination or Lys63-linked chain formation.[39] Protein monoubiquitination plays essential roles in endocytosis and transcriptional regulation processes, and is a tool for the reversible recruitment of an enzyme to a particular cellular location, which can initiate relocation of transmembrane receptor proteins from the plasma membrane to the trans Golgi network sorting compartments.[1,40] While the Lys63-linked polyubiquitination is mainly responsible for modifications of protein functions and related to a variety of different cellular pathways like endocytosis of cell surface receptors, cell signaling, mitochondrial inheritance and morphogenesis, ribosomal function, DNA repair and activation of the NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells) signaling complex.[33,41]

Following the discovery of ubiquitin in the mid-1970s, a number of distinct small proteins called ubiquitin-like (Ubl) proteins have been uncovered to function as protein modifiers as well, and new members are still being added.[42] These Ubl proteins, which include SUMO, ISG15, Nedd8, Atg8, FUBI, HUB1, FAT10, URM1, UFM1 and Atg12, function as critical regulators of many cellular processes, such as transcription, DNA repair, signal transduction, and cell-cycle control, thus the dysregulation of Ubl-substrate

modification and mutations in the Ubl conjugation machinery are implicated in the etiology and progression of a number of human diseases.[8,37]

Despite limited sequence homology of some Ubl proteins with ubiquitin, all Ubl proteins display structural homology with essentially the same characteristic β-grasp ubiquitin fold. Furthermore, for all Ubl proteins whose covalent attachments to proteins have been experimentally demonstrated, the C-terminal residue of the Ubl is a glycine, and the carboxyl group of this glycine is the site of attachment to substrates. The ε-amino groups of lysine side chains are the most common target sites within substrate proteins, forming an isopeptide bond between the Ubl and substrate.[17,40,43] Among them, small ubiquitin-like modifier (SUMO) is the most studied one and affects a similar large number of substrates as ubiquitin. However, the pathway of SUMOylation is much simpler with only one E1 (Uba2/Aos1), one E2 (Ubc9), and a few E3 ligases. Generally, Ubc9 binds substrates directly, while E3 ligases can also contribute to substrates specificity. Both ubiquitin and SUMO can form poly-modifier chains conjugated to lysine residues in substrates. Recently, it was discovered that SUMO itself may be modified by ubiquitin, resulting in mixed ubiquitin/SUMO chains.[44,45] Still, much more needs to be uncovered in the research fields of ubiquitin and Ubl proteins.

Deubiquitinases (DUBs)

Similar to other post-translational modifications, such as phosphorylation and acetylation, ubiquitination process is highly dynamic and can be reversed by a large and growing group of proteases, termed deubiquitinating enzymes (DUBs; also known as deubiquitinases), that mediate the removal of Ub molecules from target proteins by

specifically cleaving the isopeptide bond between the ε-amino group of lysine side chain in the target protein and the C-terminal carboxyl group of ubiquitin, or the peptide bond between the N-terminal amino group of the target protein and the C-terminal carboxyl group of ubiquitin.[11,12] DUBs oppose the actions of the E3 ligases, thus, it is the delicate balance between the actions of specific E3 ligases and DUBs which ultimately determine the ubiquitination status of a given target protein, resulting in DUBs acting as pivotal regulators of ubiquitin mediated signaling pathways.[46-49]

Analysis of the human genome shows the presence of at least 98 functional DUBs, which can be grouped into six families based on structural homology: ubiquitin-specific proteases (USPs), ubiquitin C-terminal hydrolases (UCHs), ovarian-tumor proteases (OTUs), Machado–Joseph disease protein domain proteases, JAMM/MPN domain-associated metallopeptidases (JAMMs) and the recently discovered monocyte chemotactic protein-induced proteins (MCPIPs).[15] All of them are cysteine (Cys) proteases with the exception of JAMMs, which are zinc metalloproteases. The reaction mechanism of Cys proteases DUB families (USPs, UCHs, OTUs, Josephins and MCPIPs) rely on two or three crucial amino acid residues, constituting a catalytic dyad or triad, respectively.[50] In these enzymes, a nearby histidine (His) side chain lowers the pKa of a catalytic Cys, enabling a nucleophilic attack on isopeptide linkages so that the carboxyl group is covalently bound to the catalytic Cys after the amino group has been cleaved. A third residue (usually asparagine (Asn) or aspartic acid (Asp)) aligns and polarizes the catalytic His. In a second step, a water molecule hydrolyses the acyl-Cys intermediate to complete the catalytic cycle. Strikingly, although displaying divergent

folds, the catalytic residues of all Cys protease DUB families superpose with only small deviations when bound to the ubiquitin C-terminus.[51,52] For JAMM DUBs, the JAMM/MPN+ motif coordinates two zinc ions, one of which activates a water molecule to attack the isopeptide bond. The amino group is subsequently released from the charged catalytic intermediate.[15] The activity of these enzymes affects the activation, recycling and localization of multiple proteins, which in turn is essential for cell homeostasis, protein stability and a wide range of signaling pathways, therefore, much progress has been made in the characterization of DUBs recently.[53,54]

USP constitutes the largest DUB family with more than 60 members, which makes up about half of all DUBs. Structures of the catalytic domains of several USPs have been determined, revealing that the USP fold is highly conserved and consists of three subdomains resembling the palm, thumb and fingers of a right hand, despite of limited sequence homology. The catalytic cysteine and histidine residues (Cys-box and His-box) are located in the cleft separating the palm and thumb, whereas the finger domain is responsible for interactions with distal ubiquitin, with the only exception of CYLD (cylindromatosis D) which lacks the finger domain. Furthermore, most USPs share the common feature of undergoing conformational changes upon ubiquitin binding, which drives the transition from an inactive form to a catalytically active state. This is well characterized for USP7, for which ubiquitin binding is required to induce changes in conformation of the catalytic domain to bring the catalytic Cys in range of the His residue.[15,55,56]

UCH is the first DUB family to be structurally characterized and is composed of four members in human cells: UCH-L1 (ubiquitin C-terminal hydrolase isozyme L1), UCH-L3, UCH-L5/UCH37 and BAP1 (BRCA1 Associated Protein-1). These enzymes are small in size and they have a confined loop preventing binding of large, fully folded ubiquitin conjugates and precluding the processing of polyubiquitin chains and large folded proteins, which is consistent with the failure to observe UCH-L1 or UCH-L3 mediated hydrolysis of tetra-ubiquitin chains.[51,57] Therefore, they can merely target small peptide conjugates from the C-terminus of ubiquitin, which may be produced as by-products of proteasomal or lysosomal degradation.

The OTU domain was first identified in an ovarian tumor gene from Drosophila melanogaster by a bioinformatics approach. So far, 15 protein-coding genes with OTU domains have been discovered in the human genome, which can be classified in 3 groups: otubains (OTUB1 and OTUB2), A20-like OTUs (A20/TNFAIP3; Cezanne, Cezanne 2, TRABID and VCPIP1) and OTUDs (OTUD1, OTUD2/YOD1, OTUD3, OTUD4, OTUD5, OTUD6A, OTUD6B and ALG13). The OTU core domain is composed of five β-strands, situated between helical domains that vary in size among OTU family members. Also, the OTU core is accompanied by ancillary ubiquitin-binding domains such as A20-type Zn fingers in A20, NPL4-type Zn fingers in TRABID, ubiquitin-interacting motif in OTUD1 and OTUD5, as well as ubiquitin-associated domain in Cezanne.[52,58-60]

The Josephin family of DUBs is composed of four different members including ataxin-3 (ATXN3), ATXN3L, JOSD1 and JOSD2. Among them, ATXN3 is the best-

studied member, which contains a poly-glutamine (poly-Gln) stretch whose extension leads to the neurodegenerative disorder Machado-Joseph disease (MJD). All four members contain the highly conserved catalytic triad consisted of one cysteine and two histidine residues. Apart from the Josephin domain, ATXN3 and ATXN3L have additional domains like ubiquitin-interacting motif (UIM), suggesting a possible interaction with two distal ubiquitins in a polymer.[15,61,62]

The JAMM forms the only family of DUBs with metalloprotease activity, and human genome encodes 12 JAMM proteins, 5 of which are catalytically inactive, whereas the rest of them have isopeptidase activity for ubiquitin or ubiquitin-like proteins (Ubl): AMSH-LP, AMSH/STAMBP, BRCC36 (BRCA1/BRCA2-containing complex subunit 36), POH1/PSMD14 (26S proteasome-associated PAD1 homolog 1), MYSM1 (Myb-like with SWIRM and MPN domains 1), MPND (MPN domain-containing protein) and CSN5/JAB1 (COP9 signalosome subunit 5). The members of the AMSH subfamily (POH1, AMSH and AMSH-LP) share high degree of sequence conservation with composition of a JAMM core and two conserved insertions which contributes to its specific recognition and DUB catalysis of Lys63-linked polyubiquitin chains, while other JAMM proteases with no specificity for Lys63-linked polyubiquitin chains lack the AMSH-specific insertions.[52,63]

Recently, DUB families have expanded due to the discovery of a novel domain exhibiting deubiquitinating activity in the MCPIP1 protein. According to a bioinformatics analysis, the sixth DUB family should be composed of at least seven members in the human genome (MCPIP1, MCPIP2, MCPIP3, MCPIP4, MCPIP5,

MCPIP6, and MCPIP7). The founding member MCPIP1 contains an N-terminal conserved region and a conserved CCCH-type zinc-finger domain in the middle region of the protein which are critical for its activity. Besides, its catalytic domain contains the Cys and Asp boxes consistent with characteristics of cysteine proteases, but lacks the conserved His box.[15,64]

The existence of six families of nearly 100 DUBs reflects the need for specificity in their functions. The small number (<100) of DUBs, compared to thousands of E3s, might at first imply a low degree of selectivity; however, DUBs can display specificity at multiple levels to distinguish between different types of ubiquitin linkage and chain structure. Currently, owing to the unavailability of well-defined ubiquitin chain preparations, other than linear, Lys48- or Lys63-linked chains, we still don't have a complete picture of DUB specificity. Once appropriate reagents are available, it is mostly likely that new DUB families will be discovered due to the existence of highly specific DUBs.[65]

First of all, each Lys in ubiquitin has a unique sequence context and can construct eight topologically different ubiquitin chains, which allows for specific recognition by the DUBs. Strikingly, the ability of DUBs to discriminate distinct chain linkages is not restricted to particular DUB families: the USPs, OTUs, and JAMMs all include linkage-specific members.[15] For example, the 26S proteasome-associated USP14 shows specificity for Lys48-linked ubiquitin chains, whereas JAMM family DUBs are often Lys63 linkage specific, as seen for AMSH, AMSHLP, BRCC36, and POH1.[66,67] In addition, linkage-specific OTU DUBs have been described, showing that OTUB1 is

specific for Lys48 linkage, Cezanne is specific for Lys11 linkages, and TRABID is specific for Lys29 and Lys33 linkages.[68,69] Besides, polyubiquitin chains can be cleaved from the end (*exo*) or within a chain (*endo*), which constitutes another layer for DUBs specificity.[52] Both mechanisms have been described with profoundly different consequences. For *endo*-DUBs, they must accommodate ubiquitin molecules on either side of the cleavage site with the ability to remove entire chains from target proteins, whereas for *exo*-DUBs, they only need to bind a single ubiquitin-the distal ubiquitin, cleaving only one ubiquitin moiety each time. Therefore, an e*ndo*-DUB would reverse polyubiquitination more efficiently in contrast with an *exo*-DUB.[15] Further, DUBs may gain specificity by targeting a select set of substrates directly, while disassembling ubiquitin chains independently of the linkage type. These DUBs can specifically recognize ubiquitinated target proteins and carry out a single-step to remove monoubiquitin or polyubiquitin chains. Most USPs belong to this large group of DUBs, which can be considered nonspecific with regard to the ubiquitin linkage but specific with respect to their substrates, and can efficiently hydrolyze the isopeptide bond between the substrate and the first ubiquitin.[55,56]

Consistent with its vast number and multiple layers of specificity, DUBs have been demonstrated to play diverse functions in distinct cellular pathways which can be classified into three major categories: ubiquitin precursor processing, ubiquitin deconjugation and editing of ubiquitin conjugates.[51,52] Firstly, ubiquitin is synthesized as inactive precursors consisting of either multiple ubiquitin copies or ubiquitin fusions to L40 and S27 ribosomal proteins, which need to be processed at their C-terminus to

expose the glycine carboxylate group that is the site of substrate conjugation. Thus, DUBs contribute to the generation of free ubiquitins through their ability to process ubiquitin precursors. Secondly, DUBs can remove the ubiquitin chains and play a critical regulatory role by counteracting ubiquitination of selective target proteins, leading to the reversion of ubiquitin signaling or to protein stabilization by rescuing them from degradative pathways, with associated activities of recycling ubiquitin molecules, thereby maintaining the cellular pool of free ubiquitins and contributing to ubiquitin homeostasis. Finally, some DUBs can trim ubiquitin chains conjugated to target proteins and alter the fates of target proteins in a more subtle way by editing the length and topology of its ubiquitin tag to change the signal (nondegradable *vs* degradable) harbored by them. For example, A20 can change receptor-interacting serine-threonine kinase 1 (RIPK1) ubiquitination status from Lys63- to Lys48-linked polyubiquitin chains, causing its degradation by the proteasome.[36,53,67,68,70]

Owing to their specific catalytic activities and pivotal roles in ubiquitin homeostasis and control of protein stability, DUB activities must be tightly controlled within the cell through a number of different mechanisms, including post-translational modifications (PTMs), allosteric interactions and subcellular localization. First of all, many DUBs are subjected to PTMs, including phosphorylation and ubiquitination.[71] Specially, multiple DUBs can be tightly controlled by phosphorylation that can switch their activity on or off. For example, phosphorylation inhibits CYLD and USP8, while it activates A20, USP7, USP15, USP16, USP19, USP28, USP34 and USP37. Ubiquitination can also regulate DUB activity either positively or negatively,

exemplified in the case of ATXN3 catalytic activity activation and UCH-L1 activity inhibition by steric hindrance.[50] Besides, certain DUBs, like UCH-L1, UCH-L3, OTU1, USP1, USP5, USP7, USP12, USP14 and USP46, contain ubiquitin-binding domains or ubiquitin-like motifs within their catalytic regions and are thought to undergo allosteric conformational changes upon interaction with ubiquitin or other proteins to obtain their active conformations, as another way to prevent uncontrolled proteolytic activity.[56] Last but not least, several DUBs can also be modulated by changes in their subcellular localization and have been shown to undergo epidermal growth factor (EGF)-dependent translocation to endosomes which would facilitate their access to specific substrates for processing. The localization of USP30 to mitochondria seems to affect the morphological properties of this organelle, whereas USP36 special nucleolar localization can regulate its structure and function.[15]

In the past decade, accumulating evidence indicates that DUBs regulate various pathways and proteins whose dysregulation is associated with numerous diseases spanning oncology, neurodegeneration, hematology and infectious diseases (**Figure 3**).[68,72-75] Representative examples are found within each DUB class, including USP1 (Fanconi anaemia), USP2 (prostate cancer), USP7 (prostate cancer, viral infection), USP16 (Down's syndrome), UCH-L1 (Parkinson's disease), A20 (lymphoma), AMSH (Microcephaly-capillary malformation syndrome), and ATXN3 (ataxia). It is thus conceivable that targeting DUBs may be useful for the treatment of various diseases, especially for cancer therapy.[70,76,77] Considerable interest in DUBs as potential drug targets has emerged and the hunt for DUB antagonists is actively carried out by

academic and pharmaceutical companies alike over the past years. Overall, the development of small molecule inhibitors for DUBs is still in its infancy, although a number of putative small inhibitor molecules for the UCH and USP family members have been identified, none of them can be used therapeutically due to problems with specificity. The future development of DUB-based therapy will largely depend on further investigations of DUBs crystal structures, improvement of better HTS-compatible, biochemical assays for compound screening, and more diversity of screening libraries.[78-81]

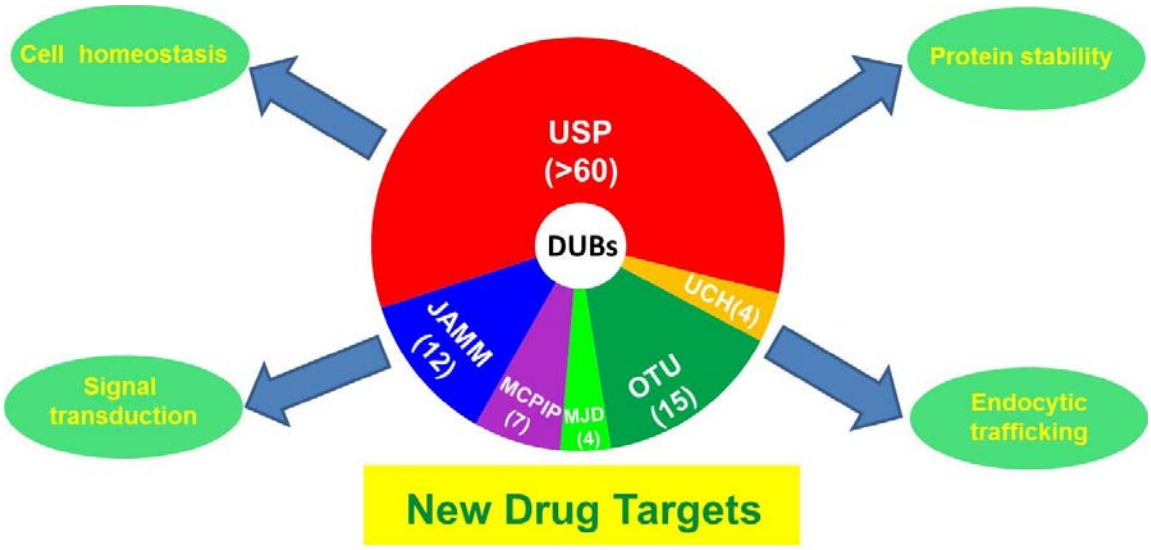

Figure 3. Six families of deubiquitinases (DUBs). DUBs can regulate numerous processes including cell homeostasis, protein stability, signal transduction and endocytic trafficking, rendering it as the emerging drug targets.

Scope of the Thesis

This dissertation was focused on the design and development of novel chemical biology tools for deubiquitinating enzymes investigations, starting from the exploitation of E1-catalyzed ubiquitin C-terminal amidation reaction (Chapter II), which could be further applied in many aspects of DUB study, including efficient syntheses of physiological-based substrates for DUBs enzyme activity study (Chapter III), construction of topologically-characterized ubiquitin polymers (poly-Ub) mimics for DUBs specificity study (Chapter IV) and establishment of a readily-adoptable novel HTS assay for DUB inhibitors discovery (Chapter V).

Firstly, in Chapter II, a straightforward single-step approach was illustrated to append numerous orthogonal functional groups (thiol, azide, alkyne, alkene, *etc.*) to the Ub C-terminus with high efficiency as well as high yield (> 90%) via E1-catalyzed ubiquitin C-terminal amidation reaction. This approach was based on the natural ubiquitination process and took full advantage of the high reactivity and universality of ubiquitin-activating enzyme (E1).The simple nature of the reaction, almost quantitative conversion rates and easy expressions of both Ub and E1 in *E. coli* allowed its wide application for functional Ub-amine conjugates production.

Then, for those Ub-amine conjugates with various functional groups at the Ub C-terminus, we further modified them (thiol, azide and alkyne) via click reactions to construct respective fluorescent ubiquitin conjugates for DUBs activity study, as described in Chapter III. The Ub-amine conjugates contained unique functionalities at their C-terminal ends that could be selectively labeled to constitute Ub-fluorophore

conjugates, which could serve as the substrates for DUBs enzymatic kinetic study based on time-dependent fluorescence polarization change. By virtue of highly efficient click reactions, Ub-fluorophore conjugates could be synthesized quantitatively in one step, with relatively flexible linkages between Ub C-terminus and fluorophore groups, mimicking the natural isopeptide bond in ubiquitin chains and rendering them as suitable physiological-based substrates for DUBs activity study.

Besides fluorescent ubiquitin conjugates, in Chapter IV, a universal method for preparing ubiquitin polymers (poly-Ub) mimics with distinct linkage types was illustrated. This method combined the high-efficient E1-catalyzed Ub C-terminal amidation reaction to append alkene groups to Ub C-terminus, together with thiol-ene coupling reaction to construct a flexible linkage between alkene and cysteine mutants in another ubiquitin molecule for the syntheses of poly-Ub mimics. Through introducing cysteine mutants to different lysine positions in the ubiquitin molecule, distinct linkage types between Ub molecules could be readily achieved, thus, this universal and straightforward method provided a novel route to construct polyubiquitin chains with desirable linkage types, which might greatly facilitate the research field of deubiquitinases cleavage specificity, whose development had been hampered by the limited availability of versatile poly-Ub chains for a long time.

In addition of preparing diverse substrates for deubiquitinases activity study, we also developed a novel luminescent assay for high-throughput screening of DUBs inhibitors, which would be discussed in Chapter V. This assay was based on the recognition and cleavage activity of DUBs to ubiquitin-D-cysteine (Ub-D-Cys) conjugate,

which would release D-cysteine molecule for further reaction with 2-cyano-6-hydroxybenzothiazole to afford luciferin for further luminescence production through luciferase enzyme catalyzed reaction at the presence of ATP and Mg^{2+}. Compared to currently widely-applied fluorogenic assay with Ub-AMC (ubiquitin-7-amido-4-methylcoumarin) as the starting substrate, our luminescent assay displayed lower signal noise and less intervention with library compounds; also, a more flexible linkage between Ub C-terminus and D-cysteine rendered Ub-D-Cys to be a better substrate than Ub-AMC for deubiquitinases recognition, which might be applied for inhibitors discovery of different DUB families.

CHAPTER II

DEVELOPMENT OF E1-CATALYZED UBIQUITIN C-TERMINAL AMIDATION REACTION FOR UBIQUITIN DERIVATIVES SYNTHESES*

Introduction

Ubiquitin (Ub) is a small but evolutionarily conserved polypeptide with 76 amino acids.[82] In eukaryotes, it is covalently conjugated to cellular proteins through protein ubiquitination process in which an amide bond (isopeptide or peptide) is formed between the Ub C-terminal carboxylate group and a lysine side-chain amino group or N-terminal amino group of a protein substrate. This process is orchestrated by sequential actions of three types of enzymes, namely, Ub-activating enzyme (E1), Ub-conjugating enzyme (E2), and Ub ligase enzyme (E3).[24,37,42] In mammalian cells, the ubiquitination cascade is pyramidal in design. One E1 enzyme can transfer Ub to dozens of E2 enzymes, which function together with hundreds of different E3 enzymes to guarantee ubiquitination specificities. Protein ubiquitination is one of the most significant post-translational modifications in eukaryotes and is the key regulator for numerous substrate proteins and various cellular processes.[43]

In the protein ubiquitination process, initially, E1 enzyme catalyzes two sequential reactions to activate Ub. The first reaction involves Ub and ATP to form Ub-

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* Reprinted (adapted) with permission from "E1-Catalyzed Ubiquitin C-Terminal Amidation for the Facile Synthesis of Deubiquitinase Substrates" by Wang, X. A.; Kurra, Y.; Huang, Y.; Lee, Y. J.; Liu, W. R. *Chembiochem* **2014**, *15*, 37-41. Copyright © 2014 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

AMP that then reacts with the E1 active-site cysteine to form an E1-Ub fusion with a thioester linkage.[16,17] In the presence of an E2 enzyme, the E2 active-site cysteine intercepts Ub from E1-Ub and then transfers Ub to a lysine side-chain amino group or N-terminal amino group of a target protein while an E3 enzyme serves as a catalyst in this transfer.[18,19] Although considered labile, the thioester bond in an E1-Ub fusion upholds relatively stable E1-Ub linkage in the absence of E2, which could be readily detected by non-reductive SDS-PAGE analysis as previously reported by Azevedo's group for determining E1 enzyme's activity.[83] Also, the same E1-Ub thioester bond was effectively employed to undergo native chemical ligation with peptides that contained a N-terminal cysteine or a mercapto-lysine entity pioneered by Ashraf Brik[84] and Chuan-Fa Liu[85,86] for functional ubiquitin derivatives syntheses. More recently, exchanging E1 in the E1-Ub thioester bond with 2-mercaptoethanesulfonate to form a Ub thioester linkage was reported by Fushman's group for polyubiquitin chains preparation.[87] Based on these previous studies, we reasoned that this E1-Ub thioester, in the absence of E2, could potentially undergo the aminolysis reaction with a primary amine to form a C-terminal functionalized Ub derivative and recover E1.[21] When this happened, E1 would essentially serve as an enzyme to catalyze Ub C-terminal conjugation in the presence of ATP and a primary amine.

With this hypothesis, we initiated our test of this E1-catalyzed ubiquitin C-terminal amidation reaction with a number of different primary amines, including cysteamine, hydrazine, hydroxylamine, azidoethylamine, propargylamine, and allylamine (**Figure 4**), and strived to achieve higher conversion rate by testing different

reaction conditions, such as pH, temperature, concentrations and reaction time. Compared to two major methods for ubiquitin derivatives preparation-total chemical synthesis and native chemical ligation, this novel and facile single-step approach is exceptionally simple and unique and can be readily adopted by a regular biochemistry and molecular biology group that is either not equipped or not capable to handle organic synthesis but work on DUBs research.[88,89]

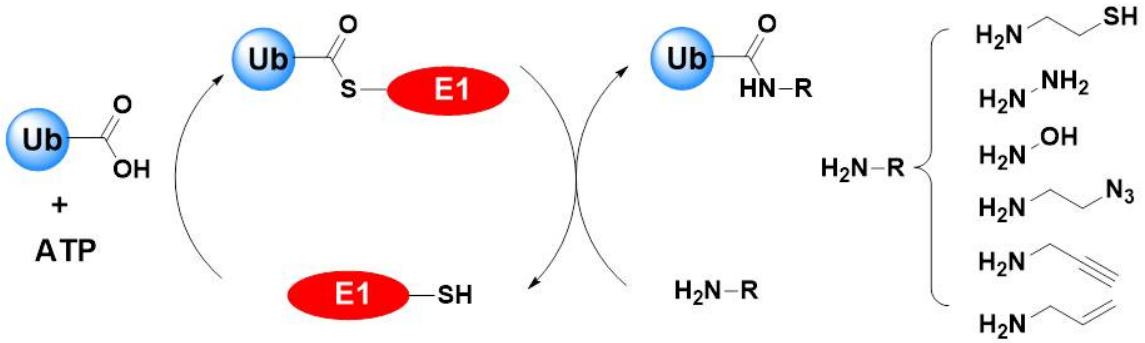


Figure 4. Scheme of E1-catalyzed amidation reaction to functionalize Ub C-terminus with different primary amine molecules.

Experimental Details

General Experimental Information

All reactions involving moisture sensitive reagents were conducted in oven-dried glassware under an argon atmosphere. Anhydrous solvents were obtained through standard laboratory protocols. Analytical thin-layer chromatography (TLC) was performed on Whatman SiO_2 60 F-254 plates. Visualization was accomplished by UV irradiation at 254 nm or by staining with ninhydrin (0.3% w/v in glacial acetic acid/*n*-butyl alcohol 3:97). Flash column chromatography was performed with flash silica gel (particle size 32-63 μm) from Dynamic Adsorbents Inc (Atlanta, GA).

Specific rotations of chiral compounds were obtained at the designated concentration and temperature on a Rudolph Research Analytical Autopol II polarimeter using a 0.5 dm cell. Proton and carbon NMR spectra were obtained on Varian 300 and 500 MHz NMR spectrometers. Chemical shifts are reported as δ values in parts per million (ppm) as referenced to the residual solvents: chloroform (7.27 ppm for ^{1}H and 77.23 ppm for ^{13}C) or water (4.80 ppm for ^{1}H). A minimal amount of 1, 4-dioxane was added as the reference standard (67.19 ppm for ^{13}C) for carbon NMR spectra in deuterium oxide, and a minimal amount of sodium hydroxide pellet was added to the NMR sample to aid in the solvation of amino acids which have low solubility in deuterium oxide under neutral or acidic conditions. ^{1}H NMR spectra are tabulated as follows: chemical shift, multiplicity (s = singlet, bs = broad singlet, d = doublet, t = triplet, q = quartet, m = multiplet), number of protons, and coupling constant(s). Mass

spectra were obtained at the Laboratory for Biological Mass Spectrometry at the Department of Chemistry, Texas A&M University.[109]

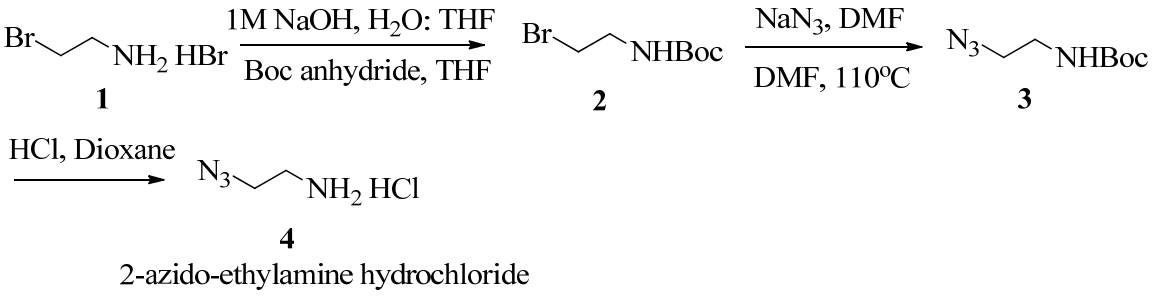
2-Azido-ethylamine hydrochloride was synthesized following the procedure below. All other reagents were obtained from commercial suppliers and used as received.

Chemical Synthesis

Synthesis of 2-azido-ethylamine hydrochloride

To a 250 mL of round-bottom-flask 2-bromoethyl amine (**1**, 5 g, 24.5 mmol) was added, followed by tetrahydrofuran (THF) (50 mL) and NaOH aqueous (1.0 M, 26.95 mL, 26.95 mmol). The reaction mixture was cooled to 0°C. Di-*tert*-butyl dicarbonate (5.87 g, 26.96 mmol) was dissolved in THF (50 mL) and added to the above mixture dropwise over 40 min. Then the resulting mixture was stirred at room temperature for 20 hours. THF was removed under reduced pressure. The residue was diluted with water (50 mL), and extracted with EtOAc (50 mL). The aqueous layer was collected and acidified to pH = 3 with HCl (1.0 M in water), then extracted with EtOAc (50 mL×3). The combined organic layers were dried over anhydrous Na_2SO_4, concentrated under reduced pressure and purified by flash chromatography to afford compound **2** (4.86 g, 90% yield). Compound **2** (4.5g, 20.07 mmol) was dissolved in *N*,*N*-dimethylformamide (DMF) (100 mL). NaN_3 (6.5 g, 100.39 mmol) was added, and the mixture was stirred at 110°C for 12 hours. The solvent was removed under reduced pressure, and the residue was dissolved in water (100 mL). The resulting aqueous solution was extracted with ethyl acetate (2×100 mL). The organic layers were combined

and dried over anhydrous $MgSO_4$(s). After filtration, the organic layer was concentrated under reduced pressure and the residue was dissolved in methylene chloride (10-20 mL) and purified by flash chromatography with 70% pure compound **3** (2.61 g). A suspension of compound **3** (2.61 g, 10.30 mmol) in dioxane and 4N HCl (7.0 mL, 2 eq.) was added as dropwise at room temperature and the reaction mixture was stirred at room temperature for 16 hours. The solid was collected by filtration to obtain 2-azido-ethylamine hydrochloride (**4**, 1.02 g, 60%). ^{1}H NMR (300 MHz, CD_3OD) δ = 3.76-3.68 (m, 2H), 3.13(t, *J* = 5.4 Hz, 2H) (**Figure 5**); ^{13}C NMR (300 MHz, D_2O) δ = 48.0, 38.8.



Scheme 1. Synthetic route of 2-azido-ethylamine hydrochloride (**4**).

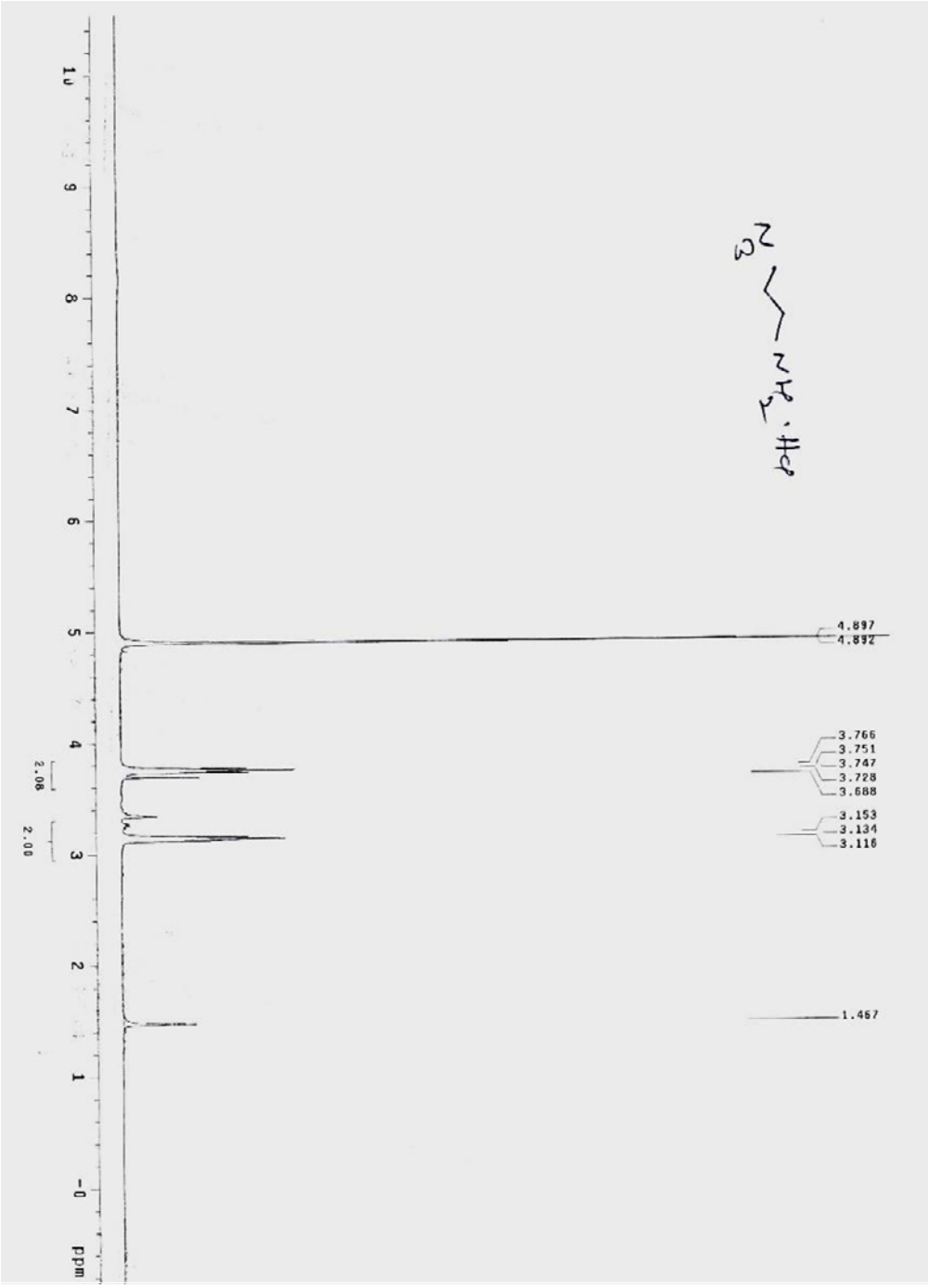


Figure 5. ^{1}H NMR spectrum for 2-azido-ethylamine hydrochloride (**4**)

DNA and Protein Sequences

DNA sequence

Ubiquitin

atgcagatcttcgtgaagactctgactggtaagaccatcactctcgaagtggagccgagtgacaccattgagaatgt
caaggcaaagatccaagacaaggaaggcatccctcctgaccagcagaggttgatctttgctgggaaacagctgga
agatggacgcaccctgtctgactacaacatccagaaagagtccaccctgcacctggtactccgtctcagaggtggtt
ga

Histidine tagged-mouse ubiquitin-activating enzyme (Histag-mE1)

atgggcagcagccatcatcatcatcacagcagcggcctggtgccgcgcggcagccatatgtccagctcgccg
ctgtccaagaaacgtcgcgtgtccgggcctgatccaaagccgggttctaactgctcccctgcacagtctgcgctgtc
cgaagtgtcctcagtgccaaccaacggaatggcgaagaacggcagtgaagcagacatagacgagagcctttactc
ccggcagctgtacgttttgggccatgaggcaatgaaaatgctccagacatccagcgtccttgtctcaggcttgcggg
gcttgggtgtagaaattgctaagaacatcatccttggtggggtcaaggctgtcaccctacatgaccaaggaactacc
cagtgggctgatctctcctcccagttttaccttcgggaggaggacattggtaaaaatcgagcggaggtatcccagcc
ccgacttgctgaactcaacagctatgtacctgtcactgcctacactgggcctcttgtcgaggacttccttagtagcttcc
aggtggtggtcctcaccaacagccccctggaagcccagctgcgagtgggggagttctgtcatagccgtggtatcaa
gctagtggtggcagatacaagaggcctgtttgggcaacttttctgtgattttggagaggaaatggtcctcacagattcc
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ggcccgacatggctttgagactggtgacttcgtctcattctcagaagtacagggcatgatccaactcaatggatgtca
gcccatggagatcaaagtgctgggtccttatacctttagtatctgtgacacttccaacttctctgactacatccgtggag
gcatcgtcagtcaggtcaaagtaccgaaaaagattagttttaaatccttgccagcatcactggtagagcctgactttgt
gatgactgactttgccaagtattctcgccctgcccaactgcacattggcttccaagctctgcaccaattctgtgctctgc

acaaccaaccacctcgaccacgaaatgaggaagatgcaacagagctggtgggcctggctcaggctgtaaacgct

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agagattgtgagccgagtgtcaaagagaaagctgggccgccatgtgcgggcactggtgcttgagctgtgctgcaa

cgatgaaagcggcgaggacgtcgaggtcccttatgtccgatataccattcgctga

Protein sequence

Ubiquitin

MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLE

DGRTLSDYNIQKESTLHLVLRLRGG

Histidine tagged-mouse ubiquitin-activating enzyme (Histag-mE1)

MGSSHHHHHHSSGLVPRGSHMSSSPLSKKRRVSGPDPKPGSNCSPAQSA

LSEVSSVPTNGMAKNGSEADIDESLYSRQLYVLGHEAMKMLQTSSVLVS

GLRGLGVEIAKNIILGGVKAVTLHDQGTTQWADLSSQFYLREEDIGKNR

AEVSQPRLAELNSYVPVTAYTGPLVEDFLSSFQVVVLTNSPLEAQLRVGE

FCHSRGIKLVVADTRGLFGQLFCDFGEEMVLTDSNGEQPLSAMVSMVTK

DNPGVVTCLDEARHGFETGDFVSFSEVQGMIQLNGCQPMEIKVLGPYTF

SICDTSNFSDYIRGGIVSQVKVPKKISFKSLPASLVEPDFVMTDFAKYSRP

AQLHIGFQALHQFCALHNQPPRPRNEEDATEVGLAQAVNARSPPSVKQN

SLDEDLIRKLAYVAAGDLAPINAFIGGLAAQEVMKACSGKFMPIMQWLY

FDALECLPEDKEALTEEKCLPRQNRYDGQVAVFGSDFQEKLSKQKYFLV

GAGAIGCELLKNFAMIGLGCGEGGEVVVTDMDTIEKSNLNRQFLFRPWD

VTKLKSDTAAAAVRQMNPYIQVTSHQNRVGPDTERIYDDDFFQNLDGV
ANALDNIDARMYMDRRCVYYRKPLLESGTLGTKGNVQVVIPFLTESYSS
SQDPPEKSIPICTLKNFPNAIEHTLQWARDEFEGLFKQPAENVNQYLTDSK
FVERTLRLAGTQPLEVLEAVQRSLVLQRPQTWGDCVTWACHHWHTQY
CNNIRQLLHNFPPDQLTSSGAPFWSGPKRCPHPLTFDVNNTLHLDYVMA
AANLFAQTYGLTGSQDRAAVASLLQSVQVPEFTPKSGVKIHVSDQELQS
ANASVDDSRLEELKATLPSPDKLPGFKMYPIDFEKDDDSNFHMDFIVAAS
NLRAENYDISPADRHKSKLIAGKIIPAIATTTAAVVGLVCLELYKVVQGH
QQLDSYKNGFLNLALPFFGFSEPLAAPRHQYYNQEWTLWDRFEVQGLQP
NGEEMTLKQFLDYFKTEHKLEITMLSQGVSMLYSFFMPAAKLKERLDQP
MTEIVSRVSKRKLGRHVRALVLELCCNDESGEDVEVPYVRYTIR

Construction of Plasmids

Plasmid pETDuet-*w.t.*Ub

The plasmid pETDuet-*w.t.*Ub bearing the gene encoding *w.t.*Ub was constructed from the pETDuet-1 vector (Stratagene Inc.). In this plasmid, *w.t.*Ub is under the control of IPTG-inducible T7 promoter.

Protein Expression and Purification

***Wild-type* ubiquitin expression and purification**

To express *wild-type* ubiquitin (*w.t.*Ub), *Escherichia coli* (*E. coli*) BL21 cells were transformed with pETDuet-*w.t.*Ub plasmid. A starter culture was grown in 2×YT(Yeast Extract Tryptone) media (5 mL) with 100 μg/mL ampicillin at 37°C

overnight. The culture was then inoculated into 2×YT media (1 L) with the same concentration of ampicillin the next morning. Isopropyl β-D-1-thiogalactopyranoside (IPTG) (0.5 mM) was added into the cell culture after the OD600 reached 0.9~1.1. The cell culture was incubated at 37°C for 8 hours, and the cells were harvested by centrifugation at 4,000 r.p.m. for 30 min at 4°C and resuspended in 50 mL of lysis buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 10 mM imidazole, pH 7.4). The resuspended cells were sonicated in an ice/water bath for four times (3 min each, 10 min interval to cool down the suspension to below 10°C before the next run) and the lysate was clarified by centrifugation at 10,000 r.p.m. for 40 min at 4°C. The supernatant was then incubated in a 75°C water bath for 1 hour and then centrifuged at 10,000 r.p.m for 40 min at 4°C for further purification. The concentration of *w.t.*Ub protein was determined by BCA (bicinchoninic acid) protein assay kit from Thermo Fisher Scientific Inc. (Rockford, IL). According to the concentration, *wild-type* ubiquitin expression yield was around 160 mg/L from the 2×YT media.

Histidine tagged-mouse ubiquitin-activating enzyme (Histag-*m*E1) expression and purification

pET28b-*m*E1 plasmid was purchased from Addgene (Plasmid #32534). To express ubiquitin-activating enzyme (E1), *E. coli* BL21 (DE3) codon plus cells were transformed with pET28b-*m*E1. The expression was adapted from the procedure stated in Andreia's paper published in 2012.[83] A starter culture was grown in 2×YT media (5 mL) with 34 μg/mL chloramphenicol and 25 μg/mL kanamycin at 37°C overnight. The culture was then inoculated into 2×YT media (1 L) with the same concentrations of

antibiotics the next morning. The culture was cooled down to 16°C after the OD600 reached 0.9~1.1 and then added isopropyl β-D-1-thiogalactopyranoside (IPTG) (0.5 mM). The culture continued to grow for another 20 hours at 16°C. Then the cells were pelleted, resuspended in 50 mL of lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% (w/v) Triton X-100, 1 mM EDTA-NaOH, 1 mM dithiothreitol (DTT), 0.1 mg/mL phenylmethylsulfonyl fluoride (PMSF), and 1:500 (v/v) Sigma mammalian protease inhibitor mixture, pH 8.0), and then sonicated in an ice/water bath for four times (3 min each, 10 min interval to cool down the suspension to below 10°C before the next run). The lysate was clarified by centrifugation at 10,000 r.p.m. for 40 min at 4°C. The clarified supernatant was incubated with 2 mL of Ni Sepharose™ 6 Fast Flow beads from GE Healthcare (Little Chalfont, United Kingdom) for 20 min for the purpose of effectively binding and then poured into a 10-mL empty purification column to allow the Ni sepharose beads to settle completely by gravity. The Ni sepharose beads were then washed with 50 mL buffer A (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 7.8) to get rid of any unspecific binding proteins and subsequently eluted out with 12 mL of buffer B (50 mM NaH_2PO_4, 300 mM NaCl, 300 mM imidazole, pH 7.8). The eluted solution was concentrated by Amicon Ultra-15 centrifugal filter units - 30,000 NMWL from Millipore (Billerica, MA) to 1 mL by centrifugation at 4,000 r.p.m. at 4°C. The buffer was then changed to the stock buffer (10 mM Tris-HCl, 1 mM EDTA-NaOH, 1 mM DTT, pH 8.0) by dialysis. Recombinant histidine-tagged mouse E1 (*m*E1) protein at 10 mg/mL was divided into small aliquots, frozen in liquid nitrogen, and stored at -80°C. The concentration of E1 enzyme was determined by BCA protein assay kit from Thermo

Fisher Scientific Inc. (Rockford, IL). According to the concentration, *m*E1 expression yield was around 10 mg/L from the 2×YT media.

E1 Activity Assay

For E1 activity assay, aliquots of nickel affinity purified mouse E1 stored at -80°C were rapidly thawed and placed on ice. Thiol ester assays were performed in 20 μL (final volume) of the buffer (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 250 mM sucrose, 50 mM KCl, 3 mM $MgCl_2$, pH 7.4) in the presence of 5 mM ATP. Reactions contained 12 μM of *w.t.*Ub and 400 nM recombinant mouse E1. Incubation was done at 37°C for 30 min. Reactions were then treated with 10 mM *N*-ethylmaleimide (NEM) for 5 min on ice and then subjected to SDS-PAGE analysis under non-reducing conditions.

E1-Catalyzed Amidation Reaction for the Syntheses of Functional Ub Derivatives

Aliquots of purified mouse E1 stored at -80°C were rapidly thawed and placed on ice. For cysteamine, the reaction solution containing 250 μM *w.t.*Ub, 2 μM E1, 2 mM ATP, 5 mM cysteamine (pre-reduced by 5 mM *tris*(2-carboxyethyl)phosphine (TCEP)) was incubated in a 1 mL (final volume) buffer (50 mM HEPES, 250 mM sucrose, 50 mM KCl, 3 mM $MgCl_2$, pH 7.4) at 4°C overnight. For hydrazine and hydroxylamine, their reactions were performed in 1 mL (final volume) buffer (50 mM HEPES, 250 mM sucrose, 50 mM KCl, 3 mM $MgCl_2$, pH 7.4) with 2 μM mouse E1, 250 μM *w.t.*Ub, 2 mM ATP, and 50 mM hydrazine or hydroxylamine, respectively. The mixtures were incubated at 37°C for overnight. For the other three primary amines (2-azido-ethylamine,

propargylamine and allylamine), their reactions were performed in the 1 mL (final volume) buffer (10 mM *N*-cyclohexyl-3-aminopropanesulfonic acid (CAPS), 250 mM sucrose, 50 mM KCl, 3 mM $MgCl_2$, pH 9.4), and consisted of 250 μM Ub, 5 μM E1, 5 mM ATP, and 750 mM respective amines. The mixtures were incubated at 37°C overnight. The conversion of each reaction was determined by ESI-MS analysis.

Protein ESI-MS Analysis for Determining E1-Catalyzed Amidation Reaction Yield

An Agilent (Santa Clara, CA) 1200 capillary HPLC system was interfaced to an API QSTAR Pulsar Hybrid QTOF mass spectrometer (Applied Biosystems/MDS Sciex, Framingham, MA) equipped with an electrospray ionization (ESI) source. The mass spectrometry (MS) data were acquired in positive ion mode (500-2,000 Da) using spray voltage of +2,700 V. BioAnalyst software (Applied Biosystems) was used for spectral deconvolution. A mass range of m/z 500-2,000 was used for deconvolution and the output range was 8,000-9,000 Da using a step mass of 0.1 Da and an S/N threshold of 2.

Results and Discussion

First of all, *wild-type* ubiquitin and mouse ubiquitin-activating enzyme (*m*E1) were expressed in *E.coli* cells and further purified by respective purification methods described in the experimental details. The purity of the proteins was judged by SDS-PAGE analysis shown in **Figure 6**. With the purified *wild-type* ubiquitin and mouse ubiquitin-activating enzyme (*m*E1), we further performed a thiol ester assay to test whether the recombinant mouse E1 produced in *E. coli* cells was active. In this assay, E1 activity was assessed by the capacity of the enzyme to form a thiol ester conjugate with ubiquitin in an ATP-dependent process. As shown in **Figure 7**, when the nickel affinity purified mouse E1 was incubated with *wild-type* ubiquitin in the presence of ATP, a species migrating slower than the E1 protein was formed (lanes 3, Ub + E1) under non-reducing SDS-PAGE conditions. It was noteworthy that essentially all E1 enzyme was able to form a thiol ester bond with *wild-type* ubiquitin, indicating that the recombinant E1 enzyme was fully active.

After confirmation of E1 enzyme activity, we further tested the hypothesized E1-catalyzed Ub C-terminal amidation reaction starting with hydrazine as a primary amine. After 2 μM mouse E1, 250 μM *wild-type* ubiquitin, 2 mM ATP, and 50 mM hydrazine were incubated at pH 7.4 and 37°C for overnight, *w.t.*Ub was almost quantitatively converted to a Ub-hydrazine conjugate. It displayed a peak at 8,578 Da detected by electrospray ionization mass spectrometry (ESI-MS) that agreed well with its theoretic molecular weight (8,578 Da) (**Figure 9**), and little *w.t.*Ub was left as unreacted with the presence of a tiny peak at 8,564 Da (**Figure 8**&**9**). With this initial success, we

embarked on testing E1-catalyzed Ub C-terminal amidation reaction with other amines including hydroxylamine, cysteamine, azidoethylamine, propargylamine, and allylamine. By optimizing reaction conditions (pH, temperature, amine concentrations, *etc.*), we successfully converted *w.t.*Ub to all five corresponding Ub-amine conjugates with yields higher than 90%. Hydroxylamine was similar to hydrazine which could be successfully appended to Ub C-terminus using the same reaction condition as hydrazine. Cysteamine reacted efficiently at 5 mM due to the existence of the β-thiol group. At physiological pH, the thiol group could perform the transthioesterification reaction with Ub-E1 thioester bond to form a Ub-cysteamine conjugate with a thioester linkage that could subsequently undergo S to N acyl transfer to regenerate the free thiol group as well as forming a stable amide linkage between Ub and cysteamine.[90,91] This process resembled the cysteine-mediated native chemical ligation (NCL) reaction,[92] eliciting a higher reactivity of cysteamine. On the contrary, for the other three primary amines (2-azido-ethylamine, propargylamine, and allylamine), the concentration of amines needed to be more than 500 mM for efficient conversion and the reactions were conducted with a pH close to 9 to maintain high levels of alkaline forms of primary amines for efficient nucleophilic attacks to the thioester bond of Ub-E1 fusion protein to generate the inert amide bond directly. With elevated concentrations of both nucleophiles and E1, efficiencies for formation of Ub-amine conjugates from 2-azido-ethylamine, propargylamine, and allylamine were achieved to levels similar as cysteamine.

The theoretical molecular weights of *wild-type* Ub, Ub-hydrazine, Ub-hydroxylamine, Ub-cysteamine, Ub-allylamine, Ub-propargylamine and Ub-

azidoethylamine were 8,564, 8,578, 8,579, 8,623, 8,603, 8,601 and 8,632 Da, respectively (Table 1). The ESI-MS analysis confirmed the successful syntheses of all six corresponding Ub-amine conjugates with yields higher than 90% (**Figure 8-14**).

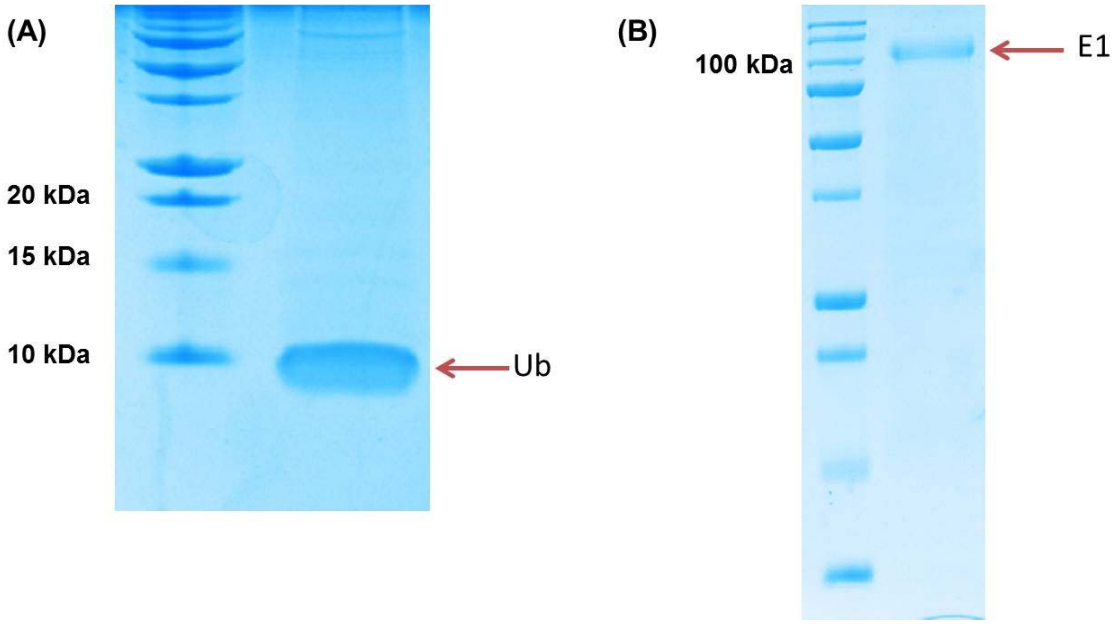

Figure 6. Coomassie-stained SDS-PAGE analyses of *wild-type* ubiquitin (A) and mouse ubiquitin-activating enzyme (*m*E1) (B). (A) *w.t.*Ub was expressed in *E. coli* BL21 cells. (B) *m*E1 was expressed in *E. coli* BL21(DE3) codon plus cells and purified with Ni Sepharose™ 6 Fast Flow beads from GE Healthcare.

Figure 7. Coomassie-stained SDS-PAGE analysis of E1 activity assay under non-reducing (no DTT) conditions. *w.t.*Ub, *m*E1, and reaction mixture of *w.t.*Ub and *m*E1 were indicated. Their theoretical molecular weights were around 8.5, 120, and 129 kDa, respectively.

Table 1. List of theoretical molecular weights (MW) and detected molecular weights by ESI-MS for *w.t.*Ub and Ub-amine conjugates.

*w.t.*Ub/Ub-amine conjugates	Theoretical MW (Da)	Detected MW (Da)
*w.t.*Ub	8,564	8,565
Ub-hydrazine	8,578	8,579
Ub-hydroxyamine	8,579	8,580
Ub-cysteamine	8,623	8,623
Ub-allylamine	8,603	8,604
Ub-propargylamine	8,601	8,601
Ub-azidoethylamine	8,632	8,633

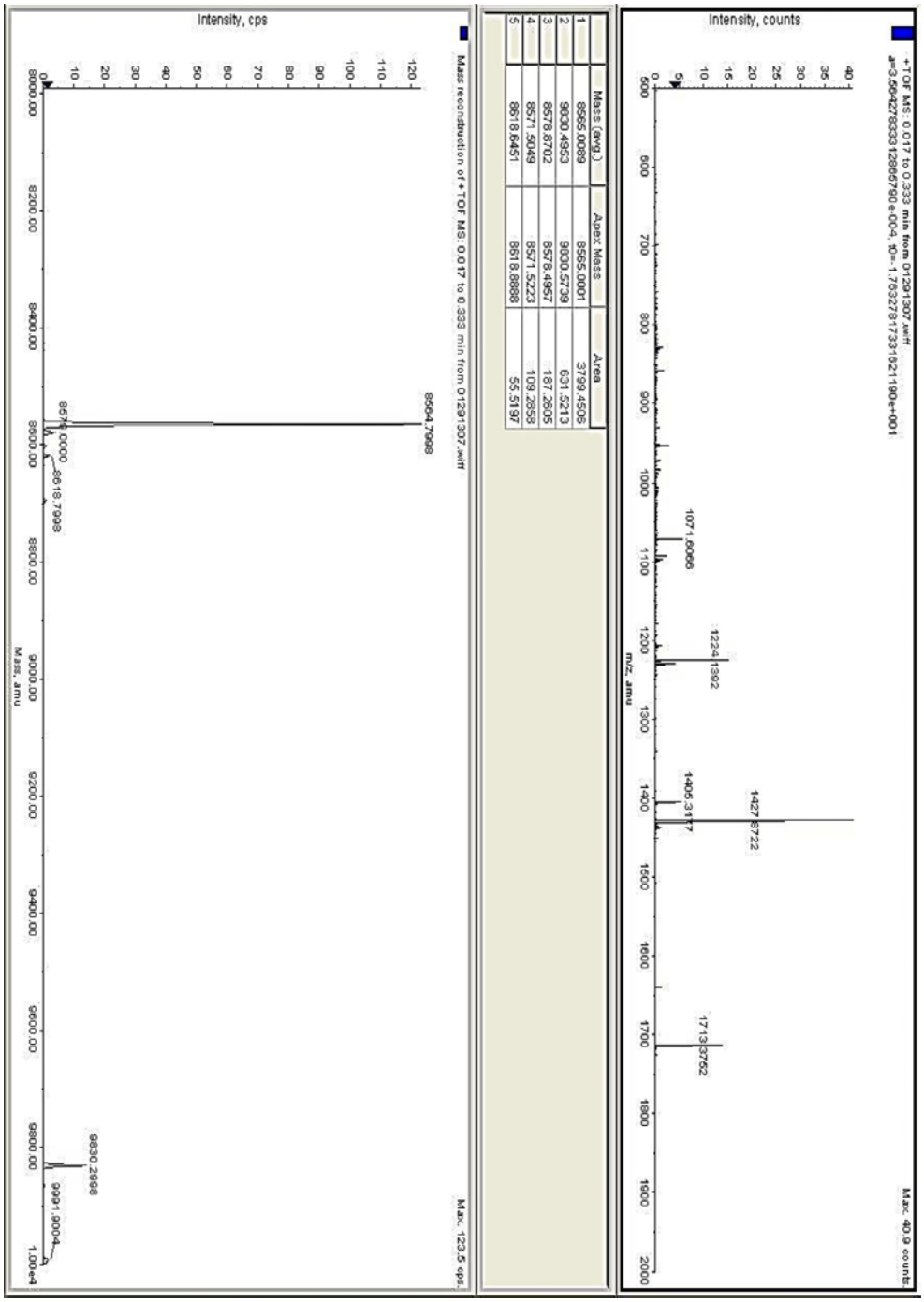

Figure 8. Deconvoluted ESI-MS spectra of *wild-type* ubiquitin (*w.t.*Ub). The theoretical molecular weight of *wild-type* Ub was 8,564 Da. The detected molecular weight of *wild-type* Ub was 8,565 Da.

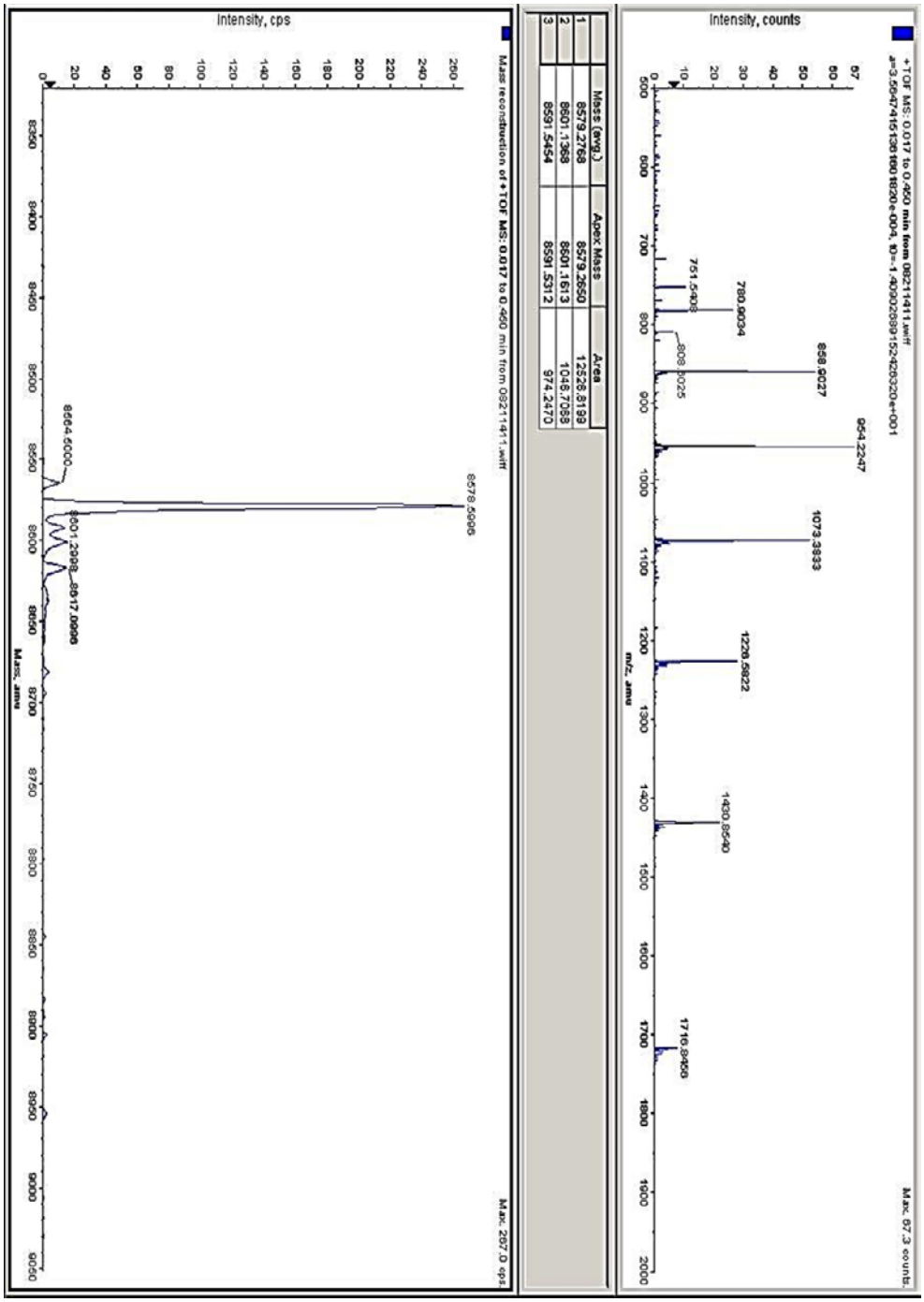

Figure 9. Deconvoluted ESI-MS spectra of Ub-hydrazine. The theoretical molecular weight of Ub-hydrazine was 8,578 Da. The detected molecular weight of Ub-hydrazine was 8,579 Da.

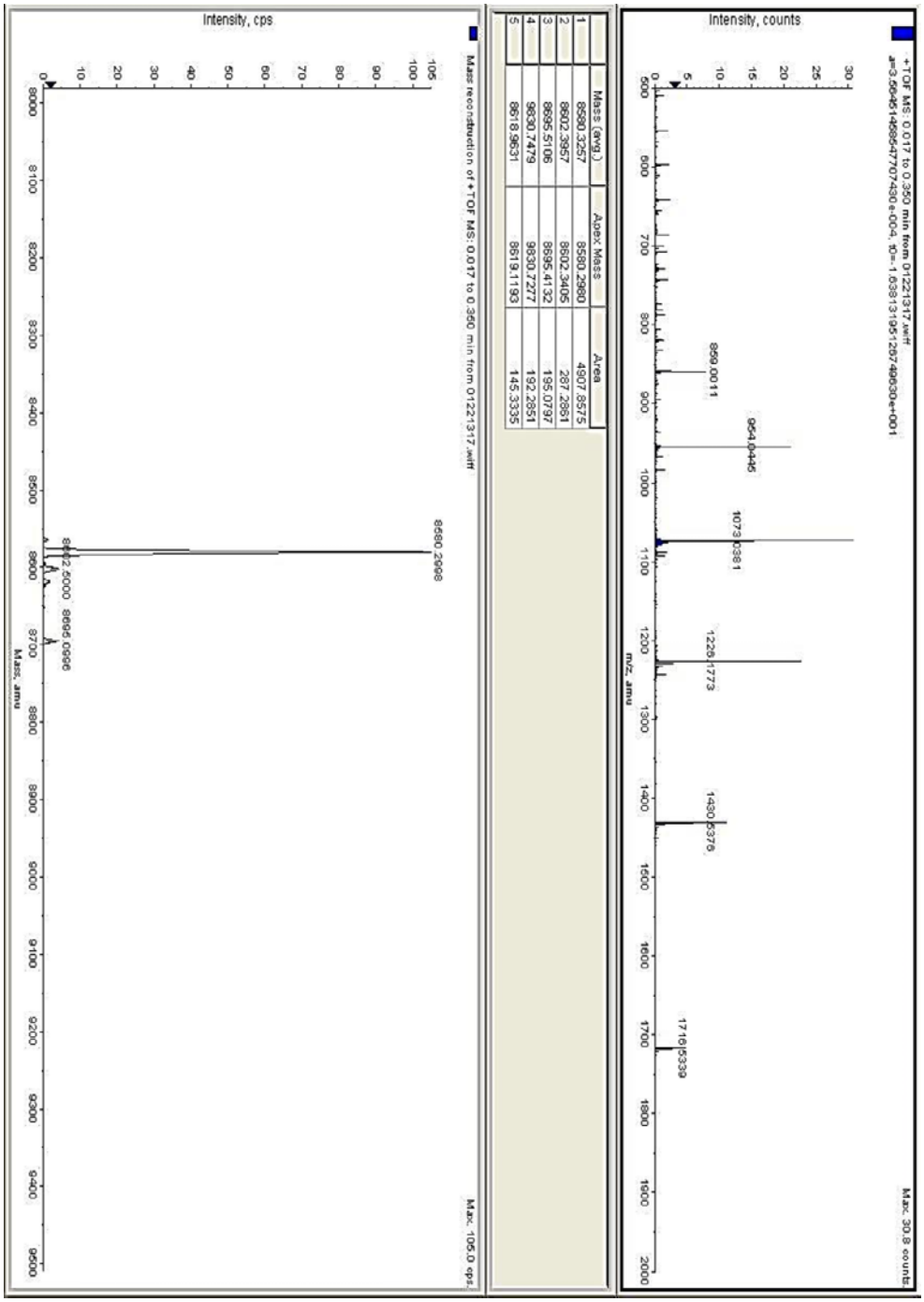

Figure 10. Deconvoluted ESI-MS spectra of Ub-hydroxylamine. The theoretical molecular weight of Ub-hydroxylamine was 8,579 Da. The detected molecular weight of Ub-hydroxylamine was 8,580 Da.

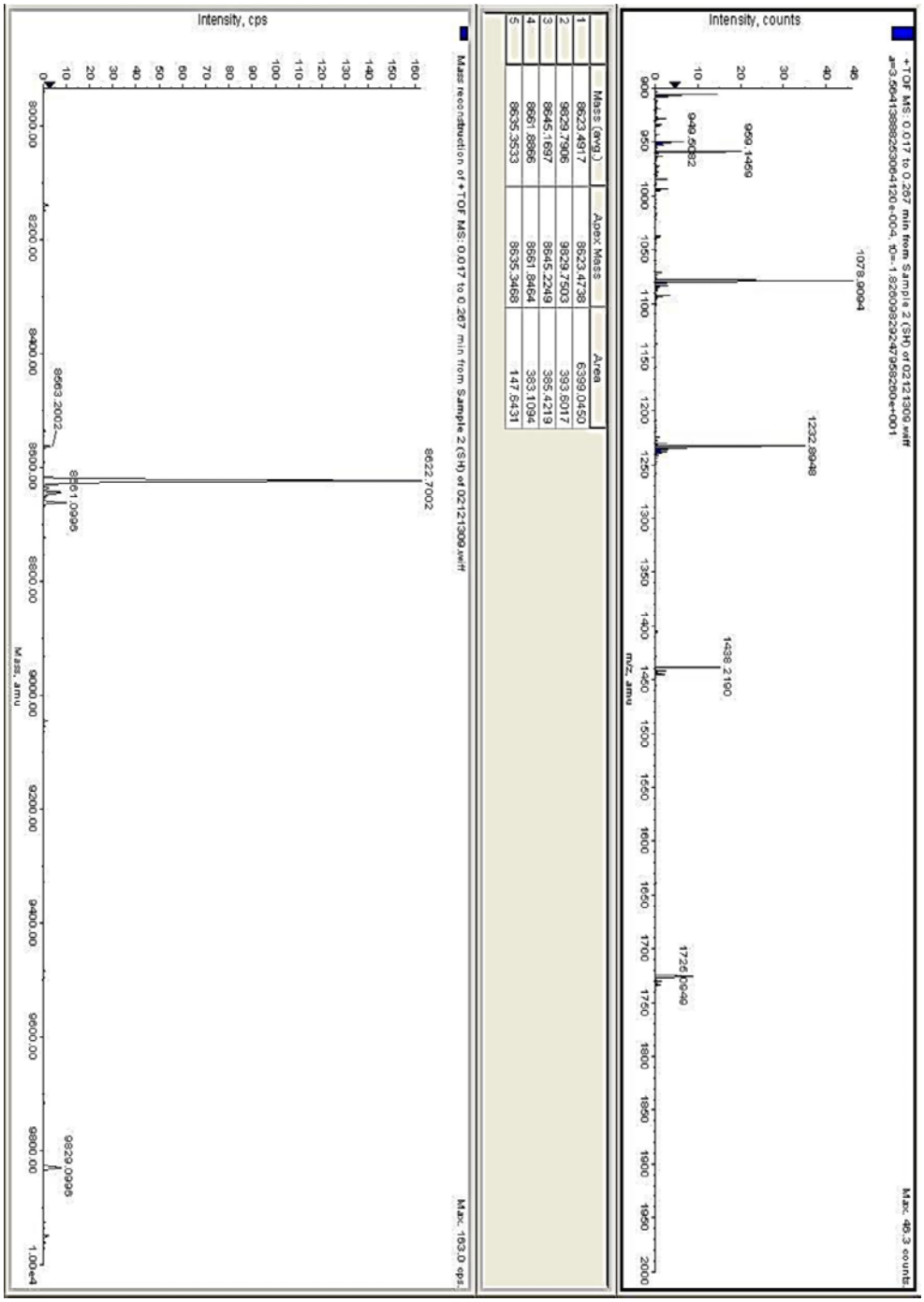

Figure 11. Deconvoluted ESI-MS spectra of Ub-cysteamine. The theoretical molecular weight of Ub-cysteamine was 8,623 Da. The detected molecular weight of Ub-cysteamine was 8,623 Da.

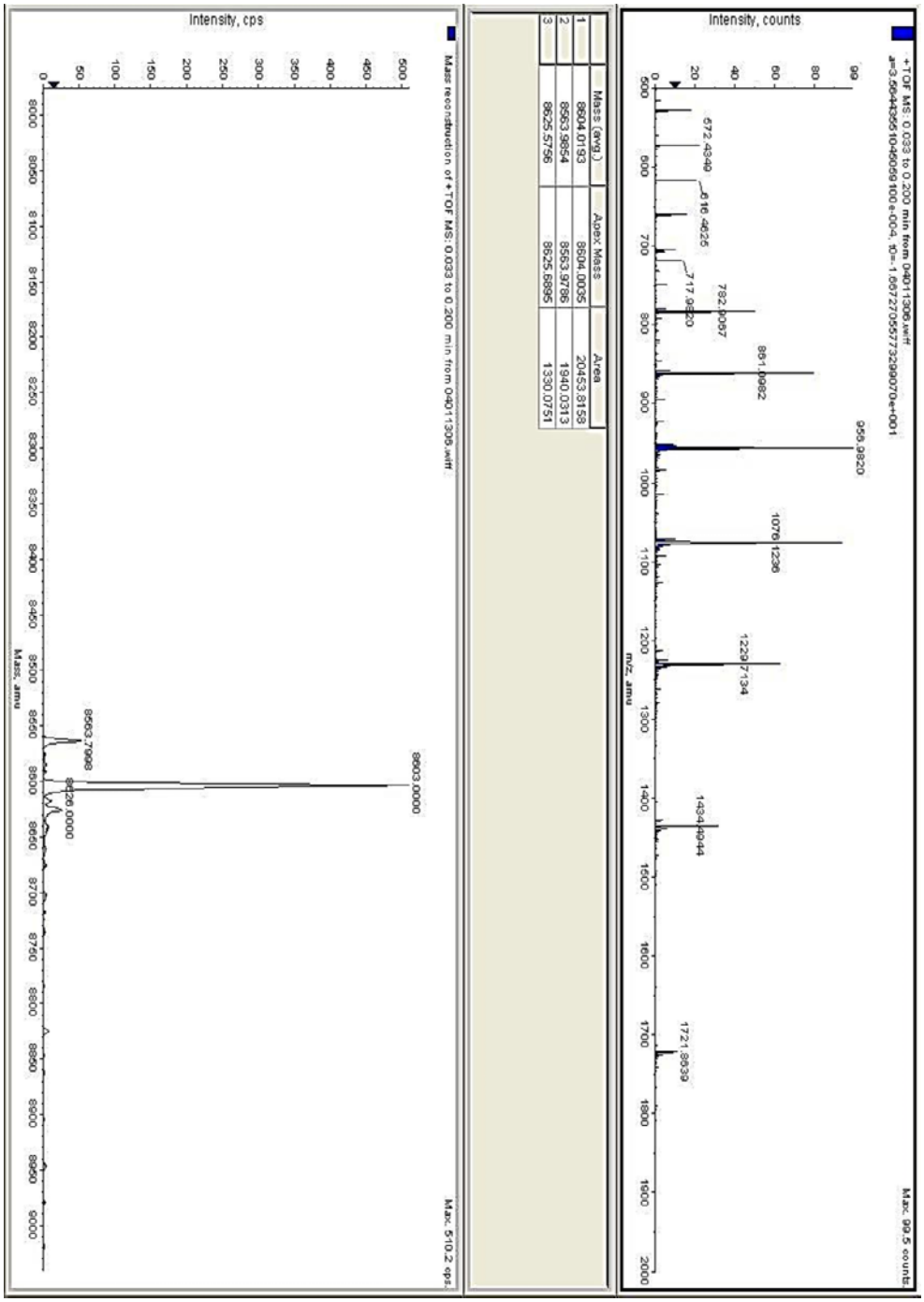

Figure 12. Deconvoluted ESI-MS spectra of Ub-allylamine. The theoretical molecular weight of Ub-allylamine was 8,603 Da. The detected molecular weight of Ub-allylamine was 8,604 Da.

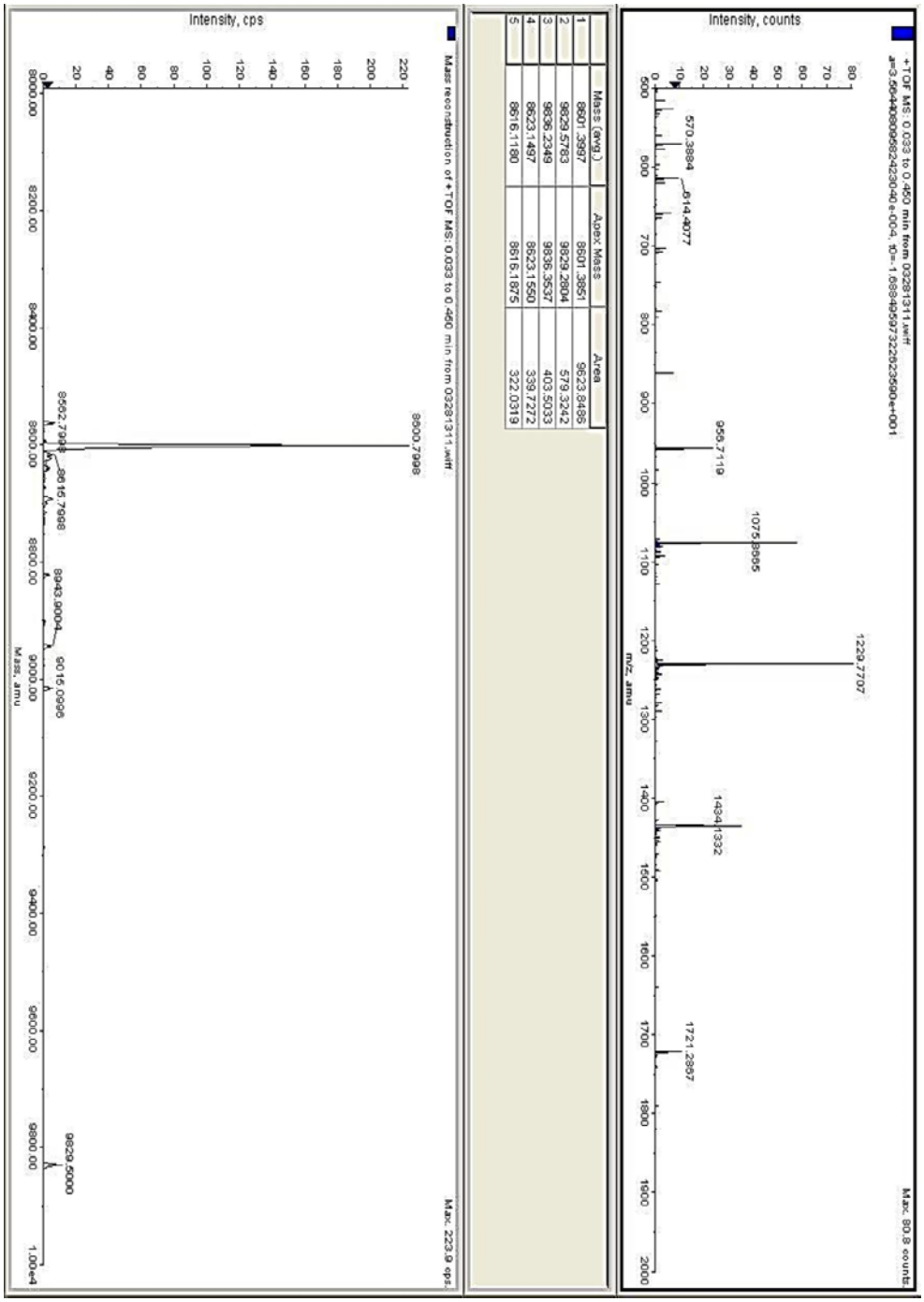

Figure 13. Deconvoluted ESI-MS spectra of Ub-propargylamine. The theoretical molecular weight of Ub-propargylamine was 8,601 Da. The detected molecular weight of Ub-propargylamine was 8,601 Da.

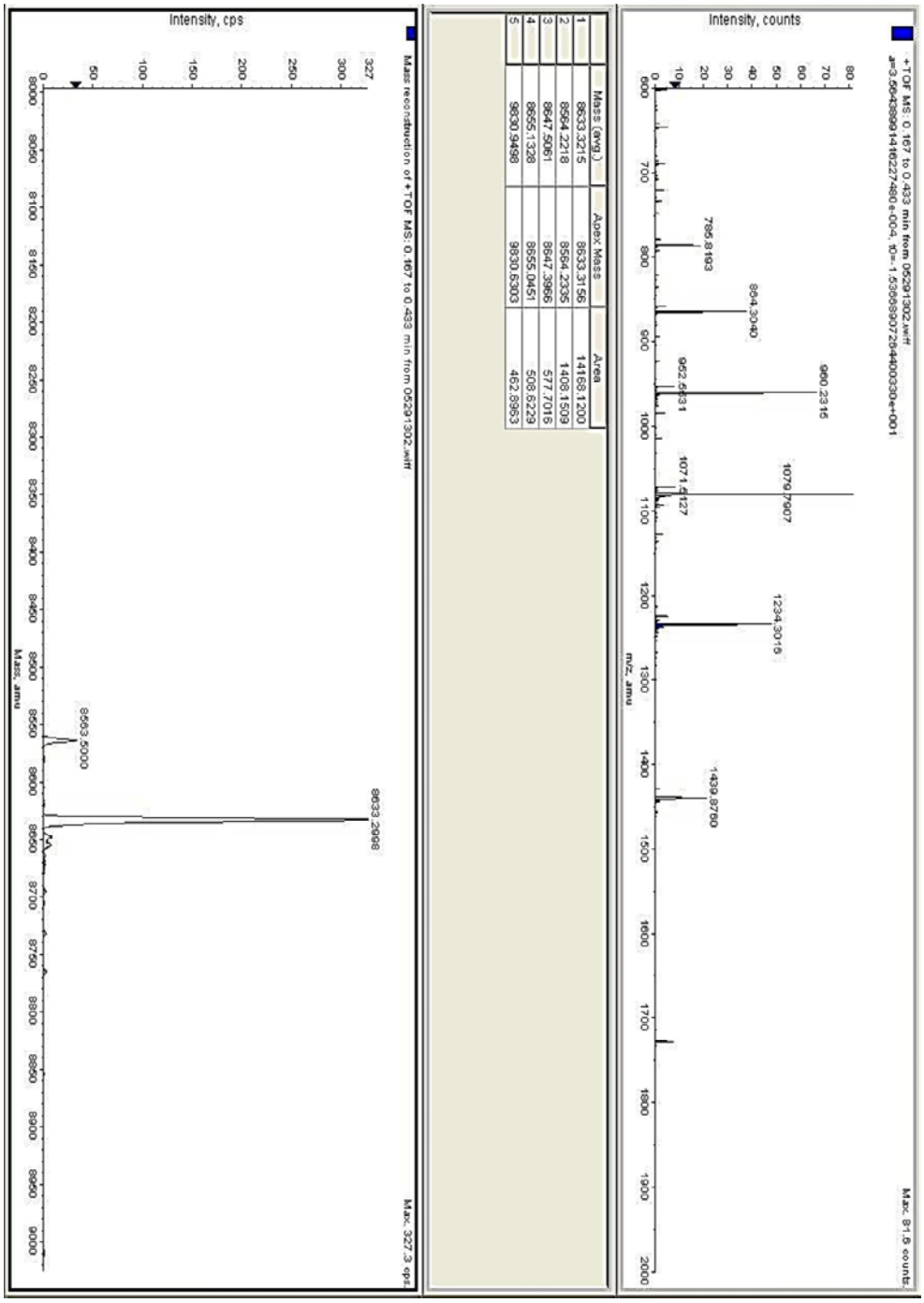

Figure 14. Deconvoluted ESI-MS spectra of Ub-azidoethylamine. The theoretical molecular weight of Ub-azidoethylamine was 8,632 Da. The detected molecular weight of Ub-azidoethylamine was 8,633 Da.

Conclusions and Outlook

In summary, by virtue of natural ubiquitination process, we took advantage of the high reactivity and universality of ubiquitin-activating enzyme (E1) to install various functional groups onto the Ub C-terminus by an amidation reaction, which provided a novel straightforward approach to generate functional ubiquitin conjugates that could be further modified into various types of DUB substrates for DUBs functional studies and high-throughput screening (HTS) of DUBs inhibitors. The simple nature of the E1-catalyzed ubiquitin C-terminal amidation process, high efficiency of the reaction itself, and the easy expression of both *wild-type* Ub[93] and E1enzyme[83] in *E. coli* cells allowed efficient large-scale production of multiple Ub-amine conjugates. We could routinely obtain Ub-amine conjugates to half-gram scales. Another important advantage of this E1-catalyzed amidation reaction was the robust and straightforward chemistry used to append Ub C-terminus with multiple unique functionalities, allowing a regular biochemistry and molecular biology research group to readily adopt this simple method. We believed that the application of this method would greatly accelerate studies of DUBs that play pivotal roles in numerous cellular pathways but whose investigations were greatly hampered by the difficulty in the accessibility of suitable DUB substrates.

Previously, the intein fusion method to recombinantly express Ub(1-75)-intein and SUMO(ΔGG)-intein thioesters followed by exchanging them with primary amines was widely used to append functional groups to Ub and SUMO (Small ubiquitin-like modifier) in which one or two C-terminal glycine(s) were removed.[94,95] Although the same approach could be applied to append functional groups to full-length Ub, the

relative low yield of intein fusion expression, the labile nature of the Ub-thioester, and the stoichiometric nature of the exchange reaction with amines would lead to low yields. In comparison to the intein fusion approach, this E1-catalyzed amidation reaction method was much more robust and practical.

Recently, other ubiquitin-like (Ubl) proteins which comprise a diverse group of evolutionarily conserved small proteins that are covalently conjugated to target proteins, thereby regulating their function, have been discovered. Although they vary in their degree of primary sequence similarity to ubiquitin, they all seem to be conjugated through a comparable enzymatic cascade: Ubl proteins are initially activated through adenylation of their C-terminal glycine-glycine motif and subsequently form a thioester bond with their specific activating E1 enzymes; then they are transferred to their respective conjugating E2 enzymes by transthioesterification reaction and are finally conjugated to ε-amino groups of lysine residues in specific target proteins with the help of ligating E3 enzymes.[96] Hence, we inferred that this E1-catalyzed amidation reaction could also be utilized to functionalize the C-termini of other Ubl proteins, which would lead to the syntheses of various functional Ubl-amine conjugates for the activity studies of their respective deconjugating enzymes. Since the small ubiquitin-like modifier (SUMO) is one of the most studied among nearly 20 types of discovered Ubl proteins so far besides ubiquitin, and has been found to be involved in diverse cellular processes (including transcription, replication, chromosome segregation and DNA repair);[44,45] also, the expression and purification procedure for SUMO-specific activating E1 enzyme (Uba2/Aos1 complex) in *E.coli* was reported recently,[97] therefore, this straightforward

E1-catalyzed amidation reaction method could be potentially applied to functionalize SUMO C-terminus as well to synthesize functional SUMO-amine conjugates for the activity study of SUMO isopeptidases (SENPs).

CHAPTER III

SYNTHESES OF FLUORESCENT UBIQUITIN CONJUGATES AS DEUBIQUITINASE SUBSTRATES FOR KINETIC STUDY*

Introduction

Protein ubiquitination is defined as the process in which a single ubiquitin (Ub) entity is tagged to one or multiple lysine sites through a concerted three-step enzymatic cascade recruiting three types of enzymes: Ub-activating enzyme (E1), Ub conjugating enzyme (E2), and Ub ligase enzyme (E3).[9,10,23,29,98] The reversed process can be achieved by deubiquitinases (or deubiquitinating enzymes, DUBs).[15] To date, it has been found that the human genome encodes around 100 DUBs, which can be divided into six structurally unrelated families (USP, UCH, OTU, MJD, MCPIP, and JAMM) for specifically deconjugating Ub from target proteins. The activities of these enzymes are crucial for cell homeostasis, protein stability, and a wide range of signaling pathways, while their misregulations have been implicated in the pathogenesis of many human diseases, including chronic inflammatory diseases and various types of cancer; thus, DUBs, as an emerging potential drug targets, have attracted increasing interest from not only researchers but also the pharmaceutical industry.[48,55,99,100]

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* Reprinted (adapted) with permission from "E1-Catalyzed Ubiquitin C-Terminal Amidation for the Facile Synthesis of Deubiquitinase Substrates" by Wang, X. A.; Kurra, Y.; Huang, Y.; Lee, Y. J.; Liu, W. R. *Chembiochem* **2014**, *15*, 37-41.

The mounting interest in DUBs has promoted DUB studies as well as DUB inhibitor discoveries.[81] One prerequisite of DUB research is the preparation of large amounts of DUB assay reagents such as fluorescent Ub-based substrates and multiple polyubiquitin chains for *in vitro* analysis.[15] Fluorogenic ubiquitin molecules that can serve as DUB substrates have been made available from several commercial providers.[101,102] The most popular substrate, Ub-AMC is the fusion protein of the ubiquitin carboxyl terminus to 7-amino-4-methylcoumarin (AMC) with an amide linkage. The major drawback of Ub-AMC is that some DUBs fail to recognize and cleave the relatively bulky and hydrophobic 7-amino-4-methylcoumarin group.[103] Another downside of most commercially available fluorogenic ubiquitin substrates is the fact that they don't have the isopeptide bond found in native ubiquitinated proteins. Fluorescent ubiquitin substrates with a native isopeptide linkage were recently synthesized through native chemical ligation in coordination with organic synthesis.[81,104] However, the relatively complicated synthetic routes prevented widespread adoption of this approach. The limited availability of suitable DUB substrates, to a large extent, hampered the development of DUB research for a long time. To facilitate functional investigations of DUBs and accelerate drug discovery for DUB-targeted disease intervention, we developed a simple but readily adoptable approach to functionalize the ubiquitin C-terminus for further syntheses of various fluorescent ubiquitin conjugates that can serve as DUB substrates.

In preliminary studies, we successfully employed ubiquitin-activating enzyme (E1) to catalyze a ubiquitin C-terminal amidation reaction for the installation of six

unique functionalities at the ubiquitin C-terminus. Theoretically, three of them (thiol, azide, and alkyne) could further undergo covalent modifications with respective fluorophores to be selectively labeled and yield fluorescent ubiquitin conjugates. For Ub-cysteamine, since *wild-type* ubiquitin naturally doesn't contain a cysteine residue itself, its thiol group could be exclusively targeted by molecules with chemical entities such as haloacetamide and maleimide.[105,106] While for Ub-propargylamine and Ub-azidoethylamine, they contained a terminal alkyne or azide group, which could undergo the Cu (I)-catalyzed azide-alkyne Huisgen cycloaddition reaction.[107,108] Thus, all these three ubiquitin-amine conjugates could be selectively labeled with respective fluorescent dyes, while *wild-type* ubiquitin itself would not react with those dyes under the same reaction conditions.[109-111]

For these three fluorescent ubiquitin conjugates, they could serve as substrates for the study of DUBs activity by following the time-dependent fluorescence polarization change after treatment with DUBs. The principle of fluorescence polarization derives from the fact that the degree of polarization of a fluorophore is inversely related to its molecular rotation. For a fluorophore that is covalently attached to a small molecule, when it is excited by polarized light, it would emit predominantly depolarized light, whereas for a fluorophore that is bound to a high molecular weight molecule such as ubiquitin, the emitted light would be much less depolarized.[112-115] During DUB-catalyzed deconjugation reaction, fluorophores conjugated to ubiquitin C-terminus would be released as small molecules, leading to a fluorescence polarization decrease (**Figure 15**). By following the fluorescence polarization change with time,

DUB cleavage activities on these substrates could be monitored effectively. Therefore, we applied the E1-catalyzed Ub C-terminus amidation reaction to the facile syntheses of three novel fluorescent ubiquitin conjugates which could serve as deubiquitinases substrates via a fluorescence polarization-based assay to investigate DUBs enzyme kinetics.

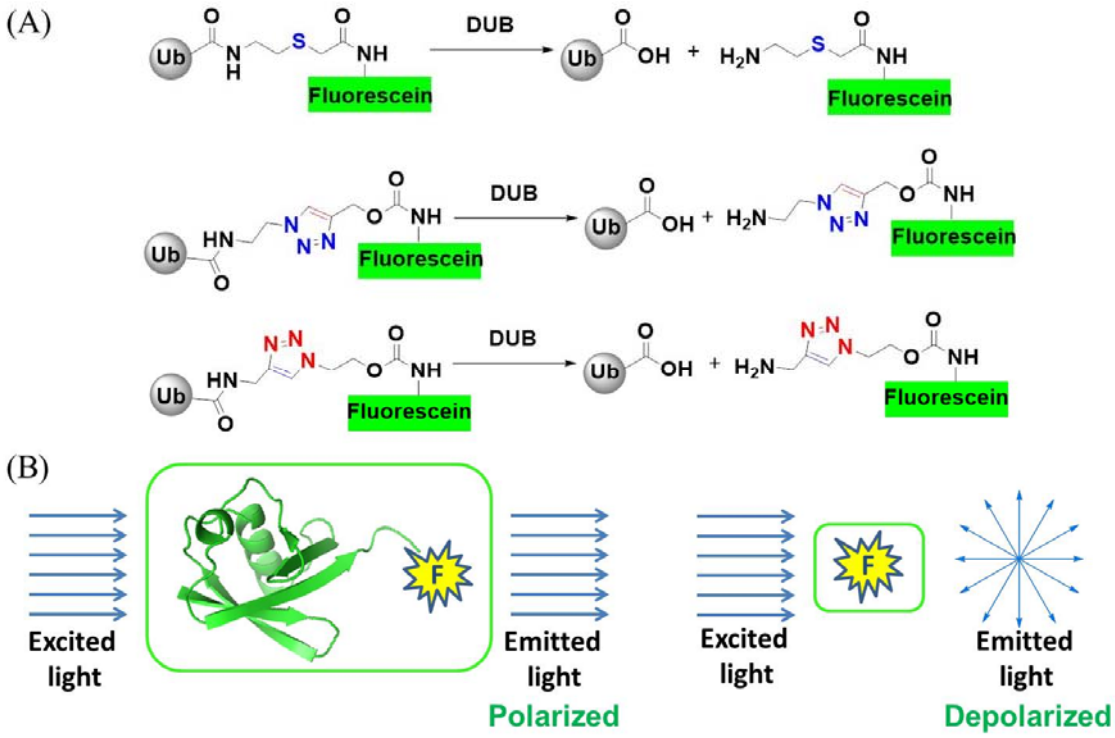


Figure 15. (A) Scheme of deconjugation reactions of fluorescent ubiquitin conjugates catalyzed by DUBs. (B) Fluorescence polarization comparison between fluorescent ubiquitin conjugates and fluorophore molecules.

Experimental Details

Chemical Synthesis

Synthesis of 2-azidoethanol (6)[109]

To a solution of 2-chloroethanol (**5**, 25.2 g, 0.31 mol) in water (80 mL), sodium azide (26.3 g, 0.40 mol) and tetrabutylammonium bromide (2.0 g, 6.2 mmol) were added, and the mixture was stirred at room temperature for 2 hours before being heated at 120°C for 2 hours. Sodium chloride was added to the cooled yellow solution until saturation, and the mixture was extracted with ethyl acetate (60 mL×3). The combined organic layers were dried (anhydrous $MgSO_4$), filtered, and evaporated to give a crude yellow oil (31 g). Distillation under an oil pump-generated vacuum (~0.1 mmHg, bp 87-90°C) afforded **6** (14.9 g, 55%) as a colorless oil. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 3.80-3.78 (m, 2 H), 3.46 (t, 2 H, J = 5.0 Hz), 1.88 (bs, 1 H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 61.7, 53.7.

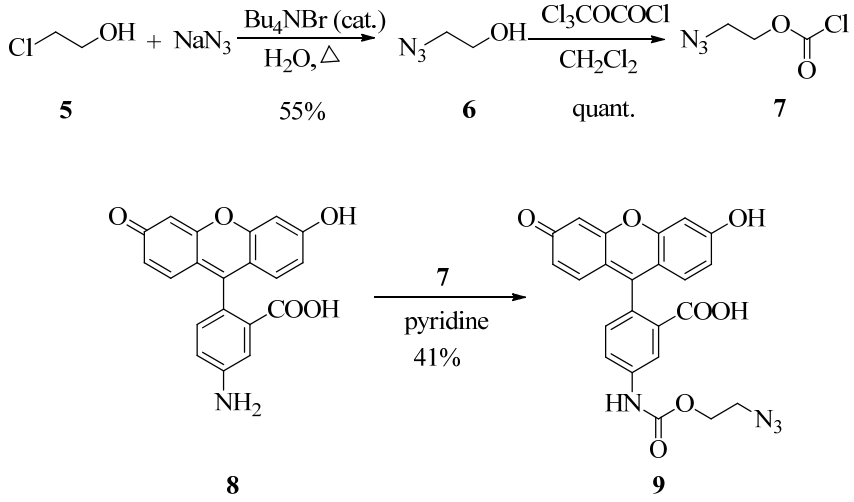
Synthesis of 2-azidoethyl chloroformate (7)[109]

To a solution of **6** (82 mg, 0.94 mmol) in anhydrous dichloromethane (0.5 mL), trichloromethyl chloroformate (0.13 mmol, 1.1 mmol) was added, and the mixture was stirred at room temperature for 12 hours. The volatiles were evaporated to leave crude **7** (0.14 g, quant.) as a yellow oil which was used without further purification. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 4.43 (t, 2 H, J = 5.2 Hz), 3.63 (t, 2 H, J = 5.0 Hz).

Synthesis of 5-((2'-azidoethyl)oxycarbonylamino) fluorescein (9)[109]

To a solution of fluorescein amine isomer I (**8**, 0.20 g, 0.58 mmol) (Aldrich) in pyridine (2.0 mL, 24.7 mmol) cooled in an ice/water bath, **7** (0.14 g, 0.94 mmol) was

added dropwise over 5 min with the aid of a small amount of anhydrous dichloromethane (0.1 mL). The mixture was then stirred at room temperature for 48 hours, and water (5 mL) was added. The mixture was diluted in ethyl acetate (50 mL), washed with water (10 mL), hydrochloric acid (0.1 N, 10 mL) and brine (10 mL), dried (Na_2SO_4), evaporated, chromatographed (EtOAc/hexanes, 1:1), and crystallized in ethyl acetate/hexanes to give **9** (0.11 g, 41%) as an orange solid. ^{1}H NMR (DMSO-d_6, 500 MHz) δ 10.32 (s, 1 H), 10.12 (s, 2 H), 8.12 (s, 1 H), 7.78 (dd, 1 H, *J* = 8.5, 1.5 Hz), 7.20 (d, 1 H, *J* = 8.5 Hz), 6.66 (d, 2 H, *J* = 2.0 Hz), 6.59 (d, 2 H, *J* = 8.5 Hz), 6.54 (dd, 2 H, *J* = 8.7, 2.2 Hz), 4.31 (t, 2 H, *J* = 4.7 Hz), 3.65 (t, 2 H, *J* = 5.2 Hz) (**Figure 16**); ^{13}C NMR (DMSO-d_6, 75 MHz) δ 168.6, 159.5, 153.3, 151.9, 146.2, 140.7, 129.1, 127.1, 125.7, 124.6, 112.6, 112.4, 109.7, 102.2, 83.3, 63.1, 49.7 (**Figure 17**); HRMS (ESI) calcd for $C_{23}H_{17}N_4O_7$ ($[M+H]^+$) 461.1097, found 461.1094.



Scheme 2. Synthetic route of 5-((2'-azidoethyl)oxycarbonylamino) fluorescein (**9**).[109]

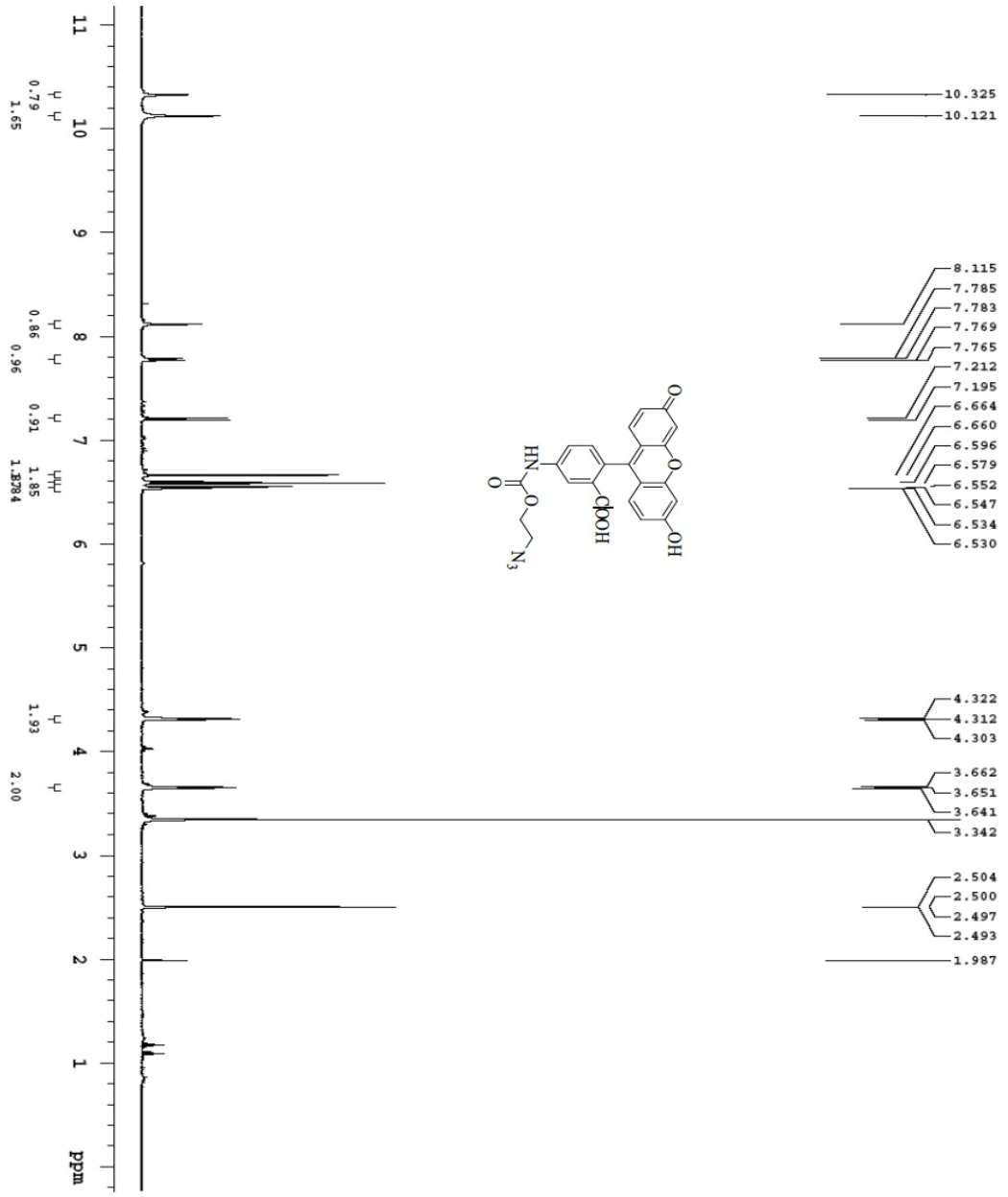


Figure 16. [1]H NMR spectrum for 5-((2'-azidoethyl)oxycarbonylamino) fluorescein (**9**).[109*]

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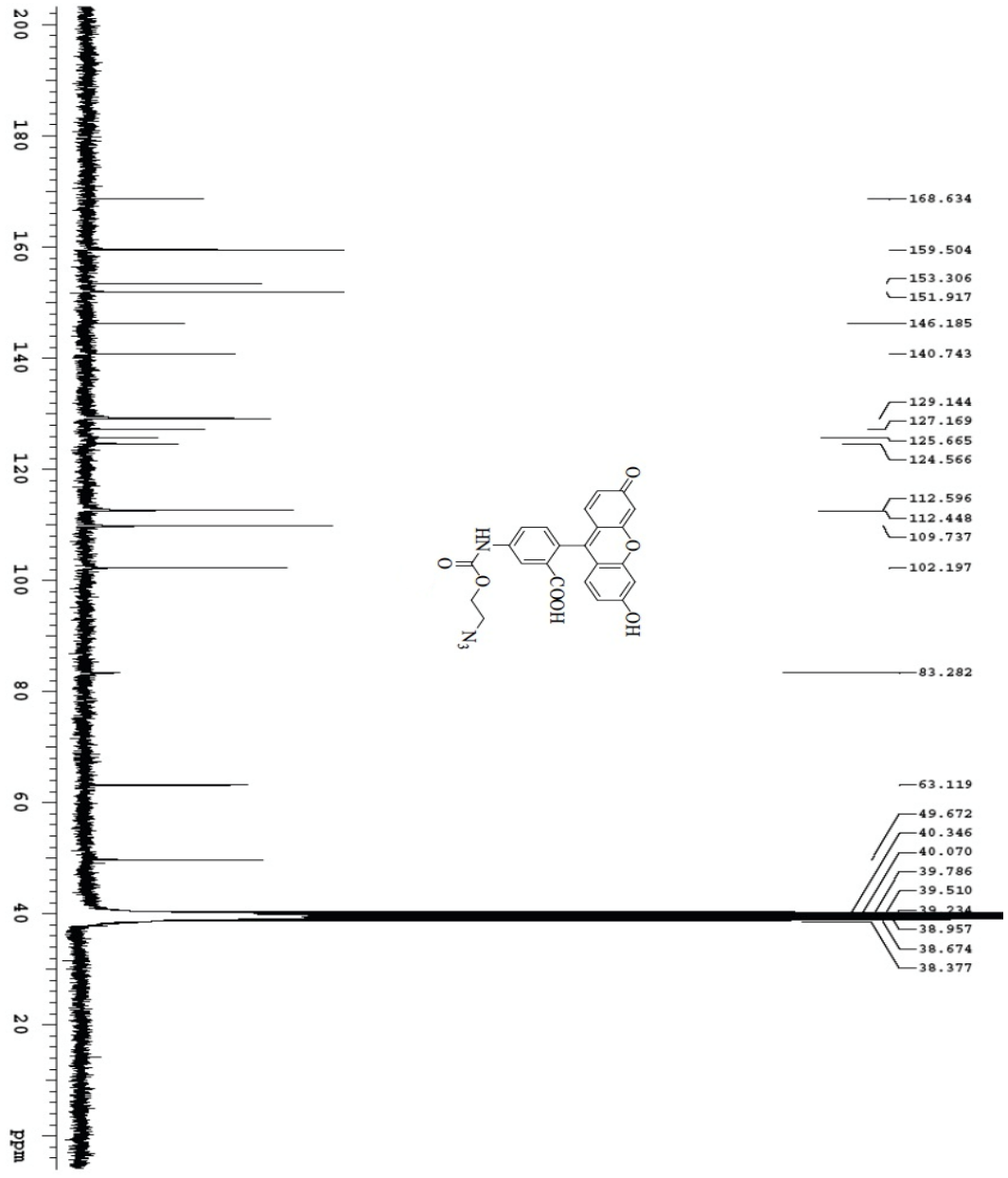

Figure 17. ^{13}C NMR spectrum for 5-((2'-azidoethyl)oxycarbonylamino) fluorescein (**9**).[109*]

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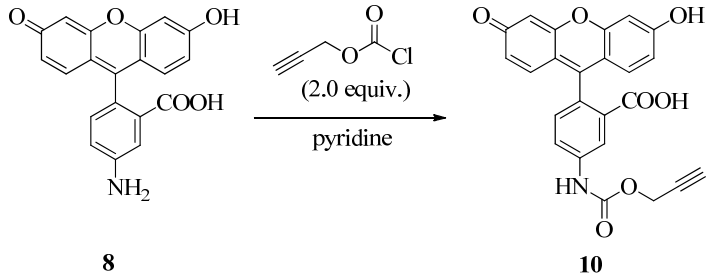
Synthesis of 5-(propargyloxycarbonylamino) fluorescein (10)[111]

To a solution of fluoresceinamine isomer I (**8**, 0.102 g, 0.29 mmol) (Aldrich) in anhydrous pyridine (1.0 mL) cooled in an ice bath, propargyl chloroformate (58 μL, 0.59 mmol) was added dropwise, and the mixture was stirred at room temperature for 30 hours. Ethyl acetate (50 mL) was then added, and the solution was washed with water (10 mL), saturated sodium bicarbonate (10 mL), hydrochloric acid (1 N, 10 mL) and brine (10 mL), dried (Na_2SO_4), evaporated, and flash chromatographed (EtOAc/hexanes, 1:1) to give a yellow oil, which was dissolved in a minimal amount of ethyl acetate (~0.5 mL) and precipitated with excessive hexanes (~10 mL) to afford **10** (66 mg, 52%) as a bright orange solid. R_f = 0.55 (EtOAc/hexanes, 2:1); ^{1}H NMR (CD_3OD, 500 MHz) δ 8.17 (s, 1 H), 7.76 (dd, 1 H, *J* = 8.5, 2.0 Hz), 7.13 (d, 1 H, *J* = 9.0 Hz), 6.67 (d, 2 H, *J* = 2.5 Hz), 6.65 (d, 2 H, *J* = 9.0 Hz), 6.54 (dd, 2 H, *J* = 8.5, 2.5 Hz), 4.82 (d, 2 H, *J* = 2.5 Hz), 2.97 (t, 1 H, *J* = 2.5 Hz) (**Figure 18**); ^{13}C NMR (CD_3OD, 75 MHz) δ 171.6, 162.3, 155.0, 154.6, 142.3, 130.5, 126.7, 126.2, 114.9, 114.1, 111.9, 103.6, 79.2, 76.4, 53.6 (**Figure 19**); HRMS (ESI) calcd for $C_{24}H_{16}NO_7$ ($[M + H]^+$) 430.0927, found 430.0931.



8 **10**

Scheme 3. Synthetic route of 5-(propargyloxycarbonylamino) fluorescein (**10**).[111]

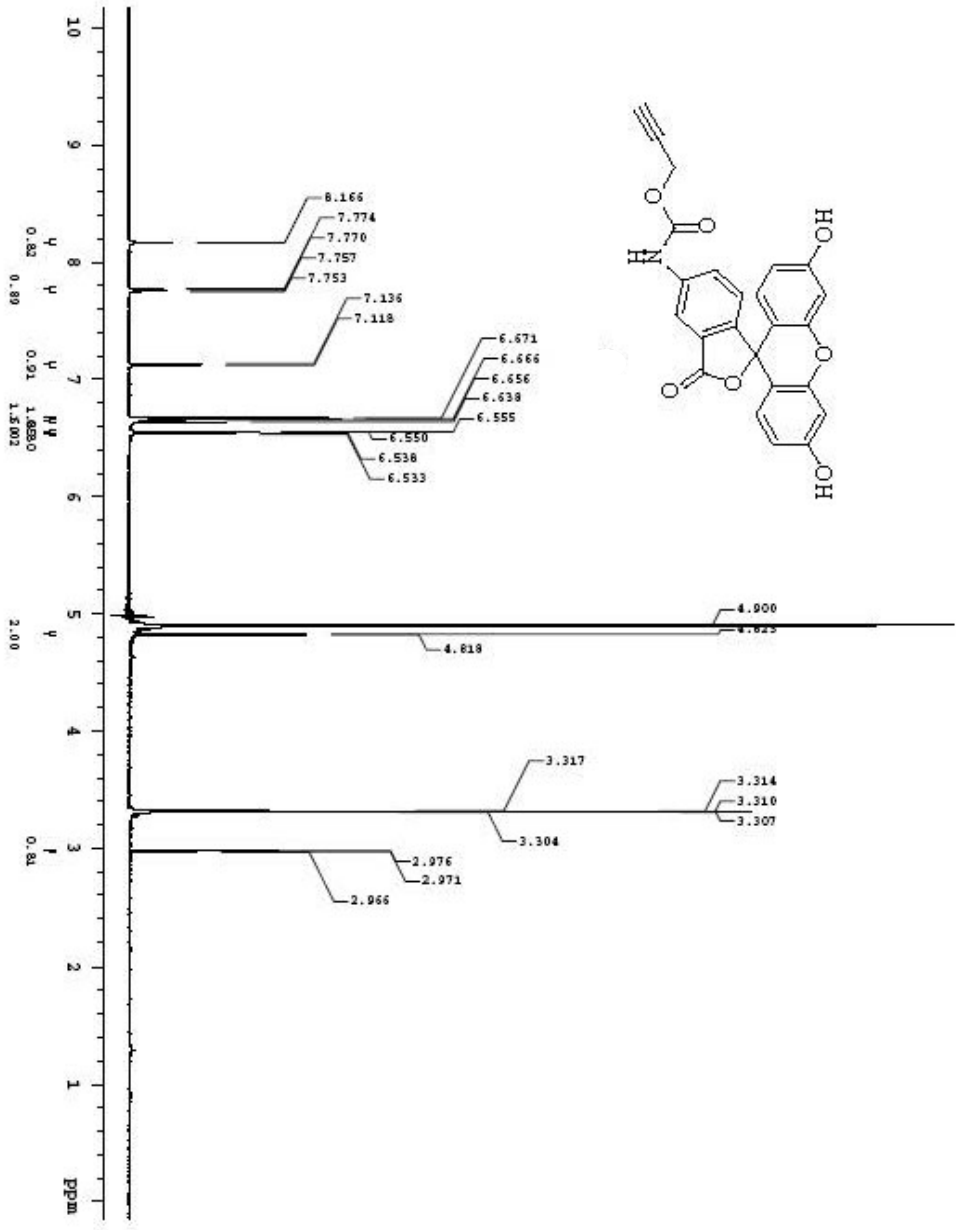


Figure 18. [1]H NMR spectrum for 5-(propargyloxycarbonylamino) fluorescein (**10**).[111*]

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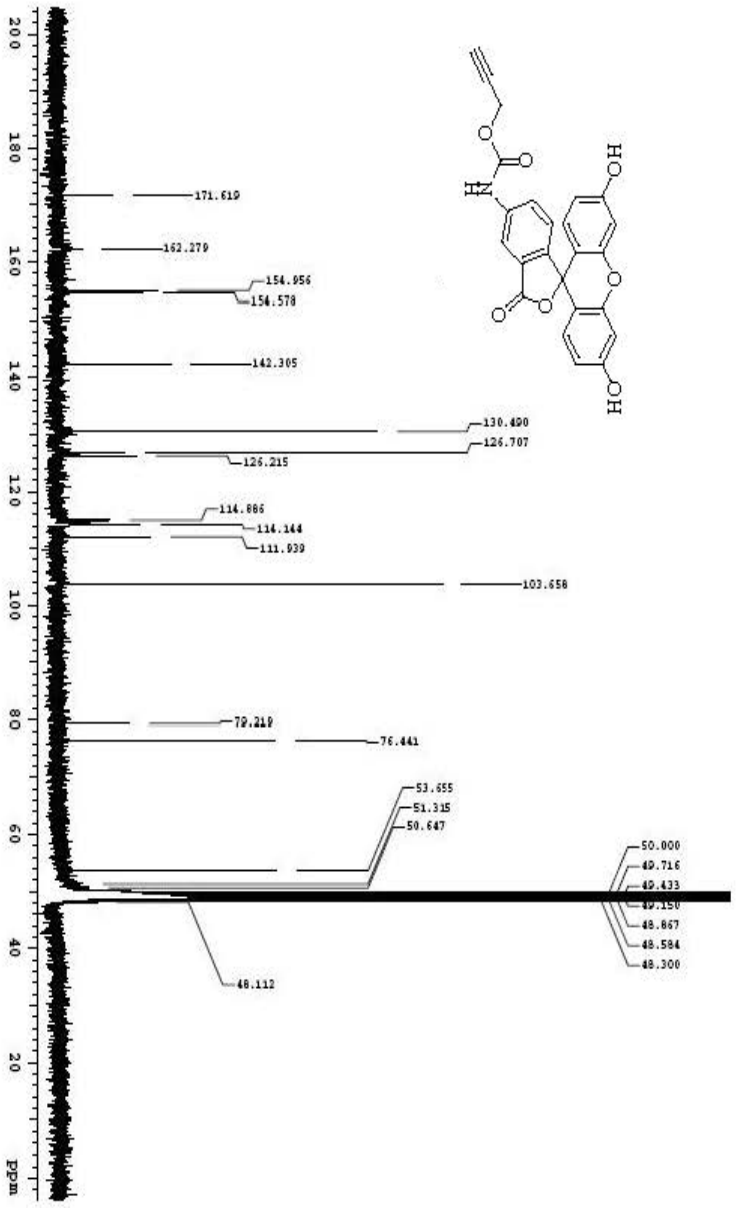

Figure 19. ^{13}C NMR spectrum for 5-(propargyloxycarbonylamino) fluorescein (**10**).[111*]

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Protein Labeling

Procedure for thiol group labeling

25 μM Ub-cysteamine in 50 mM Tris-HCl buffer (pH 7.4) was pre-reduced by 0.5 mM DTT at 37°C for 5 hours. After that, 2 mM 5-iodoacetamidofluorescein (5-IAF) (Pierce Biotechnology) (**11**, stock solutions in DMSO) was added and then incubated at 4°C for 5 hours in the dark. The labeled protein was precipitated by adding nine volumes of HPLC-grade methanol at -20°C and collected by centrifuge at 4°C at 13, 000 r.p.m. for 15 minutes. The pellets were washed with ice-cold methanol for five times and then dissolved in PBS buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4). The labeled protein was subjected to SDS-PAGE analysis and electrospray ionization mass spectrometry (ESI-MS).

Procedure for copper (I)-catalyzed azide-alkyne cycloaddition (CuAAC) labeling

To 25 μM Ub-propargylamine or Ub-azidoethylamine in 50 mM Tris-HCl buffer (pH 7.4), $CuSO_4$ (100 μM), $NiCl_2$ (1 mM), tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA, stock solution in DMSO, 500 μM) and their respective dyes (**9** or **10**, stock solutions in DMSO, 1.25 mM) were added sequentially, followed by sodium ascorbate (5 mM). The reactions were performed at room temperature for 5 hours. Then ethylenediaminetetraacetic acid (EDTA, pH 8.0, 50 mM final concentration) was added to the reaction mixture to chelate the two metals to quench the reaction. The labeled proteins were precipitated by adding nine volumes of HPLC-grade methanol at -20°C and collected by centrifuge at 4°C at 13, 000 r.p.m. for 15 minutes. The pellets were washed with ice-cold methanol for five times and then dissolved in PBS buffer (140 mM

NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4). The labeled proteins were subjected to SDS-PAGE analysis and electrospray ionization mass spectrometry (ESI-MS).

Fluorescence Polarization-based Assays for UCH-L3 Activity Study

Fluorescence polarization-based assays were performed on a FuoroMax®-4 spectrofluorometer (Horiba Scientific) which allowed direct injection of substrate or enzyme into a cuvette for immediate measurement after reactions started. Anisotropy values, which indicated the extent of fluorescence polarization, could be read out directly and the reactions were monitored by the decrease in anisotropy values with excitation wavelength at 490 nm and emission wavelength at 520 nm. The enzymatic assays were performed in the reaction buffer (20 mM Tris-HCl, 5 mM DTT, 100 mM NaCl, 1 mM EDTA, 0.05% bovine serum albumin, pH 7.5). Buffer and substrate (at various concentrations) were predispensed and the reactions were started by adding the enzyme, UCH-L3 (ubiquitin C-terminal hydrolase isozyme L3) (human recombinant, BostonBiochem, MA). Anisotropy values were collected every 30 seconds. The obtained anisotropy values were converted into concentrations of processed substrates [P] with the following equation:

$$[P] = \frac{r_0 - r}{r_0 - r_{final}}$$

[P] is the amount of processed substrates; r_0 is the anisotropy of 100% unprocessed substrate (determined for each reagent at different substrate concentrations before adding UCH-L3); r is the anisotropy values obtained at different time points; r_{final} is the

anisotropy of 100% processed substrate. Values for initial velocities of individual reactions were calculated with Origin 8.5 from OriginLab (Northampton, MA, USA) and used to determine maximal velocity V_{max} and the affinity constant K_m following the equation:

$$v = \frac{V_{max} \times [S]}{K_m + [S]}$$

v is initial velocity; V_{max} is maximal velocity (k_{cat}•E); K_m is affinity constant; [S] is total substrate concentration; E is enzyme concentration.

Protein ESI-MS Analysis for Fluorescent Ubiquitin Conjugates

An Agilent (Santa Clara, CA) 1200 capillary HPLC system was interfaced to an API QSTAR Pulsar Hybrid QTOF mass spectrometer (Applied Biosystems/MDS Sciex, Framingham, MA) equipped with an electrospray ionization (ESI) source. The MS data were acquired in positive ion mode (500-2,000 Da) using spray voltage of +2,700 V. BioAnalyst software (Applied Biosystems) was used for spectral deconvolution. A mass range of m/z 500-2,000 was used for deconvolution and the output range was 8,000-10,000 Da using a step mass of 0.1 Da and an S/N threshold of 2.

Results and Discussion

Firstly, all three Ub-amine conjugates with unique functionalities at the ubiquitin C-terminus were labeled with corresponding fluorescein dyes at their respective optimized conditions through robust and straightforward click chemistry. The SDS-analysis of the labeling reactions with *w.t.*Ub as a control showed the successful conversion of all three Ub-amine conjugates to respective fluorescent ubiquitin conjugates **I**-**III** by comparing the results between Coomassie blue stained SDS gel and the fluorescent image of the same gel before Commassie blue staining (**Figure 20**). Furthermore, all ubiquitin-amine conjugates showed nearly quantitative conversion to their correspondingly fluorescent ubiquitin conjugates whose detected molecular weights by ESI-MS agreed well with their theoretical values (**Figure 21-23**). The theoretical molecular weights of **I**, **II**, **III** were 9,011, 9,062, 9,062 Da, respectively.

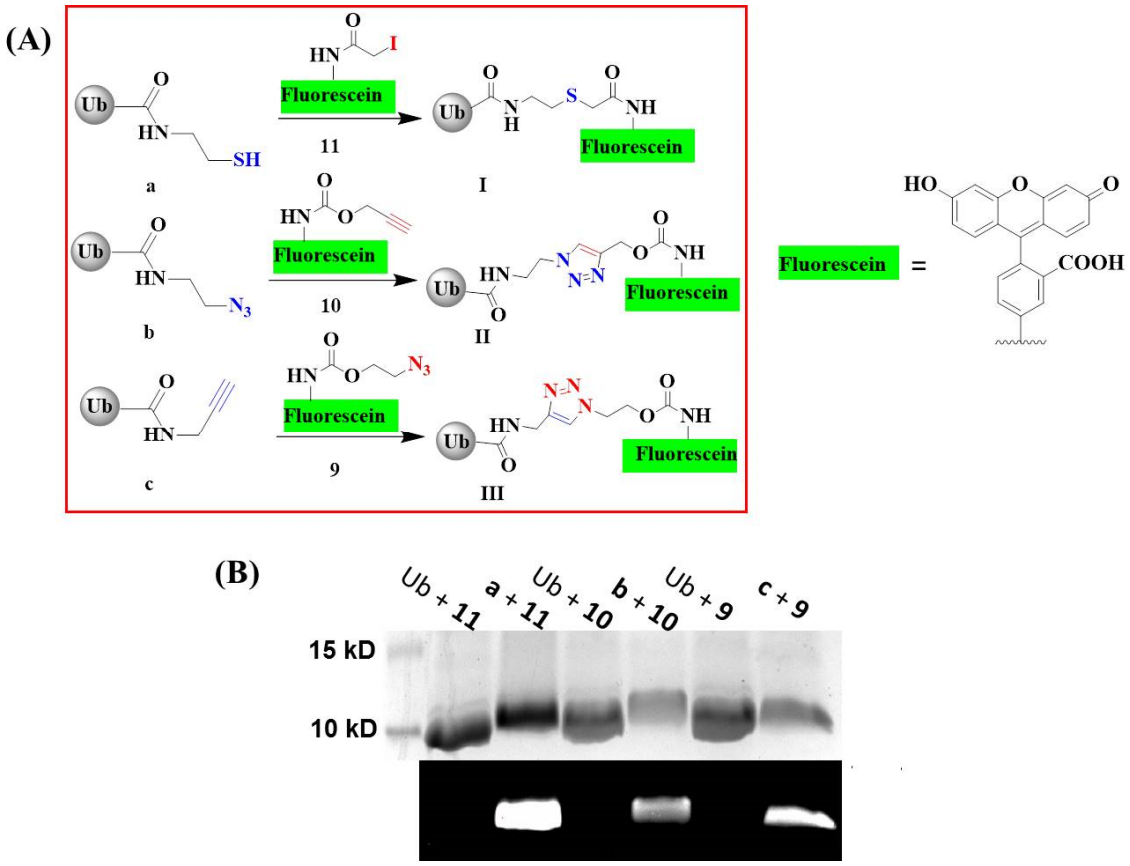


Figure 20. (A) Synthetic route of three Ub-fluorophore conjugates (**I**, **II**, **III**) via selective labeling of Ub-amine conjugates (**a**, **b**, **c**) with respective fluorescein dyes (**11**, **10**, **9**). (B) SDS analysis of selective labeling of **a**-**c** with fluorescein dyes using *w.t.*Ub as control. The top panel showed the Coomassie blue stained SDS gel and the bottom panel showed the fluorescent image of the same gel before Commassie blue staining.

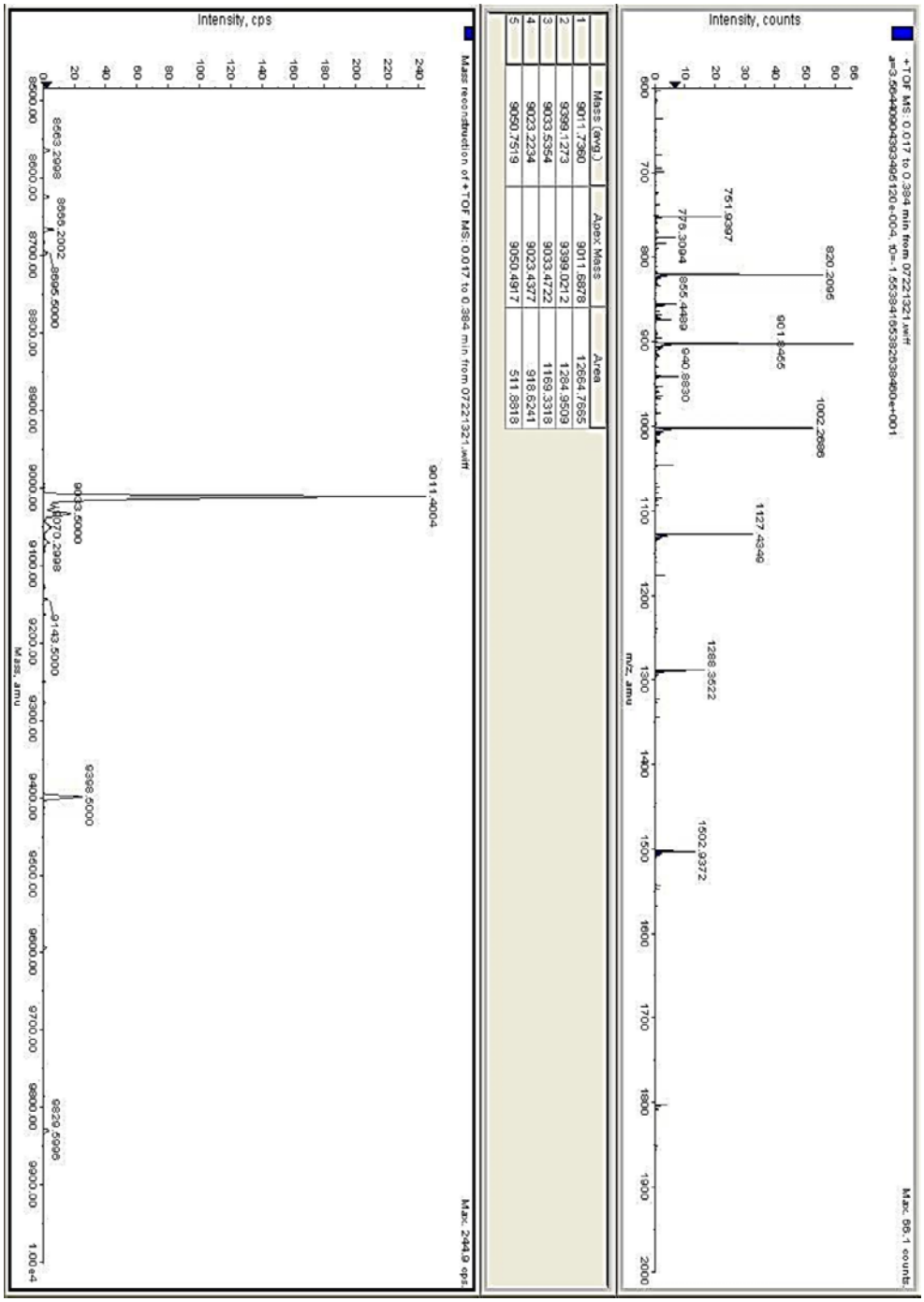


Figure 21. Deconvoluted ESI-MS spectra of Ub-fluorophore conjugates **I**. The theoretical molecular weight of **I** was 9,011 Da. The detected molecular weight of **I** was 9,011 Da.

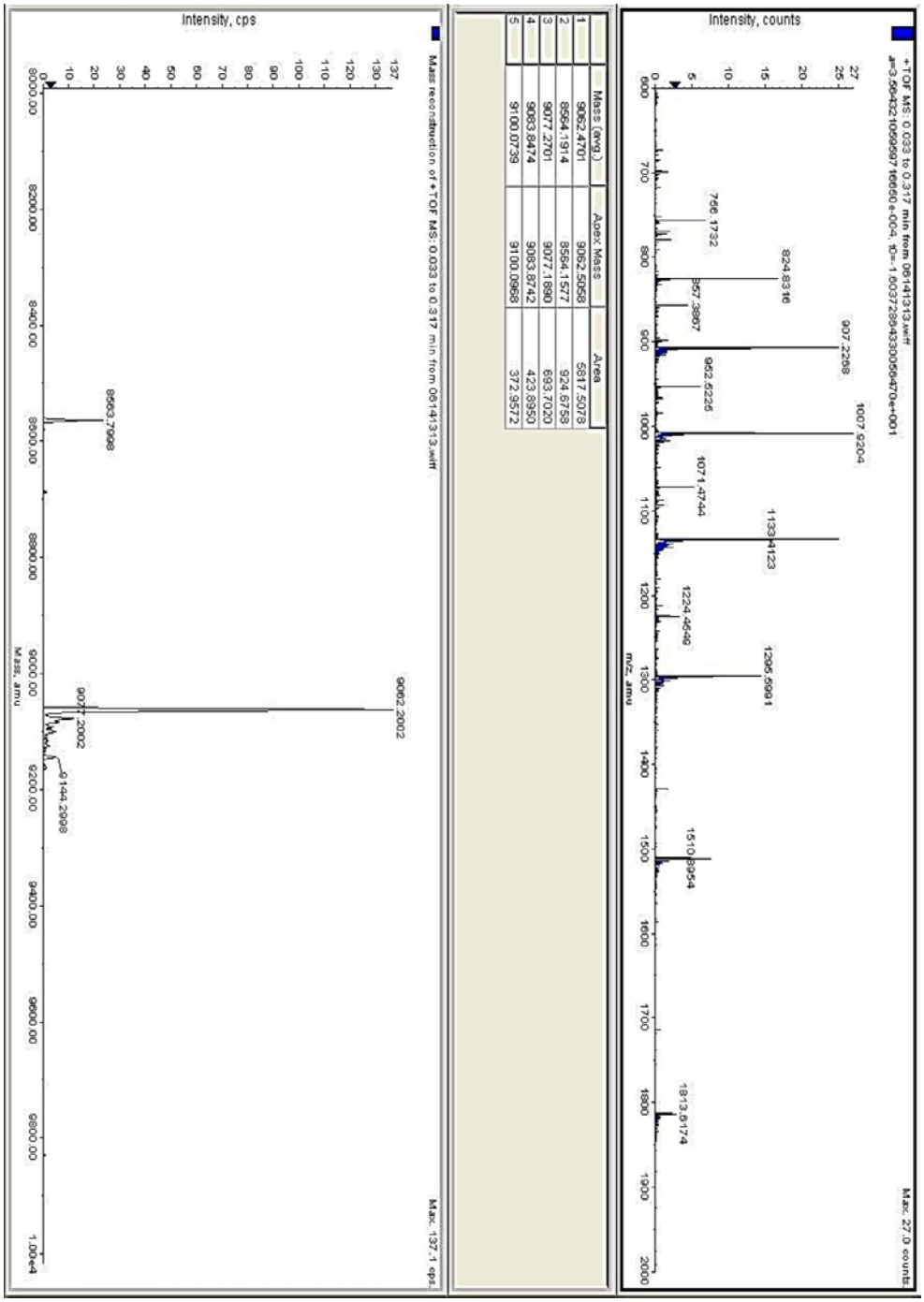


Figure 22. Deconvoluted ESI-MS spectra of Ub-fluorophore conjugates **II**. The theoretical molecular weight of **II** was 9,062 Da. The detected molecular weight of **II** was 9,062 Da.

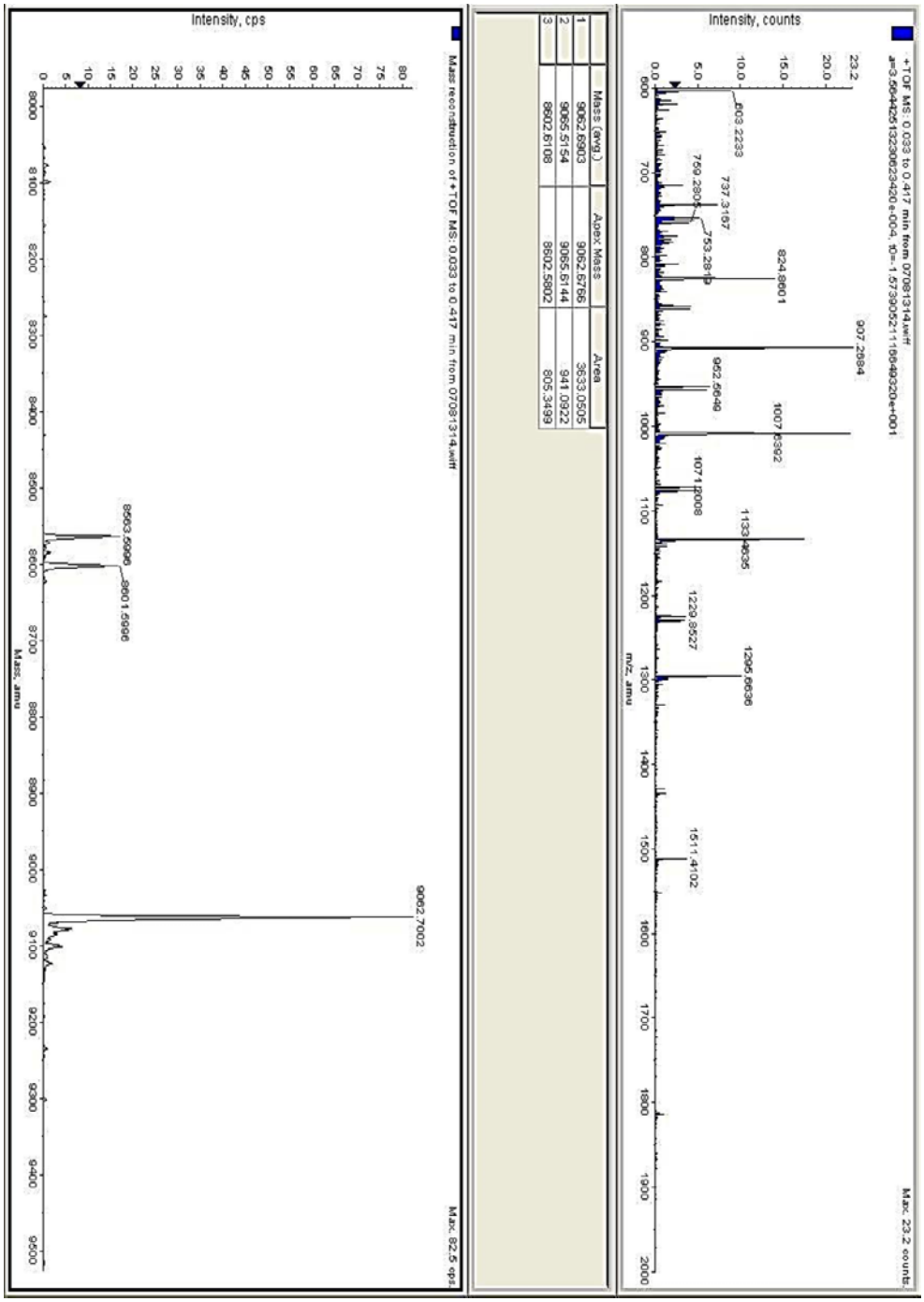


Figure 23. Deconvoluted ESI-MS spectra of Ub-fluorophore conjugates **III**. The theoretical molecular weight of **III** was 9,062 Da. The detected molecular weight of **III** was 9,062 Da.

Since DUB-catalyzed deconjugation reactions of Ub-fluorophore conjugates would release small-molecule fluorescein entities that emitted predominantly depolarized fluorescence different from their corresponding large-molecule Ub-fluorophore conjugates whose emitted fluorescence was much less depolarized, these reactions could be easily monitored by following the fluorescence polarization change with time. We chose to demonstrate this application of Ub-fluorophore conjugates **I-III** using Ub C-terminal hydrolase isozyme L3 (UCH-L3). When 250 nM **I-III** were used as substrates, increasing UCH-L3 concentrations led to rising rates of fluorescence anisotropy decrease at 520 nm emission wavelength (excited at 490 nm) (**Figures 24A**, **24C**, and **24E**). The results indicated that all three Ub-fluorophore conjugates could be efficiently cleaved by UCH-L3 in a concentration-dependent manner, validating **I-III** as suitable substrates for UCH-L3. Then we also characterized Michaelis-Menten parameters of UCH-L3 with **I-III** as substrates by performing kinetic studies at different substrate concentrations in the presence of 100 pM UCH-L3 (**Figures 24B**, **24D**, and **24F**). The resulting anisotropy change was transformed to the extent of product formation as a function of time following the procedure described in experimental details part. Initial velocities were then calculated and plotted against substrate concentrations. The determined parameters were presented in Table 1 and comparable to values reported in the literature for other UCH-L3 substrates such as Ub-AMC,[101] UbR110[116] and Ub-Lys-TAMRA.[102] These results vindicated **I-III** as efficient substrates for DUB activity study.

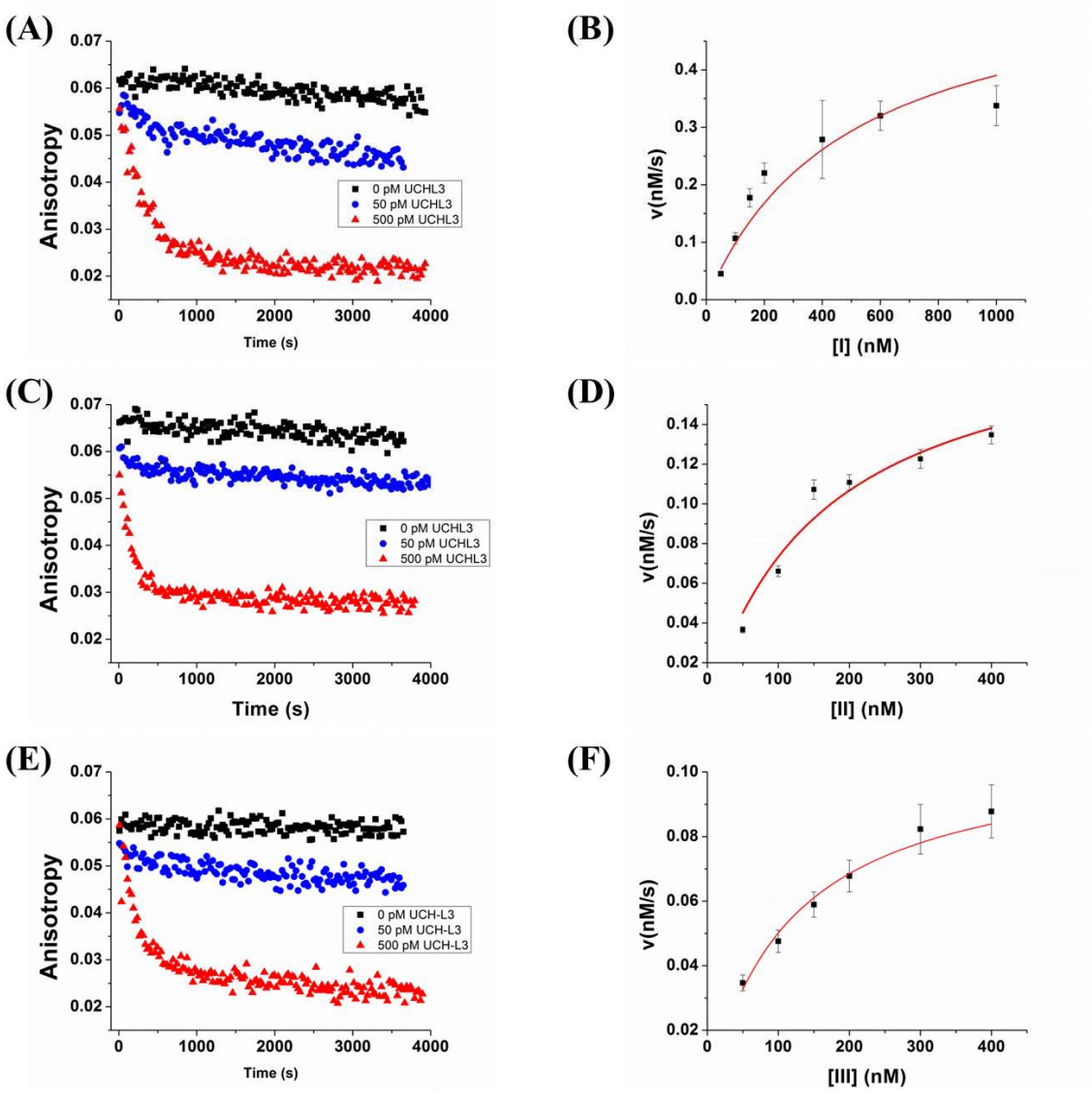


Figure 24. Fluorescence polarization assays of UCH-L3 catalyzed deconjugation reactions of **I-III**. (A), (C) and (E) presented reaction time courses of deconjugation reactions of 250 nM of **I**, **II** and **III**, respectively, by 0 pM, 50 pM, and 500 pM UCH-L3. (B), (D) and (F) presented Michaelis-Menton plots of UCH-L3 catalyzed deconjugation reactions of **I**, **II** and **III**, respectively. For (B), (D), and (F), different substrate concentrations were used to undergo deconjugation reactions at the presence of 100 pM UCH-L3.

Table 2. Kinetic parameters of UCH-L3 with **I-III** as substrates.

Substrate	k_{cat} [s^{-1}]	K_m [nM]	k_{cat}/K_m [M^{-1} s^{-1}]
I	5.8 ± 1.5	492 ± 174	(1.2 ± 0.8) ×10^7
II	2.0 ± 0.3	167 ± 53	(1.2 ± 0.5) ×10^7
III	1.1 ± 0.1	116 ± 18	(0.9 ± 0.6) ×10^7

Conclusions and Outlook

In conclusion, based on our newly-developed one-step approach to modifying ubiquitin C-terminus, we employed it with robust click reactions to synthesize three types of Ub-based fluorescent substrates for the activity study of DUBs. Their applications for DUB kinetic study have been tested with one common DUB-namely, UCH-L3 (ubiquitin carboxyl-terminal hydrolase isozyme L3). This facile method is suitable for large-scale production of various fluorescent ubiquitin conjugates with distinct flexible linkages. The simplicity and high yield of this approach cannot be matched by any other current methods. Because research on DUBs, as well as high-throughput screening for DUB inhibitors hampered by the difficult of producing suitable quantities of DUB substrates, this method has the potential to impact several research fields.

Currently, ubiquitin-7-amino-4-methylcoumarin (Ub-AMC) and ubiquitin rhodamine110 (UbR110) are two widely used fluorogenic Ub-based substrates for the kinetic study of deubiquitinases. Unlike the natural isopeptide linkage in ubiquitinated proteins, Ub-AMC and UbR110 have a bulky hydrophobic fluorophore group linked to the Ub C-terminus, preventing their hydrolysis by some DUBs. Nevertheless, the Ub-fluorophore conjugates **I-III** each contain a linkage between Ub and a fluorophore that mimics the native isopeptide linkage of ubiquitinated proteins. Therefore, they are expected to be recognized and serve as substrates by most DUBs.

Furthermore, due to the similarities in both the structures and the conjugation mechanism for Ub and Ubl (ubiquitin-like) proteins, it is likely that the approach

described here will provide a method to fluorescently label other Ubl proteins, including NEDD8 and SUMO, which will enlarge its application to other research fields as well. Altogether, this newly-developed method should greatly facilitate activity studies of DUBs that regulate varieties of cellular pathways by providing physiologically relevant substrates.

CHAPTER IV

A FACILE AND UNIVERSAL METHOD FOR PREPARATION OF VERSATILE UBIQUITIN POLYMERS

Introduction

Ubiquitin (Ub) is a small 76-residue protein modifier[27,82] with seven lysines which can be conjugated to lysine residues of diverse target proteins to regulate many biological processes,[24] including protein degradation, signal transduction, embryonic development, endocytic trafficking, and the immune response.[28,43] Ub can be attached to target proteins either as a monomer or as a polyubiquitin (poly-Ub) chain (polyubiquitination),[23,24] which is diversified by the generation of chains assembled through isopeptide/peptide bond between the C-terminal carboxylate group of one Ub and any ε-amino group of seven internal Lys residues or the N-terminal amino group of another Ub.[10,29,33,98,117] This diverse isopeptide/peptide bond formation process produces distinct linkage types of linear (homo- and hetero-typic) and branched polyubiquitin chains with different topological and cellular characteristics.[9,29] Deubiquitinases (DUBs) are enzymes that remove Ub from target proteins,[15] thus, they regulate most Ub-dependent cellular processes.[12,26] Consistent with this, their misregulation has been involved in the pathogenesis of various diseases.[38,48,99,100,118,119] Therefore, DUBs, as emerging drug targets, have attracted mounting interests from researchers in both academia and the pharmaceutical industry.[13,55,66]

The human genome encodes more than 100 DUBs.[52] To deal with the structural complexity of polyubiquitin chains, many DUBs display multiple layers of specificity for the recognition of different structural and topological features of diverse polyubiquitin chains.[28,31,32,120,121] The most striking layer of DUB specificity is the ability to select among eight different ubiquitin chain linkages. Besides that, when catalyzing the Ub removal, DUBs can potentially remove one Ub at a time from the end of polyubiquitin chains (*exo*-) or cleave within polyubiquitin chains (*endo*-) to remove Ub oligomers or even entire chains from target proteins (**Figure 25**).[67,122-124] Although an *endo*-DUB can theoretically process the Ub removal much more efficient than an *exo*-DUB and therefore efficiently balances Ub homeostasis, the *endo*-hydrolase feature of DUBs has been little explored due to the difficult accessibility of polyubiquitin chains with structurally defined linkage types. Currently, however, only diubiquitins (di-Ub) and several types of homo-typic polyubiquitin (poly-Ub) chain types (Lys11-, Lys48-, Lys63-, and Met1-linked) are available for *in vitro* studies of DUB specificity.[15,125,126] With only limited types of polyubiquitin chains available, the research in the field of DUB specificity has been hindered for a long time. Hence the overall picture remains incomplete and requires development of new and better reagents and assays to explore deeper aspects of the entire DUB specificity system.

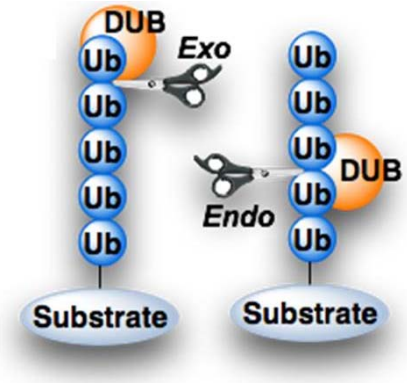


Figure 25. Scheme of *exo*- and *endo*-DUB activities to polyubiquitinated substrates.

Several methods including total synthesis and native chemical ligation have been applied for the synthesis of different ubiquitin polymers (poly-Ubs).[81,84,86,121,127-133] Pioneered by Ashraf Brik and Chuan-Fa Liu, a mercapto-lysine entity in combination with native chemical ligation and desulfurisation has been utilized to synthesize di-Ub and poly-Ub molecules for more physiological-based functional studies of particular DUBs about their linkage type specificities.[84-86,104,134] Alternative poly-Ubs synthetic methods which comprised the genetic incorporation of a noncanonical amino acid[135-139] and its following chemical manipulation have also been developed in research groups such as Chin, Fushman, Cropp, and Rubini.[31,87,121,127,130,140,141] Recently, Strieter and coworkers developed a fairly simple method in which a Ub K→C mutant underwent a thio-ene photo-click reaction with a Ub-allylamine conjugate for the syntheses of di-Ub mimics.

The isopeptide linkage installation is more or less the most critical step and also a major obstacle in the poly-Ubs syntheses.[104,142] In order for simple and efficient functionalization of the Ub C-terminus with high yields, we invented a facile single-step approach to append numerous unique chemical functionalities to Ub C-terminus.[89] This method was based on E1-catalyzed amidation reaction of the Ub C-terminus. E1 naturally catalyzes its own thioester linkage formation with Ub (E1-Ub) and subsequently transfers Ub to Ub conjugating enzyme (E2) for further Ub transfer. We showed that E1-Ub thioester bond could be intercepted efficiently with primary amines to form Ub-amine conjugates. One of these primary amines was allylamine. Its conjugate with Ub (Ub-Al) readily underwent a thiol-ene photo-click reaction with seven

Ub K→C mutants to afford di-Ub (Ub_2) variants with linkages closely analogous to the seven native isopeptide linkage types, which was reported by Strieter's group previously.[129] The smallest subunits that represent topological complexities of poly-Ubs are Ub_3 variants.[25,26] Therefore, we would like to expand our methodology to synthesize structurally defined Ub trimer (Ub_3) variants and would like to use them to characterize activities (such as *endo*- and *exo*- hydrolytic activities) of DUBs. The E1-catalyzed amidation reaction could be employed to functionalize the proximal Ub C-terminus of Ub_2 variants with allylamine molecule to form Ub_2-Al that would further undergo the thiol-ene photo-click reaction with Ub K→C mutants for the second time to afford Ub_3 variants (**Figure 26**).[20,22] With our developed E1-catalyzed amidation reaction in combination with thiol-ene coupling chemistry, we could readily acquire seven types of di-Ub (Ub_2) mimics and as many as 49 kinds of Ub trimer (Ub_3) mimics with mixed linkage types. With these versatile ubiquitin polymers as substrates, we could continue to study the multiple specificity layers of numerous DUBs. Given its exceptional simplicity and universality, our method was expected widely employed in the research field of DUBs structural and functional investigations.

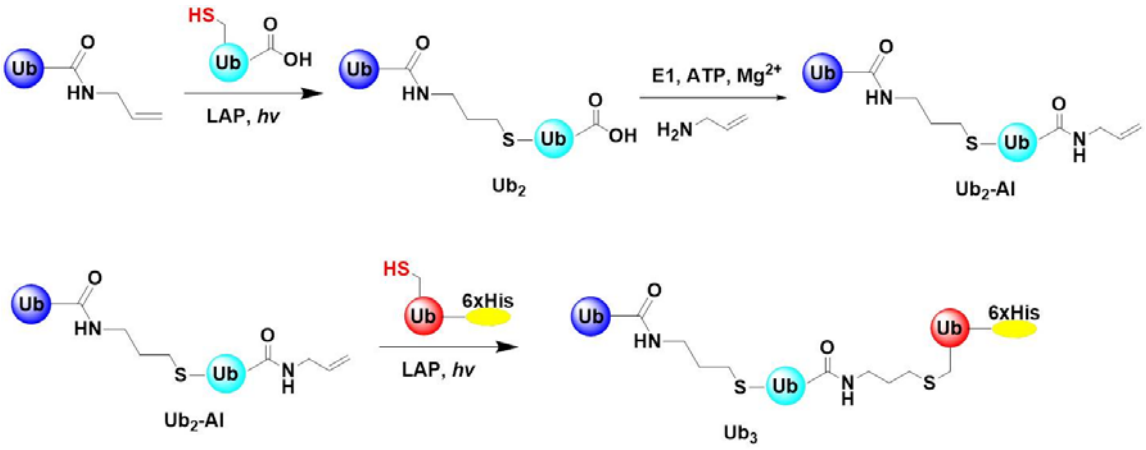


Figure 26. Scheme of a cascade of reactions to synthesize Ub_3 mimics with two isopeptide linkages.

The superfamily of OTU DUBs contains 16 members.[51,60] They have emerged as regulators of important signaling cascades and been implicated in human diseases such as inflammation and cancer.[59,68,73-75] Recent comprehensive study indicated that most OTU DUBs displayed intrinsic isopeptide linkage specificities, preferring one or a small subset of isopeptide types.[117,143-146] In addition, most OTU DUBs have several Ub-associating domains that potentially facilitate their binding of several Ub subunits in polyubiquitin chains with unique topologies for cleavage of Ub polymers at *endo* sites.[71,147] For example, Komander and coworkers showed that OTUD2 could cleave K11-, K27-, K29- and K33-linked Ub_2 molecules preferentially and displayed a much more enhanced hydrolytic activity when a homo-typic K11-linked Ub_3 instead of other homo-typic Ub_3 variants to be used as its substrate. The structure of an inactive OTUD2 complexed with K11-linked Ub_2 also showed that Ub_2 was not directly bound at the active site.[58] Based on these observations, we proposed a mechanism of OTUD2-catalyed poly-Ubs cleavage shown in **Figure 27**. A K11-linked Ub_2 formed between the distal and middle Ubs recruited OTUD2 that rapidly hydrolyzed K11, K27, K29, and K33 isopeptide linkages between the middle and proximal Ubs. To examine this model, Ub_3 variants as shown in **Figure 27B** that contained the K11 linkage between distal and middle Ubs and all other linkage types between middle and proximal Ubs would be used as substrates for OTUD2 cleavage specificity study.[69] *Endo*- and *exo*- DUB activities of OTUD2 would lead to different hydrolytic products that could be easily differentiated by comparing the size difference (around 1,000 dalton) between *wild-type* Ub and His-tagged Ub, which could be readily displayed in SDS-PAGE analysis. As predicted, if

OTUD2 did have the preference to Lys11 linkage and had the *endo*-cleavage capability, then we would only obtain His-tagged Ub K→C with Lys11-linked di-Ub mimics, while if not, its *exo*-cleavage capability would be evidenced by the presence of the *w.t.* Ub with His-tagged di-Ub mimics.[148] Furthermore, different types of second linkage with distinct topological features were supposed to affect the efficiency of its cleavage activity as well, thereby giving a more comprehensive overview of various interactions in polyubiquitin chains.[149,150]

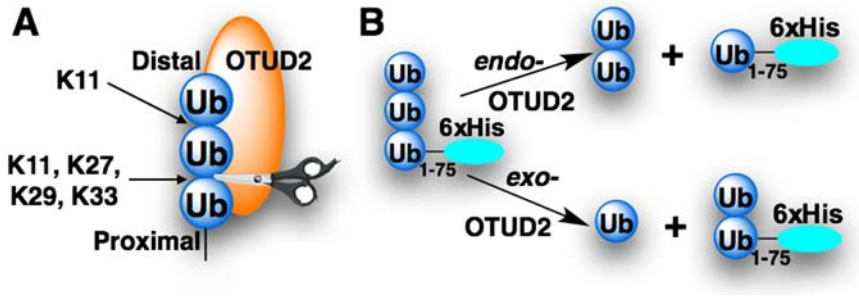


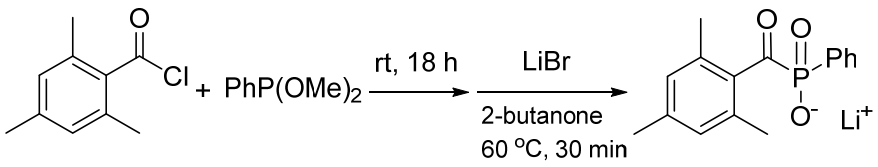
Figure 27. (A) A proposed linear Ub_3 hydrolysis by OTUD2. (B) Potential products of OTUD2-catalyzed hydrolysis of a 6×His-tagged linear Ub_3.

Experimental Details

Chemical Synthesis

Synthesis of lithium acylphosphinate (LAP)

In a round bottom flask, 2,4,6-trimethylbenzoyl chloride (0.52 mL, 3.2 mmol) was added dropwise to a solution of dimethyl phenylphosphonite (0.50 mL, 3.2 mmol) in an ice bath. The mixture was then warmed to room temperature and stirred under inert atmosphere for 20 hours. The solution of lithium bromide (1.1 g, 12.7 mmol) in 2-butanone (10 mL) was added to the mixture from the previous step. The mixture was heated and stirred at 60°C for 30 min before cooled down to room temperature and left stirred for 10 hours. The resulting white suspension was collected by filtration, washed with 2-butanone and dried under reduced pressure to give a white powder as the desired product (0.94 g, quant.). ^{1}H NMR (D_2O, 300 MHz) δ 7.74 (m, 2H), 7.62 (m, 1H), 7.50 (m, 2H), 6.92 (s, 2H), 2.27 (s, 3H), 2.05 (s, 6H)



Scheme 4. Synthetic route of lithium acylphosphinate (LAP).

DNA and Protein Sequences

DNA sequence

Ub-K6C-Histag

atgcaaatattcgtgtgcaccctaactggtaagaccatcactctcgaagtggagccgagtgacaccattgagaatgtc

aaggcaaagatccaagacaaggaaggcatccctcctgaccagcagaggttgatctttgctgggaaacagctggaa

gatggacgcaccctgtctgactacaacatccagaaagagtccaccctgcacttggtccttaggctgagaggaggag

agctccatcaccatcaccatcactaa

Ub-K11C-Histag

atgcaaatattcgtgaaaaccctaactggttgcaccatcactctcgaagtggagccgagtgacaccattgagaatgtc

aaggcaaagatccaagacaaggaaggcatccctcctgaccagcagaggttgatctttgctgggaaacagctggaa

gatggacgcaccctgtctgactacaacatccagaaagagtccaccctgcacttggtccttaggctgagaggaggag

agctccatcaccatcaccatcactaa

Ub-K27C-Histag

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ctgcgcaaagatccaagacaaggaaggcatccctcctgaccagcagaggttgatctttgctgggaaacagctgga

agatggacgcaccctgtctgactacaacatccagaaagagtccaccctgcacttggtccttaggctgagaggagga

gagctccatcaccatcaccatcactaa

Ub-K29C-Histag

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caaggcatgcatccaagacaaggaaggcatccctcctgaccagcagaggttgatctttgctgggaaacagctgga

agatggacgcaccctgtctgactacaacatccagaaagagtccaccctgcacttggtccttaggctgagaggagga

gagctccatcaccatcaccatcactaa

Ub-K33C-Histag

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gagctccatcaccatcaccatcactaa

Ub-K48C-Histag

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Ub-K63C-Histag

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agatggacgcaccctgtctgactacaacatccagtgcgagtccaccctgcacttggtccttaggctgagaggagga

gagctccatcaccatcaccatcactaa

Ub-K11C

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caaggcaaagatccaagacaaggaaggcatccctcctgaccagcagaggttgatctttgctgggaaacagctgga

agatggacgcaccctgtctgactacaacatccagaaagagtccaccctgcacctggtactccgtctcagaggtggtt

ga

Protein sequence

Ub-K6C-Histag

MQIFVCTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLE

DGRTLSDYNIQKESTLHLVLRLRGGHHHHHH

Ub-K11C-Histag

MQIFVKTLTGCTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLE

DGRTLSDYNIQKESTLHLVLRLRGGHHHHHH

Ub-K27C-Histag

MQIFVKTLTGKTITLEVEPSDTIENVCAKIQDKEGIPPDQQRLIFAGKQLE

DGRTLSDYNIQKESTLHLVLRLRGGHHHHHH

Ub-K29C-Histag

MQIFVKTLTGKTITLEVEPSDTIENVKACIQDKEGIPPDQQRLIFAGKQLE

DGRTLSDYNIQKESTLHLVLRLRGGHHHHHH

Ub-K33C-Histag

MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDCEGIPPDQQRLIFAGKQLE

DGRTLSDYNIQKESTLHLVLRLRGGHHHHHH

Ub-K48C-Histag

MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGCQLE

DGRTLSDYNIQKESTLHLVLRLRGGHHHHHH

Ub-K63C-Histag

MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLE

DGRTLSDYNIQCESTLHLVLRLRGGHHHHHH

Ub-K11C

MQIFVKTLTGCTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLE

DGRTLSDYNIQKESTLHLVLRLRGG

Construction of Plasmids

Construction of plasmids pETDuet-UbK×C-Histag (× = 6, 11, 27, 29, 33, 48, 63)

Plasmids pETDuet-UbK×C-Histag (× = 6, 11, 27, 29, 33, 48, 63) were constructed from the pETDuet-Ub-Histag by quick change PCR (polymerase chain reaction) using respective forward and reverse oligodeoxynucleotide primers for each site:

K6C forward: 5'-TGCACTCTGACTGGTAAGACCATCACTCTC-3';

K6C reverse: 5'-CACGAAGATCTGCATATGTATATCTCCTTC-3';

K11C forward: 5'-TGCACCATCACTCTCGAAGTGGAGCCGAGT-3';

K11C reverse: 5'-ACCAGTCAGAGTCTTCACGAAGATCTGCAT-3';

K27C forward: 5'-TGCGCAAAGATCCAAGACAAGGAAGGCATC-3';

K27C reverse: 5'-GACATTCTCAATGGTGTCACTCGGCTCCAC-3';

K29C forward: 5'-TGCATCCAAGACAAGGAAGGCATCCCTCCT-3';

K29C reverse: 5'-TGCCTTGACATTCTCAATGGTGTCACTCGG-3';

K33C forward: 5'-TGCGAAG GCATCCCTCCTGACCAGCAGAGG-3';

K33C reverse: 5'-GTCTTGGATCTTTGCCTTGACATTCTCAAT-3';

K48C forward: 5'-TGCCAGCTGGAAGATGGACGCACCCTGTCT-3';

K48C reverse: 5'-CCCAGCAAAGATCAACCTCTGCTGGTCAGG-3';

K63C forward: 5'-TGCGAGTCCACCCTGCACCTGGTACTCCGT-3';

K63C reverse: 5’-CTGGATGTTGTAGTCAGACAGGGTGCGTCC-3’.

In these plasmids, UbK×C-Histag is under the control of IPTG-inducible T7 promoter.

Construction of plasmid pETDuet-UbK11C

The plasmid pETDuet-UbK11C was constructed from the pETDuet-*w.t.*Ub by quick change PCR using forward and reverse oligodeoxynucleotide primers (forward: 5’-TGCACCATCACTCTCGAAGTGGAGCCGAGT-3’; reverse: 5’-ACCAGTCAGAG TCTTCACGAAGATCTGCAT-3’). In this plasmid, UbK11C is under the control of IPTG-inducible T7 promoter.

Protein Expression and Purification

UbK×C-Histag (× = 6, 11, 27, 29, 33, 48, 63) expression and purification

To express ubiquitin (Ub) K→C mutants, *E. coli* BL21 cells were transformed with respective pETDuet-UbK×C-Histag (× = 6, 11, 27, 29, 33, 48, 63) plasmids. A starter culture was grown in 2×YT media (5 mL) with 100 μg/mL ampicillin at 37°C overnight. The culture was inoculated into 2×YT media (200 mL) with the same concentration of ampicillin the next morning. Isopropyl β-D-1-thiogalactopyranoside (IPTG) (0.5 mM) was added into the cell culture after the OD600 reached 0.9~1.1. The cell culture was incubated at 37°C for 8 hours, and the cells were harvested by centrifugation at 4,000 r.p.m. for 30 min at 4°C and resuspended in 50 mL of lysis buffer (50 mM Tris-HCl, 0.5 mM EDTA, 1 mM DTT, pH 7.5). The resuspended cells were sonicated in an ice/water bath four times (4 min each, 10 min interval to cool down the suspension to below 10°C before the next run) and the lysate was clarified by

centrifugation at 10,000 r.p.m. for 40 min at 4°C. The clarified supernatant was then incubated with 5 mL of Ni Sepharose^{TM} 6 Fast Flow beads from GE Healthcare (Little Chalfont, United Kingdom) for 20 min for the purpose of effectively binding and then poured into a 10-mL empty purification column to allow the Ni sepharose beads to settle completely by gravity. The Ni sepharose beads were then washed with 50 mL buffer A (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 7.8) to get rid of any unspecific binding proteins and subsequently eluted out with 12 mL of buffer B (50 mM NaH_2PO_4, 300 mM NaCl, 300 mM imidazole, pH 7.8). The eluted solution was concentrated by Amicon Ultra-15 centrifugal filter units - 3,000 NMWL from Millipore (Billerica, MA) to 1 mL. The purity of expressed ubiquitin mutants was confirmed by SDS-PAGE analysis. The buffer was then changed to 100 mM ammonium acetate (pH 4.5) by dialysis for further reactions. The concentration was determined by BCA protein assay kit from Thermo Fisher Scientific Inc. (Rockford, IL). According to the concentration, Ub K→C mutants expression yield were around 150 mg/L from the 2×YT media.

UbK11C expression and purification

To express UbK11C, *E. coli* BL21 cells were transformed with pETDuet-UbK11C plasmid. A starter culture was grown in 2×YT media (5 mL) with 100 μg/mL ampicillin at 37°C overnight. The culture was inoculated into 2×YT media (200 mL) with the same concentration of ampicillin. Isopropyl β-D-1-thiogalactopyranoside (IPTG) (0.5 mM) was added into the cell culture after the OD600 reached 0.9~1.1. The cell culture was incubated at 37°C for 8 hours, and the cells were harvested by centrifugation

at 4,000 r.p.m. for 30 min at 4°C and resuspended in 50 mL of lysis buffer (50 mM Tris-HCl, 0.5 mM EDTA, 1 mM DTT, pH 7.5). The resuspended cells were sonicated in an ice/water bath four times (4 min each, 10 min interval to cool down the suspension to below 10°C before the next run) and the lysate was clarified by centrifugation at 10,000 r.p.m. for 40 min at 4°C. The clarified supernatant was then acidified by adding glacier acetic acid to adjust pH to 3.7 to eliminate the impurities. The purity of expressed UbK11C was confirmed by SDS-PAGE analysis. The buffer was then changed to 100 mM ammonium acetate (pH 4.5) by dialysis for further reactions. The concentration was determined by BCA protein assay kit from Thermo Fisher Scientific Inc. (Rockford, IL). According to the concentration, Ub K11C expression yield was around 180 mg/L from the 2×YT media.

Syntheses of Ub Dimers by Thio-ene Coupling (TEC) Chemistry

Reactions contained UbK×C-Histag (Ub K→C mutants) (1 mM), Ub-allylamine (Ub-Al) (1 mM), LAP (1 mM) in 100 mM ammonium acetate buffer (pH 4.5) (20 μL reaction volume). Samples were placed on ice and irradiated from above with 365 nm light for 1 hour using an OmniCure series 1500 light source. Control reactions contained *w.t.*Ub (1 mM) instead of Ub-Al. For UbK11C reaction with Ub-Al, 1 mM UbK11C was mixed with 1 mM Ub-Al and 1 mM LAP in 100 mM ammonium acetate buffer (pH 4.5). The mixture was placed on ice and irradiated from above with 365 nm light for 1 hour using an OmniCure series 1500 light source. The synthesized K11-linked Ub_2 was further purified by cation exchange chromatography.

Cation Exchange Chromatography for Ub Dimer Purification

TEC reaction mixture for K11-linked Ub_2 was collected and purified by cation exchange chromatography with fast protein liquid chromatography (FPLC) system (Bio-Rad Laboratories, Inc.). The reaction mixture was dialyzed to buffer A (100 mM ammonium acetate, 1 mM DTT, pH 4.5) and injected into a 20 mL HiPrep SP HP 16/10 column (GE Healthcare Life Sciences, Little Chalfont, UK). The program was set as followed: 1) the column was washed with a gradient of 0-60% B (100 mM ammonium acetate, 1 M NaCl, 1 mM DTT, pH 4.5) over 10 column volumes; 2) then washed with a gradient of 60-100% B for one column volume; 3) finally washed with 100% B for one column volume. Pure fractions for each peak were collected and concentrated by Amicon Ultra-15 centrifugal filter units - 3,000 NMWL from Millipore (Billerica, MA) for SDS-PAGE analysis.

E1-Catalyzed Amidation Reaction for the Syntheses of Ub_2-Al (DiUb-allylamine)

Aliquots of purified mouse E1 stored at -80°C were rapidly thawed and placed on ice. The reaction contained 250 μM purified K11-linked Ub_2, 20 μM E1, 5 mM ATP, 750 mM allylamine and was incubated in 1 mL (final volume) of the buffer (10 mM CAPS, 250 mM sucrose, 50 mM KCl, 3 mM $MgCl_2$, pH 9.4) at 37°C overnight. The conversion rate of the reaction was determined by ESI-MS analysis. The reaction mixture was then changed to buffer of 100 mM ammonium acetate (pH 4.5) by dialysis for further reaction.

Syntheses of Ub trimers by Thio-ene Coupling (TEC) Chemistry

Reaction mixtures contained UbK×C-Histag (Ub K→C mutants) (2 mM), diUb-allylamine (Ub_2-Al) (0.5 mM), LAP (1 mM) in 100 mM ammonium acetate buffer (pH 4.5). Samples were placed on ice and irradiated from above with 365 nm light for 1 hour using an OmniCure series 1500 light source. The reaction results were further analyzed with SDS-PAGE analysis.

Purification of Ub Trimers from Polyacrylamide Gels

22 cm (h) × 16.5 cm (w) 12% SDS-PAGE gel was casted and ran by Adjustable Slab Gel Kit (C.B.S. Scientific Company, CA, USA). After gel electrophoresis, a clean scalpel was used to cut off the region of the protein lane at around 25 kDa according to the molecular weight marker lane. The excised gel piece was placed in a clean screw-cap culture tube. Then 1 mL of elution buffer (50 mM Tris-HCl, 150 mM NaCl, and 0.1 mM EDTA; pH 7.5) was added into the tube so that the gel pieces were completely immersed. The gel piece was crushed using a clean pestle and incubated in a rotary shaker at 37°C overnight. The next morning, the tube was taken out and centrifuged at 10,000 × g for 10 minutes. The supernatant was carefully pipetted into a new microcentrifuge tube and concentrated using Amicon Ultra-4 centrifugal filter units - 3,000 NMWL from Millipore (Billerica, MA). An aliquot of the supernatant was tested for the presence of pure Ub trimer by subjecting it to a 15% SDS-PAGE gel.

Results and Discussion

Recently, Streiter and coworkers demonstrated that Ub-Al could undergo a thiol-ene photo-click reaction with a cysteine-containing Ub mutant to generate a Ub dimer mimic. In their method, the synthesis of Ub-Al took advantage of the reversible nature of reactions catalysed by yeast ubiquitin C-terminal hydrolase (YUH1), and in the presence of YUH1, allylamine was added in large excess relative to a Ub variant harbouring a C-terminal aspartate cap. Under these conditions, Ub-Al could be obtained in 30% yield.[129] By contrast, our E1-catalyzed amidation approach was more advantageous, providing close to quantitative conversion of *wild-type* Ub to Ub-Al. To test our synthesized Ub-Al in the construction of Ub dimer mimics with seven isopeptide linkage types, firstly, seven C-terminal Histagged Ub K×C mutants (× = 6, 11, 27, 29, 33, 48, 63) were expressed in *E. coli* and purified by nickel affinity purification (**Figure 28**). After obtaining both purified cysteine-containing Ub mutants and Ub-Al, we further tested the thiol-ene photo-click reaction following the Streiter group's protocol. Equal amounts (1 mM) of Ub-Al and a Ub K→C mutant were mixed in the presence of a photoinitiator, lithium acylphosphinate (LAP, 0.5 mM) and placed under UV irritation for 30 min at 4°C. The formation of a Ub dimer mimic was determined by SDS-PAGE analysis (**Figure 29**). According to the SDS-PAGE analysis, for all seven Ub K×C mutants (× = 6, 11, 27, 29, 33, 48, 63), corresponding Ub dimer mimics were synthesized with varied efficiencies, comparable to what were reported by Streiter *et al.* Streiter and coworkers stated that Ub K27C mutant was refractory toward coupling with Ub-Al, which was not observed in our experiments.

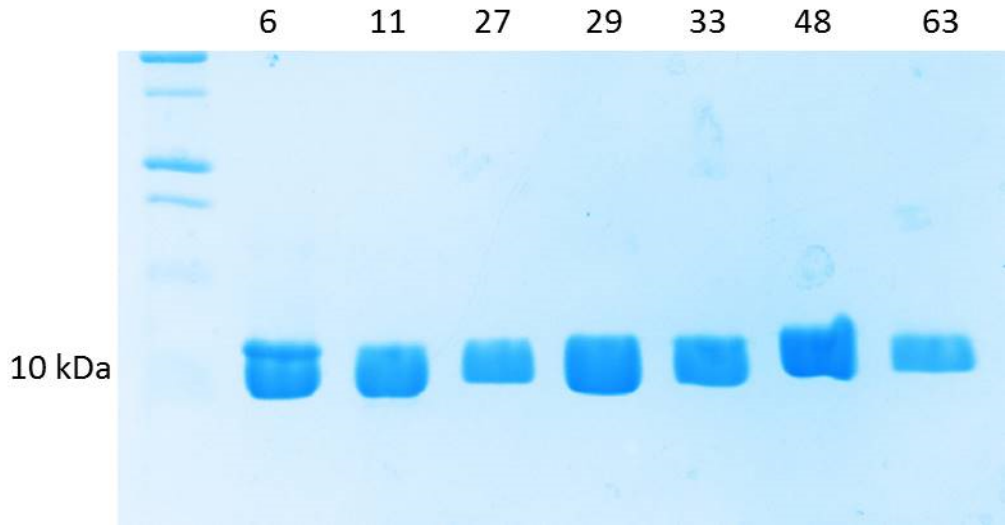


Figure 28. Coomassie-stained SDS-PAGE analysis of UbK×C-Histag (× = 6, 11, 27, 29, 33, 48, 63). UbK×C-histag proteins were expressed in *E. coli* BL21 cells and purified with Ni Sepharose^{TM} 6 Fast Flow beads from GE Healthcare.

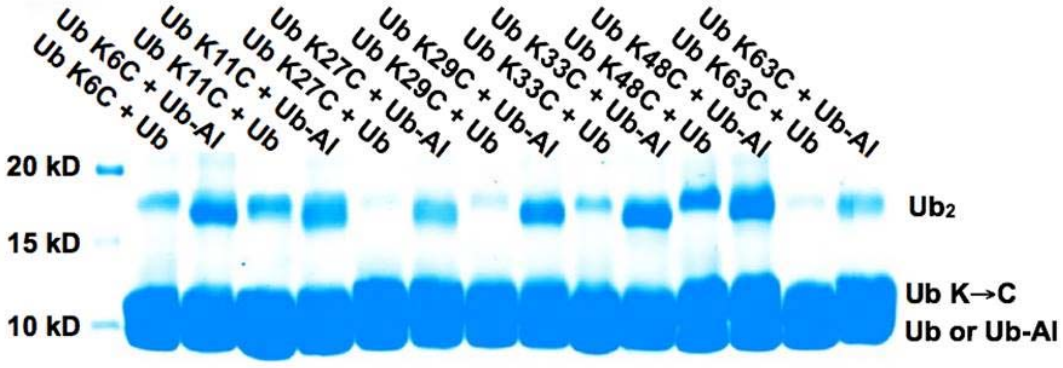


Figure 29. Coomassie-stained SDS-PAGE analysis of thiol-ene photo-click reaction products of Ub-allylamine (Ub-Al) with seven 6×Histidine-tagged Ub K×C mutants (Ub K→C). Reactions involving *w.t.*Ub (Ub) served as controls. A weak band with a slightly larger size than Ub_2 showing in both control and Ub-Al experiments is the disulfide-conjugated homodimer of a 6×Histidine tagged Ub K×C mutant.

With the initial success of the di-Ub mimics syntheses, we further hypothesized that Ub dimers with a free C-terminus could be modified using an E1 enzyme as well in order to perform the second amidation reaction and afford numerous Ub_2-amine conjugates, including diUb-allylamine (Ub_2-Al). Also, the *exo*- cleavage activity study of OTUD2 required Ub trimers that contained the K11 linkage between distal and middle Ubs and all other linkage types between middle and proximal Ubs as substrates. Therefore, we constructed and expressed the Ub K11C mutant with a free C-terminus, and performed the thiol-ene photo-click reaction with purified Ub K11C and Ub-allylamine as substrates in the presence of LAP. Since the conversion rate was only around 30%, the reaction mixture was then subjected to FPLC (fast protein liquid chromatography) purification using a cation exchange column. The K11-linked Ub_2 was eluted at around 30% buffer B concentration, and its purity was judged by SDS-PAGE analysis (**Figure 30**).

Based on the universality of E1 enzymes in the ubiquitination system, we reasoned that Ub dimers could also be recognized by an E1 enzyme. We therefore conducted the E1-catalyzed C-terminal amidation reaction to functionalize the C-terminus of Ub dimers. With FPLC purified K11-linked Ub_2, we tested E1-catalyzed amidation reactions following similar conditions with optimized E1 enzyme concentrations and determined the conversion rate by ESI-MS analysis (**Figure 31**). Theoretical molecule weights of Ub_2 and Ub_2-Al were 17,143 and 17,182 Da, respectively. According to the ESI-MS results, more than 80% Ub_2 was successfully converted to Ub_2-Al, which proved the applicability of E1 catalytic activity to Ub dimers.

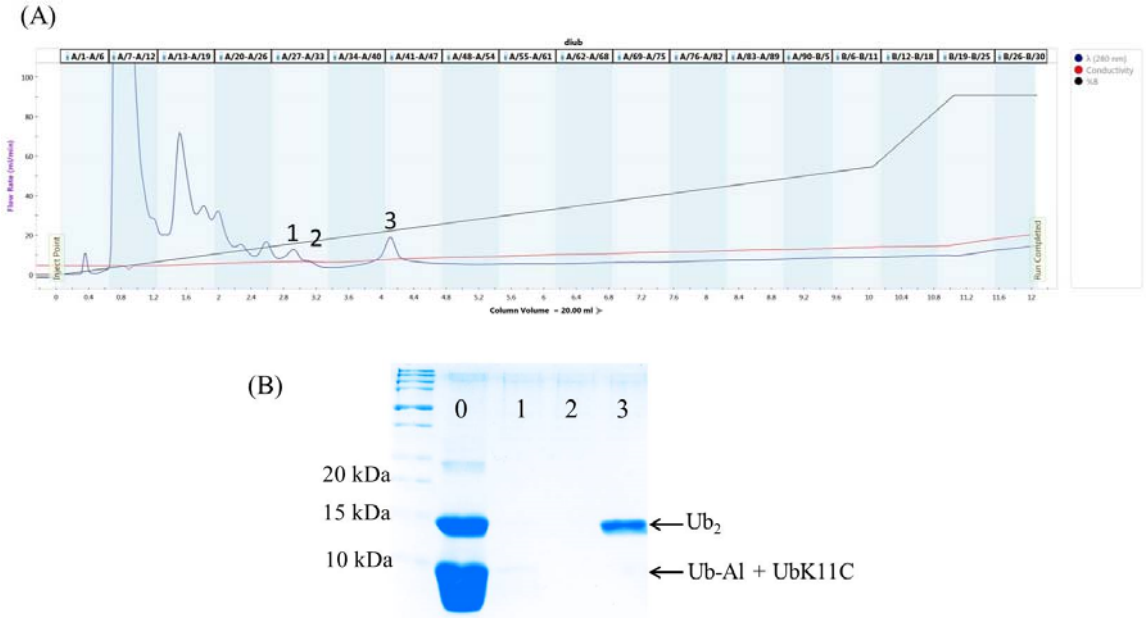
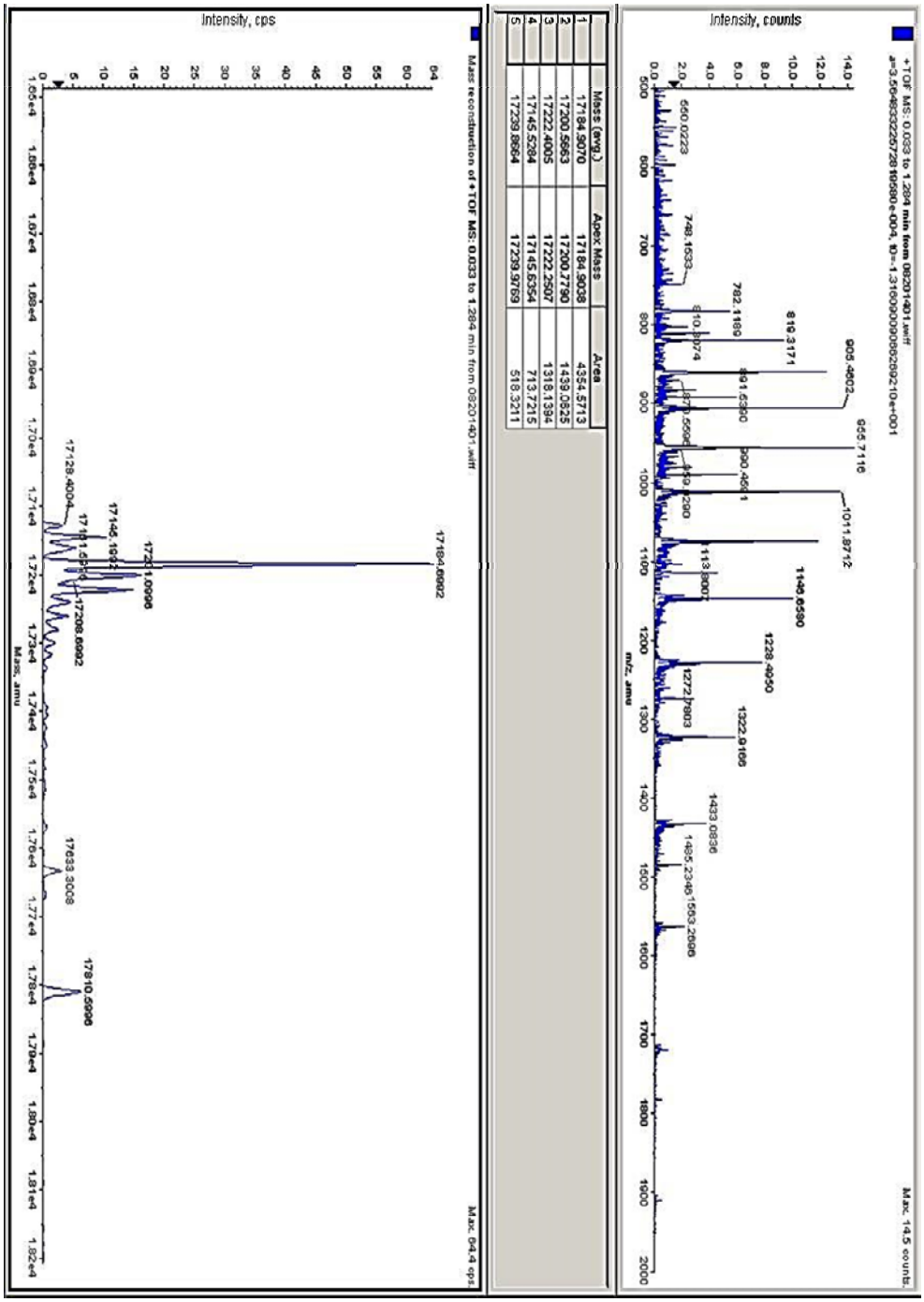


Figure 30. (A) FPLC purification chromatogram for the TEC reaction mixture for K11-linked Ub_2. (B) Coomassie-stained SDS-PAGE analysis for each peak observed in the chromatogram: peak 3 contains the purified K11-linked Ub_2.



	Mass (avg.)	Apex Mass	Area
1	17184.9070	17184.9038	4354.5713
2	17200.5663	17200.7790	1439.0625
3	17222.4005	17222.2507	1318.1394
4	17145.5284	17145.6554	713.7215
5	17239.8664	17239.9769	518.3211

Figure 31. Deconvoluted ESI-MS spectra of K11-linked Ub_2 after its E1-catalyzed amidation with allylamine. Theoretical molecule weights of Ub_2 and Ub_2-Al were 17,143 and 17,182 Da, respectively. Detected molecule weights of Ub_2 and Ub_2-Al were 17,145 and 17,184 Da, respectively.

Through an E1-catalyzed amidation reaction, alkene functional group could also be appended to the C-terminus of Ub dimers, which was supposed to react with a thiol group under the conditions of thiol-ene photo-click reaction as well. Therefore, we adopted the similar reaction conditions using Ub_2-Al and seven C-terminal Histagged UbK×C mutants (× = 6, 11, 27, 29, 33, 48, 63) as substrates and conducted a series of thiol-ene photo-click reactions to synthesize Ub trimers. The efficiency of these reactions was determined by the SDS-PAGE analysis (**Figure 32**). According to the SDS analysis, we found that for all seven positions, respective Ub trimers were successfully formed; however, the conversion rate of the thiol-ene coupling reaction was much lower than that of using Ub-Al as a substrate. A possible explanation was that, compared to Ub-Al, modified Ub_2-Al was much more difficult to obtain, considering the loss during the first thiol-ene reaction and the FPLC purification, as well as the lower efficiency of E1 amidation reaction to the C-terminus of Ub dimers. Thus, a lower concentration of Ub_2-Al (0.5 mM) was employed to perform the second thiol-ene coupling reaction. Although the concentration of UbK×C-Histag mutants was increased to 2 mM, the yield was still much lower than the first thiol-ene reaction when 1 mM of both Ub-Al and UbK11C were used. Hence, the availability of large amount of purified Ub_2-Al was one of the key factors for the successful preparation of Ub trimers.

The low yield of Ub trimers also led to the difficulty in Ub trimer purification. We tried cation exchange chromatography following the similar elution conditions as the Ub dimers purification. However, mixtures of tri-Ub and mono-Ub always eluted together, and the same results were obtained as well when we tried to use reverse-phase

high performance liquid chromatography (HPLC) purification with a C4 reverse phase column (Phenomenex, USA). After testing numerous purification conditions and columns, we decided to extract the protein band directly from the polyacrylamide gel to recover the proteins. By following this method, we attempted the purification of seven types of Ub trimers with different linkages and subjected them to SDS-PAGE analysis for purity determination (**Figure 33**). Based on the gels, Lys11-, Lys33- and Lys48-linked Ub trimers were more than 90% pure, while Lys6-, Lys27- and Lys63-linked Ub trimers still contained some impurities with similar size as *w.t.*Ub and di-Ub. For Lys29-linked Ub trimer, its recovery yield was too low to show clearly on the gel to determine its purity.

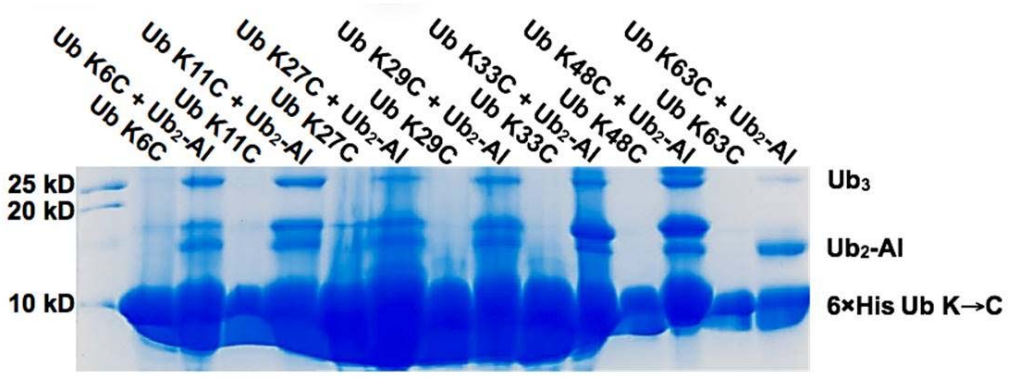


Figure 32. Coomassie-stained SDS-PAGE analysis of thiol-ene photo-click reaction products of diUb-allylamine (Ub_2-Al) with seven 6×His-tagged Ub K×C mutants (Ub K→C). A weak band with a slightly larger size than Ub_2-Al is the disulfide-conjugated homodimer of a 6×His tagged Ub K→C mutant.

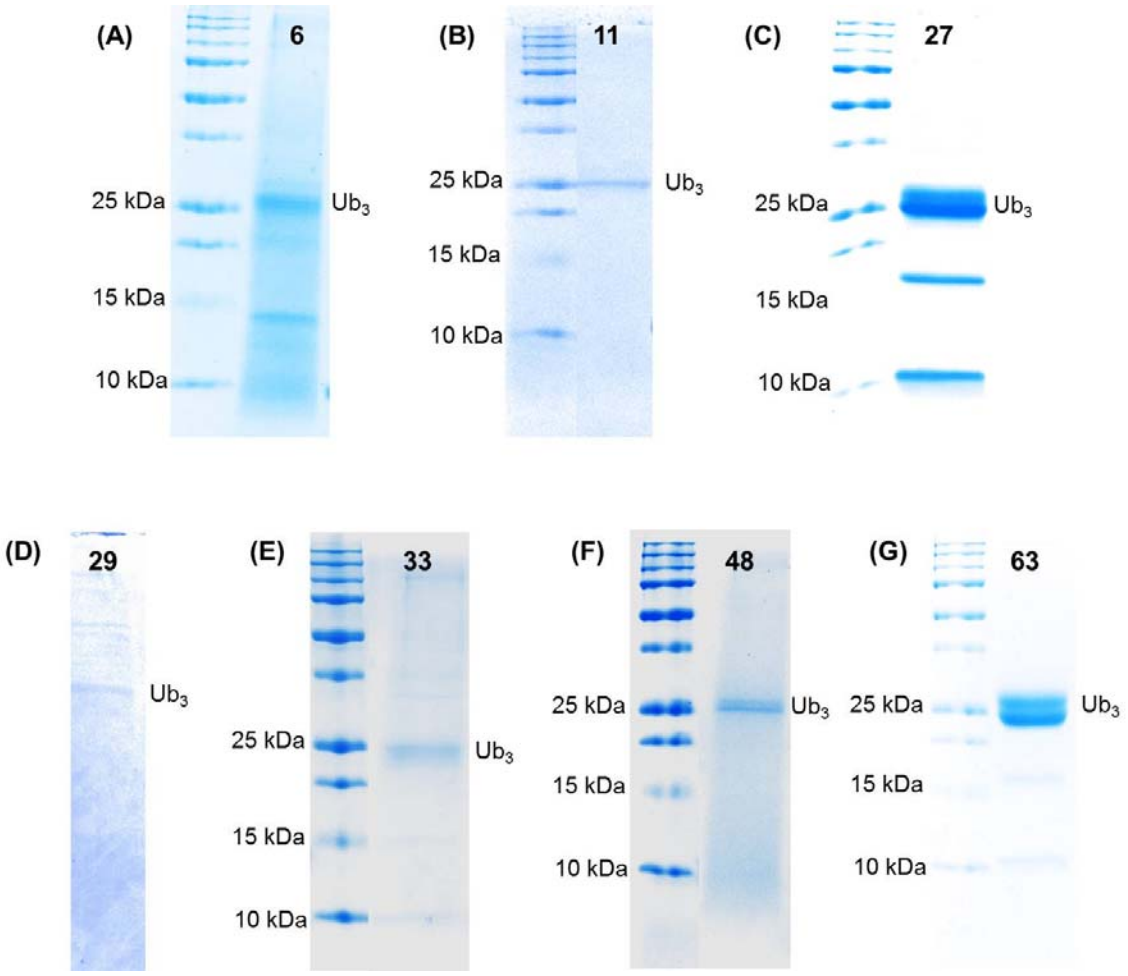


Figure 33. SDS-PAGE analysis of purity of Ub trimers after polyacrylamide gel extraction. The position of Ub trimers (Ub_3) was marked and (A) - (G) indicated seven 6×His tagged Ub K×C mutants (× = 6, 11, 27, 29, 33, 48, 63) were used to synthesize Ub trimers with different linkage types between middle and proximal Ubs.

Conclusions and Outlook

In summary, we developed a universal and straightforward method to synthesize Ub dimers and Ub trimers with distinct linkage types by conducting a E1-catalyzed amidation reaction followed by a thiol-ene photo-click reaction. With our newly-developed E1-catalyzed amidation reaction, we could successfully functionalize the Ub C-terminus with allylamine to prepare the Ub-Al conjugate, which was able to react with a thiol group in Ub K→C mutants in the presence of photoinitiator lithium acylphosphinate (LAP) under UV light. This approach produced all seven linkage types of Ub dimers, which can be recognized by diverse families of deubiquitinases including USPs, OTUs and JAMMs, according to a previous report by Strieter's group. Besides the syntheses of Ub dimers, we further expanded the combination of these two reactions to prepare Ub trimers with desirable linkages in a controllable way, which could further serve as versatile substrates for the study of deubiquitinases specificity, including both linkage specificity and *exo*-/*endo*- cleavage specificity. However, with the extended length of the reactants in E1 amidation reaction and thiol-ene reaction, the total reaction efficiency was substantially reduced. Moreover, the purification of Ub trimer from its respective monomer and dimer also proved to be much more difficult due to the largely-decreased thiol-ene coupling reaction efficiency.

Furthermore, beyond the syntheses of Ub dimers and Ub trimers, this method can be further adopted as a universal way to prepare Ub polymers with distinct desirable linkage types due to its universal applicability. However, owing to its decreased yield for the syntheses of longer polyubiquitin chains, still there are several obstacles to overcome,

especially the development of efficient purification methods for Ub polymers. With more advanced purification strategies, this method can be applied to large-scale productions of desirable Ub polymers. These distinct Ub polymers can serve as a large library that contains various linkage types and topological characteristics for the comprehensive study of deubiquitinase specificity, which will provide valuable information and dramatically improve our understanding of the ubiquitin system.

CHAPTER V

A NOVEL LUMINESCENT ASSAY FOR HIGH-THROUGHPUT SCREENING OF DEUBIQUITINASES INHIBITORS AS CANCER THERAPY

Introduction

Deubiquitinating enzymes (DUBs), also known as deubiquitinases, catalyze the removal of attached ubiquitin molecules from lysine residues of target proteins.[15] Deubiquitinating enzymes are a large (106 human enzymes currently, about 20% of all human proteases) but catalytically unusual family of proteases, whose physiological function is to hydrolyze an isopeptide bond rather than the "classical" peptide bond.[102] The important role of the deubiquitinating enzymes in the regulation of the intracellular homeostasis of different proteins and influences in key events such as cell division and apoptosis has emerged during the past few years.[46] Due to their wide-ranging involvement in key pathways such as protein stability regulation, ubiquitin homeostasis, and ubiquitin signaling, DUBs might provide new targets for therapeutic interventions of various diseases, such as cancer and neurodegeneration.[49,70,151-154] For example, ubiquitin specific peptidase 2 (USP2), one of the DUB family member, is believed to regulate the stability of fatty acid synthase (FAS) in prostate cancer, protect cells from apoptosis and interact with mouse double minute 2 homolog (MDM2). USP2 is found to be overexpressed in prostate cancer. It deubiquitinates and stabilizes fatty acid synthase (FAS), a novel oncogene in prostate cancer and possibly other cancers. Depletion of USP2 by RNA interference (RNAi) or ectopic expression results in a lower level of FAS

and enhanced apoptosis. [155-157] Therefore, inhibitors of these oncogenic DUB enzymes, such as USP2, could lead to enhanced degradation of oncoproteins and may thus stop tumor growth.[47,48,78-80]

With the increasing interest in DUBs by researchers and the pharmaceutical industry, a number of DUB substrates for *in vitro* analysis are available,[34,158-161] many relying on naturally occurring DUB substrates such as ubiquitin-60S ribosomal protein L40 precursor (ubiquitin-L40) or polyubiquitin chains and designed fusion proteins. Cleavage is reflected by a change in molecular weight and is easily monitored by HPLC or SDS–PAGE analysis.[15] However, this straightforward but laborious assay is not suitable for the determination of kinetic parameters of DUBs or high-throughput screening (HTS) for DUB inhibitors. HTS of compound libraries against pharmacological targets is a key strategy in contemporary drug discovery.[46] To generate meaningful data with the required throughput, high-quality assay methods are needed for translating specific biomolecular phenomena into observable parameters, which is achieved primarily through fluorescence and luminescence.[162]

In order to derive kinetic parameters of DUBs or perform high-throughput screening of small molecule libraries for DUB inhibitors, several fluorescent ubiquitin molecules that can serve as DUB substrates have been made available from several commercial providers. These DUB substrates rely on the generation or depletion of a fluorescent group upon DUB action (**Figure 34**). The most popular substrate, ubiquitin 7-amido-4-methylcoumarin (Ub-AMC) is a fusion of the ubiquitin carboxyl terminus to 7-amino-4-methylcoumarin with an amide linkage. Release of the fluorophore by DUB-

mediated cleavage relieves quenching of fluorescence.[101] However, Ub-AMC has two critical disadvantages when used for drug discovery screening and compound profiling campaigns. The major drawback is its unfavorable spectroscopic properties with an excitation wavelength (~355 nm) in the UV range, which can excite a significant number of screening compounds and thus will generate a large fraction of false positives. Another downside of Ub-AMC is that some DUBs fail to cleave the relatively bulky and hydrophobic 7-amino-4-methylcoumarin group which is totally different from the Gly-Lys isopeptide bond found in native ubiquitinated substrates.[102,163]

To overcome the shortcomings of Ub-AMC, another fluorogenic substrate, rhodamine-based substrate ubiquitin rhodamine110 (UbR110) was designed and developed. This substrate is designed to release a monosubstituted rhodamine after cleavage by DUBs, which permits the excitation wavelength to be moved to 480 nm, reducing the risk of artifacts in screenings due to autofluorescence of compounds.[163] However, UbR110 does not mimic the naturally occurring isopeptide linkage between Ub C-terminal carboxylate group and an ε-amino group of a lysine residue of an ubiquitinated protein. Recently, fluorescent ubiquitin substrates with native isopeptide linkages were synthesized, such as the rhodamine based ubiquitin-$^{\varepsilon}$N-($^{\alpha}$N-tetramethyl-rhodamine)-lysine (Ub-Lys-TAMRA). Cleavage of the isopeptide bond resulted in a decrease of fluorescence polarization, which made Ub-Lys-TAMRA suitable for high-throughput screenings.[102] However, the moderate dynamic range of fluorescence polarization based assay still limits the use of Ub-Lys-TAMRA for compounds profiling and kinetic studies of DUBs.

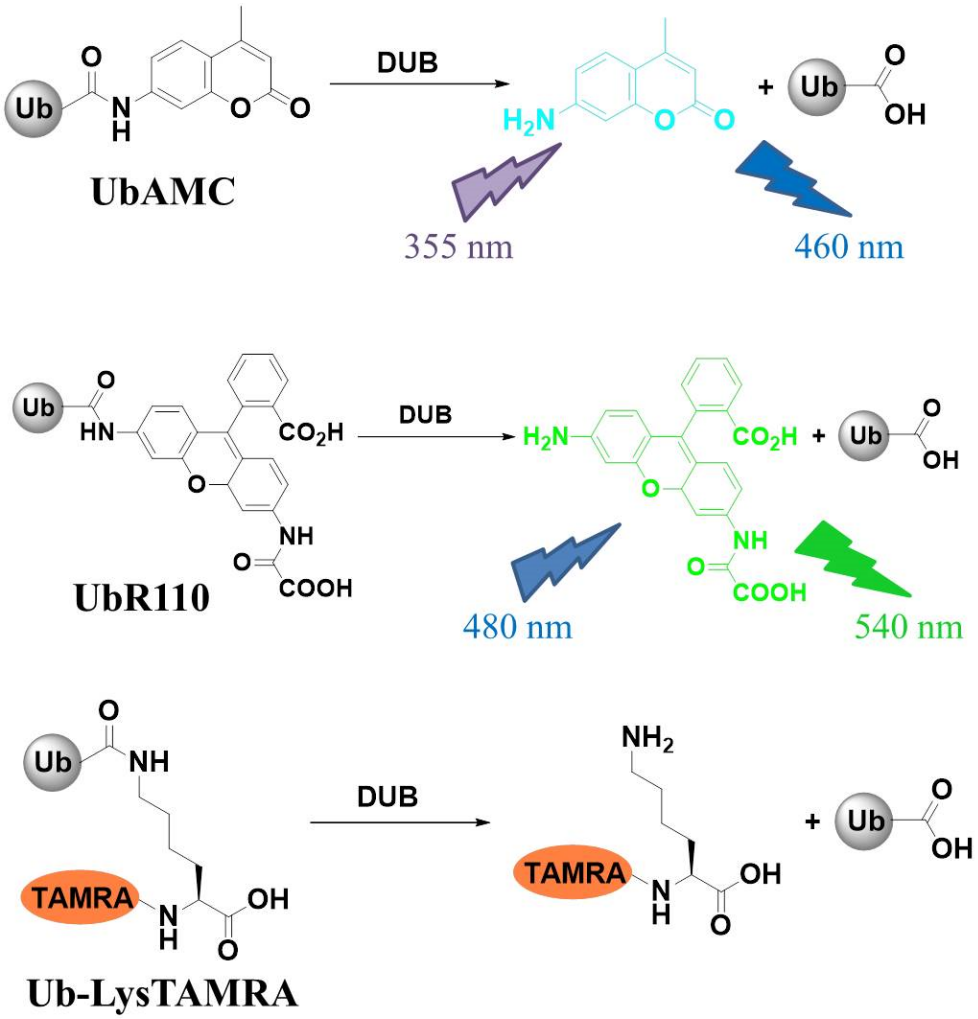


Figure 34. Scheme of deconjugation reactions of fluorogenic substrates (Ub-AMC and UbR110) and fluorescent ubiquitin conjugate (Ub-Lys-TAMRA) catalyzed by DUB.

Clearly, better model substrates for DUBs inhibitors screening are needed. Based on our simple and readily adoptable E1-catalyzed amidation reaction, we synthesized ubiquitin-D-cysteine conjugate (Ub-D-Cys) and developed a luminescence-based assay for HTS assays of DUBs inhibitors. Compared to fluorescent assays, luminescent assays typically show rather low background, which allows for a better signal-to-noise ratio and thus higher assay sensitivity.[162,164] Bioluminescence is a special form of luminescence found in living organisms, which is based on light-emitting enzymes (luciferases) and requires specific substrates (luciferins) for light production. The most widely-used luminescence assay employs the luciferase enzyme (Luc) from the North American firefly, *Photinus pyralis*. Luc catalyzes the conversion of the natural substrate D-luciferin, in the presence of oxygen, Mg^{2+}, and ATP, to oxyluciferin in a high-energy state. The subsequent relaxation to the ground state yields yellow-green light with a spectral maximum of 560 nm.[165,166] Our designed luminescence-based assay for HTS was based on this natural process (**Figure 35**). For the Ub-D-Cys, deubiquitinases (DUBs) could cleave the amide bond to release the *wild-type* ubiquitin and free D-cysteine molecules, while the D-cysteine molecules could subsequently react with 6-hydroxybenzothiazole-2-carbonitrile to form luciferin, which was reported previously. With the presence of ATP, Mg^{2+}, and luciferase enzyme, luminescence should then be observed. Therefore, Ub-D-Cys could serve as a novel substrate for luminescence-based HTS assays for DUBs inhibitors. We believed that this novel assay would facilitate functional investigations of DUBs and accelerated drug discovery for DUB-targeted disease interventions.

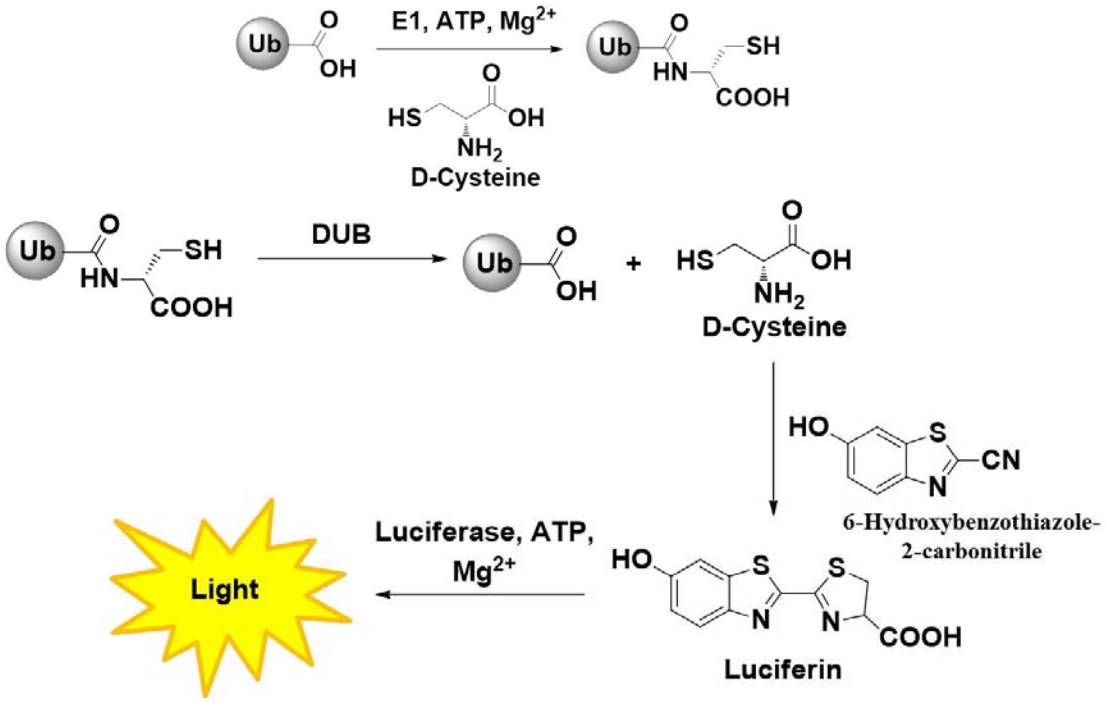


Figure 35. Scheme of designed luminescence-based high-throughput screening (HTS) assay for deubiquitinases inhibitors discovery. For the Ub-D-Cys, deubiquitinases (DUBs) could cleave the amide bond to release the *wild-type* ubiquitin and free D-cysteine molecules, while the D-cysteine molecules could react with 6-hydroxybenzothiazole-2-carbonitrile to obtain luciferin. With the presence of ATP, Mg^{2+}, and luciferase enzyme, the luminescence would be radiated.

Experimental Details

DNA and Protein Sequences

DNA sequence

Histidine tagged-ubiquitin specific protease 2 (USP2)

atgggcagcagccatcaccatcatcaccacagccaggatccgaattcaaattctaagagtgcccagggtctggctg
gtcttcgaaaccttgggaacacgtgcttcatgaactcaattctgcagtgcctgagcaacactcgggagttgagagatt
actgcctccagaggctctacatgcgggacctgcaccacggcagcaatgcacacacagccctcgtggaagagtttg
caaaactaattcagaccatatggacttcatcccccaatgatgtggtgagcccatctgagttcaagacccagatccaga
gatatgcaccgcgctttgttggctataatcagcaggatgctcaggagttccttcgctttcttctggatgggctccataac
gaggtgaaccgagtgacactgagacctaagtccaaccctgagaacctcgatcatcttcctgatgacgagaaaggcc
gacagatgtggagaaaatatctagaacgggaagacagtaggatcggggatctctttgttgggcagctaaagagctc
gctgacgtgtacagattgtggttactgttctacggtcttcgacccccttctgggacctctcactgcccattgctaagcgag
gttatcctgaggtgacattaatggactgcatgaggctcttcaccaaagaggatgtgcttgatggagatgaaaagcca
acatgctgtcgctgccgaggcagaaaacggtgtataaagaagttctccatccagaggttcccaaagatcttggtgct
ccatctgaagcggttctcagaatccaggatccgaaccagcaagctcacaacatttgtgaacttccccctaagagacc
tggacttaagagaatttgcctcagaaaacaccaaccatgctgtttacaacctgtacgctgtgtccaatcactccggaac
caccatgggtggccactatacagcctactgtcgcagtccagggacaggagaatggcacactttcaacgactccagc
gtcactcccatgtcctccagacaagtgcgcaccagcgacgcctacctgctcttctacgaactggccagcccgccctc
ccgaatgtga

Protein sequence

Histidine tagged-ubiquitin specific protease 2 (USP2)

MGSSHHHHHHSQDPNSNSKSAQGLAGLRNLGNTCFMNSILQCLSNTREL
RDYCLQRLYMRDLHHGSNAHTALVEEFAKLIQTIWTSSPNDVVSPSEFKT
QIQRYAPRFVGYNQQDAQEFLRFLLDGLHNEVNRVTLRPKSNPENLDHL
PDDEKGRQMWRKYLEREDSRIGDLFVGQLKSSLTCTDCGYCSTVFDPFW
DLSLPIAKRGYPEVTLMDCMRLFTKEDVLDGDEKPTCCRCRGRKRCIKK
FSIQRFPKILVLHLKRFSESRIRTSKLTTFVNFPLRDLDLREFASENTNHAV
YNLYAVSNHSGTTMGGHYTAYCRSPGTGEWHTFNDSSVTPMSSRQVRT
SDAYLLFYELASPPSRM

Protein Expression and Purification

Histidine tagged-ubiquitin specific protease 2 (USP2)

E. coli BL21 cells were transformed with human USP2 expression plasmid (pETDuet-Histag-USP2). A starter culture was grown in 2×YT media (5 mL) with 100 μg/mL ampicillin at 37°C overnight. The culture was inoculated into 2×YT media (1 L) with the same concentration of ampicillin. Isopropyl β-D-1-thiogalactopyranoside (IPTG) (0.5 mM) was added into the cell culture after the OD600 reached 0.9~1.1. The cell culture was incubated at 37°C for 6 hours, and the cells were harvested by centrifugation at 4,000 r.p.m. for 30 min at 4°C and resuspended in 100 mL lysis buffer (10 mM Tris-HCl, 100 mM NaCl, 10 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM Tris (2-carboxyethyl) phosphine (TCEP), 1 mM $MgCl_2$, and 100 mg/ml lysozyme, pH 8.0) and sonicated in an ice/water bath four times (3 min each, 10 min interval to cool down the

suspension to below 10°C before the next run) and the lysate was clarified by centrifugation at 10,000 r.p.m. for 40 min at 4°C. The clarified supernatant was then incubated with 2 mL of Ni Sepharose™ 6 Fast Flow beads from GE Healthcare (Little Chalfont, United Kingdom) for 20 min for the purpose of effectively binding and then poured into a 10-mL empty purification column to allow the Ni sepharose beads to settle completely by gravity. The Ni sepharose beads were then washed with 50 mL buffer A (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 7.8) to get rid of any unspecific binding proteins and subsequently eluted out with 12 mL of buffer B (50 mM NaH_2PO_4, 300 mM NaCl, 300 mM imidazole, pH 7.8). The eluted solution was concentrated by Amicon Ultra-15 centrifugal filter units - 10,000 NMWL from Millipore (Billerica, MA) to 1 mL. The purity of expressed USP2 was confirmed by SDS-PAGE analysis. The buffer was then changed to the stock buffer (45 mM potassium phosphate, 300 mM NaCl, pH 6.0) by dialysis. The concentration was determined by BCA protein assay kit from Thermo Fisher Scientific Inc. (Rockford, IL). According to the concentration, USP2 expression yield was around 50 mg/L from the 2×YT media. Purified USP2 protein at 10 mg/mL was divided into small aliquots, frozen in liquid nitrogen, and stored at -80°C.

E1-Catalyzed Amidation Reaction for the Synthesis of Ub-D-Cysteine

Aliquots of purified mouse E1 stored at -80°C were rapidly thawed and placed on ice. The reaction contained 250 μM Ub, 2 μM E1, 2 mM ATP, 5 mM D-cysteine (pre-reduced by 5 mM TCEP) and were incubated in 1 mL (final volume) of the buffer (50

mM HEPES, 250 mM sucrose, 50 mM KCl, 3 mM $MgCl_2$, pH 7.4) at 4°C overnight. The conversion rate was determined by ESI-MS analysis.

Cation Exchange Chromatography for Ub-D-Cysteine Purification

E1 amidation reaction mixture was dialyzed to buffer A (100 mM ammonium acetate, 1 mM DTT, pH 4.5) and further purified by cation exchange chromatography with fast protein liquid chromatography (FPLC) system (Bio-Rad Laboratories, Inc.). The reaction mixture was concentrated to 1 mL and injected into a 20 mL HiPrep SP HP 16/10 column (GE Healthcare Life Sciences, Little Chalfont, United Kingdom). The program was set as followed: 1) the column was washed with a gradient of 0-60% B (100 mM ammonium acetate, 1 M NaCl, 1 mM DTT, pH 4.5) over 10 column volumes; 2) then washed with a gradient of 60-100% B for one column volume; 3) finally washed with 100% B for one column volume. Pure fractions for each peak were collected and concentrated by Amicon Ultra-15 centrifugal filter units - 3,000 NMWL from Millipore (Billerica, MA) for SDS-PAGE analysis.

Luminescent Assay Procedure for Z-Factor Determination

With our Biotek® Synergy H1 Multi-Mode Reader (Winooski, VT), the LUMITRAC™ 600-384 well plate from Greiner bio-one (Kremsmünster, Austria) was used to study the ubiquitin specific peptidase 2 (USP2) cleavage activities to Ub-D-Cys based on the designed luminescent assay.

The reaction buffer consisted of 25 mM Tris, 200 mM glycine, 10 mM $MgCl_2$ and 10 mM KCl with pH at 7.6. Aliquots of purified USP2 stored at -80°C were rapidly

thawed and placed on ice. The stock solutions for each reactant were listed as followed: *N*-ethylmaleimide (NEM, 10 mM in DMSO), 6-hydroxybenzothiazole-2-carbonitrile (1 mM in DMSO, Sigma-Aldrich), Ub-D-Cys (10 μM in reaction buffer with 5 mM DTT), ubiquitin specific peptidase 2 (USP2, 2 μM in reaction buffer with 5 mM DTT), ATP (100 mM in water, pH = 7), luciferase from *Photinus pyralis* (firefly) (1 mg from Sigma-Aldrich).

To validate the design of the luminescent assay, Z-factor was determined by using *N*-ethylmaleimide (NEM) as positive control while DMSO as negative control, and calculated using the following equation:

$$Z\text{-}factor = 1 - \frac{3(\sigma_p + \sigma_n)}{|\mu_p - \mu_n|}$$

σ_p is the standard deviations of positive controls; σ_n is the standard deviations of negative controls; μ_p is the means of positive controls; μ_n is the means of negative controls.

Firstly, 2 μL DMSO or NEM (10 mM in DMSO) and 20 μL reaction buffer containing 150 nM USP2 were added to the LUMITRAC™ 600-384 well plate and mixed well. The plate was incubated at room temperature for 20 min. Then 20 μL reaction buffer containing 600 nM Ub-D-Cys and 60 μM 6-hydroxybenzothiazole-2-carbonitrile was added into each well and the plate was incubated at 37°C for 1 hour. After that, 20 μL reaction buffer containing 100 μM ATP and 0.2 μg luciferase was added to each well and mixed together. Total reaction volume for each well was 62 μL. For both positive and negative groups, 23 parallel assays were performed to obtain the

value for Z-factor. Then the Biotek® Synergy H1 Multi-Mode Reader was used to determine the luminescence signal for each well. Luminescence fibers were choosen for the measurement and the Gain was set at 150, while the integration time was set as every one second.

Luminescent Assay for High-throughput Screening (HTS)

For the high-throughput screening assay, we used NIH Clinical Collection which was a group of 727 compounds with known activity against many cancers to study their effects on USP2. The CyBio® Well Vario Channel Simultaneous Pipettor System (Jena, Germany) was adopted for automatic pipetting into the LUMITRAC™ 600-384 well plate (Greiner bio-one, Kremsmünster, Austria). The Biotek® Synergy H1 Multi-Mode Reader (Winooski, VT) which provided detection capabilities in luminescence was employed to study the compounds interactions with USP2 based on the designed luminescent assay.

The layout for the 384-well plate was as followed: for the first two columns, 2 μL DMSO was added manually for each well as the negative controls, while on the other hand, 2 μL NEM (10 mM in DMSO) was added manually into the wells in last two columns as the positive controls. For those wells between, 2 μL small compounds stock solution (1 mM in DMSO) were transferred from NIH Clinical Connection plates into each well automatically by the CyBio® Well Vario Channel Simultaneous Pipettor System, respectively.

After that, 20 μL reaction buffer (25 mM Tris, 200 mM glycine, 10 mM $MgCl_2$, 10 mM KCl, pH 7.6) containing 150 nM USP2 were added to each well and mixed well

automatically by the CyBio® Well Vario Channel Simultaneous Pipettor System. The plate was incubated at room temperature for 20 min. Then 20 μL reaction buffer containing 600 nM Ub-D-Cys and 60 μM 6-hydroxybenzothiazole-2-carbonitrile was added into each well and mixed together automatically. The plate was placed into incubator at 37°C for 1 hour. Finally, 20 μL reaction buffer containing 100 μM ATP and 0.2 μg luciferase was added to each well and mixed together automatically. Total reaction volume for each well is 62 μL. Then the Biotek® Synergy H1 Multi-Mode Reader was used to determine the luminescence signal for each well. Luminescence fibers were choosen for the measurement and the Gain was set at 150, while the integration time was every one second.

Results and Discussion

USP2 belongs to the USP family of deubiquitinating enzymes, which is believed to inhibit the degradation of various tumor-growth-promoting proteins by removing the trigger for degradation. USP2 is thus a promising drug target for cancer therapy. Due to its easy-accessibility and relatively high expression levels, we chose USP2 as a target and applied our designed luminescent assay for the discovery of USP2 inhibitors. N-terminal Histidine tagged USP2 was expressed in *E.coli* BL 21 cells and purified by the nickel affinity method. The purity of USP2 was estimated to above 90% by SDS-PAGE analysis (**Figure 36**).

Using our efficient E1-catalyzed amidation reaction, we functionalized *w.t.*Ub C-terminal carboxyl group with a primary amine molecule, D-cysteine, with a near quantitative conversion rate, which was indicated by the presence of a peak at 8,668 Da from ESI-MS consistent with the predicted molecular weight for a Ub-D-cysteine (Ub-D-Cys) conjugate (**Figure 37**). Since our designed luminescent assay was based on the reaction between D-cysteine and 6-hydroxybenzothiazole-2-carbonitrile, to remove the free D-cysteine molecule in E1 amidation reaction mixture, we conducted additional cation exchange chromatography using FPLC to purify the Ub-D-Cys conjugate. Ub-D-Cys was eluted at around 20% NaCl concentration, as shown in **Figure 38**. After FPLC purification, fractions of Ub-D-Cys were collected and concentrated to 8 mg/mL prior to further reaction.

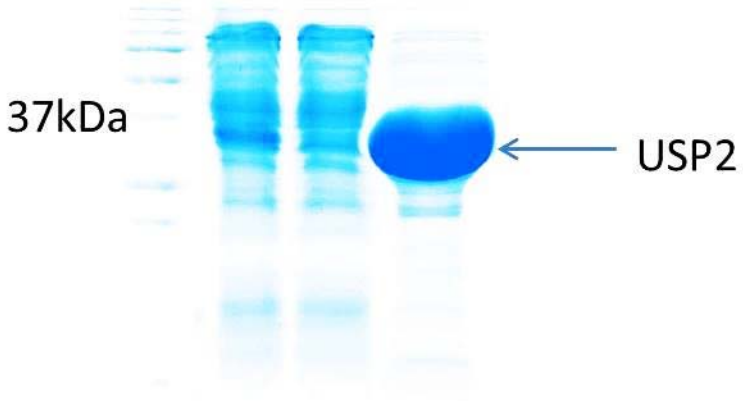


Figure 36. Coomassie-stained SDS-PAGE analysis of USP2. USP2 was expressed in *E. coli* BL21 cells and purified with Ni Sepharose^{TM} 6 Fast Flow beads from GE Healthcare.

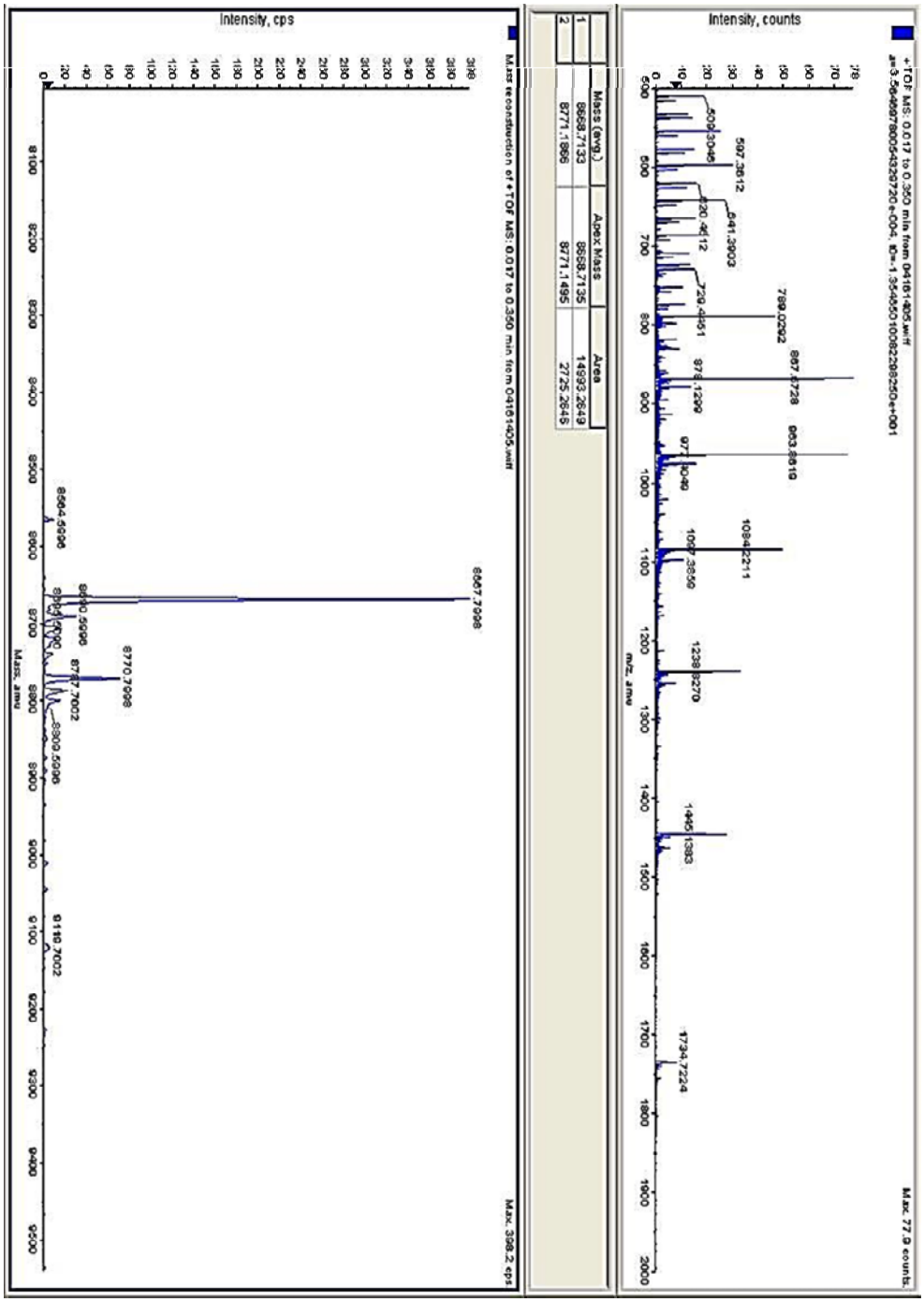


Figure 37. Deconvoluted ESI-MS spectra of Ub-D-Cys. The theoretical molecular weight of Ub-D-Cys was 8,667 Da. The detected molecular weight of Ub-D-Cys was 8,668 Da.

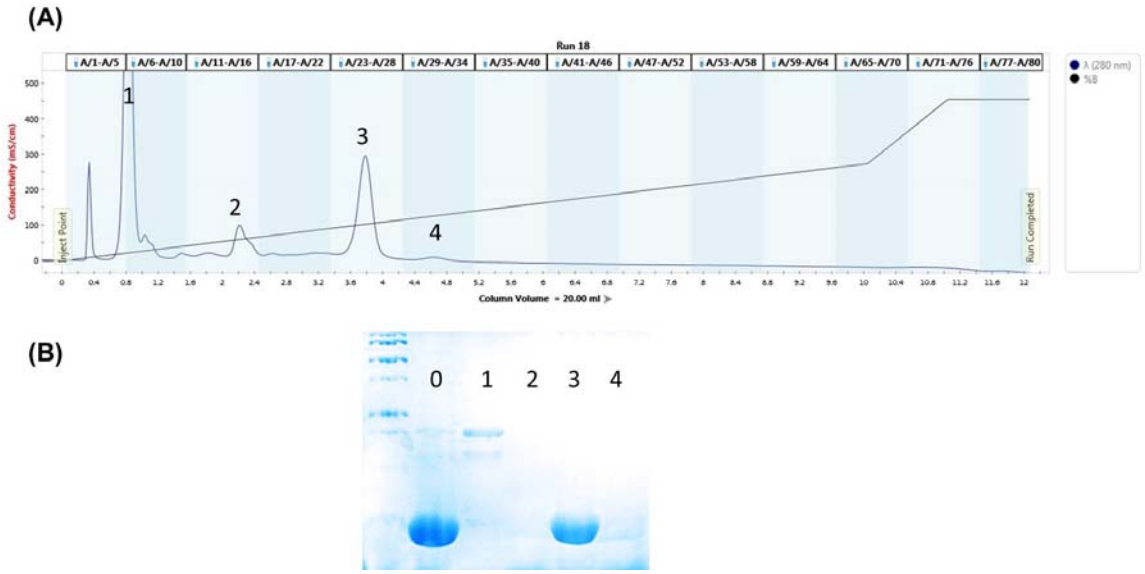


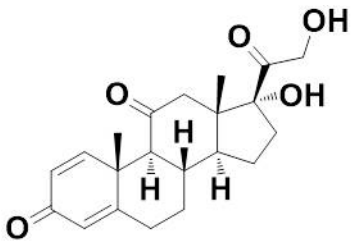
Figure 38. (A) FPLC purification chromatogram for Ub-D-Cys. (B) Coomassie-stained SDS-PAGE analysis for each peak observed in the chromatogram: peak 3 contained the purified Ub-D-Cys. Lane 0 was the Ub-D-Cys before FPLC.

With purified Ub-D-Cys as the substrate, and purified USP2 as the study target, we tested our designed luminescence-based assay with commercially available 6-hydroxybenzothiazole-2-carbonitrile and luciferase from *Photinus pyralis* (Sigma-Aldrich, St. Louis, MO). To validate the applicability of this assay, we employed the general irreversible inhibitor of cysteine peptidases, *N*-ethylmaleimide (NEM), as the positive control compared with DMSO as the negative control. 23 parallel assays were performed to obtain the value of Z-factor, which was calculated to quantify the suitability of an assay for the purpose of high-throughput screening. Firstly, DMSO or NEM was incubated with USP2 at room temperature for 20 minutes, respectively, in order to interact with the enzyme. Then the substrate Ub-D-Cys was added together with 6-hydroxybenzothiazole-2-carbonitrile and the reaction mixture was placed at 37°C. The USP2 deconjugation reaction was conducted for one hour. During this process, D-cysteine should be released from the ubiquitin C-terminus and further react with 6-hydroxybenzothiazole-2-carbonitrile to yield luciferin. The yield of the luciferin was dependent on USP2 cleavage activity toward Ub-D-Cys. After one hour, ATP and luciferase were added to the reaction system. The resulting luminescence signal was measured by the plate reader and a Z-factor value was calculated accordingly. Based on the values obtained and listed in Table 3, σ_p (standard deviations of positive controls), σ_n (standard deviations of negative controls), μ_p (means of positive controls), and μ_n (means of negative controls) were calculated as 3.9, 487.9, 16.4 and 29.9, respectively. Therefore, the value of Z-factor was equal to 0.78, which indicated the excellent applicability of this assay as a HTS.

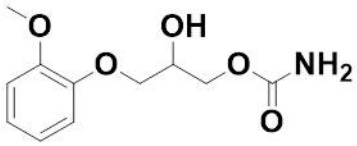
Table 3. Luminescence value for 23 parallel assays of positive controls (NEM) and negative controls (DMSO).

No.	Lum (NEM)	Lum (DMSO)	**No.**	Lum (NEM)	Lum (DMSO)
1	12	451	**13**	12	501
2	19	473	**14**	19	541
3	22	471	**15**	18	464
4	15	562	**16**	14	506
5	18	490	**17**	12	441
6	17	501	**18**	17	484
7	13	522	**19**	11	495
8	15	491	**20**	14	442
9	23	461	**21**	17	495
10	24	507	**22**	16	481
11	12	509	**23**	23	477
12	14	457			

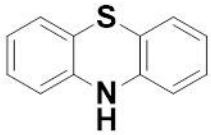
After initial validation of the assay, we chose to run a test screen with the NIH Clinical Collection (NCC), which consists 727 compounds with a history of use in human clinical trials. The clinically tested compounds in the NCC were highly drug-like with known safety profiles and undiscovered bioactivities for potential therapeutic, which could provide excellent starting points for medicinal chemistry optimization and might be appropriate for direct human use in treating human disease. Similar procedures were adopted for this HTS as employed for the Z-factor determination. Of the 727 compounds screened, several dozens were found to display inhibitory effects on USP2. Among them, several displayed very strong inhibitory effects on USP2 with more than ten-fold decreased luminescence signal (**Figure 39**), and represent excellent candidates for further IC_{50} (half maximal inhibitory concentration) measurement to study their respective effectiveness in inhibiting USP2.



Prednisone



Methocarbamol



Phenothiazine

Figure 39. The structure of the selected small molecules displaying the highest inhibitory effects on USP2 through our developed luminescent HTS assay using Ub-D-Cys as the substrate.

Conclusions and Outlook

In conclusion, we designed and developed a novel luminescent assay for high-throughput screening of deubiquitinase inhibitors, and tested it with one common DUB, USP2, to validate the applicability of this assay. This innovative assay was built on the utilization of E1-catalyzed amidation reaction for the easy preparation of a Ub-D-Cys conjugate and the efficient formation of luciferin from D-cysteine to release luminescence in the presence of luciferase. The accuracy and suitability of this assay for HTS was confirmed by the high Z-factor (0.78). In addition, several small molecules from the NIH Clinical Collection were selected for further study due to their relatively high inhibitory effects in this assay.

Compared with currently widely-used fluorogenic ubiquitin conjugates, this assay was based on the measurement of luminescence, which resulted in a lower background and higher sensitivity. Further, the synthesized DUB substrate, Ub-D-Cys, mimicked the native isopeptide linkage compared to currently widely-used substrates-Ub-AMC and UbR110, and thus should provide a more realistic substrate for the study of many other DUBs. Therefore, this assay could be broadly adopted for DUB inhibitor discovery. In addition, the implementation of this assay was straightforward, involving simple addition and mixing reagents at different time points, a protocol that could be readily adopted by virtually any research group. This substantive departure from the status quo was expected to overcome limitations associated with current DUB studies, accelerating our understanding of DUB functional roles, and facilitating identification of DUB-targeting drugs. Currently, more oncogenic DUB members have been discovered,

including USP2, USP7, USP9, USP28, *etc.* With this assay and these oncogenic DUBs as drug targets, more small molecules could be screened as potential specific inhibitors for preclinical study and should lead to faster identification of promising drugs for cancer therapy.

CHAPTER VI

CONCLUSIONS

Post-translational modifications (PTMs) dramatically diversify the proteome. Among them, phosphorylation, acetylation, methylation, hydroxylation and glycosylation are common types which covalently add respective small chemical groups to one or several side chain residues in a protein. Besides, there is another major type of PTMs, ubiquitination, which occurs with covalent additions of another protein (ubiquitin) to the lysine side chain amino group of a protein substrate. Ubiquitin (Ub) is a small but evolutionarily conserved polypeptide with 76 amino acids containing seven lysines (K6, K11, K27, K29, K33, K48, and K63) as well as N-terminal amino group, which can act as the acceptors for further ubiquitination. Therefore, besides monoubiquitination and multi-monoubiquitination defined by the attachment of ubiquitin to one or multiple lysine side chain amino groups of substrates, eight types of homo-typic (one linkage type per ubiquitin polymer) polyubiquitination as well as various combinations of hetero-typic (multiple linkage types per ubiquitin polymer) polyubiquitination are also indispensable components of the ubiquitination family. The reversed process is achieved by deubiquitinases (DUBs), also known as deubiquitinating enzymes. Notably, recent studies have revealed that the activities of these enzymes play pivotal roles in numerous cellular processes including signal transduction, embryonic development, endocytic trafficking, and the immune response. Malfunction of deubiquitinases attributes to significant cellular alteration and is highly related to the occurrence of various human

diseases, thus, DUBs, as an emerging potential drug targets, have attracted increasing interests from not only researchers but also the pharmaceutical industry. However, currently, the development of DUB research has been largely hampered by the difficulty in the accessibility of diversified DUB substrates, including both suitable ubiquitin conjugates for high-throughput screening (HTS) assays and various polyubiquitin chains for *in vitro* and *in vivo* analysis.

Recently, we developed a novel and facile approach to append numerous orthogonal functional groups (thiol, azide, alkyne, alkene, *etc.*) to the Ub C-terminus with high efficiency as well as high yield (>90%) according to electrospray ionization mass spectrometry (ESI-MS) by exploiting the catalytic activity of the ubiquitin-activating enzyme (E1). Beyond this approach, we further developed these functionalized Ub derivatives into multiple DUB substrates for the activity study and inhibitors discovery of DUBs. Firstly, based on the difference in fluorescence polarization, we covalently labelled several functionalized ubiquitin derivatives with their respective fluorophores to generate novel fluorescent ubiquitin conjugates and adopted them as starting substrates for DUB kinetic study by measuring the fluorescence polarization change with reaction time. Besides, inspired by the use of thiol-ene coupling reaction for syntheses of Ub dimers described by Strieter's group, one of the functionalized Ub derivatives, Ub-Al, was site-specifically coupled with Ub K→C mutants to yield di-Ub mimics. These synthesized di-Ub molecules could be recognized by an E1 as well and conducted another E1-catalyzed reaction to produce Ub_2-allylamine, which would be followed by a thiol-ene coupling reaction for the assembly

of Ub trimer molecules. Ideally, this synthetic route might be further applied to longer Ub polymers preparation, and these synthesized Ub polymers could constitute a large library for the activity and specificity study of DUBs. Furthermore, considering multiple types of DUBs acting as oncoproteins, the discovery of their inhibitors would become promising cancer therapies. Thus, a novel high-throughput screening (HTS) assay for DUB inhibitors discovery was developed on the basis of the bioluminescence process. Ub-D-cysteine (Ub-D-Cys) was designed as the starting substrate and produced through an E1-catalyzed amidation reaction. The released D-cysteine upon the DUB hydrolytic action would cause the production of luminescence after a series of chemical reactions in the presence of other substances; thereby the inhibitory effects of different small molecules could be readily detected by the luminescence intensity. Altogether, those previous chapters were mainly focused on the development and optimization of the E1-catalyzed amidation reaction and its application in developing novel chemical biology tools for investigating deubiquitinating enzymes.

The initial hypothesis of E1-catalyzed amidation reaction was proposed in reference to the pyramidal design of the natural ubiquitination process, E1-E2-E3 cascade, in which one ubiquitin-activating enzyme (E1) could initiate the concerted three-step enzymatic cascade and function together with dozens of ubiquitin conjugating enzymes (E2s) and hundreds of ubiquitin ligase enzymes (E3s) to guarantee substrate specificities. The universality and high-efficiency of the E1 enzymes to initiate the frequent ubiquitination processes in eukaryotic cells inspired us to consider its interaction with primary amine molecules. As a result, we tested our hypothesis with a

series of primary amines and all of them could be attached to the ubiquitin C-terminus carboxylate group with high yields (>90%), which greatly mimicked the natural ubiquitination process. For hydrazine, hydroxylamine and cysteamine, due to their relatively low pKa and high reactivities, the conjugation of them to Ub C-terminus required relatively lower concentrations of small molecules at 50 mM or less, as well as physiological pH at around 7.4 at the presence of *wild-type* ubiquitin, E1 enzyme and ATP. On the other hand, for allylamine, propargylamine and azidoethylamine molecules, owing to their relatively higher pKa and stability, the attachment of them to Ub C-terminus had to be conducted under harsher conditions including higher pH at around 9.4 for their efficient nucleophilic attacks and 10-fold higher concentrations at above 500 mM. Nevertheless, all of these tested primary amines could be efficiently appended to the Ub C-terminus at their respective optimized reaction conditions. Therefore, this E1-catalyzed amidation reaction provided us an easier approach to functionalize Ub C-terminus with various functional groups, which could not be achieved previously without complicated total chemical synthesis and native chemical ligation. Also, besides these reported amine molecules, other primary amines were also supposed to be attached to Ub C-terminus efficiently by optimizing their reaction conditions including concentrations, pH, *etc.* In one word, a simple and efficient one-step reaction called E1-catalyzed amidation reaction was developed by us recently, which could be potentially applied for desired Ub C-terminus functionalization.

Using this straightforward E1-catalyzed amidation reaction, several functional groups could be introduced to Ub C-terminus, which could be further applied to various

aspects. Firstly, we successfully functionalized Ub C-terminus with multiple orthogonal groups (thiol, azide, alkyne, *etc.*), which could be selectively modified to constitute different types of Ub-fluorophore conjugates. These Ub-fluorophore conjugates could be used as substrates for DUB kinetic study by measuring the time-dependent fluorescence polarization change starting from less depolarized large Ub-fluorophore conjugates to more depolarized small fluorescein molecules. Among them, the terminal alkyne and azide groups could undergo the Cu (I)-catalyzed azide-alkyne Huisgen cycloaddition reaction with corresponding clickable dyes at optimized reaction conditions, resulting in the generation of desired Ub-fluorophore conjugates with yields above 90%; while the thiol group could be efficiently labeled by 5-iodoacetamidofluorescein (5-IAF) and achieve a near quantitative conversion to yield a fluorescent ubiquitin derivative with a flexible linkage between ubiquitin and fluorophore that greatly mimicked the native isopeptide linkage in ubiquitinated proteins. For all three fluorescent ubiquitin conjugates, their suitability to serve as DUB substrates was confirmed by testing with one common DUB, Ub C-terminal hydrolase isozyme L3 (UCH-L3), and the determined kinetic parameters via the fluorescence polarization-based assays were comparable to the values previously reported in the literature, which vindicated these three Ub-fluorophore conjugates as efficient substrates for DUB activity assays. Compare to currently widely-used fluorogenic DUB substrates for DUB kinetic study, ubiquitin-7-amino-4-methylcoumarin (Ub-AMC) and ubiquitin rhodamine110 (UbR110), our newly-developed three fluorescent ubiquitin conjugates possessed more physiologically-based flexible linkages for better recognition and action by most DUBs, while Ub-AMC and

UbR110 contained rather bulky and hydrophobic fluorophores linked to the Ub C-terminus directly, preventing them from hydrolysis by many DUBs. Besides, the synthetic routes of these fluorescent ubiquitin conjugates employed the robust and straightforward chemistry for the fluorescent labeling, which rendered them readily adoptable for large-scale preparations by a regular biochemistry and molecular biology research group. Therefore, these novel fluorescent ubiquitin conjugates were expected to become widely-used substrates for DUB kinetic study in future due to their easy-accessibility and universal-applicability for most DUBs.

In addition to the application in the field of fluorescent Ub conjugates syntheses for DUB kinetic study, the E1-catalyzed amidation reaction could also be exploited for Ub polymers (poly-Ub) generation. The one-step high-efficient E1-catalyzed amidation reaction was able to attach allylamine to Ub C-terminus, leading to a ubiquitin conjugate with the terminal alkene functionality, Ub-allylamine (Ub-Al). Ub-Al was previously reported by Strieter's group that could undergo the thiol-ene coupling (TEC) photo-click reaction with Ub K→C mutants as a new approach for the syntheses of Ub dimers. Considering the universality of the E1 enzyme in the ubiquitin system, we inferred that the E1-catalyzed amidation reaction could also act on a di-Ub molecule to functionalize its C-terminus with multiple functional groups through the nucleophilic attack of primary amine molecules. Thus, we extended the E1-catalyzed amidation reaction to di-Ub molecule that was obtained through the thiol-ene photo-click reaction between a Ub-Al and a Ub K→C mutant, and subsequently functionalized the di-Ub molecule with a terminal alkene group, which would be able to react with Ub K→C mutants for a second

thiol-ene coupling reaction to generate tri-Ub molecules. Theoretically, E1-catalyzed amidation reaction could be applied to any ubiquitin polymer as long as they contained a free C-terminus for the nucleophilic attack. Moreover, the thiol-ene coupling reaction depended on the concurrent presence of a thiol and an alkene group. Thus, the E1-catalyzed amidation reaction and the thiol-ene coupling reaction could be combined together to serve as a general approach for desired ubiquitin polymer preparation in a controllable way. In practice, due to the relatively low reaction efficiency of thiol-ene coupling reaction, we had encountered many difficulties in tri-Ub molecules preparation and purification, and thus didn't continue to explore the applicability of this general approach for longer Ub polymer preparation. Still, tri-Ub molecules contained the basic structure of a Ub polymer, containing a proximal Ub, a middle Ub, and a distal Ub, which would be enough for the preliminary *exo-/endo-* cleavage specificity study of DUBs. Besides, there are two linkage positions in tri-Ub molecules, each with seven possible linkage types, which would provide abundant materials for the cleavage specificity study of DUBs. All in all, a general approach for Ub polymers preparation was developed by integrating a E1-catalyzed amidation reaction with a thiol-ene coupling reaction, and tri-Ub molecules with desirable linkage types were synthesized by following this approach. Current DUB cleavage specificity study was greatly hindered by the difficulty in large-scale production of versatile Ub polymers with distinct linkage types as well as topological characteristics, and the appearance of this approach would become the promising standard method for desired Ub polymers

preparation and hopefully accelerate the research progress in the field of DUB specificity study.

Besides applying the E1-catalyzed amidation reaction to different types of substrates syntheses for DUB activity study, we also broadened its application into the field of drug discovery for cancer therapy by developing a novel high-throughput screening (HTS) assay. Recent research in DUB field has revealed that many of them were overexpressed in various types of cancers and more detailed study uncovered that several DUBs, such as USP2, USP7, USP9, *etc.*, acted as oncoproteins in cellular pathways. Discovery of specific inhibitors for these oncogenic DUBs could become a potential route for future cancer therapy, thus, these oncogenic DUBs have emerged as new drug targets in clinical trials. However, in contrast with the increasing attention to the drug discovery for these oncogenic DUBs, still, current HTS methods of DUB inhibitors had several drawbacks which hindered the progress in the discovery of DUB inhibitors. To solve this problem, we developed a novel HTS assay based on luminescence detection with the ubiquitin-D-cysteine (Ub-D-Cys) synthesized using E1-catalyzed amidation reaction as the DUB substrate. After DUB hydrolytic cleavage, D-cysteine would be released from Ub C-terminus, and it could then react with a compound, 6-hydroxybenzothiazole-2-carbonitrile, to generate a luciferin. The luciferin would undergo a series of chemical reactions at the presence of ATP, Mg^{2+}, and luciferase to release luminescence as the detection signal. The design of this assay was especially straightforward and the large-scale preparation of the substrate, Ub-D-Cys, could be readily implemented by following the E1-catalyzed amidation reaction.

Moreover, compared to other current methods, this assay was based on the luminescence signal, with much lower background and higher sensitivity than other fluorescence-based assays; also, the excited states of luminescence were generated from the luciferase-catalyzed reaction, which would not intervene with small molecules in the compound library, while on the other hand, the production of the excited states in fluorescence was gained through the absorption of light, which might also excite a significant number of screening compounds to cause pseudo-positive signals. Therefore, this high-performance and straightforward assay displayed many advantages over many current methods and would be promising to become the general method for DUBs inhibitors screening and widely applied to drug discovery field for cancer therapy.

In conclusion, this dissertation systematically illustrated our recent research progress in design and development of novel chemical biology tools for investigating the deubiquitinating enzymes, initiating from exploiting the catalytic capacity of the ubiquitin-activating enzyme (E1) for ubiquitin C-terminus functionalization, to further applying this optimized E1-catalyzed amidation reaction for DUB activity study and inhibitors discovery, including the development of preparation methods for desired fluorescent ubiquitin conjugates as well as versatile ubiquitin polymers, and establishment of a readily-adoptable HTS assay. The most significant merit of our approach was its exceptional simplicity and broad applicability for the large-scale syntheses of multiple substrates for DUB research by a regular biochemistry and molecular biology group that was either not equipped or not capable to handle organic synthesis. With its potential widespread application, this straightforward method would

dramatically facilitate functional investigations of DUBs as well as accelerate drug discovery for DUB-targeted disease intervention.

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