

Peptidic Activity Based Probes to Understand the Deubiquitination Machinery



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Declaration

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Abstract

Ubiquitination is a highly conserved post-translational modification (PTM) that governs numerous critical cellular events such as protein degradation and membrane import/export. Such process is tightly regulated by a complex enzymatic network. Deubiquitinating enzymes (DUBs) are a group of isopeptidases that remove ubiquitin from their target proteins, providing a further control point for protein ubiquitination. Eukaryotes express over 100 DUBs, and the dysregulation of these enzymes has been linked to several diseases, including but not limited to cancer and neurogenerative disorders. Activity Based Probes (ABPs) have emerged as useful tools to understand DUBs and unravel their mechanism of action.

Typically, ABPs targeting DUBs are designed on either a mono- or multi-ubiquitin scaffold and include an electrophilic moiety that is positioned to come in close proximity to the active site cysteine, enabling covalent interaction with the targeted DUB. While these probes remarkably advanced the mechanistic and structural understanding of DUBs, they suffer from onerous synthesis. This work aims to develop a new strategy to study DUBs with previously unexplored peptidic ABPs. Such novel peptide-based probes were designed, synthesised and their activity was tested against DUBs.

The first set of peptide probes was designed to capture the DUBs removing ubiquitin from the p53 protein. p53 is a highly versatile transcription factor; its activity is modulated by different PTMs. In healthy cells, ubiquitination and subsequent degradation by the proteasome keep p53 basal levels low. However, following stress or DNA damage p53 is saved from proteasomal degradation by DUBs, triggering a stress-specific response. With respect to p53, nearly 15 DUBs have been reported to interact with p53, either as positive or negative regulators. However, the DUB (or set of DUBs) responsible for deubiquitination at recently discovered K291/K292 sites has not yet been characterized. In a section of this study, ABPs were generated using a portion of the p53 sequence that was synthetically modified to incorporate different electrophilic moieties. Being designed to target p53 deubiquitination only, this approach identified new functions of DUB enzymes and broadened our knowledge on p53 PTMs.

The second set of peptide probes was designed to covalently label OTUB1, a highly specific DUB responsible for cleavage of K48 polyubiquitin chains. In a section of this work, a set of peptide probes was designed and synthesised, and their labelling activity towards OTUB1 was evaluated.

Overall, this work outlines a novel strategy for targeting deubiquitinating enzymes using peptidic Activity Based Probes. While these probes were not specifically designed to outperform existing DUB-targeting probes, they represent the first attempt to incorporate peptide chemistry into ABPs design for DUB targeting.

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Abbreviations

ABP activity based probe

ABPP Activity Based Protein Profiling

AcOH acetic acid

Ala; A alanine

aq. aqueous

Arg; R arginine

Asp; D aspartic acid

ATP adenosine triphosphate

Boc *tert*-butoxycarbonyl

Boc₂O Di-*tert*-butyl dicarbonate

BPB bromophenol blue

br. broad

BSA Bovine Serum Albumin

cat. catalytic

CD circular dichroism

CDCl₃ Deuterated chloroform

Cys; C cysteine

Cy5 Cyanine 5 fluorescent dye

Da dalton

DCM dichloromethane

DEPT Distortionless Enhancement by Polarization Transfer

DIPEA *N,N*-diisopropylethylamine

DMAP 4-dimethylaminopyridine

DMF *N,N'*-dimethylformamide

DMP Dess-Martin Periodinane

DMSO dimethyl sulfoxide

DMSO-**d**₆ deuterated dimethyl sulfoxide

DNA deoxyribonucleic acid
DUB Deubiquitinating enzyme
equiv. equivalents
ESI electron spray ionisation
EtOAc ethyl acetate
Et₂O Diethyl ether
Fmoc fluorenyl methoxycarbonyl
Fmoc-OSu *N*-(9-fluorenylmethoxycarbonyl)succinimide
Glu; E glutamic acid
Gly; G glycine
Hex hexane
His; H histidine
HMBC heteronuclear multiple bond correlation
HPLC high performance liquid chromatography
HRMS high resolution mass spectrometry
HSQC heteronuclear single quantum coherence
Hse homoserine
Hz hertz
Ile; I isoleucine
IPTG Isopropyl β-D-1-thiogalactopyranoside
IR infrared
J coupling constant
Leu; L leucine
Lys; K lysine
m/z mass per unit charge
MeCN acetonitrile
MeOH methanol
Met; M methionine

min. minute

MS mass spectrometry

NMM *N*-methyl morpholine

NMR nuclear magnetic resonance

PCC Pyridinium Chloro Chromate

PDB protein data bank

Pet. Ether petroleum ether

Phe; F phenylalanine

PMSF phenylmethanesulphonyl chloride

ppm parts per million

Pro; P proline

PTM Post Translational Modification

PyBOP benzotriazol-1-yl-oxytripyrrolidinophosphoniumhexafluorophosphate

R_f retention factor

RNA ribonucleic acid

rt room temperature

sat. saturated

Ser; S serine

SPPS solid phase peptide synthesis

TBAF tetra-butyl ammonium fluoride

TBSCl *tert*-butyl dimethyl silyl chloride

***t*Bu** *tert*-butyl

TES triethyl silane

TFA trifluoroacetic acid

THF tetrahydrofuran

Thr; T threonine

TLC thin layer chromatography

TOCSY Total Correlation Spectroscopy

Trp; W typtophan

Trityl; Trt triphenylmethyl

Tyr; Y tyrosine

UAA unnatural amino acid

Ubls Ubiquitin-like proteins

UPP Ubiquitin Proteasome Pathway

VME Vinyl Methyl Ester

Chapter 1

Introduction

1.1 The ubiquitin signalling pathway

Ubiquitination is a well-understood Post Translational Modification (PTM) consisting in the conjugation of ubiquitin to substrate proteins. This ubiquitous 76 amino acids protein was discovered in 1975 by G. Goldstein,¹ and the mechanism of its bioconjugation was elucidated in the 80's by A. Ciechanover, A. Hershko and I. Rose, with their research being awarded the Nobel Prize in 2004.² The ATP-dependent ubiquitin conjugation machinery, known as the ubiquitin cascade, requires the sequential action of three classes of enzymes: ubiquitin-activating enzymes (E1s), ubiquitin-conjugating enzymes (E2s), and ubiquitin-protein ligases (E3s, Fig. 1.1).

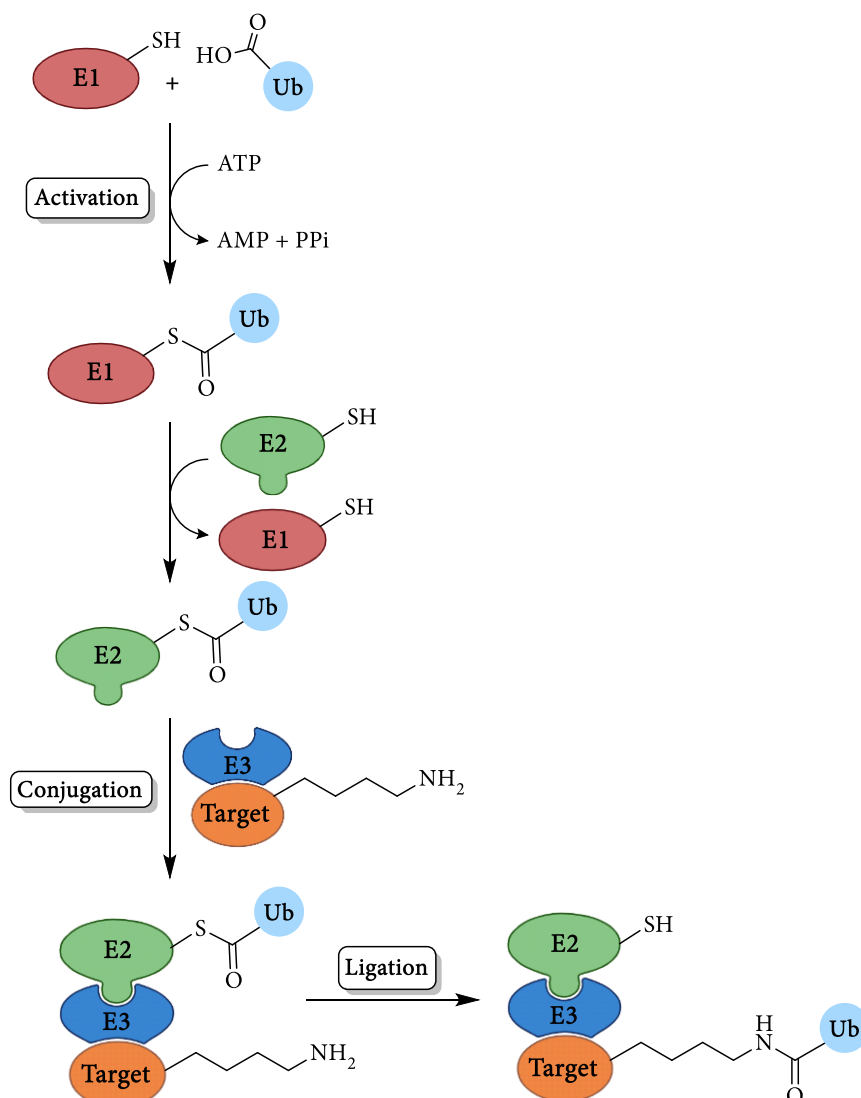


Figure 1.1: The ubiquitin cascade. A target protein is ubiquitinated after a series of transthioesterification steps, forming an isopeptide bond with a lysine side chain.

First, an E1 enzyme catalyses the formation of a high-energy thioester bond between the C-terminal carboxyl group of ubiquitin and its active site cysteine. Next, the activated ubiquitin is transferred to the active-site cysteine of an E2 enzyme via trans-thiolation, forming a new thioester bond. Finally, an E3 ligase facilitates the transfer of the activated ubiquitin thioester to a target protein, typically creating an isopeptide bond at the ϵ -amino group of a lysine side chain on the target substrate.³ E3s can assist this final trans-thiolation by interacting with the target protein or the E2~Ub complex.

The process of monoubiquitination is of great physiological significance as it has been shown to regulate chromatin level and cellular localization of various proteins, including transcription factors such as p53.^{4, 5} To date, the human genome encodes for 8 E1s, ~40 E2s and ~620 E3s.⁶ The relatively large number of E3 ligases compared to E1s and E2s confers the ubiquitin cascade an exceptional specificity and selectivity, surpassing that of the phosphorylation pathway, which involves 538 different kinases.⁷

Depending on the structure of the target protein, additional ubiquitin moieties can be attached, resulting in multiubiquitinated substrates.⁸ Moreover, each ubiquitin contains seven different lysines in its sequence (K6, K11, K27, K29, K33, K48, K63) and an N-terminal residue (M1, **Fig. 1.2A**) where the ubiquitination process can be reiterated, allowing proteins to be modified with linear or branched polyubiquitin chains. Polyubiquitin chains are defined as 'homotypic' if they present the same linkage type, whereas they are defined as 'heterotypic' if they present different linkage types. A further level of complexity is added when polyubiquitin chains can be marked with Ubiquitin-like proteins (Ubls) such as NEDD-8⁹ or SUMO¹⁰ or even with non-ubiquitin PTMs, such as phosphorylation of tyrosine and acetylation of lysine residues (**Fig. 1.2B**). All the aforementioned topologies of ubiquitin chains have been observed.⁸ They are responsible for the exquisite diversity of the ubiquitin signalling, giving rise to what is commonly known as the 'ubiquitin code'.¹¹

The most abundant and well-characterized of the linkage types is the K48 linked polyubiquitin chain. Proteins tagged with a minimum of four Lys48 linked polyubiquitin chains exhibit an increased affinity for the 26S subunit of the proteasome, making this linkage type the main signal for proteasomal degradation.¹² The second most abundant linkage type

is K63 polyubiquitination, that has been shown to influence protein-protein interactions and is believed to play a key role in immune signalling. Moreover, many pathogens (including SARS-CoV-2) are known to be able to suppress host's immune response *via* suppression of K63 ubiquitination.¹³

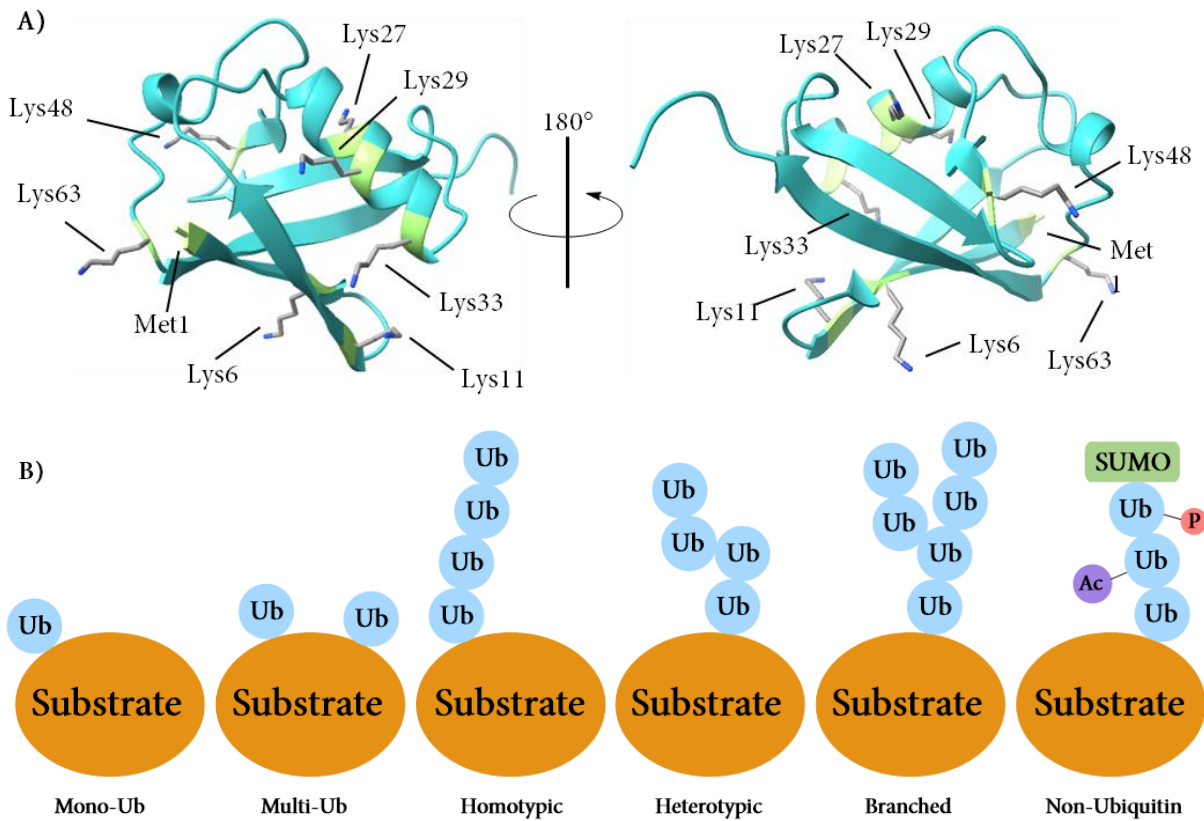


Figure 1.2: A) X-Ray crystal structure of ubiquitin (light blue, PDB: 1UBQ). Lysine residues are highlighted in green and side chains are coloured by heteroatom. B) Representation of different types of ubiquitination; P = phosphorylation, Ac = acetylation.

Although the roles of ‘atypical’ linkages (K6, K11, K27, K29, K33, and M1) are not as well-defined as the typical K48 and K63 linkages, ubiquitination is considered as a key PTM, and several different diseases are linked with malfunction of this process. These include but are not limited to neurodegenerative diseases (such as Alzheimer’s and Parkinson’s),¹⁴ genetic disorders,^{15, 16} and cancer.¹⁷ Therefore, understanding how this process is regulated in the human cell plays an important role in the development of new therapeutics.

1.2 Deubiquitinating enzymes

A pivotal characteristic of the ubiquitination machinery lies in the reversibility of this process. The diversity of the ubiquitin code would not exist without the presence of deubiquitinating enzymes (DUBs). In the cell, ubiquitin is expressed either as a pro-protein or as a linear polyubiquitin chain that must be processed in order to obtain the functional ubiquitin monomer.^{18, 19} Consequently, the first cellular role of DUBs is to carry out ubiquitin activation by breaking ubiquitin polymers.²⁰ Moreover, DUBs possess ubiquitin C-terminal hydrolytic activity and can disassemble or edit ubiquitin chains, providing a further regulation point of ubiquitin conjugation that can dramatically impact cell homeostasis (**Fig. 1.3**).^{21, 22}

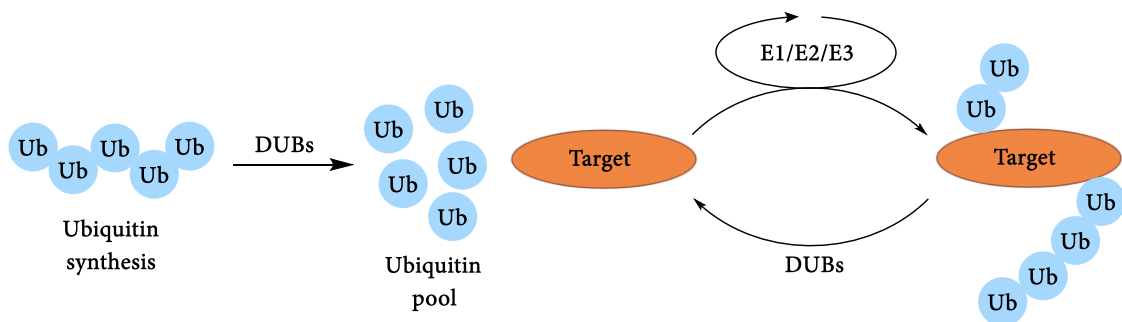


Figure 1.3: Principal functions of DUBs in the cell. Upon translation, DUBs convert ubiquitin precursors into ubiquitin monomers that can be used in the signalling pathway as PTM. In addition, DUBs can balance the ubiquitin cascade by reversing the action of E1, E2 and E3 enzymes.

The human genome encodes a family of ~100 DUBs, divided in seven subfamilies. Six of these subfamilies are known to be cysteine proteases: the ubiquitin-specific proteases (USPs, with 54 members in humans), the ovarian tumour proteases (OTUs, with 16 members), the ubiquitin C-terminal hydrolases (UCHs; 4 members), the Josephin family (4 members), the recently discovered motif interacting with ubiquitin (MIU) found in a novel DUB family (MINDYs, 4 members),²³ and the zinc finger with UFM1-specific peptidase domain protein (ZUFSP, 1 member). The seventh subfamily of DUBs consists of 16 different Zn-dependent metalloproteases, known as JAB1/MPN/MOV34 proteases (JAMMs).²⁴

Mechanistically, the thiol-based protease families rely on a highly conserved catalytic triad consisting of a cysteine, responsible for the nucleophilic attack on the isopeptide bond, an adjacent basic histidine, that increases the nucleophilicity of the active cysteine by lowering the thiol pK_a , and frequently a third acidic residue (usually an asparagine, a glutamate or an aspartate) that further polarizes the histidine (**Fig. 1.4**).^{21, 22, 25} Interestingly, although the arrangement of these residues is highly conserved, some DUBs exhibit variations in their active sites. For example, members of the newly discovered MINDY DUBs do not possess a third acidic residue but avail of a Thr residue to coordinate a water molecule in the active site.²⁶ Moreover, all the OTU DUBs possessing an A20 core rely on a catalytic dyad that does not include a third acidic residue.^{27, 28}

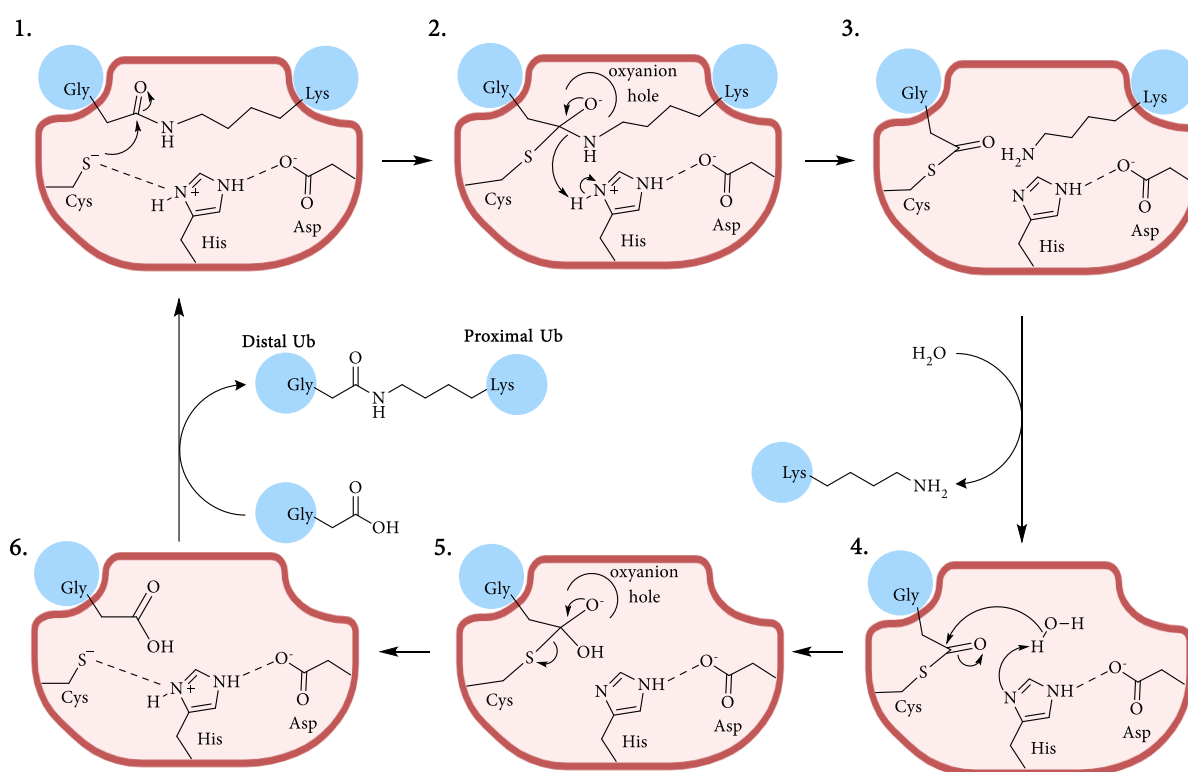


Figure 1.4: Mechanism of cysteine protease DUBs. After the substrate is bound in the active site of the apoenzyme, the activated cysteine thiolate attacks the isopeptide linkage (1). The tetrahedral intermediate is stabilised in the oxyanion hole (2) followed by formation of an acyl enzyme intermediate and simultaneous release of the proximal ubiquitin (3). Deacylation is carried out by water (4) via a stabilised carboxyl intermediate (5). Release of the distal ubiquitin restores the activity of the catalytic triad (6).

Differently, the catalysis operated by JAMM metalloproteases involves a Zn^{2+} ion that is coordinated by two histidines, an aspartate and a water molecule. A glutamate residue is responsible for water activation that results in nucleophilic attack of the isopeptide bond. The tetrahedral intermediate is stabilised by a nearby serine (Fig. 1.5).^{29, 30}

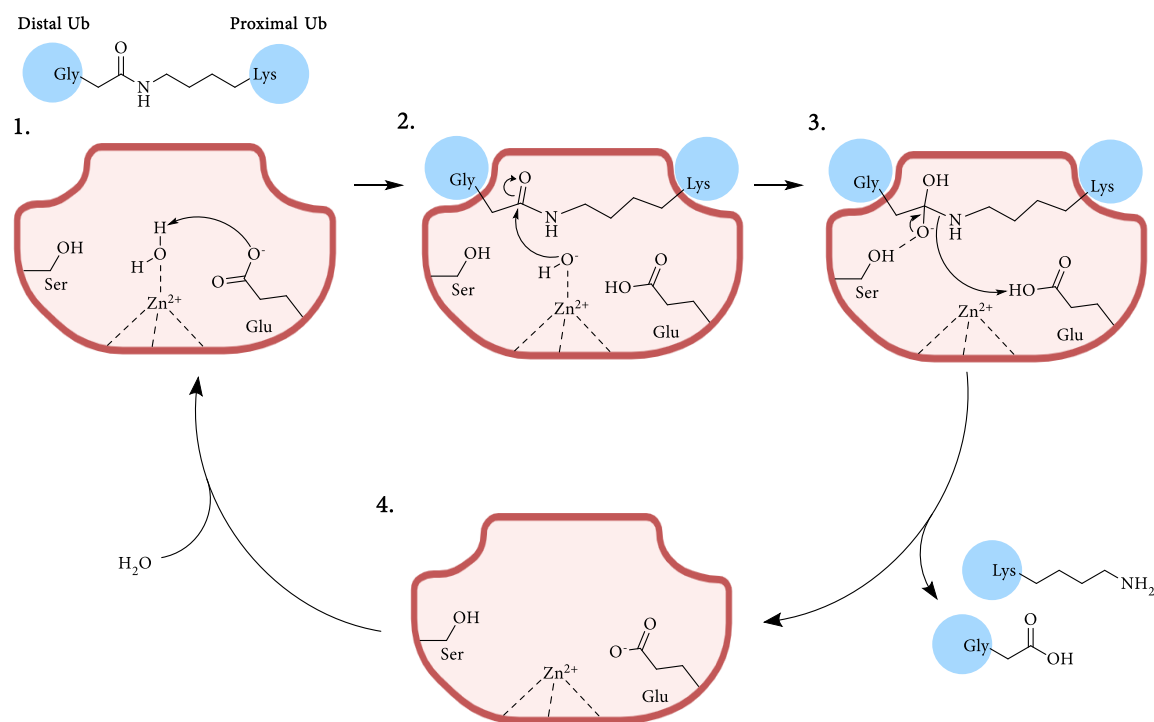


Figure 1.5: Mechanism of metalloprotease DUBs. Activation of a water molecule (1) allows the nucleophilic attack (2) with subsequent formation of a tetrahedral intermediate stabilised via hydrogen bond (3). The apoenzyme is regenerated after the simultaneous expulsion of both the ubiquitin moieties (4).

Given the high complexity of the ubiquitin code, some DUBs possess substrate specificity and can direct their activity towards specific ubiquitinated substrates. All the DUBs possess at least one ubiquitin-binding site, called the S1 site, that engulfs the ubiquitin C-terminus and projects the isopeptide bond into the active site where the hydrolysis is performed. The interface at the S1 site can cover up to 40% of the bound ubiquitin, while the presence of additional ubiquitin binding sites (S1', S2, S2' or more) enables interactions between the DUB and specific substrates or ubiquitin linkage types.³¹ Generally, DUBs can edit ubiquitin

modifications in two different ways, by directly binding the substrate protein or by binding the ubiquitin chain (Fig. 1.6).²¹ For example, the majority of the USP DUBs can regenerate an unmodified protein by removing ubiquitin chains regardless of their linkage type. Moreover, polyubiquitin chains present different points of attack, and DUBs have been shown to perform exo-proximal cleavages (producing monoubiquitinated substrates), exo-distal cleavages (removing one ubiquitin from the chain), endo cleavages (releasing a polyubiquitin chain) or even debranching cleavages (reducing chain complexity). A well-established example of a linkage specific DUB is OTULIN.³² This cysteine isopeptidase can selectively cleave Met1-linked polyubiquitin chains, even when other types of chains are overexpressed in a cell.³³ The field of DUBs specificity is not fully understood but rapidly growing with new functions yet to be uncovered.²¹

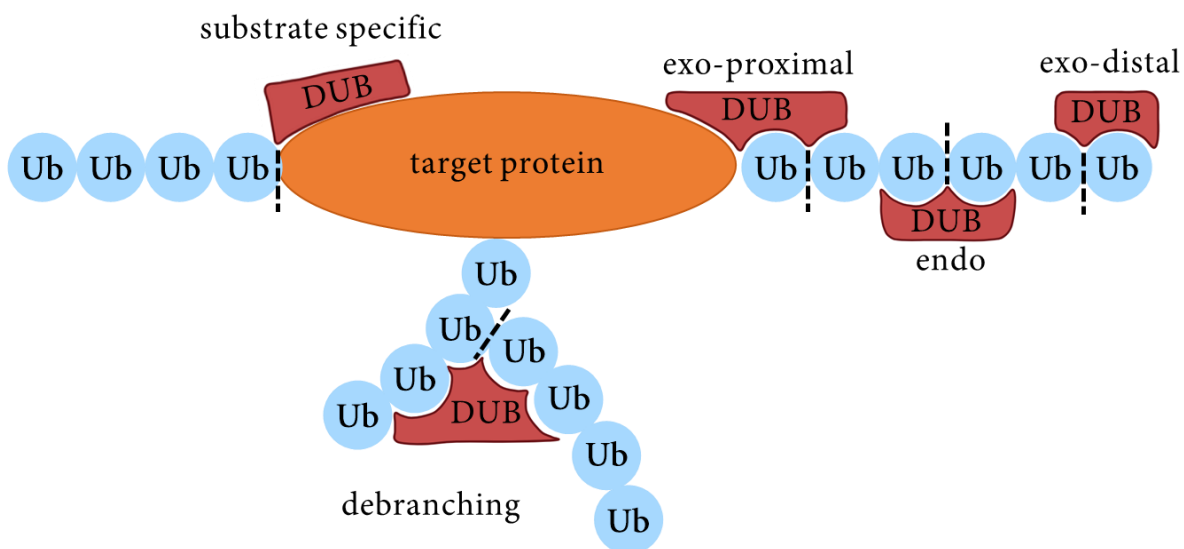


Figure 1.6: Substrate recognition strategies of DUBs. Linkage specificity of DUBs (indicated with a dotted black line) enables a high selectivity for certain polyubiquitin scaffolds.

The balance between ubiquitination and deubiquitination plays a major role in maintaining the cell homeostasis, providing a tightly controlled response to cellular stress or stimuli. Therefore, it is not surprising that perturbations of this balance can lead to diseases or dramatically affect the cell viability. In the last decade, all the hallmarks of cancer have been associated to DUBs,³⁴ and several new therapeutic approaches rely on new discoveries on

DUBs activities.^{35, 36} Moreover, DUBs dysregulation have been implicated in other processes such as autoimmune diseases,³⁷ radiosensitivity,³⁸ and in the regulation of the circadian clock.³⁹

1.2.1 Otubain 1

Otubain 1 (OTUB1) is the most important representative of the OTU family of cysteine protease deubiquitinating enzymes. This ~35 kDa protein was discovered in the early 2000s,⁴⁰ and found to be abundantly expressed in almost all humans cells.⁴¹ Preliminary structural studies revealed that OTUB1 can accommodate up to two ubiquitin moieties in two different sites: a distal allosteric site and a proximal catalytic site, comprising ~50 essential N-terminal residues.⁴² Akin to other DUBs, human OTUB1 performs its enzymatic activity availing of a catalytic triad consisting of Cys91, His265 and Asp267 (Fig. 1.7),⁴³ which is highly conserved across multiple species. However, the activity of OTUB1 is remarkably linkage specific, being solely directed towards the cleavage of Lys48 linked polyubiquitin chains (Fig. 1.8A).⁴⁴ To date, several proteins directly targeted by this canonical mechanism of action have been identified, and perhaps unsurprisingly, the topology of their ubiquitin chain is Lys48 linked. OTUB1 is responsible for the stabilisation of those ubiquitinated targets, aligning with the prototypical role of Lys48 linked ubiquitin chains in promoting protein degradation.⁴⁵

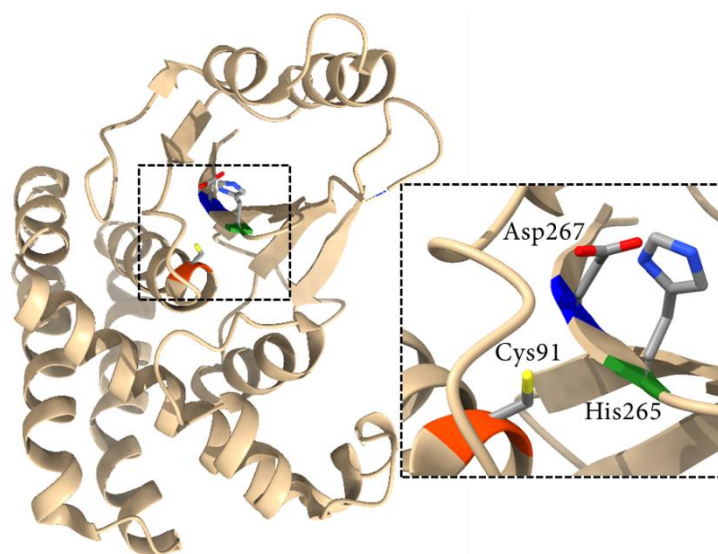


Figure 1.7: Human Otubain 1 crystal structure. The residues involved in the catalytic triad are coloured in red, blue and green. PDB structure: 2ZFY.

Intriguingly, OTUB1 possess a second non-canonical function that can result in inhibition of protein ubiquitination (Fig. 1.8B).⁴⁶ Recent studies have unveiled that OTUB1 can also bind ubiquitinated E2 enzymes such as UBC13⁴² and UBCH5B⁴⁷, preventing their participation in ubiquitination reactions, thus avoiding unnecessary accumulation of polyubiquitin chains. Structural insights into this mechanism revealed a profound allosteric communication between OTUB1 proximal and distal sites. Upon binding of a free ubiquitin in OTUB1 distal site, the unstructured N-terminal residues in the proximal site undergoes a substantial rearrangement into a ubiquitin binding α -helix that drastically increases the affinity towards the charged E2~Ub substrate (Fig. 1.8C). Moreover, the relative arrangement of proximal and distal ubiquitin mimics a Lys48 diubiquitinated scaffold, further confirming OTUB1 specificity for such isopeptide linkage.⁴⁴ Taken together, these results confirm that OTUB1 can not only prevent protein degradation via a canonical isopeptidase mechanism but also sense ubiquitin cellular concentration and balance ubiquitination by inhibition of E2 enzymes.⁴⁸ These two activities are closely intertwined and regulated by the fluctuations in E2s charging and mono/Lys48 polyubiquitin chains concentrations. An uncharged OTUB1-E2 complex will enhance canonical Lys48 chain cleavage, whereas an increase in free ubiquitin levels will promote noncanonical E2 conjugating activity, blocking isopeptidase activity.⁴⁸

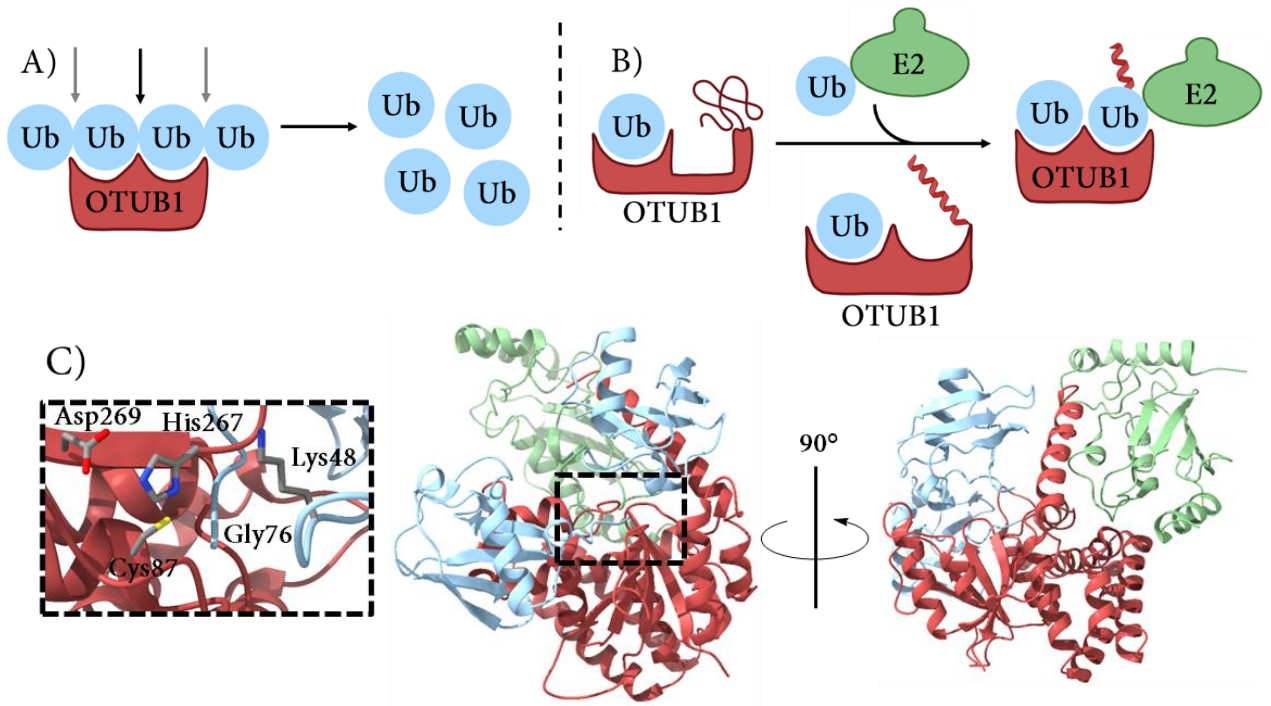


Figure 1.8: OTUB1 mechanisms of action. A) The canonical mechanism consists in accommodation of a diubiquitin moiety and subsequent isopeptide bond cleavage at the preferred Lys48 residue. B) The noncanonical mechanism occurs when ubiquitin acts as an allosteric modulator upon binding in the distal site, increasing the affinity of the proximal site for a Ub~E2 complex. C) Crystal structure showing two ubiquitin like proteins (turquoise) bound to OTUB1 (red) and the E2 enzyme UBC13 (green). On the right, a rotation of 90° reveals the structurally induced α -helix of OTUB1. The left image shows a close up of the active site where the Gly76 of the distal ubiquitin and the Lys48 of the proximal ubiquitin are brought in close proximity to the catalytic triad. PDB structure: 4DHz.

Across the last decade OTUB1 has become a target of increased attention owing to its ability to participate in key cellular processes. For example, global OTUB1 knockout in mouse models led to defects in lung⁴⁹ and bone development.⁵⁰ Moreover, OTUB1 overexpression has been observed in many cancer types and is currently believed to be a biomarker for tumorigenesis.⁵¹ In addition, OTUB1 has been linked to regulation of DNA damage via stabilisation of p53-mediated apoptosis. Although the importance of OTUB1 and DUBs has

been highlighted by the scientific community, their mechanism of action is still far from being completely understood.^{21, 51} Consequently, there is an increasing demand for new methods and tools to interrogate these biological systems.

1.3 Activity Based Probes to capture elusive enzymes

Traditional methods aiming to profile new enzymes or define their function within a cellular environment rely on the *a priori* assumption of a direct correlation between messenger ribonucleic acid (mRNA) expression levels and enzymatic activity. Although this can be true in some applications,⁵² the expression level of an enzyme is often not correlated with its activity.⁵³ Following translation of a protein from its mRNA, PTMs (like ubiquitination) as well as protein-protein interactions could modulate their activity without affecting their levels.⁵⁴ For example, phosphorylation of OTUB1 at Ser16 by casein kinase 2 results in nuclear import,⁵⁵ whereas hydroxylation by FIH (factor inhibiting HIF) at Asn22 alters the DUB interactome.⁵⁶ As a result, PTMs dramatically impact the DUB activity while maintaining its cellular concentration. Another interesting case regards p53, which has been shown to be modified by a much broader panel of PTMs such as ubiquitination, acetylation, phosphorylation, methylation, or hydroxylation, all resulting in the modulation of its activity.⁵⁷ Thus, in order to design an assay to unravel new functions of p53 or discover new interactions with other enzymes such as DUBs, the active population of p53 or of the DUB of interest has to be considered. That is because the active population of an enzyme determines its actions, not its concentration or expression within the cellular environment. As an example, p53 is continuously expressed in human cells but it is kept inactive by different pathways including proteasomal degradation and modulated only when a cell sustains DNA damage or any kind of stress, so that its concentration does not reflect its activity.⁵⁸

Overcoming such challenges is one of the main targets of Activity Based Protein Profiling (ABPP). ABPP is an excellent strategy to assess enzymes activities directly in their native environment.⁵⁹ This technique allows to selectively target the *active* form of a desired enzyme (or class of enzymes) by using chemical probes named Activity Based Probes (ABPs). ABPs

are designed to trap a targeted enzyme by forming a covalent bond within their active site in a mechanism-based manner, allowing for the selective labelling of the active form.⁶⁰ The first description of an ABP targeting esterase enzymes was performed in 1961 by Barnard and Ostrowski.⁶¹ Since then, the enormous advances in genomics and proteomics, as well as in protein separation technologies and Mass Spectrometry (MS) allow the use of ABP for the analysis of a multitude of uncharacterized enzyme functions, providing useful insights into their biology.⁶²

1.3.1 Structure and specificity of Activity Based Probes

An Activity Based Probe is composed of three critical structural components, a warhead, a recognition scaffold and a visualization tag (Fig. 1.9A). The warhead is responsible for covalently labelling the catalytic nucleophilic residue within the enzyme's active site. To facilitate this reaction, the warhead usually contains an electrophilic moiety. The reactivity of the warhead requires precise modulation: excessive reactivity can cause off-target labelling, while insufficient reactivity leads to incomplete or ineffective labelling.⁶³

The recognition scaffold provides selectivity to the ABP towards its target. In addition to the warhead tuning, the recognition scaffold must be carefully selected to promote the irreversible labelling event by bringing the electrophile moiety in close spatial proximity to the enzyme active site.

Finally, a detectable agent allows the visualisation of the captured enzyme or its isolation from the assay environment. Upon binding to the ABP to the desired enzyme (or class of enzymes), an adduct is formed, which can be revealed using three common types of detectable tags, which are radionuclides, fluorescent and affinity tags. Radionuclides were widely used in the early stages of ABPP and consisted in the covalent modification of protein residues with a radioactive atom (for example ^{125}I) to generate a detectable complex.⁶⁴ Fluorescent tags such as fluorescein can allow visualisation by SDS-PAGE or fluorescence microscopy.⁶⁵ Although fluorescence has been used for more than 25 years to visualize and analyse the dynamics of living organisms, its use is limited by major drawbacks such as poor photostability or

insufficient membrane permeation during *in vivo* studies.^{66, 67} For this reason, quenched fluorophore tags have been developed in recent years to become fluorescent after the covalent modification of the targeted enzyme. This effect increases cell permeation and improves the understanding of the active enzyme population in living cells.⁶⁸ Lastly, affinity tags like biotin can be used to visualize labelled compounds through blotting experiment or even by isolation using a solid-support resin, enabling downstream MS and proteomics analysis (Fig. 1.9B).⁶⁹ Alternatively, the reporter tag can be installed using a small biorthogonal group, such as an azide or an alkyne, that allows subsequent attachment of the tag.⁷⁰ This approach is commonly known as 'two-step' labelling and can be particularly useful when larger tags affect the probe's reactivity, selectivity, or physicochemical properties. Certain large ABPs, such as the ubiquitin probes discussed below, significantly increase the target protein's molecular weight, allowing a detectable mass shift on an SDS/PAGE gel to confirm probe labelling.

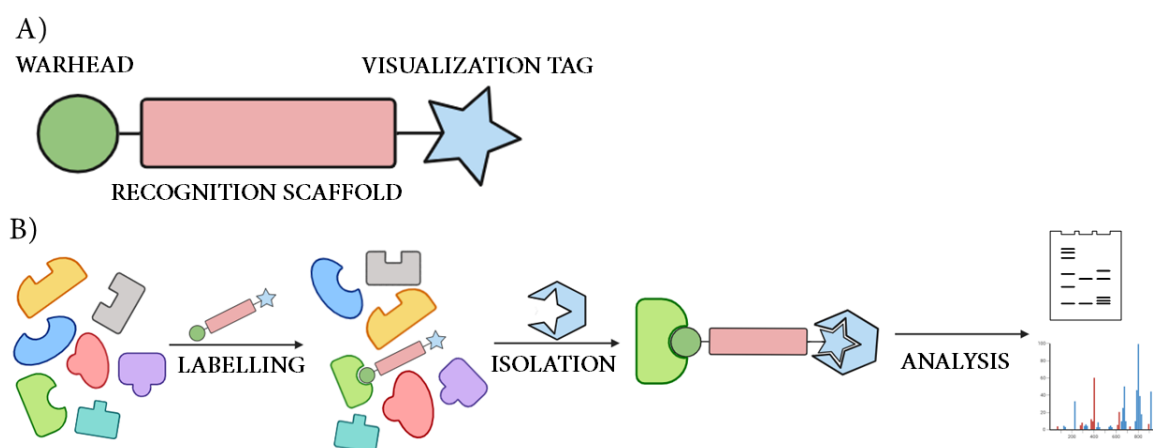


Figure 1.9: A) General structure of an Activity Based Probe. B) Workflow of activity based protein profiling experiment.

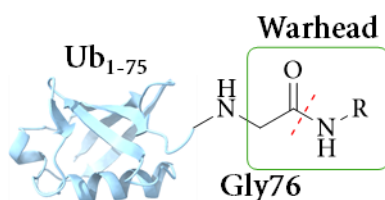
1.3.2 Probes for profiling Deubiquitinating enzymes

As mentioned in section 1.2 (p. 5), the majority of DUBs are cysteine proteases. Upon recognizing their target protein, these enzymes perform their isopeptidase activity availing of a nucleophilic cysteine (Cys) in their active site. Consequently, ABPs designed to capture DUBs typically feature a reactive group strategically positioned to react with the catalytic Cys

in a covalent fashion. This approach offers a significant advantage over traditional expression-based quantification methods, as it relies on the catalytic activity of the targeted DUB rather than its expression level.⁷¹ A significant challenge in designing ABPs for DUBs is balancing reactivity with selectivity. Both the warhead and recognition scaffold must be carefully engineered to achieve specificity for a particular DUB or group of DUBs.⁷² Typically, warheads incorporate an electrophilic moiety near the targeted ϵ -amino group of a lysine residue or on the C-terminus of another ubiquitin, depending on the recognition scaffold structure. This allows for the development of ABPs aimed at capturing either mono-ubiquitin or polyubiquitin chains.

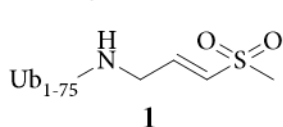
The first generation of ABPs to interrogate DUBs was based on a mono ubiquitin recognition scaffold. Nearly 40 years ago, Rose *et al.* developed Ubiquitin-aldehyde (Ubal), the earliest DUB-targeting ABP, featuring a full-length ubiquitin with a C-terminally modified Gly76 residue.⁷³ Ubal was a crucial tool in early studies exploring DUB mechanism. It was used to demonstrate the mechanism of the UCH subfamily,⁷⁴ and also helped to obtain the first crystal structure of a DUB in complex with ubiquitin, revealing the profound conformational changes undergoing upon ubiquitin binding.⁷⁵ However, the labelling carried by Ubal on DUBs is reversible and therefore incompatible with common applications such as the strongly reducing conditions of SDS-PAGE.⁷⁶ To overcome this limitation, ABPs featuring C-terminal reactive groups that can label DUBs in an irreversible fashion were developed in the following years, with the first examples being described by Borodovsky *et al.* featuring a recombinant ubiquitin that is C-terminally modified availing of an innovative intein-based chemical ligation methodology (Fig. 1.10).⁷⁷ Various warheads, each with distinct electrophilic groups, were tested, enabling selectivity among different DUBs. For instance, probe 4 bearing a bromoalkyl warhead selectively reacted with DUBs from the OTU family, whereas the less reactive vinyl sulfone probe 1 did not.⁶³

General scaffold:

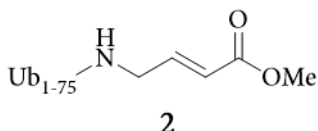


Ubiquitin ABPs:

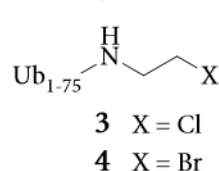
R = vinylsulfone



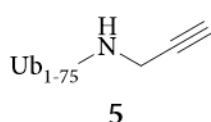
R = vinylmethylester



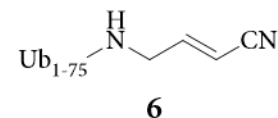
R = haloethylamine



R = propargyl amine



R = vinylcyanide



R = lactone

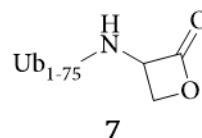


Figure 1.10: Structure of ABPs based on a Ub scaffold with a warhead at the C-terminal residue. The reactivity of the warhead (green box) can be modified with different electrophiles resulting in a different DUB specificity for the ABP. Scissile bond cleaved by DUBs is indicated by a dotted red line.

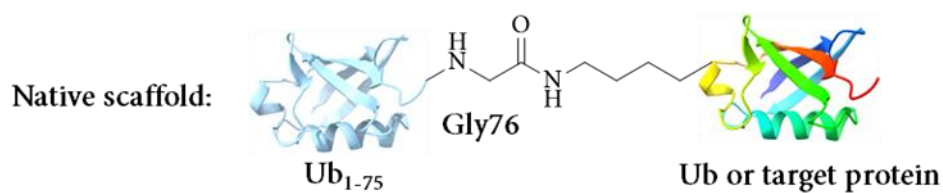
To date, a wide variety of electrophiles have been installed at the C-terminus of recombinant ubiquitin, and they can be classified based on the type of reaction they undergo in the active site of the targeted DUB (**Table 1.1**). Examples of probes reacting with either direct or conjugate addition have been reported. Notably, vinyl methyl ester (VME, **Table 1.1**, entry 5) warheads showed great reactivity whereas propargyl warheads (**Table 1.1**, entry 2) exhibits less reactivity but a higher degree of selectivity, being able to selectively label subsets of DUBs.^{63, 78, 79} Probes reacting via nucleophilic substitution such as the acyloxymethyl ketone (**Table 1.1**, entry 10) warhead have also been developed, although they possess much broader activity and can label several E1, E2, and E3 enzymes in addition to DUBs.⁸⁰

Native scaffold:				
	Reaction type	Warhead	Structure	Ref.
1	Conjugate 1-4 addition	Aldehyde		73
2		Propargyl amide		78, 79
3		Vinyl methyl sulfone		63, 77
4		Vinyl ethyl sulfone		80
5		Vinyl methyl ester		63
6		Vinyl cyanide		63
7	Nucleophilic substitution	Dehydroalanine		81
8		Chloroethylamine		63
9		Bromoethylamine		63
10		Acyloxymethyl ketone		80
11	Disulfide exchange	Disulfide		82
12	Radical	Vinyl amide		83

Table 1.1: Selection of different warheads installed on monoubiquitin probes for DUBs. *Ub₁₋₇₅* describes a native ubiquitin scaffold lacking the terminal Gly76. Warheads are classified based on their mechanism of action at the active site.

Although they provided valuable insights into DUBs, the first generation of mono-ubiquitin probes relies solely on the interaction between ubiquitin and the S1 site of the targeted DUB. As mentioned in section 1.2 (p. 5), some DUBs, notably those of the USP and OTU families, possess more than one ubiquitin binding pocket and can therefore cleave polyubiquitin chains (exo, endo or debranching) or ubiquitin-target isopeptide bonds (substrate specific). To target such DUBs and gather information about their linkage specificity a second generation of ABPs featuring a di-Ub or a ubiquitinated substrate recognition scaffold was developed (Table 1.2). These probes follow mostly the same design of monoubiquitin probe, featuring a warhead installed between two recognition elements.

For example, Li *et al.* developed a semisynthetic approach using recombinant ubiquitin scaffolds to make diubiquitin probes that features a Michael acceptor as a warhead (Table 1.2, entry 1).⁸⁴ On the contrary, McGouran *et al.* developed a click chemistry approach that successfully mimicked all the eight different linkage types and allowed quantification of DUB selectivity (Table 1.2, entry 2).⁸⁵ Click chemistry was also employed to synthesise diubiquitin ABP directed at the S1-S2 pocket of DUBs (Table 1.2, entry 4) in which the propargyl warhead was positioned on the proximal ubiquitin rather than on the isopeptide bond.⁸⁶ Apart from Michael acceptors (Table 1.2, entry 5),⁸⁷ diubiquitin ABPs also feature a dehydroalanine α - β unsaturated system as the warhead (Table 1.2, entry 3).⁸⁸ All the probes discussed so far employ ubiquitin scaffolds in both the recognition elements. To further interrogate DUBs specificity towards selected proteins, ubiquitin-substrate proteins have been designed (Table 1.2, entry 6-7).^{89, 90} Extending their dehydroalanine strategy, Meledin *et al.* created an ABP for α -globin, conjugating this protein to a ubiquitin moiety in a complex that mimics the native one. With this strategy they were able to discover USP15 as a DUB targeting ubiquitinated α -globin. The same design directed the Zhuang lab towards the synthesis of an ABP specific for DUBs interacting with Proliferating Cell Nuclear Antigen (PCNA). The group mimicked the native isopeptide bond with a Michael acceptor and was able to identify DUBs involved in PCNA deubiquitination



	Structure	DUB specificity	Ref.
1		K48, K63	84
2		M1, K6, K11, K27, K29, K33, K48, K63	85
3		M1, K48, K63	88
4		K6, K11, K27, K29, K33, K48, K63	86
5		K6, K11, K27, K29, K33, K48, K63	87
6		α-Globin	89
7		PCNA	90

Table 1.2: Selected examples of second-generation probes for DUBs. Ub₁₋₇₅ describes a native ubiquitin scaffold lacking the terminal Gly76, whereas Ub denotes the full-length sequence.

It is important to note that although all the approaches discussed improved our understanding of DUBs specificity and selectivity, they suffer from onerous and complex synthetical processes (see **Table 1.2**, entry 1-7). Moreover, ABP stability and specificity might be compromised in cell systems because of the presence of native isopeptide bonds near the warhead. Thus, in order to improve selectivity towards targeted DUBs, further studies focusing on the ABP structure have to be conducted.

1.3.3 Peptidic Activity Based Probes

A valuable strategy to reduce complexity and costs of synthesising ABPs involves simplifying the recognition scaffold. Peptidic ABPs utilise a portion of the protein chosen as recognition scaffold and can be efficiently synthesised via Solid Phase Peptide Synthesis (SPPS). However, Probes with a peptide scaffold have not been used widely to interrogate biological systems, especially to target DUBs. The only known ABPs to target DUBs that features a peptidic recognition scaffold are the ones proposed by Iphofer *et al.* (**Fig. 1.11**).⁹¹ Ubiquitin sequences flanking Lys48 and Lys63 were installed on probes **8-9** to test their specificity, and linkage specificity was described for several DUBs like USP15, ataxin-3 and UCH-3. Although the peptide is part of the recognition scaffold, the probes also feature a full-length ubiquitin monomer, thus they cannot be considered peptide based ABPs. Moreover, synthetically achieving compounds **8-9** is laborious and the probes do not feature any reporting tag, thus they are difficult to use in proteomic applications.

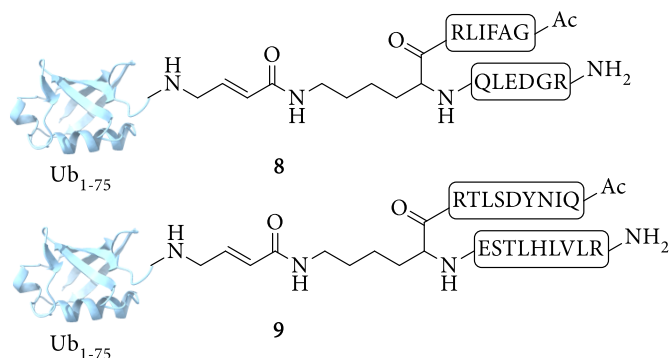


Figure 1.11: Structure of an ABP with a peptide recognition scaffold. Probe **8** features ubiquitin peptidic sequence near Lys48, whereas probe **9** features ubiquitin peptidic sequence near Lys63.

An investigation on the performance of several ubiquitin peptide fragments was detailed by Borodovsky *et al* (Fig. 1.12).⁹² To define a minimum binding motif, a panel of vinyl sulfone peptides resembling the ubiquitin C-terminus sequence were synthesised and their labelling activity towards DUBs was evaluated. It has been observed that inhibition of labelling in lysate could only be achieved when preincubating with a 12-mer peptide based on the ubiquitin C-terminal sequence. Shorter sequences proved to be ineffective while longer sequences did not improve labeling inhibition, indicating that the 12 C-terminal might be essential for probe efficacy.

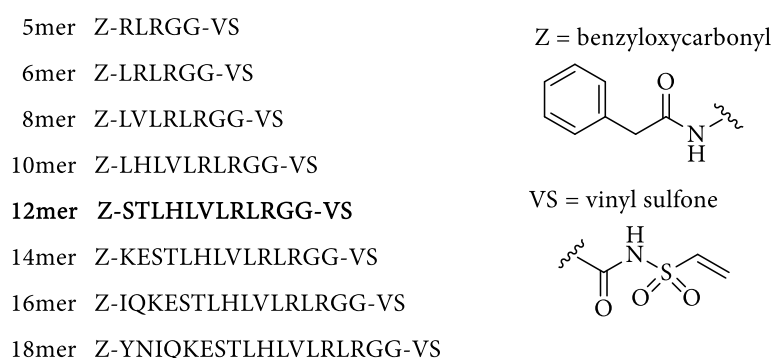
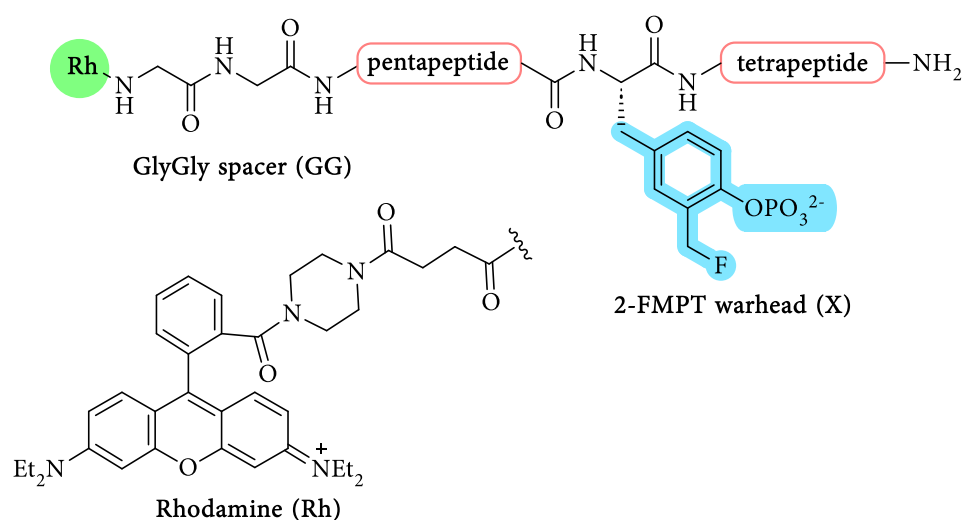


Figure 1.12: Study on small ubiquitin peptide inhibitors. Different lengths of the C-terminal ubiquitin to investigate minimum binding region to DUBs are shown. The benzyloxycarbonyl moiety (Z) allows detection via radioiodination whereas the vinyl sulfone (VS) acts as a warhead.

Overall, these studies indicate several reasons why a full-length ubiquitin recognition scaffold would be required when designing a probe to interrogate DUBs. Firstly, catalytic domains of DUBs have extensive ubiquitin-binding surfaces, meaning numerous interactions will contribute to their affinity for ubiquitin.⁹³ This intricate pattern of interactions is more challenging to replicate with a smaller recognition element. Moreover, the majority of DUBs undergo significant structural rearrangements upon ubiquitin binding. Many apo DUBs catalytic domains exist in catalytically inactive conformations and would not react with ubiquitin unless after ubiquitin binds, as seen for USP7⁹⁴ or USP14.⁹²

Although targeting cysteine proteases such as DUBs with peptidic ABPs has proven challenging, other proteases enzymes can be efficiently targeted with this strategy. This is due to the fact that shorter recognition sequences are necessary to achieve the required degree of selectivity and specificity. A notable example of a peptidic ABP was brought by Kalesh *et al.* which reported the synthesis of a probes to target protein tyrosine phosphatases (PTP), a class of enzymes that carries the dephosphorylation of tyrosine residues (Fig. 1.13).⁹⁵ To generate a probe specific for a certain PTP, peptidic sections of their known substrates were used as a recognition scaffold. As a warhead, the native phosphotyrosine residue is efficiently mimicked by using an innovative 2-fluoromethyl phenyl phosphate which generates the highly reactive quinone methide intermediate upon elimination of fluorine. Finally, all the probes were decorated with a C-terminal rhodamine fluorophore to allow fluorescence labelling profiling of the probe. Overall, the probes were observed to label the PTP they were designed for, which indicates that the peptide-based recognition scaffold enhances selectivity through the establishment of sequence-specific interactions.



PTP target	Native substrate	Sequence
SHP1	Siglec	Rh-GGDEGIHXSELI-NH ₂
PTP1B	Stat3	Rh-GGGSAAPXLKTK-NH ₂
PTP1B	Stat5b	Rh-GGKAVDGXVKPQ-NH ₂
SHP2	Ptk2b	Rh-GGLPPEGXVVVV-NH ₂
LMWPTP	Zap70	Rh-GGLNSDGXTPEP-NH ₂

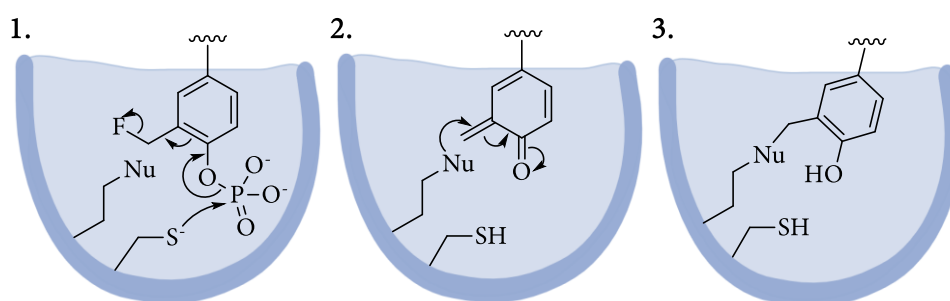


Figure 1.13: Selection of peptidic ABPs for tyrosine phosphatases reported by Kalesh et al and their mechanism of action. The two peptide sequences (red) flanking the reactive 2-FMPT warhead (light blue) were derived from the native substrate of the targeted PTP. Reporter tag indicated in green. Steps (1-3) indicate the proposed warhead mechanism of action. Upon attack of the active site cysteine of the PTP enzyme (1), a reactive quinone moiety is produced (2), and subsequent attack of a nearby nucleophile (3) covalently binds the probe to its target.

Another class of enzymes that has efficiently been targeted by peptidic ABPs is serine proteases. Recent studies demonstrated that peptide based serine protease probes had superior reactivity and selectivity compared to small molecules due to their increased affinity with the target enzyme.⁹⁶ Some studies indicate that such effect can be achieved without recurring to long peptide sequences. For example, the Gütschow lab developed an efficient elastase probe with a modified Gly-Val-Pro-Val tetrapeptide (Fig. 1.14).⁹⁷ Probe 10 featured a clickable BODIPY fluorogenic tag and a phosphonate warhead and was able to selectively bind elastase even in complex cell lysates.

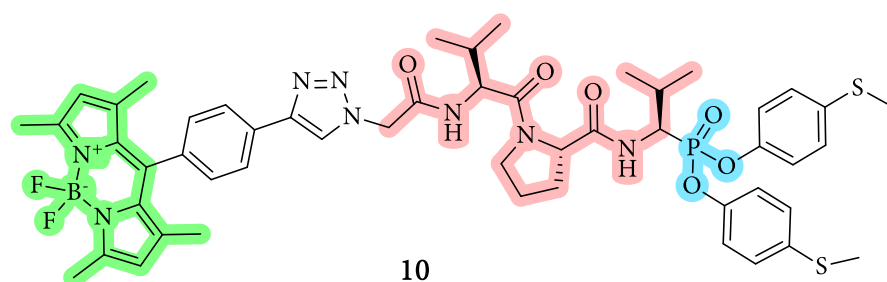


Figure 1.14: Human elastase peptide ABP proposed by Gütschow *et al.* The N-terminal nitrogen of the recognition scaffold (light red) is fused into the triazole ring formed upon click addition of the BODIPY reporter tag (green). The warhead is indicated in light blue.

Fluorescent ABPs were recently developed to study Granule-associated serine proteases (Grs), a small class of enzymes (consisting of 5 members, Gr A, B, K, H, M) involved in immune system regulation. Grs possess broad and overlapping substrate specificity, therefore selectivity constitutes a significant challenge when targeting a specific member of the enzyme family. Kolt *et al.* screened and tested a vast panel of different natural and unnatural tetrapeptides and identified the best binding substrate for Gr A (Fig. 1.15).⁹⁸ The fluorescent cyanine derivative (Cy5) tag and the warhead could be installed on the N and C-terminus at a later stage availing of standard solid phase peptide chemistry. The obtained peptidic probe 11 was the first specific ABP for Gr A, allowing detection of the enzyme both in-gel and in complex biological samples such as NK92 cell lysates.

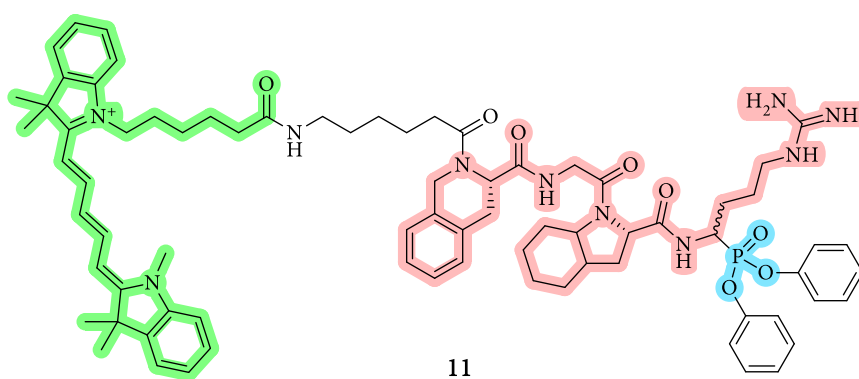


Figure 1.15: Structure of a peptidic Gr A ABP. The warhead is positioned at the C-terminus and consists of an arginine diphenyl phosphonate derivative (light blue). The rest of the recognition scaffold (red) features a *L*-octahydroindole-2-carboxylic acid (Oic, a proline analogue) and an unnatural 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic, a tryptophan analogue). The latter is linked to the fluorescent Cy5 tag (green).

Taken together, these examples showcase the versatility and efficacy of peptidic ABPs. Although targeting some enzyme classes represent an ongoing challenge, the activity of several proteases has been successfully probed with an increasing amount of new applications such as FRET-based probes⁹⁹ or fluoromethyl ketone warheads.¹⁰⁰ In all cases, ABPs, particularly the more specific peptic ones, represent an invaluable tool to monitor the dynamics of biological processes.

1.4 p53: the “Guardian of Immunity”

Upon its discovery in 1979, p53 was initially believed to be an oncogene due to its elevated levels in tumour cells and immortalized or transformed mouse fibroblasts.¹⁰¹⁻¹⁰³ However, over a decade of subsequent research revealed that most of the p53 clones identified up to that point were expressing mutated forms of the protein. It became evident that the wild type p53 is, in fact, a bona fide tumour suppressor protein.¹⁰⁴ Over the past 30 years, the role of p53 has evolved from being considered a simple cancer-associated protein to being recognized as a crucial transcription factor that regulates a wide array of essential cellular processes, including stress response, tumour suppression, cell cycle control, and apoptosis.¹⁰⁵⁻¹⁰⁷ To date, over a hundred thousand publications have provided new insights into the intricate mechanisms governing p53's functions and regulation in both normal and cancer immunity, leading to its designation as the "guardian of Immunity" in 2020.¹⁰⁸

1.4.1 p53, structure and mediated pathways

p53 is a transcription factor encoded by the *TP53* gene, playing a pivotal role in the transcription of DNA into RNA.¹⁰⁹ Transcription factors such as p53 regulate gene expression by binding to specific DNA regions, where they either promote or inhibit transcription by facilitating or obstructing the recruitment of the RNA polymerase II transcription machinery towards a specific gene portion. p53 is particularly crucial in determining cell fate in response to various sources of stress, including but not limited to oncogene activation, telomere erosion, ribosomal stress, and hypoxia (**Fig. 1.16**).¹¹⁰⁻¹¹³ Upon activation, p53 can be imported in the nucleus and tetramerize to bind DNA portions. This has major consequences in all aspects of the cell life and can substantially impact its cycle. The diversity of p53's stress-induced responses is largely dependent on the specific patterns of post-translational modifications (PTMs) that tag the protein. This can influence p53 localization in the cell, and therefore modulate its activity.

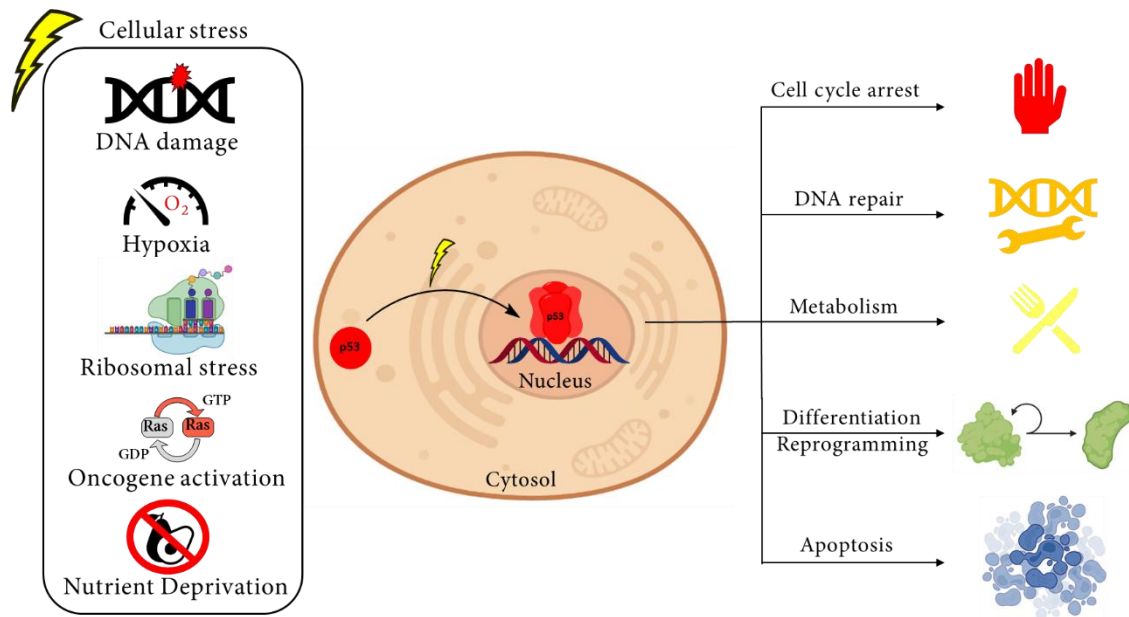


Figure 1.16: p53 activation pathways. Different types of cellular stress trigger p53 responses.

Within the nucleus, p53 functions as a bona fide transcription factor, activating genes that govern processes such as cellular senescence, apoptosis, cell cycle arrest, DNA repair, cell metabolism, and autophagy.^{108, 114} In the cytoplasm, p53 interacts with different proteins to mediate tumour suppression and can also initiate transcription-independent pathways that lead to cell death.^{115, 116}

Structurally, p53 is a highly conserved protein of 393 amino acids with a modular domain structure consisting of five distinct domains (**Fig. 1.17**): the trans-activation domain (TAD, residues 1-60), the proline-rich domain (PRD, residues 61-94), the DNA-binding domain (DBD, residues 95-292), the tetramerization domain (TD, residues 325-356), and the C-terminal regulatory domain (CTD, residues 357-393).^{114, 117} Notably, both the N-terminal and C-terminal regions of p53 are intrinsically disordered, and unfolded regions account for nearly 40% of the full-length peptide sequence. This is not an uncommon feature among signalling proteins because it confers the structural flexibility needed for interactions with a broad panel of binding partners. These interactions are often further modulated by PTMs such as ubiquitination, phosphorylation, methylation, and acetylation.¹¹⁸ The TAD domain,

despite being natively unfolded, contains two regions of nascent helical structure, known as TAD1 (residues 1-40) and TAD2 (residues 41-60), which can transition into fully folded motifs upon binding to partner proteins. These regions serve as promiscuous binding sites for a wide range of proteins, including the core negative regulators Mouse Double Minute 2/X (MDM2/MDMX)¹¹⁹ and the transcriptional coactivators Histone acetyltransferase 300 (p300)¹²⁰ and CREB Binding Protein (CBP).¹²¹ Similarly, the C-terminal domain is intrinsically disordered,¹²² but can adopt various conformations such as an α -helix¹²³ or a β -strand¹²⁴ depending on the binding partner it interacts with. The PRD domain, which acts as a spacer between the TAD1/TAD2 subdomains and the DBD,¹²⁵ contains five PXXP (where P is proline and X is any amino acid) motifs with high flexibility and conformational mobility. However, the exact role of the PRD is not yet fully understood. The DNA-binding domain (DBD) is the core functional region of p53, responsible for binding to a specific double-stranded DNA consensus sequence composed of two decameric palindromes of the form 5'-RRRCWWGYYY-3' (R = A, G; W = A, T; Y = C, T).^{114, 126} The tetramerization domain, another region with a defined secondary structure, enables p53 to exert its transcriptional activity as a tetramer. The p53/DNA complex forms with a 4:1 stoichiometry, inducing a 60° axial bend in the DNA double helix,¹²⁷ which subsequently leads to the promotion or repression of target genes and influences multiple cellular outcomes.¹⁰⁵

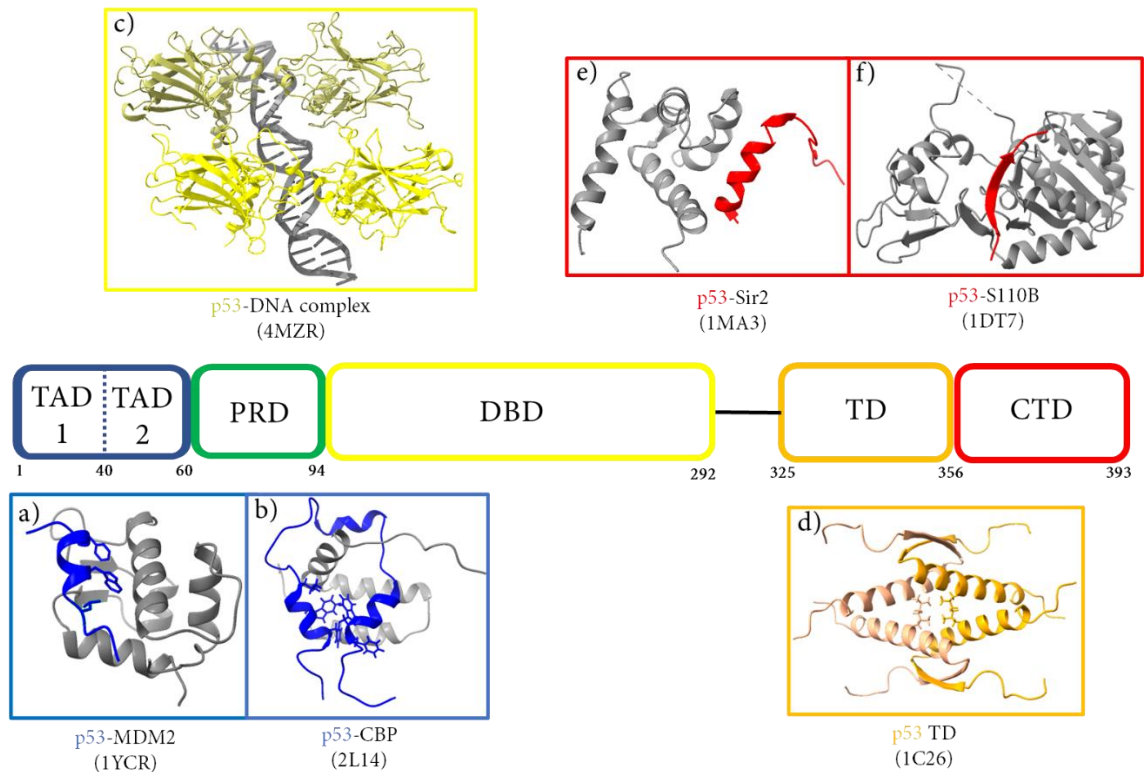


Figure 1.17: Structure and domain organization of human p53. a) p53 Transactivation domain bound to its negative regulator Mouse Double Minute 2 (MDM2). b) p53 transactivation domain bound to CREB Binding Protein (CBP). c) Four different p53 proteins with their core domain bound to DNA. d) Four p53 tetramerization domains cocrystallized together. e) p53 C-terminal domain bound to Sirtuin2 (Sir2) adopts an α -helix configuration, whereas a β -sheet is formed (f) when bound to S100 calcium binding protein (S100B).

1.4.2 Regulation and stability of p53

Given p53's involvement in numerous cellular processes, it is evident that a sophisticated and intricate regulatory network is essential for its control in both the cellular nucleus and cytoplasm. The regulation of p53 occurs at multiple levels. Firstly, at the genetic level, regulation can occur through single point mutations in the *TP53* gene that result in loss of function of the wild-type enzyme.¹²⁸ It is important to note that such genetic mutations occur in almost every type of cancer with frequencies ranging from approximately 10% to almost 100% in some ovary carcinomas.¹²⁹ Secondly, transcriptional regulation is facilitated through

interactions with various transcription factors such as miRNAs¹³⁰ or mRNAs.¹³¹ However, the most well-characterized mechanisms governing p53 stability and function operate at the protein level. Following translation in the cytoplasm, p53 is subject to a wide array of post-translational modifications, that tightly regulate its folding, quaternary structure, cellular localization, and protein-protein interactions.^{57, 132, 133} Common PTMs include ubiquitination via the Ubiquitin Proteasome Pathway (UPP), phosphorylation by kinases, acetylation by histone acetyltransferases, and methylation by methyltransferases. Additionally, less common PTMs such as SUMOylation, NEDDylation, O-GlcNAcylation, ADP-ribosylation, hydroxylation, and β -hydroxybutyrylation have also been observed (Fig. 1.18).⁵⁷

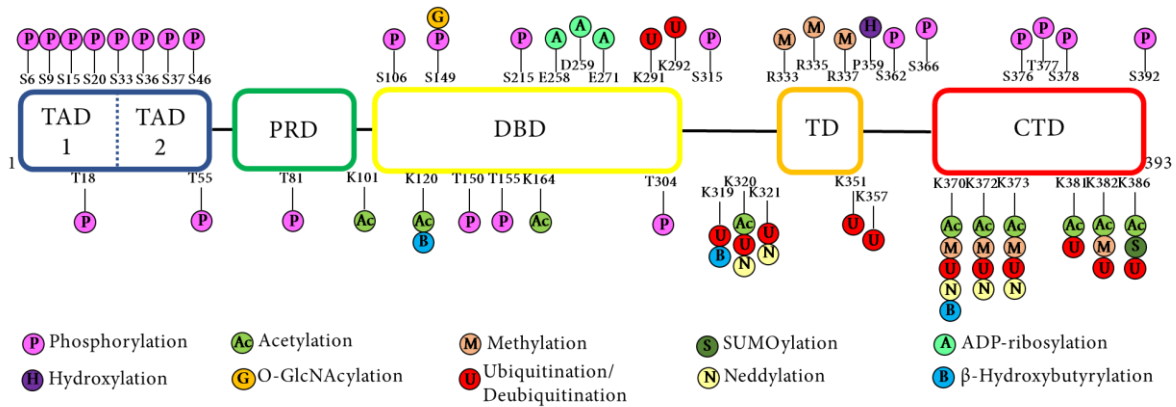


Figure 1.18: Schematic representation of p53 PTMs. Different colours are used for each modification.

In general, PTMs exhibit four key characteristics that are also applicable to p53. The same PTM can occur on different amino acids, and a single amino acid can bear multiple PTMs (multiple sites). For instance, CBP and its paralog p300 can efficiently acetylate six C-terminal lysines (K370, K372, K373, K381, K382, K386),¹³⁴ which can also be ubiquitinated by the E3 ligase MDM2.¹³⁵ A single PTM at different sites can lead to varying functions, while different PTMs can achieve the same function even at distinct sites (multiple functions). For example, in response to different stress types, specific serine (Ser) and threonine (Thr) residues within the TAD domain are phosphorylated, each triggering its respective cellular protective pathway.¹³⁶ One PTM can influence the effect of another at a different site (widespread crosstalk). This synergy is particularly relevant for phosphorylation, as this PTM alters the local electrostatic environment around p53, inducing conformational changes that

modify its affinity for other target proteins, as described for Pin1.¹³⁷ Each PTM has an enzyme responsible for reversing it (reversibility). For instance, deubiquitinating enzymes (DUBs) can remove mono-, multi-, and poly-ubiquitin tags from various lysine residues, significantly impacting cellular signalling and homeostasis.¹³⁸

1.4.2.1 p53 regulation by E3 ligases

As discussed in section 1.1 (p. 2) , E3 ligases provide the UPP with remarkable precision and specificity. With respect to p53, E3 ligases can either function as scaffolds, mediating the transfer of ubiquitin from an E2 enzyme to the target protein, or directly transferring ubiquitin to the substrate.¹³⁹ Currently, five of the ten classes of E3 ligases have been implicated in the regulation of p53 levels, namely RING-domain E3s, U-box-type E3s, F-box-type E3s, RBR-type E3s, and HECT-domain E3s.¹⁴⁰ The majority of p53-interacting E3s either mark it for degradation by the 26S proteasome or modulate its nuclear concentration through monoubiquitination. In unstressed cells p53 is maintained at very low levels due to continuous degradation by the UPP, resulting in a cellular half-life of less than 20 minutes.¹⁴¹ Such process ensures the cell proceeds efficiently through its life cycle.

The primary regulators of p53 degradation are MDM2 and its close homolog MDMX.¹⁴² Although MDMX lacks E3 ligase activity, it stabilises MDM2 through heterodimerization, preventing MDM2 auto-ubiquitination and enhancing its affinity for p53.¹⁴³ Consequently, MDM2 tags p53 at six lysine residues within its C-terminal domain, acting as a negative regulator.^{135, 144} Interestingly, MDM2 is also a transcriptional target of p53, leading to the overexpression of MDM2 in response to p53 activation by stress or DNA damage. This establishes a tightly regulated negative feedback loop in which p53 controls the expression of its own inhibitor. Furthermore, MDM2 can ubiquitinate both itself and MDMX, adding another layer of complexity to what is commonly known as the p53 regulatory pathway (**Fig. 1.19A**).

Although MDM2 and MDMX are central to p53 degradation and regulation, accumulating evidence over recent decades has suggested that several other E3 ligases can also modulate

p53 activity.¹⁴⁵ To date, nearly 20 E3 ligases have been shown to influence p53 levels both in vivo and in vitro.¹⁴⁶ One noteworthy example is the Makorin RING finger protein 1 (MKRN1), a member of the RING-domain E3 subfamily. Akin to MDM2, MKRN1 can target p53 for proteasomal degradation. Interestingly, MKRN1 also serves as an E3 ligase for the universal cyclin-dependent kinase inhibitor p21, a well-known tumour suppressor protein that inhibits cell proliferation and arrests the cell cycle.¹⁴⁷

In unstressed cells, MKRN1 acts as a negative regulator of both p53 and p21, promoting cell survival by facilitating their degradation via the UPP. Consequently, cells lacking MKRN1 exhibit elevated levels of p53 and p21, leading to cell cycle arrest and apoptosis (**Fig. 1.19B**). However, under conditions of severe DNA damage, MKRN1 selectively ubiquitinates p21 while its affinity for p53 decreases, thereby enhancing p53-dependent apoptosis.¹⁴⁸⁻¹⁵⁰ MKRN1 thus plays a dual role: it maintains cell survival under normal conditions by suppressing p53 and induces cell death under stress by downregulating p21. Preventing the excessive accumulation of p21 appears to be necessary for sensitizing cells to apoptotic stimuli. Intriguingly, MKRN1 ubiquitinates p53 at two previously unidentified post-translational modification sites, K291 and K292, located in an unstructured region at the end of the p53 DNA-binding domain (DBD).

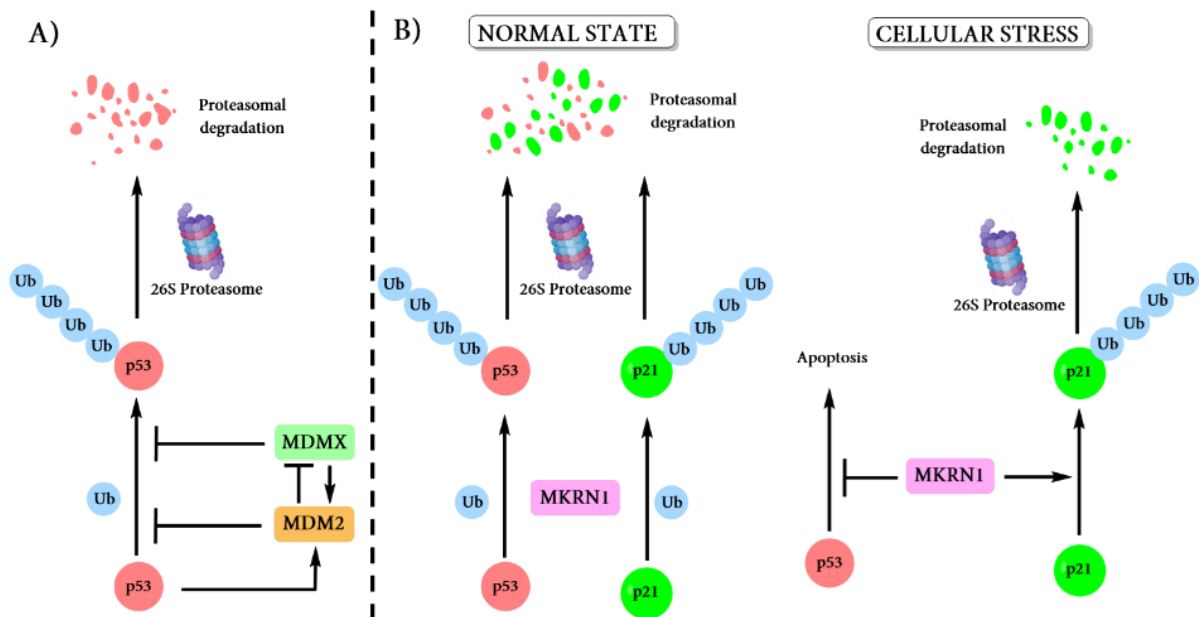


Figure 1.19: Schematics of two p53 mediated pathways. A) Simplified view of the p53 pathway. MDM2 and MDMX can downregulate p53 but p53 can also promote their transcription, forming a negative feedback loop. B) Schematic view of the p53/p21 pathway mediated by Makorin RING Finger Protein 1 (MKRN1). In unstressed cells, p53 and p21 are both downregulated by MKRN1. However, in stressed cells MKRN1 selectively targets p21 for proteasomal degradation, allowing p53-mediated apoptosis.

1.4.2.2 DUBs are p53 modulators

As previously noted in sections 1.1 (p. 5) and 1.2 (p. 5), ubiquitination is a crucial PTM which is reversed by the action of Deubiquitinating enzymes (DUBs). The action of DUBs serves as an additional control point for the level and activity of several proteins, resulting in either their rescue from proteasomal degradation or the modification of their cellular levels.¹⁵¹⁻¹⁵⁴ To date, several studies have unveiled a deep interconnection between p53 and DUBs, particularly in diseases such as cancer, and some hold promise for the development of effective therapeutic strategies. With respect to p53, nearly 15 DUBs have been reported to act as positive or negative regulators, respectively promoting or hampering its tumour suppression functions (Fig. 1.20A).¹³⁸

When p53 is tagged with mono- or polyubiquitin Lys48 linked chains, it undergoes proteasomal degradation. Maintaining p53 at low levels ensures that the cell can continue its

life cycle in the absence of additional stress. Deubiquitinating enzymes, which utilise a cysteine in their active site, can either directly cleave ubiquitin from p53 (direct activation of p53) or indirectly remove ubiquitin from the associated E3 ligase (indirect activation of p53). For instance, USP7, a member of the ubiquitin-specific protease (USP) subfamily, performs both functions within the p53-MDM2 pathway by deubiquitinating both p53 and MDM2 (Fig. 1.20B).^{155, 156} The action of USP7 is crucial for proper cell cycle maintenance. In stressed cells, USP7 ensures that p53 remains deubiquitinated at lysine residues targeted by MDM2, thereby circumventing negative regulation by MDM2. In USP7-deficient cells, MDM2 levels are drastically reduced, leading to improper MDM2 self-ubiquitination, and resulting in undesired p53 stabilisation and apoptosis.¹⁵⁷ Moreover, overexpression of USP7 counteracts MDM2-mediated ubiquitination of p53, thereby stabilising it and triggering cell apoptosis.¹⁵⁶ Consequently, USP7 has become an increasingly studied target in cancer therapy.¹⁵⁸ In addition to members of the USP subfamily, the OTU subfamily member Otubain 1 is reported to act as an indirect activator of p53.^{159, 160} OTUB1 directly inhibits MDM2-mediated p53 ubiquitination in cells and in vitro, independently of its catalytic activity. This inhibition occurs mainly through the suppression of the MDM2-associated ubiquitin-conjugating E2 enzyme UbcH5. Overexpression of OTUB1 leads to significant apoptosis and reduced cell proliferation in a p53-dependent manner. Conversely, inhibiting OTUB1 severely impairs p53 activation in response to DNA damage.

Negative regulation of p53 notoriously occurs with E3 ligases such as MDM2 or MKRN1. Interestingly, at least six DUBs have been also described to impair p53 expression by stabilising E3 ligases that interact with p53, rather than stabilising p53 itself. This can have harmful effects on the cellular environment, as it inhibits the tumour suppressor function of p53 by promoting its ubiquitination. To date, only members of the USP subfamily have been shown to interact with various E3 ligases, including MDM2. A well-known DUB for the MDM2-MDMX complex is USP2a.¹⁶¹ Unlike USP7, USP2a selectively deubiquitinates MDM2, leading to p53 suppression via MDM2-mediated degradation. Although USP2a overexpression has been associated with prostate cancer, further studies are necessary to fully

understand how this DUB regulates the MDM2-p53 pathway and the specific p53 lysine residues from which USP2a removes ubiquitin.

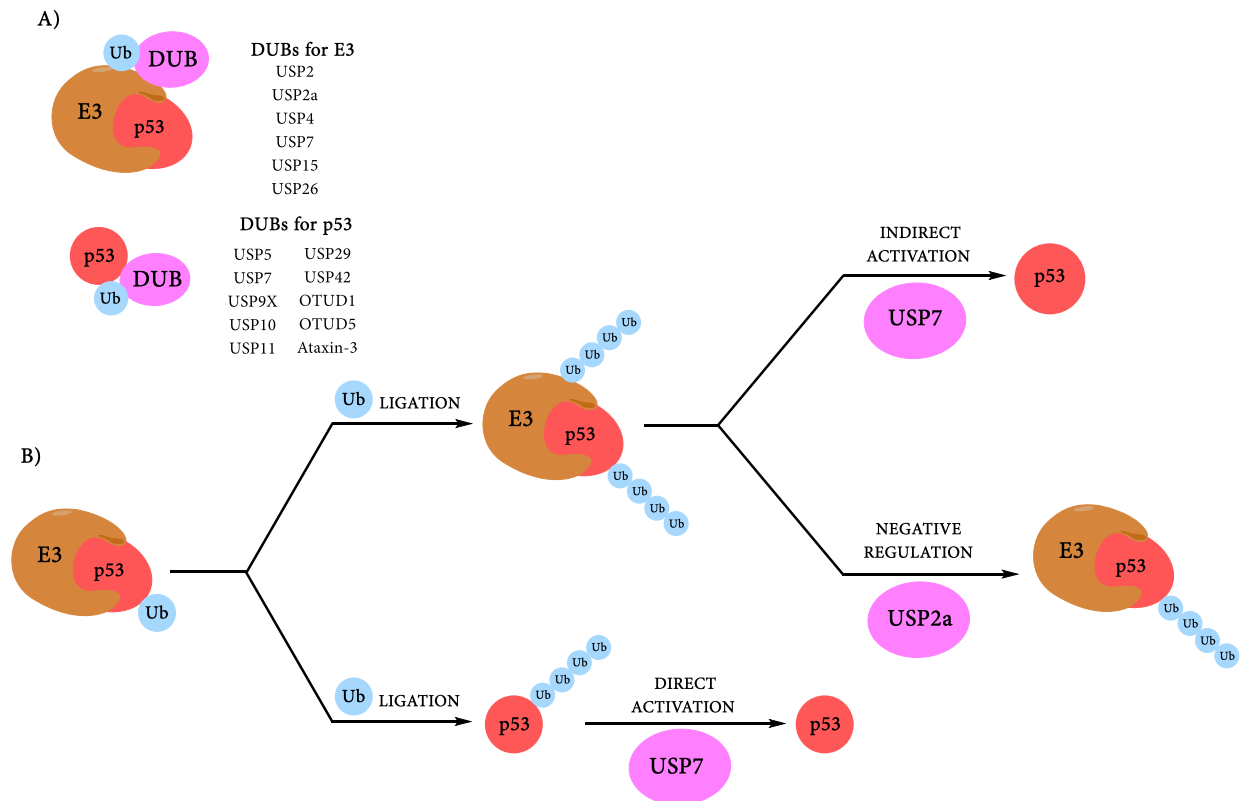


Figure 1.20: A) list of DUBs interacting with p53 alone or complexed to an E2 or E3. B) overview of different p53 regulation pathways triggered by DUBs. Deubiquitination by USP7 helps the cell maintaining its regular cell life, whereas USP2a acts as a negative p53 regulator.

1.5 Project aims

The critical role of DUBs and the ubiquitin conjugation machinery in maintaining proper cellular function is well established and extensively documented.^{11, 34} Additionally, ABPs have proven to be valuable tools for investigating these systems, as they covalently label active enzymes by targeting their active sites.^{60, 162} To date, the design of probes for DUBs has predominantly utilised mono- or polyubiquitin scaffolds, incorporating a reactive warhead and a reporter element.⁹⁰ The aim of this project was to expand the panel of peptidic ABPs to interrogate specific DUB (or subset of DUBs) within the cell. This concept was used to design

a set of peptide probes, one targeting the DUBs interacting with p53 at its K291/292 sites and the other to target the DUB Otubain1.

The purpose of the work described in Chapter 2 was to synthesise a set of peptidic ABPs which can selectively target the group of DUBs that remove ubiquitin from residues K291/K292 of p53. It has been recently reported that the E3 ligase that attaches Ub to K291/K292 of p53 is MKRN1,¹⁴⁹ a RING-domain E3 (**Fig. 1.21**). MKRN1 acts as a negative regulator of p53 by promoting its ubiquitination and subsequent degradation via the UPP. Although limited information is available on MKRN1, the DUB that reverses this action has yet to be identified.¹⁶³ This unidentified DUB could play a critical role in cellular processes by activating p53 through the removal of ubiquitin, thereby inducing apoptosis. The probes **12-14** were designed on a portion of the p53 sequence wherein the lysine residues K291/K292 were synthetically modified to include an electrophilic warhead at the isopeptide bond site. While previous studies have struggled to achieve specificity for a particular DUB,^{81, 164} this approach is specifically designed to target the DUB responsible for deubiquitinating p53. Once synthesised, the ABP can be employed in both normal and cancerous human cells to isolate the DUB that removes ubiquitin from p53 under different environmental and stress conditions, thus advancing our understanding of p53 post-translational modifications and potentially uncovering novel DUB functions.

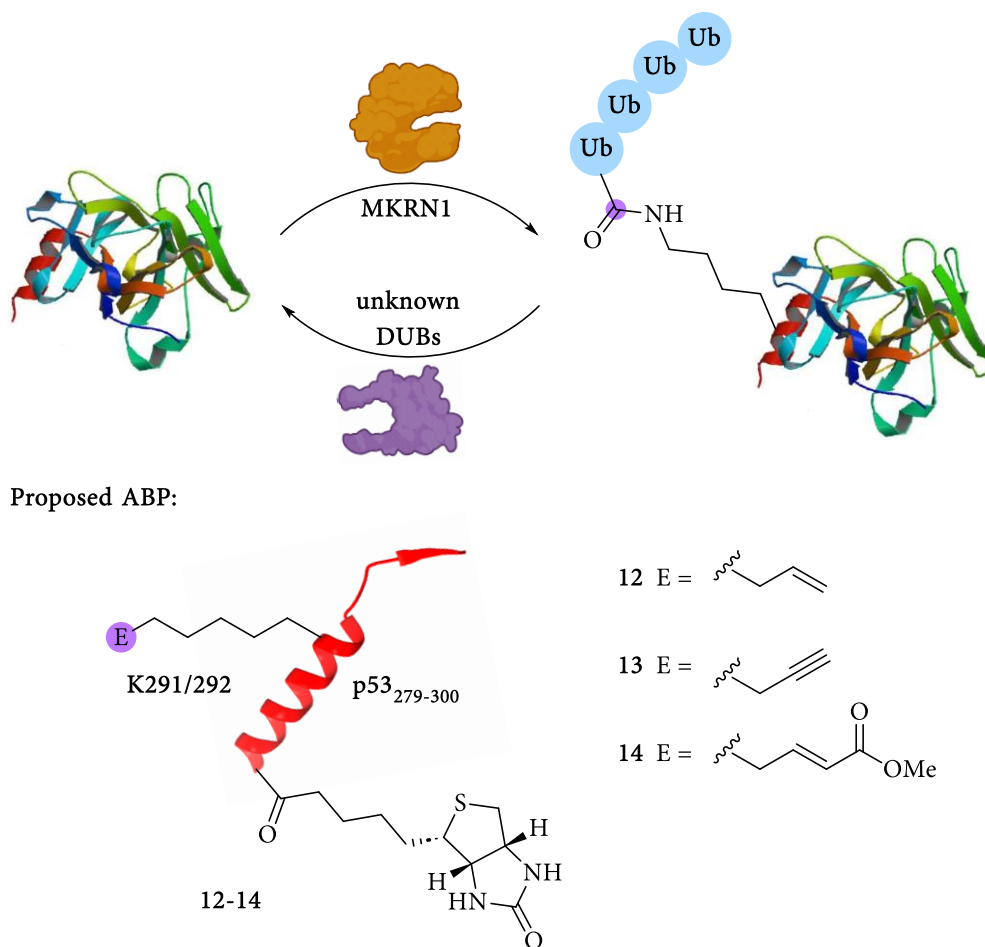


Figure 1.21: schematic view of the ubiquitination and deubiquitination processes for p53 at residues K291/292. The proposed trap will mimic the DUB natural substrate and will be trapped by the electrophilic warhead (E) placed in the appropriate position (highlighted in purple).

OTUB1 is a linkage specific DUB which is responsible for Lys48 linked polyubiquitin chains cleavage. This hydrolytic activity and the ability to interact with different E2 enzymes allow OTUB1 to save a target protein from proteasomal degradation and balance its expression levels.⁴³ To selectively target OTUB1 with peptidic ABPs, the short peptides 15-16 were designed, synthesised, and tested (Fig. 1.22). Moreover, a second set of probes 17-18 featuring a more reactive vinylmethylester (VME) warhead was also proposed to enhance reactivity. This work is presented in Chapter 3.

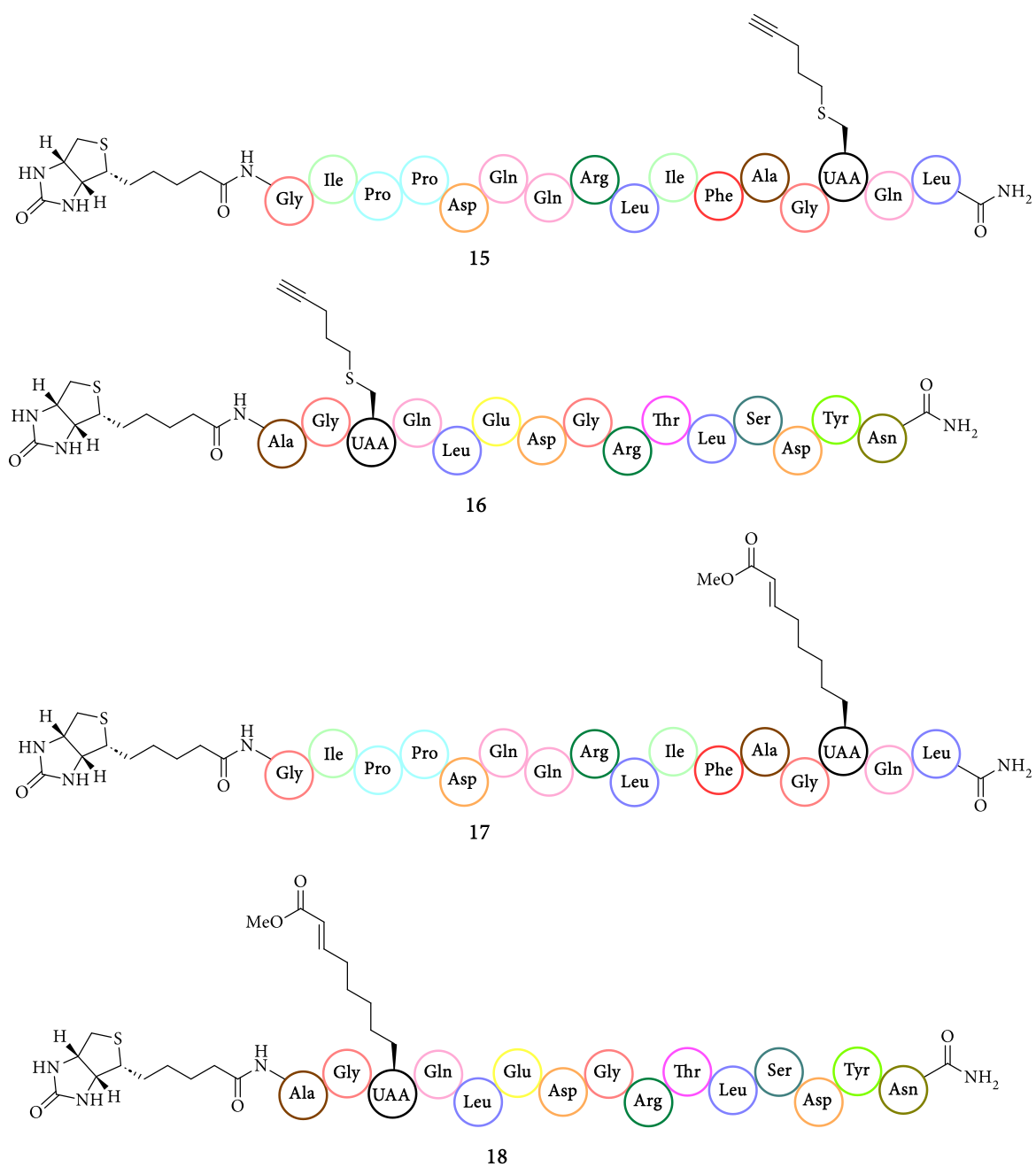


Figure 1.22: set of proposed probes 15-18 to target OTUB1. UAA = unnatural amino acid.

Chapter 2

**Activity Based Probes to reveal
new insights into p53 deubiquitination**

2.1 Introduction

The DUB (or subset of DUBs) responsible for the removal of ubiquitin from p53 at lysines 291/292 was postulated to be a cysteine protease. To perform its catalytic activity, it exploits an active site cysteine which specifically targets the isopeptide bond between the ϵ -amine group of K291/292 side chains and the Ub C-terminus (Fig. 2.1A). In order to covalently label the thiol group of the active site cysteine, a warhead must replace the native isopeptide bond and position an electrophilic group at the ζ position, mimicking the amide carbonyl carbon. The proposed warheads **19-24** feature 3 distinct electrophiles, each of which has been previously reported to label DUBs in activity-based protein profiling studies (Fig. 2.1B). The vinylmethylester (VME) probes **21** and **24** reacts via [1-4] Michael addition and is reported as a reactive warhead.^{63, 84} The propargyl warheads **20** and **23** label following a milder electrophilic addition,^{78, 79} whereas the alkene probe **19** and **22** are based on a radical mechanism of activation.⁸³ These warheads were synthesised as Fmoc-protected Unnatural Amino Acids (UAA) and later incorporated into the peptidic recognition scaffold via solid phase peptide synthesis (SPPS). The modified peptides were decorated with a biotin tag to allow visualisation of the labelled targets and their quantification using proteomics.

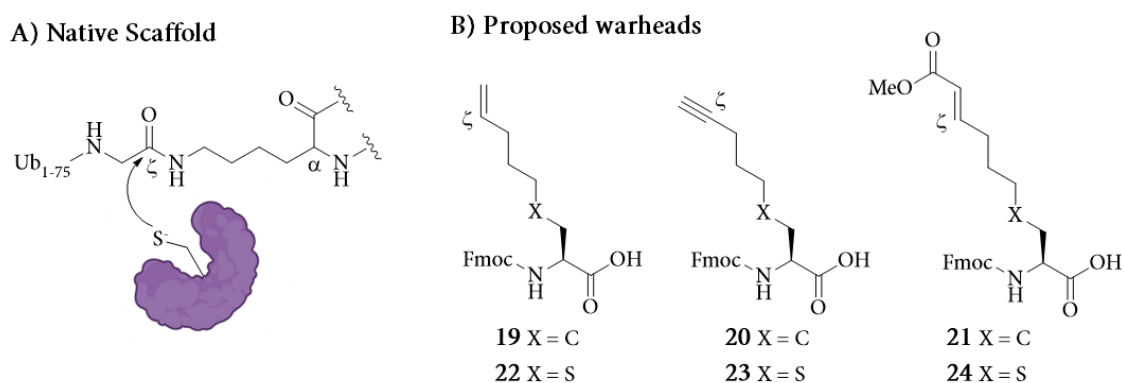


Figure 2.1: A) depiction of p53-Ub native isopeptide linkage. DUB enzyme is shown in purple. B) Proposed warheads. The electrophile moiety is placed at the ζ position, emulating the isopeptide bond.

2.2 Synthesis of warheads

Different retrosynthetic schemes were considered for the preparation of warheads **19-24** (Fig. 2.2). For the C-derivatives **19-21**, a radical addition with a suitable alkyl bromide was carried out on the dehydroalanine intermediate **25**¹⁶⁵ that could be obtained performing a dialkylation-elimination on cysteine **26**.¹⁶⁶ For the S-derivatives **22-24**, Fmoc protection was performed on **27-29** that could be obtained following alkylation of **26** at a suitable pH.¹⁶⁷

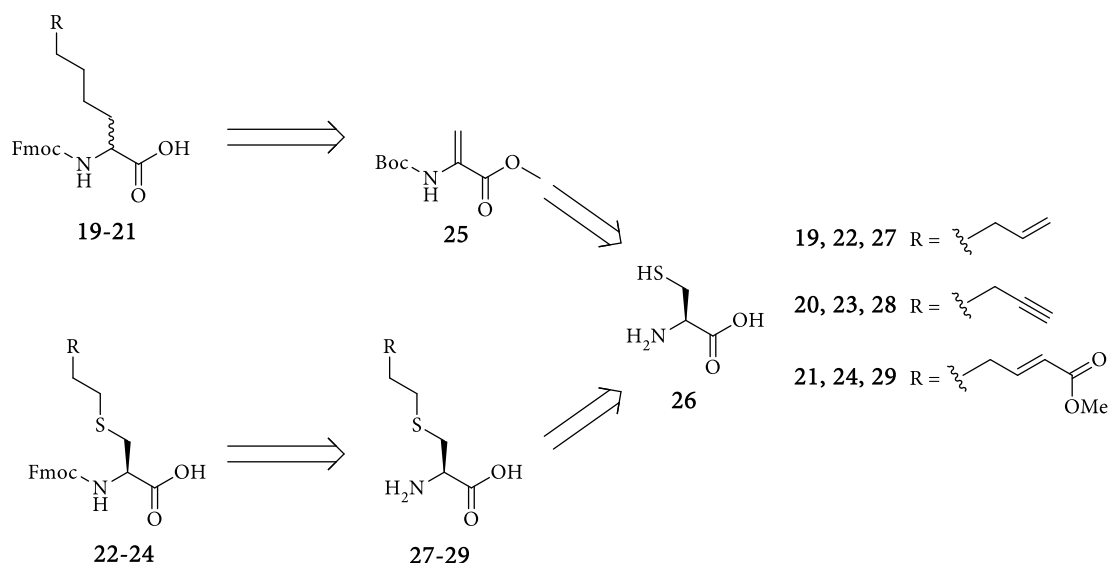


Figure 2.2: retrosynthetic routes for C-derivatives **19-21** and S-derivatives **22-24**.

2.2.1 Synthesis of C-Derivatives

Cysteine **26** was simultaneously N-Boc and C-OMe protected by carrying out a literature procedure,¹⁶⁸ followed by a dialkylation-elimination reaction to yield the common precursor dehydroalanine **25** via a cyclic intermediate **31** (Fig. 2.3).¹⁶⁶

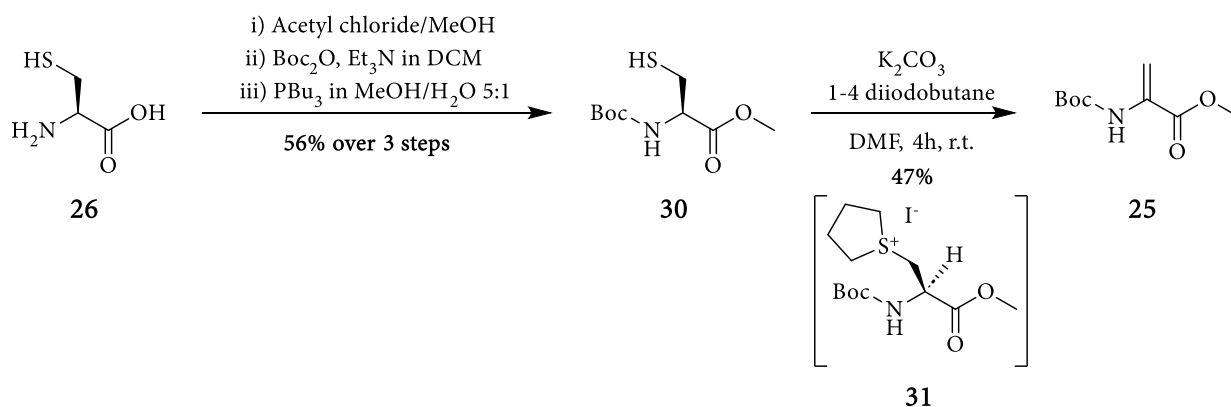


Figure 2.3: Synthetic scheme for dehydroalanine precursor **25**.

Hydrochloric acid was safely generated *in situ* by adding acetyl chloride to MeOH to trigger an acid catalyzed esterification. The crude product was reacted with Boc anhydride and was used for the following reaction without further purification. Tributylphosphine was used to reduce disulfide traces giving Boc-Cys-OMe **30** in 56% yield over 3 steps. Dialkylation of **30** generated the intermediate **31**, and base catalysed elimination afforded dehydroalanine compound **25** in a moderate yield of 47%.

Prior to carrying out the radical alkylation, the non-commercial bromide **34** was synthesised starting from the corresponding alcohol **32** (Fig. 2.4).

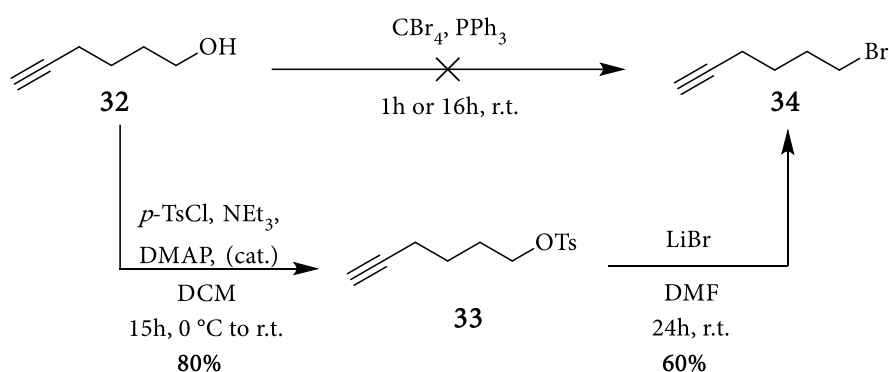


Figure 2.4: Synthetic scheme for alkyl bromide **34**.

Unfortunately, a 1 hour Appel reaction to synthesise bromide **34** in one step failed to yield the product, and subsequent attempts where the reaction time was increased up to 16 hours were not successful.¹⁶⁹ Consequently, 5-hexyn-1-ol **32** was converted into a tosylate derivative **33** which was found to be a better leaving group for the following S_N2 displacement that afforded the desired compound **34** in decent yield of 60%.¹⁷⁰

The alkyl bromide **38** bearing a vinylmethylester (VME) moiety was synthesised from alcohol **35** in a two steps synthesis (Fig. 2.5). This bromide is one carbon unit shorter than the desired chain length and was synthesised to test if the conditions of the radical alkylation were suitable for the installment of a VME moiety.

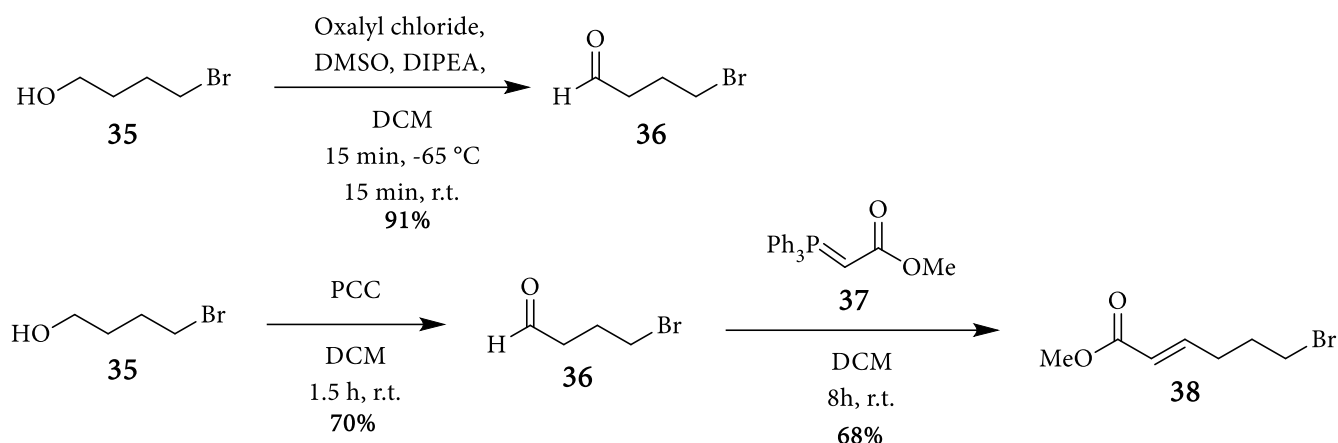
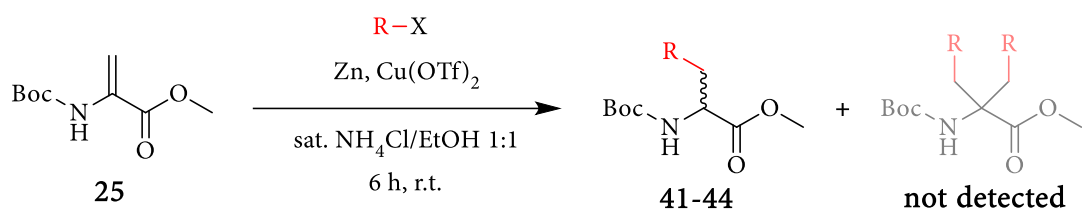


Figure 2.5: Synthetic scheme for VME bromide **38**.

Aldehyde **36** was synthesised from 4-Br-1-butanol **35** by performing a Swern oxidation or using Pyridinium Chloro Chromate (PCC). The latter method was preferred as it did not require low temperatures, which made the reaction operationally easier without a drastic negative impact in the yield. A Wittig olefination with the stable ylide **37** was exploited to access the desired VME bromide **38** with 68% yield.

Once the desired alkyl bromides **34** and **38** were successfully synthesised, the radical alkylation was carried out. To obtain the desired alkylated compounds **41-44**, dehydroalanine **25** was employed in a Zn/Cu(OTf)₂-mediated radical addition readapted from a procedure described by Shin *et al.* (Scheme 2.1).¹⁶⁵ The corresponding alkyl bromides **38-40** and **34** were added in 3 portions over 3 hours to avoid accumulation of the stable C α radical that could result in undesired dialkylation byproducts. 5-Bromo-1-pentene **39** was added to dehydroalanine **25** to generate **41** in a test reaction (Scheme 2.1, entry 1), and the 6-carbon unit homologue 6-Bromo-1-hexene **40** was employed to generate compound **42** with excellent yield of 80% and no dialkylation product detected (Scheme 2.1, entry 2). The propargyl moiety was installed to obtain **43** with a modest yield of 55% (Scheme 2.1, entry 3). Unfortunately, all the attempts to employ the VME **38** to access compound **44** with the radical addition reaction were unsuccessful (Scheme 2.1, entry 4).



Entry	Bromide R-X	Product	Yield
1	 39	 41	67%
2	 40	 42	80%
3	 34	 43	55%
4	 38	 44	-

Scheme 2.1: radical addition of different alkyl bromides to dehydroalanine 25.

A literature procedure was modified to devise a general three-step protecting group exchange to access Fmoc-protected unnatural amino acids (UAA) **19** and **20** from their Boc-protected methyl ester derivatives **42** and **43** (Fig. 2.6).¹⁷¹

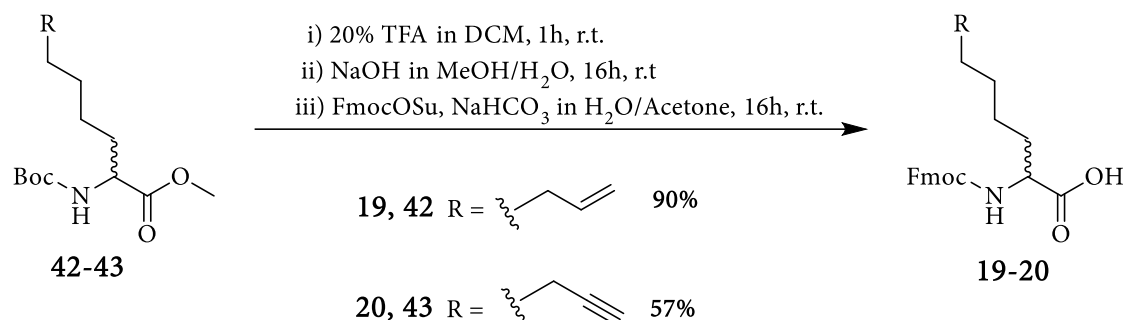


Figure 2.6: Synthetic scheme for the one-pot synthesis of Fmoc protected UAA **19** and **20**.

Deprotection of Boc protecting group is nearly quantitative in 20% TFA, and the crude can be immediately used in the following saponification reaction. Installation of the Fmoc protecting group is carried out using Fmoc-succinimide (Fmoc-Osu) in basic aqueous environment. The alkene and alkyne UAAs **19** and **20** were obtained with modest to excellent yields and were ready to be used in solid phase peptide synthesis for the synthesis of the probe recognition scaffold.

2.2.2 Synthesis of S-Derivatives

In order to perform cysteine alkylation, a set of alkyl halides was synthesised (Fig. 2.7).

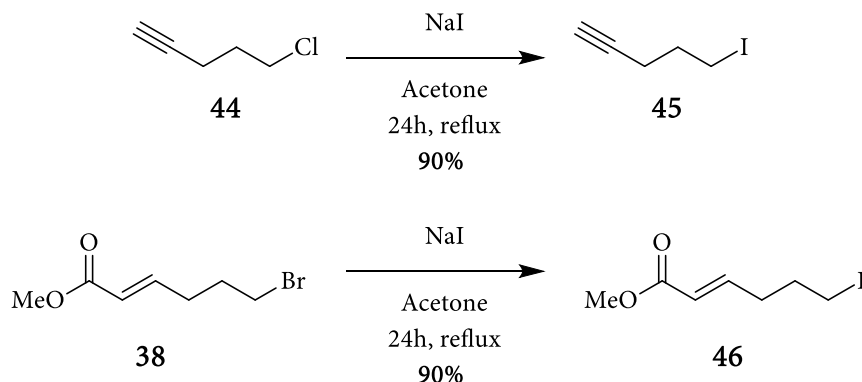
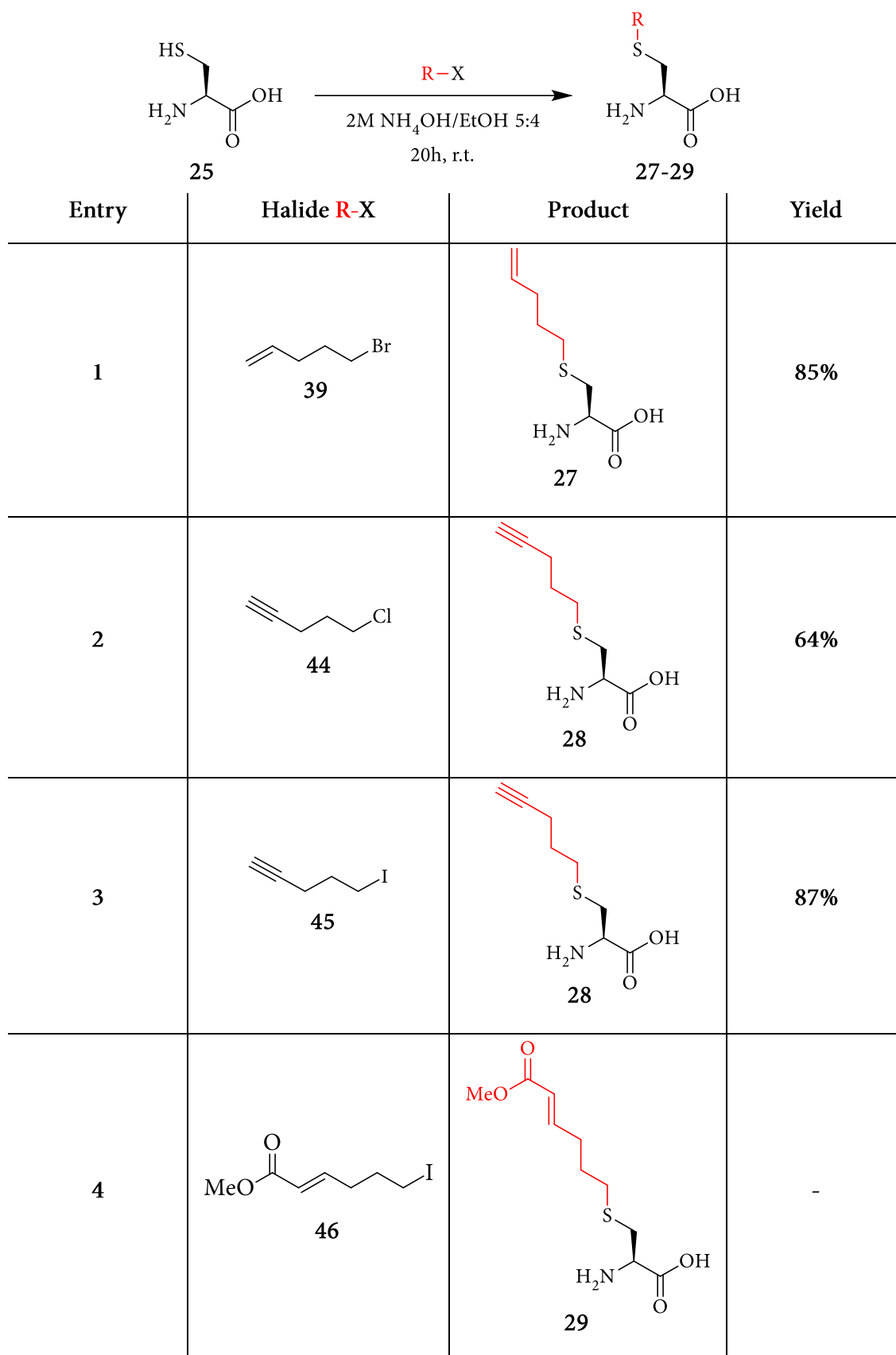


Figure 2.7: Finkelstein reaction on compounds 44 and 38.

A Finkelstein reaction was employed to furnish iodides 45 and 46 in 90% yield. These compounds would feature a better leaving group for the following nucleophilic displacement.

To access compounds 27-29 with the correct side chain, cysteine was directly alkylated with the corresponding alkyl halide (Scheme 2.2). In an attempt to increase the yield of the alkylation reaction, EtOH was exchanged for THF, but the reaction yielded no product. Thus, a 5:4 v/v mixture of 2M NH_4OH /EtOH was used to carry out cysteine thiol alkylation.



Scheme 2.2: synthesis of alkylated cysteine with different alkyl bromides.

The alkylated products **27** and **28** were synthesised with commercially available 5-Br-1-pentene (**39**, **Scheme 2.2**, entry 1) and 5-Cl-1-pentyne (**44**, **Scheme 2.2**, entry 2) with a yield of 85% and 64% respectively. As expected, when 5-I-1-pentyne **45** was used as alkylating reagent (**Scheme 2.2**, entry 3) yield improved to 87%. However, all the attempts to carry out alkylation with the VME iodide **46** failed to give the desired product **29** (**Scheme 2.2**, entry 4).

Installment of Fmoc protecting group was performed following a literature procedure and was employed to synthesise compounds **22** and **23** with 79% and 29% yield respectively (**Fig. 2.8**).¹⁷¹ These Fmoc-protected UAA bearing an electrophilic warhead were implemented in the recognition scaffold of ABPs using SPPS.

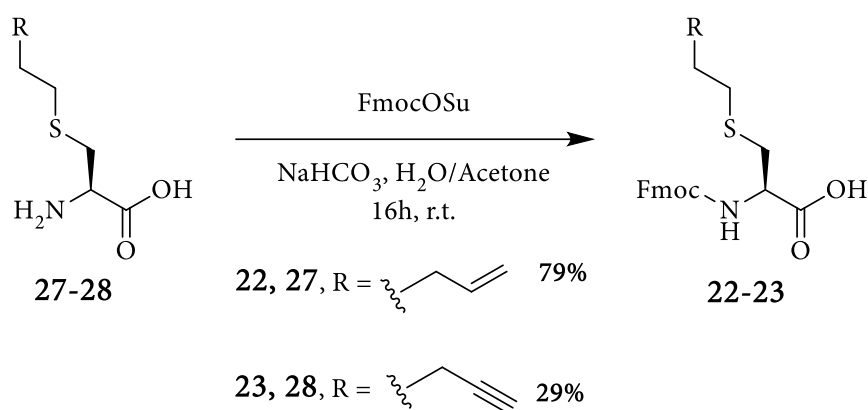


Figure 2.8: Fmoc protection of compounds **27** and **28**.

2.3 Design of the peptide recognition scaffold

To generate an ABP capable of selectively targeting the DUB that interacts with p53 ubiquitinated at Lys291 and Lys292, a short fragment of the p53 sequence covering residues 279-300 has been chosen as the minimal recognition scaffold. Due to the high structural flexibility of transcription factors like p53,¹¹⁴ the exact secondary structure of this region has only been partially resolved. While residues 277-294 form an α -helix, the remainder of the sequence (residues 294-300) is believed to be unstructured. The selected sequence includes the two crucial lysines, K291 and K292, which are the specific targets of the DUB(s) of interest. The 22-mer peptide **47**, consisting of the native p53 sequence has been successfully synthesised using standard Fmoc based SPPS (**Fig. 2.9**). To understand which of the two

lysines interacts preferentially with the targeted DUB/s, each was individually replaced by an UAA bearing a warhead on its side chain. The two mutants **48** and **49** were obtained when the native lysines in positions 291 and 292 respectively were replaced by the previously synthesised UAA **23** that bears a propargyl warhead.

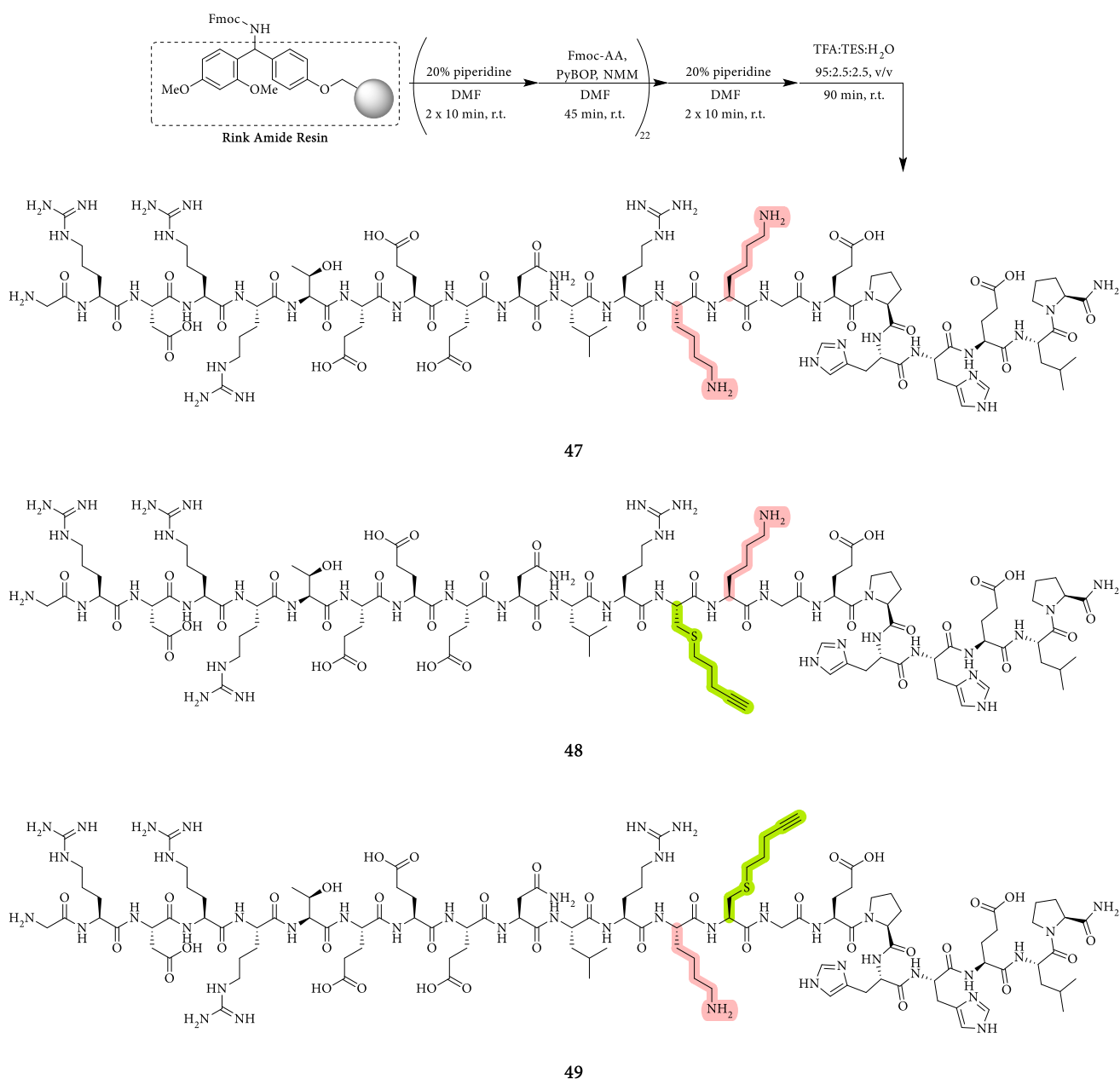


Figure 2.9: p53 based peptides **47-49**. The native lysine residues are highlighted in pink whereas the modified lysines bearing the probe warhead are highlighted in green.

Twenty-two iterations of Fmoc protection and amino acid coupling were conducted to assemble the peptide on resin. The side chains of Glu and Asp were protected using *tert*-Butyl groups whereas Lys side chains were protected with Boc groups. Moreover, Pbf and Trt groups were used to protect Arg and Asn/His residues, respectively. The rationale behind the protecting group selection lies in the stability of such groups in basic conditions and their ease of removal with TFA. After the final Fmoc protection, a mixture of TFA/TES/H₂O (95:2.5:2.5, v/v/v) was used to both remove all the protecting groups and cleave the peptide from the resin support. TES was employed to scavenge the cationic species generated during global deprotection, preventing undesirable side-reactions. Following cleavage, the reaction mixture was concentrated, and crude peptides **47-49** were precipitated with Et₂O. High Resolution Mass Spectrometry (HRMS) analysis of the crude mixture confirmed the presence of the desired products.

2.4 Tag installation

Biotin was chosen as the tag for multiple reasons. Biotinylated macromolecules can be readily visualized through immunoassays using a streptavidin-conjugated IR dye. Furthermore, streptavidin-coated beads can be used to perform affinity enrichment experiments to isolate biotinylated peptides and their adducts allowing for proteomic analysis of the targeted DUB(s). Moreover, after the final Fmoc deprotection the free carboxylic group on biotin can be coupled to the N-terminus of the peptide grown during SPPS. This on-resin biotinylation simplifies the probe synthesis process, allowing a more direct approach requiring only a single HPLC purification step (**Fig. 2.10**).

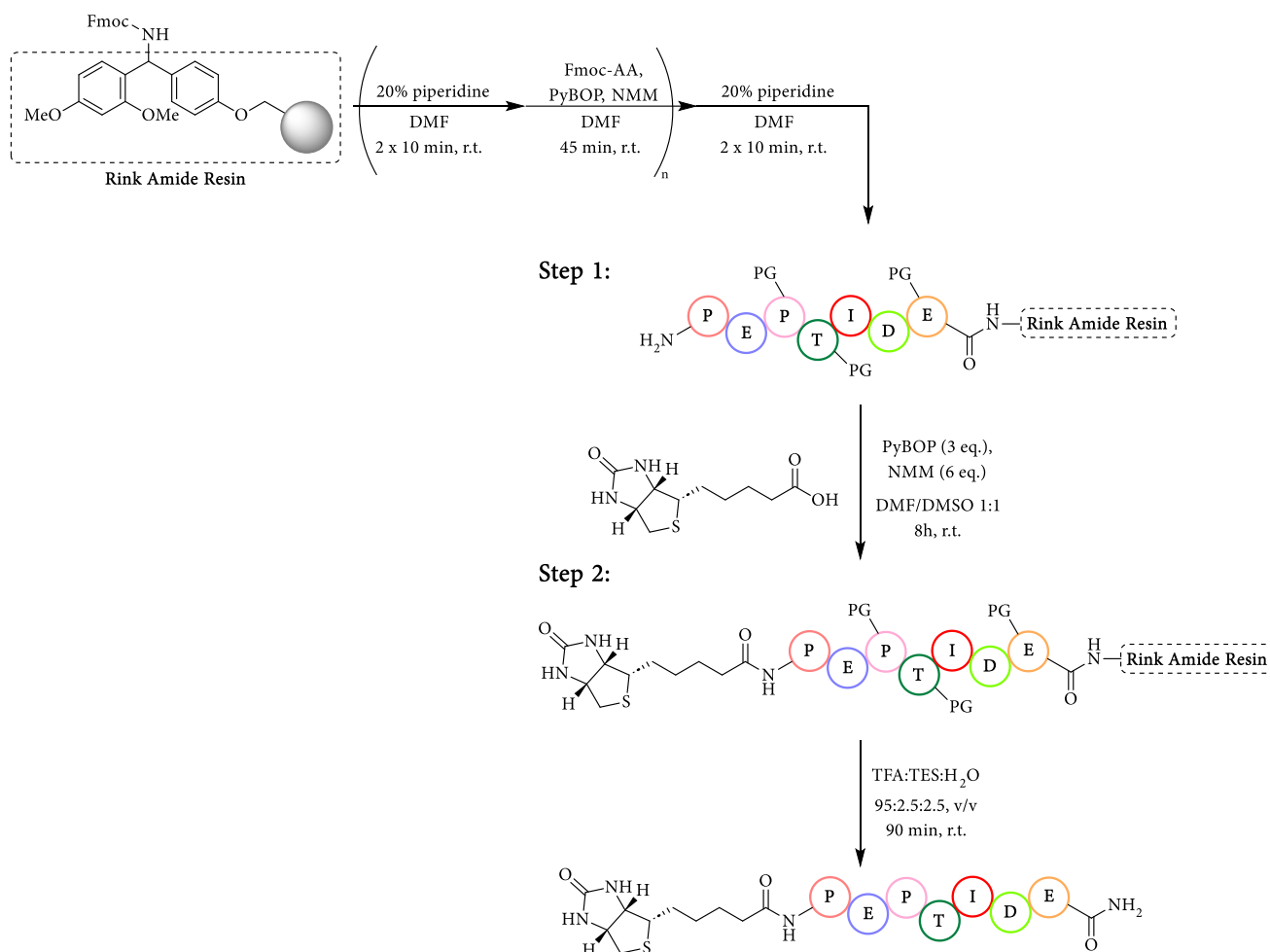


Figure 2.10: synthetic strategy for on-resin biotinylation. Following SPPS, the N-terminus of the growing peptide is biotinylated using standard coupling reagents (step 1). Cleavage and HPLC purification of the crude yield the desired biotinylated peptide (step 2).

Following SPPS, the N-terminus of the growing peptide was biotinylated using PyBOP and NMM as coupling reagents (Fig. 2.10, step 1). Global deprotection and cleavage followed by HPLC purification of the crude yielded the desired biotinylated peptide (Fig. 2.10, step 2). Availing of this strategy, biotin was efficiently installed on peptides 47–49 to generate a set of probes (Fig. 2.11). The biotinylated probe 50 does not feature any reactive warhead and so will act as a negative control. The probes 51 and 52 will react at the terminal alkyne forming a covalent adduct with the active site cysteine residue of the targeted DUB/s.

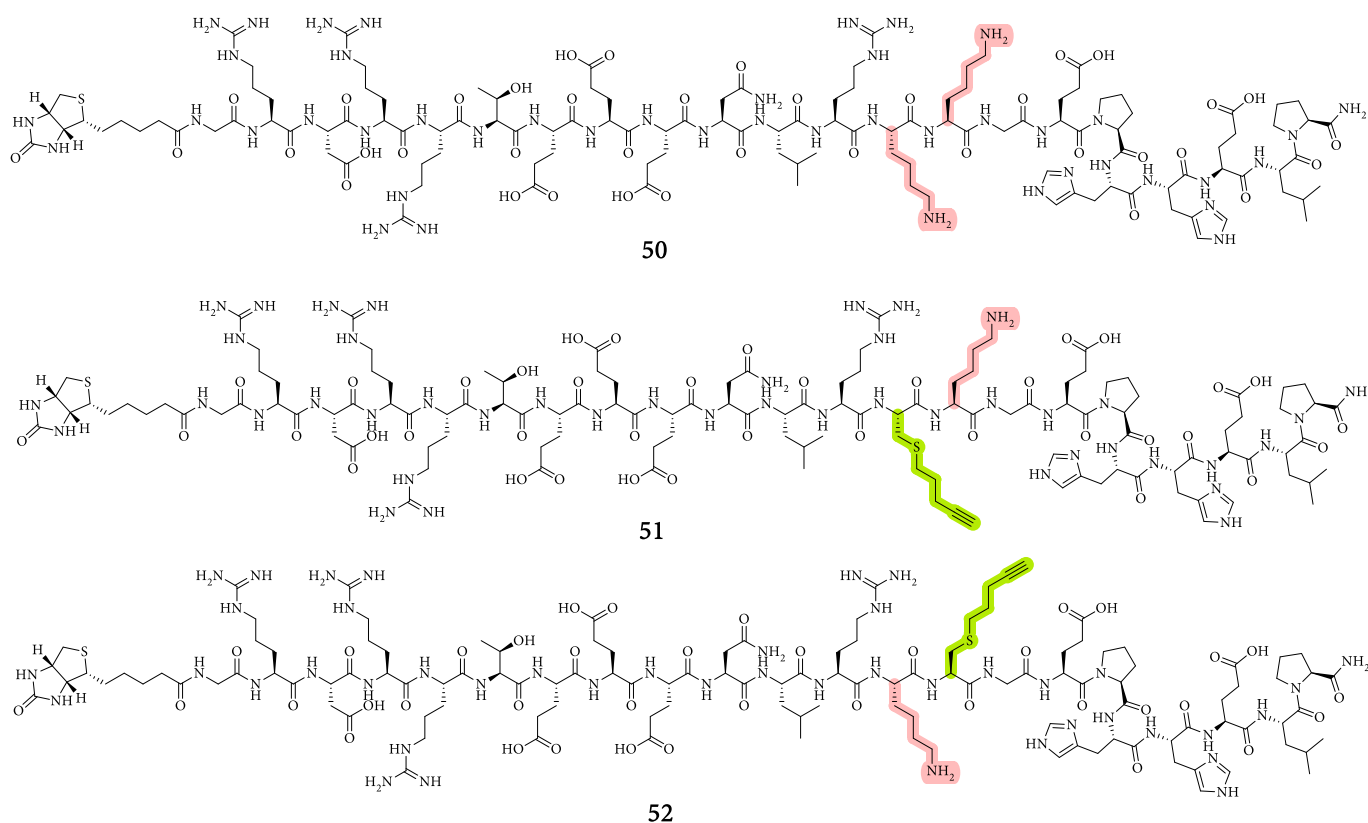


Figure 2.11: set of biotinylated probes to target DUBs interacting with ubiquitinated p53.

2.5 Probe labelling assays and affinity enrichment experiments

2.5.1 Labelling assays on cell lysates

Once the warhead, scaffold and recognition tag are assembled in an ABP, activity must be evaluated by testing the labelling in a complex mixture such as a cell lysate. To fulfil this purpose, Human Embryonal Kidney 293T (HEK293T) cell lysate was employed for preliminary labelling assays. HEK293T cells were chosen as they are known to express active p53,¹⁷² and consequently the DUB/s that activate p53 by removing ubiquitin at K291/292 were also postulated to be expressed. An equal amount of HEK293T lysate was titrated against alkyne probes 51 and 52 (Fig. 2.12). Following separation by gel electrophoresis the labelled proteins were imaged with an anti-biotin western blot that selectively visualizes biotinylated adducts.

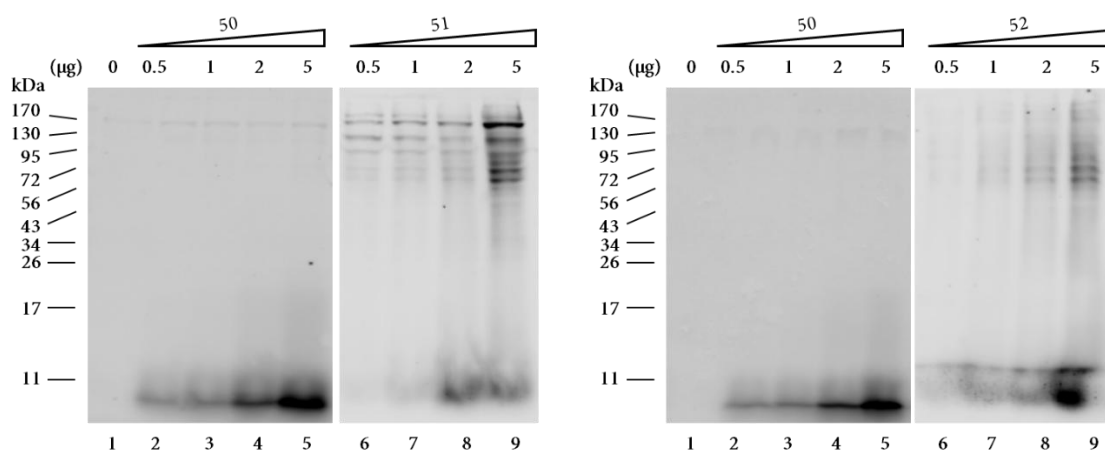


Figure 2.12: Western Blot of HEK293T cell lysate incubated for 1h at 37°C with increasing concentrations of ABP 51 (left, lanes 6-9) and 52 (right, lanes 6-9) bearing a terminal alkyne warhead at position K291 and K292 respectively. Native sequence 50 (left and right, lanes 1-5) is used as a negative control.

Interestingly, an increasing amount of labelling was observed when HEK293T cell lysate was incubated with increasing amounts of the alkyne ABPs 51 and 52 (Fig. 2.12, lanes 6-9) whereas no proteins were covalently labelled by the native biotinylated sequence 50 that does not bear any alkyne moiety at Lys 291/292. The labelling experiment was also repeated with Human Colorectal Tumour cancer cells (HCT116, Fig. 2.13). The advantage of utilising HCT116 lysate stems from the fact that cancer cells are stressed and exhibit elevated levels of active p53, which is activated by various enzymes, including DUBs. In contrast, HEK293T cells are immortalized, healthy cells. Moreover, HEK293T cells express the simian virus 40 (SV40) large T antigen, which is known to bind and inactivate p53, thereby reducing its availability to DUBs.¹⁷³

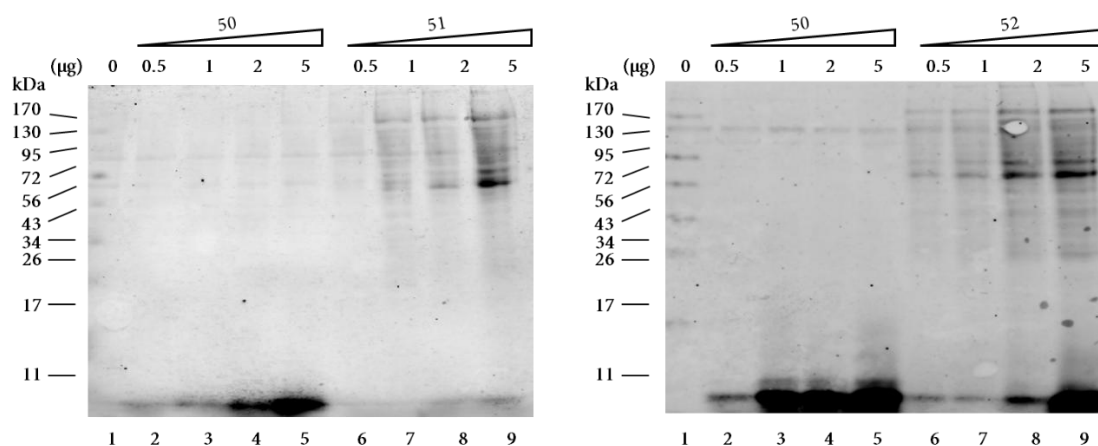


Figure 2.13: Western Blot of HCT116 cell lysate incubated for 1h at 37°C with increasing concentrations of ABP 51 (left, lanes 6-9) and 52 (right, lanes 6-9) bearing a terminal alkyne warhead at position K291 and K292 respectively. Native sequence 50 (left and right, lanes 1-5) is used as a negative control.

As shown with HEK293T cell lysate, a progressively increased labelling was observed when HCT116 cell lysate was incubated with increasing concentrations of the alkyne ABPs 51 and 52 (Fig. 2.13, lanes 6-9). In contrast, no covalent labelling of proteins occurred with the native biotinylated sequence 50. Interestingly, the labelling pattern was different across the two cell lines (Fig. 2.12, lanes 6-9, Fig. 2.13, lanes 6-9). The results from the labelling assay demonstrated that the alkyne probes 51 and 52 functioned as effective ABPs, successfully labelling target proteins in complex environments.

2.5.2 Affinity enrichments

Following labelling assays, pulldown experiments were optimised to separate the labelled targets from the rest of the cell lysate. As a general strategy, labelling was first carried out by incubating an ABP with a cell lysate, followed by treatment with streptavidin-coated agarose beads to capture any biotinylated compound (Fig. 2.14). Elution from the beads after washing steps affords a purified sample enriched in biotinylated material that can be used for downstream analysis such as gel electrophoresis and proteomic analysis.

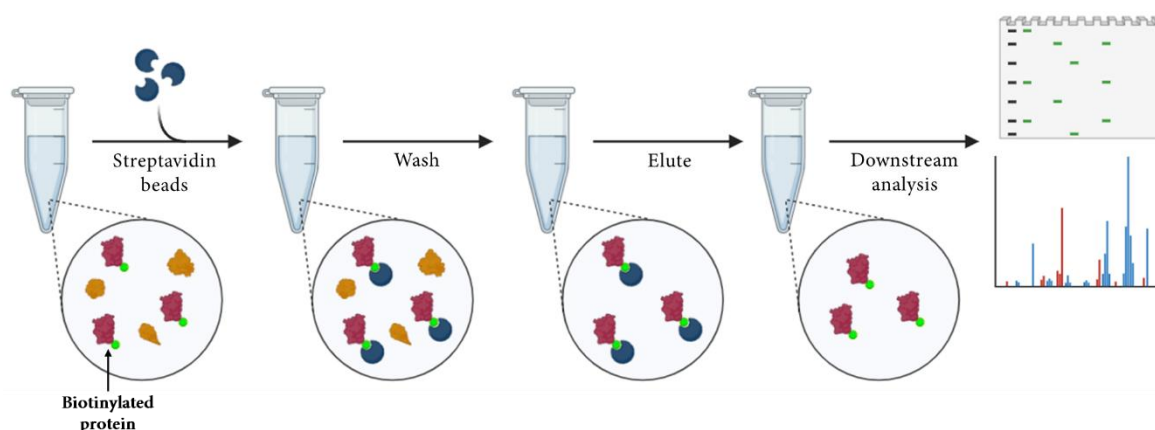


Figure 2.14: general scheme of a pulldown protocol.

Labelling was carried out by incubating probe 51 with HEK293T lysate. An equivalent fraction (20%) of the sample was taken at multiple stages throughout the whole protocol to allow for the process to be followed and failed steps to be identified. These samples were analysed by anti-biotin western blot to selectively image biotinylated substrates, or silver staining to visualize all the proteins in the sample (Fig. 2.15).

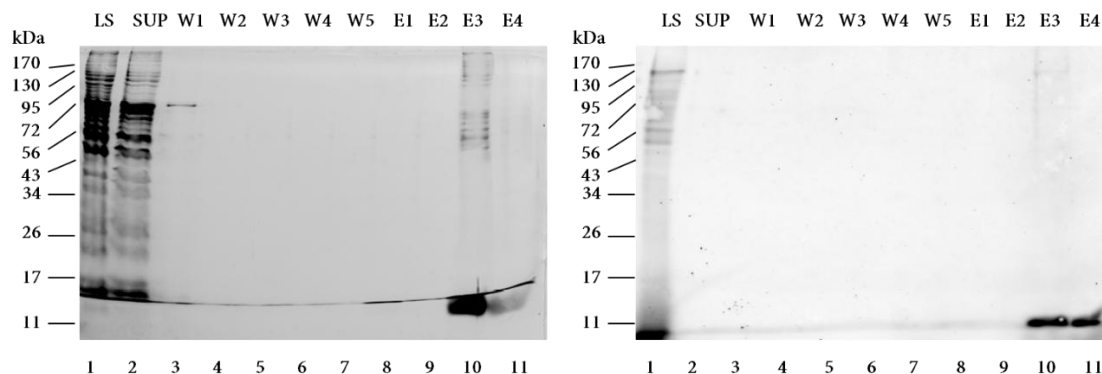


Figure 2.15: Labelling was carried out by incubating HEK293T cell lysate (50 μ g) and probe 51 (10 μ g) for 1.5h at 37°C. Enrichment of biotinylated proteins was performed with streptavidin agarose beads (20 μ L settled beads) and visualised by silver staining (left) and anti-biotin western blot (right). Equivalent fractions (10%) of labelling solution (LS), supernatant (SUP), washes (W1-5) and eluate samples (E1-4) were taken.

As expected, biotinylated material was captured by the beads after incubation (Fig. 2.15, lane 2). It was observed that incubation with reducing buffer for 5 minutes was not sufficient to elute from the beads (Fig. 2.15, lanes 8-9) and that simultaneous heating at 95 °C is necessary to destroy the stable biotin-streptavidin interaction (Fig. 2.15, lanes 10-11). In the following pulldown experiments the amount of cell lysate employed in the labelling was increased from 50 µg to 150 µg to allow for a better identification of the biotinylated material with western blot. Moreover, wash steps were reduced from 5 to 4. These improved conditions were employed for all the following pulldown experiments. Biotin enrichment was performed again on HEK293T cell lysate, and biotinylated material was efficiently eluted from streptavidin beads upon incubation and heating with reducing buffer (Fig. 2.16).

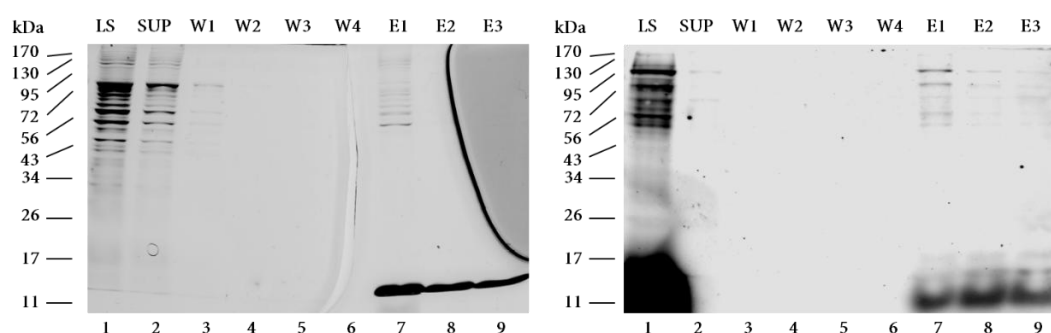


Figure 2.16: Labelling was carried out by incubating HEK293T cell lysate (50 µg) and probe 51 (10 µg) for 1.5h at 37°C. Enrichment of biotinylated proteins was performed with streptavidin agarose beads (50 µL settled beads) and visualised by silver staining (left) and anti-biotin western blot (right). Equivalent fractions (20%) of labelling solution (LS), supernatant (SUP), washes (W1-4) and eluate samples (E1-3) were taken.

The major limitation of using streptavidin beads lies in the harsh denaturing conditions that must be employed to elute biotinylated proteins from streptavidin-conjugated beads as biotin and streptavidin exhibit an exceptionally strong affinity for each other.¹⁷⁴ All the previously shown pulldown experiments showed that boiling the beads in reducing buffer denatures the streptavidin moiety on the agarose beads, leading to contamination of the enriched biotinylated material. Streptavidin leakage from the beads, which can be identified as a band around 17 kDa, can interfere with downstream applications, including proteomic analysis. In

an attempt to reduce streptavidin contamination, a lower amount of beads was employed in a test pulldown carried out on HCT116 cell lysate incubated with probe 51 (Fig. 2.17).

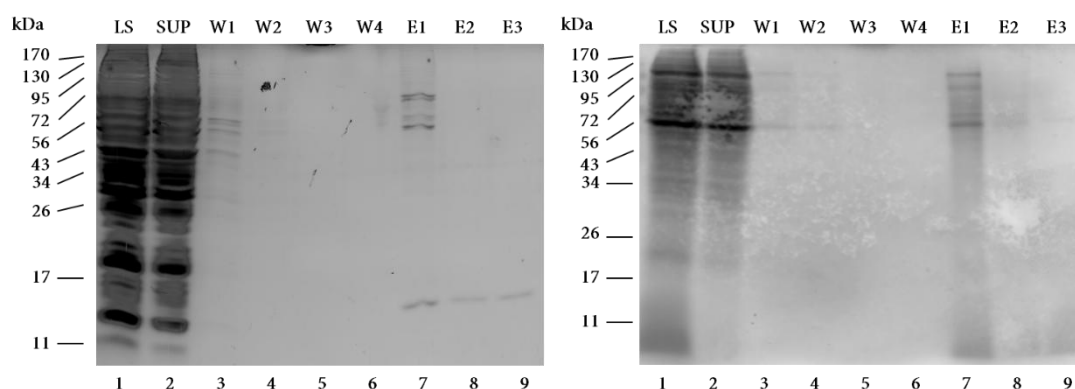


Figure 2.17: Labelling was carried out incubating HCT116 cell lysate (380 μ g) and probe 51 (20 μ g) for 1.5h at 37°C. Enrichment of biotinylated proteins was performed with streptavidin agarose beads (10 μ L settled beads) and visualised by silver staining (left) and anti-biotin western blot (right). Equivalent fractions (20%) of labelling solution (LS), supernatant (SUP), washes (W1-4) and eluate samples (E1-3) were taken.

Although streptavidin contamination was reduced (Fig. 2.17, lanes 7-9), western blot showed incomplete capture of biotinylated material (Fig. 2.17, lane 2), suggesting an excess of beads should be used to avoid oversaturation and improve enrichment. Therefore, alternative elution methods were evaluated to reduce streptavidin contamination during the elution steps.

An elution buffer containing 8M guanidine HCl was explored as an alternative to reducing buffer but unfortunately compromised the electrophoresis protocol and failed to elute biotinylated proteins from the beads. (Fig. 2.18, lanes 7-8). Biotinylated material could be eluted only when reducing buffer was employed (Fig. 2.18, lanes 9).

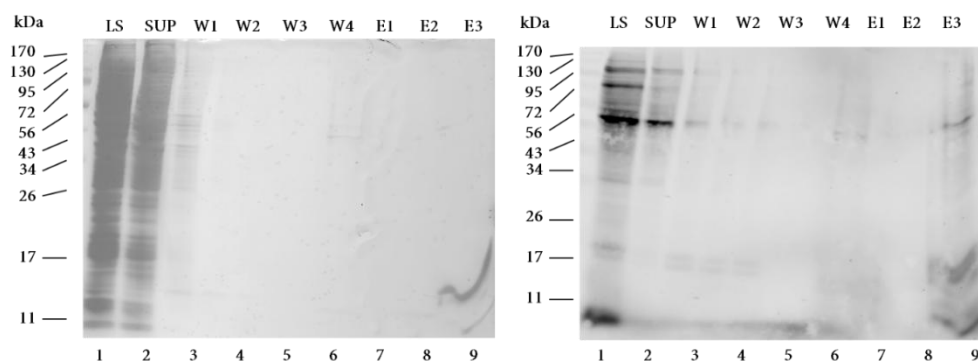


Figure 2.18: Labelling was carried out by incubating HCT116 cell lysate (380 μ g) and probe 51 (16 μ g) for 1.5h at 37°C. Enrichment of biotinylated proteins was performed with streptavidin agarose beads (60 μ L settled beads) and visualised by silver staining (left) and anti-biotin western blot (right). Equivalent fractions (20%) of labelling solution (LS), supernatant (SUP), washes (W1-4) and eluate samples (E1-3) were taken.

Following an extensive literature research, different procedures consisting of the use of excess biotin and heat were tested as alternative eluting buffers for the affinity enrichment protocol (Fig. 2.19 A-C).¹⁷⁵⁻¹⁷⁷

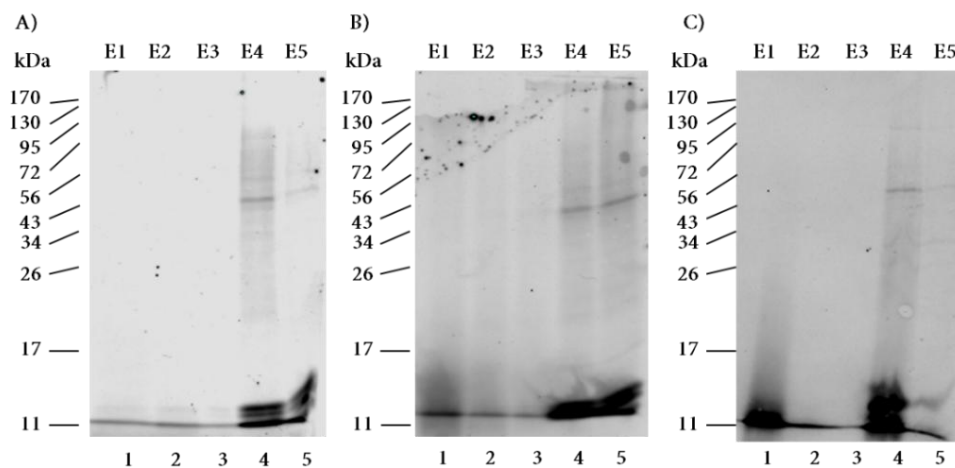


Figure 2.19: Labelling was carried out by incubating HCT116 cell lysate (380 μ g) and probe 51 (16 μ g) for 1.5h at 37°C. Enrichment of biotinylated proteins was performed with streptavidin agarose beads (60 μ L settled beads) and visualised by anti-biotin western blot (A-C). Equivalent fractions (20%) of eluate samples (E1-5) were taken. A) elution with 25 mM biotin at room temperature followed by heating at 95°C for 5 min. B) elution with 25 mM biotin at 95°C followed by heating at 95°C for 5 min. C) elution with 25 mM biotin in homogenate buffer 2 at 95°C followed by heating at 95°C for 5 min.

After labelling HCT116 lysate with probe 51, elution was attempted first with the addition of a 25 mM solution of biotin followed by heating for 5 min at 95 °C (Fig. 2.19 A). In a second attempt the solution was preheated to 95 °C prior to addition to the beads to improve biotin solubility in water (Fig. 2.19 B). A third attempt employed a different buffer with increased detergent composition (Fig. 2.19 C). Unfortunately, none of these conditions (Fig. 2.19 A-C, lanes 1-3) improved elution of biotinylated material when compared to the use of reducing buffer (Fig. 2.19 A-C, lanes 4-5). Consequently, reducing buffer was identified as the most effective elution buffer for biotinylated compounds bound to streptavidin-agarose beads.

Other beads were tested to evaluate their streptavidin leaking when exposed to reducing buffer. Pulldown experiments were carried out using four different types of Dynabeads™ Streptavidin beads (Invitrogen, Fig. 2.20 A-D).

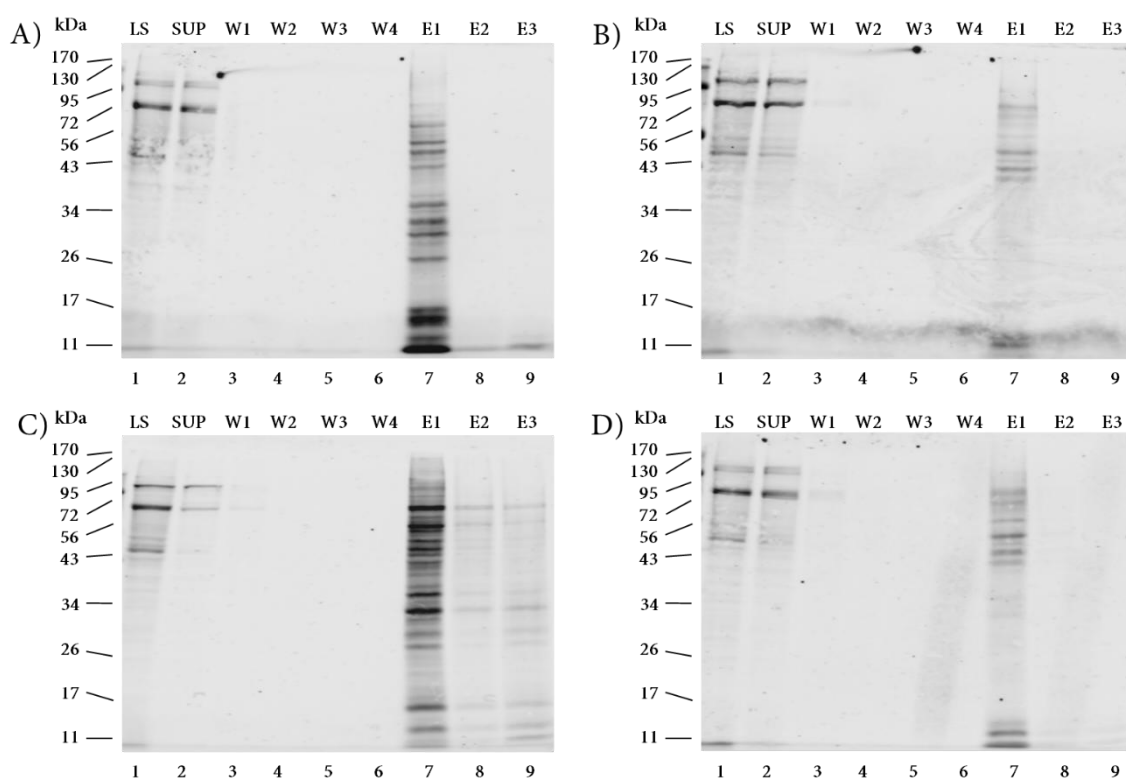


Figure 2.20: Labelling was carried out by incubating HCT116 cell lysate (200 µg) and probe 51 (8 µg) for 1.5h at 37°C. Enrichment of biotinylated proteins was performed with Dynabeads (50 µL settled beads) and visualised by anti-biotin western blot (A-D). Equivalent fractions (20%) of labelling solution (LS), supernatant (SUP), washes (W1-4) and eluate samples (E1-3) were taken. Elution was performed with reducing buffer and: A) Dynabeads M-270. B) Dynabeads M-280. C) Dynabeads MyOne C1. D) Dynabeads MyOne T1.

Dynabeads M-270 and M-280 are 2.8 μm monodispersed polystyrene beads covalently coupled to streptavidin, with M-270 being hydrophilic (Fig. 2.20 A) and M-280 being hydrophobic beads (Fig. 2.20 B). Dynabeads MyOne C1 and T1 are 1 μm monodispersed polystyrene beads covalently coupled to streptavidin, with C1 being hydrophilic (Fig. 2.20 C) and T1 being hydrophobic beads (Fig. 2.20 D). The small and uniform size of these beads provides a large surface area that results in high binding capacity, making them suitable for enrichment of biotinylated targets. Unfortunately, none of the tested beads were able to fully capture all the biotinylated material following labelling with probe 51 (Fig. 2.20 A-D, lane 2). Additionally, appearance of new bands in the eluted fractions suggests undesired non-specific enrichment (Fig. 2.20 A-D, lanes 7-9). Despite this, the eluted material still exhibits streptavidin contamination. As a conclusion, streptavidin agarose beads were used for all the following affinity enrichment experiments.

Following the optimisation and attempts to reduce streptavidin leaking from the beads, a reproducible biotin enrichment protocol was developed (Fig. 2.21 A-C).

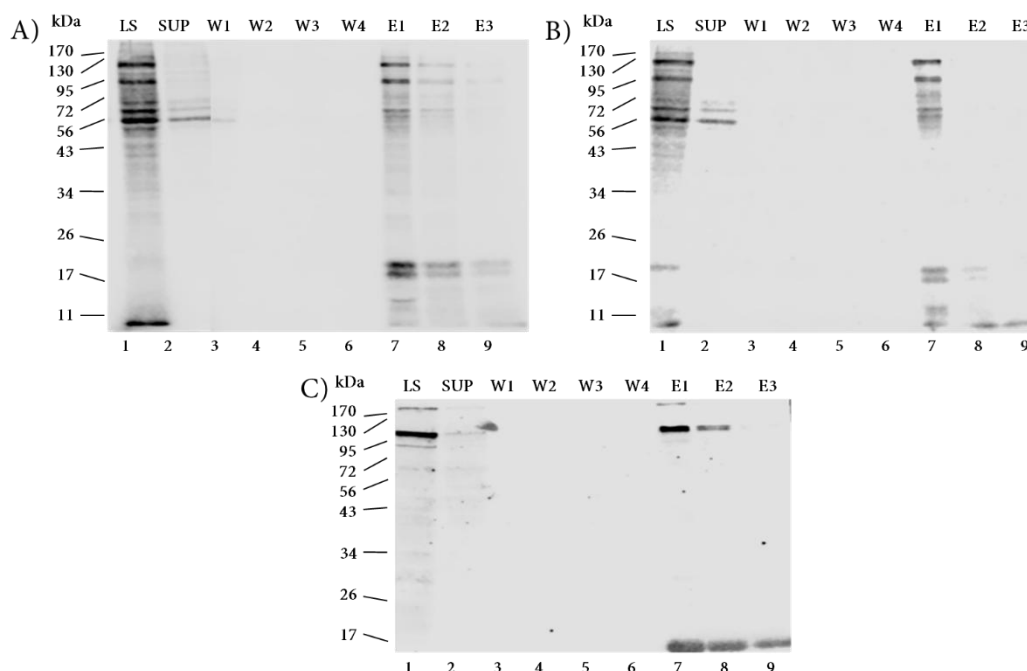


Figure 2.21: Labelling was carried out by incubating HCT116 cell lysate (380 μg) and probe 51-52 (16 μg) for 1.5h at 37°C. Enrichment of biotinylated proteins was performed with streptavidin-agarose beads (60 μL settled beads) and visualised by anti-biotin western blot (A-C). Equivalent fractions (20%) of labelling solution (LS), supernatant (SUP), washes (W1-4) and eluate samples (E1-3) were taken. Enrichment with: A) probe 51. B) probe 52. C) no probe.

Such protocol entailed the use of streptavidin agarose beads to enrich the sample in biotinylated material and washes with reducing buffer to efficiently elute the adducts from the beads after performing washing steps.

The labelling was carried out on HCT116 cells treated with alkyne probes 51 and 52 (probe/lysate ratio of 1:25, **Fig. 2.21 A-B**), and a mock pulldown was performed to assess endogenous biotinylated material (**Fig. 2.21 C**). Most of the biotinylated material present in the labelling solution (**Fig. 2.21 A-B**, lane 1) was effectively captured by the beads (**Fig. 2.21 A-B**, lane 2) and fully recovered during the elution process (**Fig. 2.21 A-B**, lanes 7-9). The eluted material from the resin was subsequently analysed using proteomics.

2.5.3 Proteomic analysis

Having identified that ABPs 51 and 52 were capable of labelling discrete bands in HCT116 lysate (**Fig. 2.13**) and having developed a working pulldown to enrich in biotinylated material (**Fig. 2.21 A-B**), proteomic analysis was carried out to identify labelled proteins. A Filter Assisted Sample Preparation (FASP) protocol was employed to perform proteomic analysis on material eluted from streptavidin beads.¹⁷⁸ Following buffer exchange, samples were subjected to trypsin digestion and submitted for analysis by LC-MS/MS. Identification, assignment and analysis were performed using MaxQuant and Perseus. The samples were analysed by label-free quantification (LFQ), a technique that scales the abundance of each detected protein in relation to a mock pulldown. The results are represented as relative intensities and visualized with volcano plots (**Fig. 2.22 A-B**). A hit located in the upper right side indicates statistically significant enrichment in the probe samples when compared to the control.

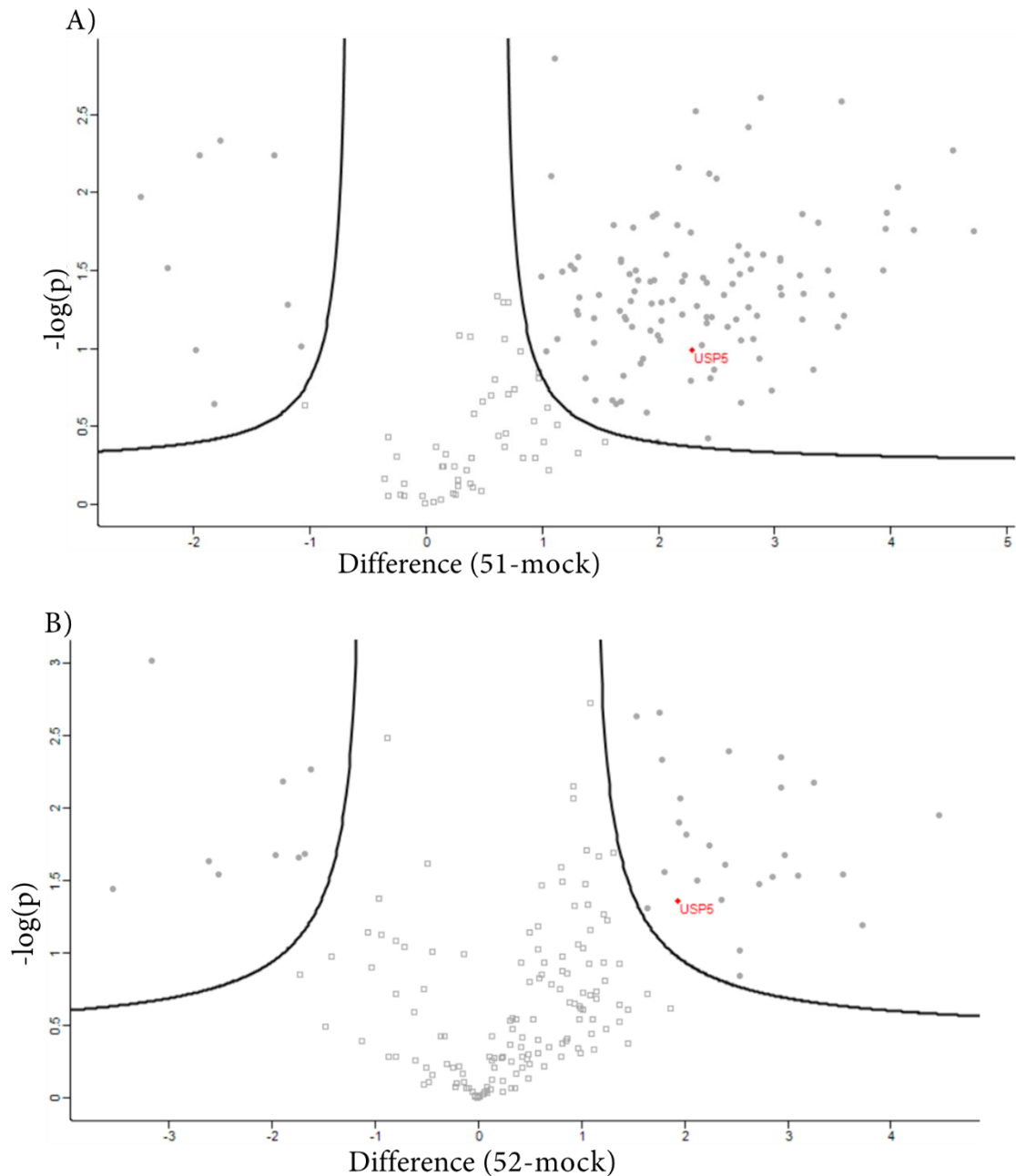


Figure 2.22: Volcano plots of HCT116 lysate (380 μ g) enriched with **A)** probe 51 (16 μ g) and **B)** probe 52 (16 μ g). The enrichment is measured in relation to a mock enrichment with no probe. 122 proteins were labelled by probe 51 labelled whereas probe 52 labelled 35 proteins.

In a preliminary proteomic analysis probe 51 enriched 113 proteins whereas probe 52 showed increased selectivity enriching 35 proteins in HCT116 lysate. Gratifyingly, the DUB Ubiquitin Specific Peptidase 5 (USP5) was found to be enriched by both probes 51 and 52. USP5 is a known DUB involved in the activation of p53, though the specific interaction sites are not

known.¹⁷⁹ Recognition of USP5 by probes 51-52 could suggest that its role would be deubiquitinating p53 at K291/292 thereby contributing to its stabilisation. However, streptavidin counts in the experiment were notably elevated (<1100 combined unique spectral counts), further confirming its presence as a contaminant in the analysis. Subsequent experiments seeking to validate USP5 and other DUBs as hits were not successful in reproducing the previously acquired results.

2.6 Conclusion

In this study, two ABPs were designed, synthesised and tested to target the DUB(s) interacting with ubiquitinated p53 at its K291/292 sites. The Fmoc protected UAA 19-20 and 22-23 were successfully synthesised and the terminal alkyne S-derivative UAA 23 was successfully installed into a peptidic recognition scaffold. A strategy was devised for on-resin biotinylation allowing for efficient installation of the biotin tag. HPLC purification afforded the peptidic probes 51-52 that were found to be working as ABPs in preliminary labelling assays. Discrete bands were observed when probes 51-52 were incubated with HEK293T and HCT116 cell lysates, suggesting these probes possess the ability to label DUB(s) in complex environments. After this qualitative assessment of ABPs 51-52, an affinity enrichment protocol using streptavidin-coated agarose beads was devised and optimised to quantify and identify the labelled proteins. Interestingly, the deubiquitinating enzyme USP5 was enriched by both probes 51-52, suggesting that USP5 could play a significant role in carrying out p53 deubiquitination at its K291/292 sites. Unfortunately, this result could not be further validated due to streptavidin contamination hampering the reproducibility of the proteomic analysis. Further work in this study should be aimed in reducing streptavidin contamination by carrying out alternative protocols for protein digestion that do not subject the streptavidin beads to harsh conditions such as boiling in reducing buffer.

Chapter 3

Peptidic Activity Based Probes to target Otubain1

3.1 Introduction

Otubain 1 (OTUB1) is a ~35 kDa deubiquitinating enzyme of the OTU subfamily (see section 1.2.1, p. 9). The catalytic activity of OTUB1 is extremely linkage specific, being uniquely directed towards the hydrolysis of K48 linked polyubiquitin chains. OTUB1 features two binding sites for Ub, a proximal and a distal site (Fig. 3.1 A). Upon binding in the distal site, ubiquitin acts as an allosteric modulator of OTUB1 and enhances its affinity for the donor ubiquitin which is accommodated in the proximal site. The di-ubiquitin scaffold adopts a conformation that positions the Lys48 residue of the donor ubiquitin in close proximity to the active site cysteine of OTUB1 enhancing its catalytic activity (Fig. 3.1 B). Moreover, computational and crystallographic studies showed that ubiquitin residues 54-60 interact with OTUB1 either via hydrogen bonding or van der Waals interactions (Fig. 3.1 C).⁴²

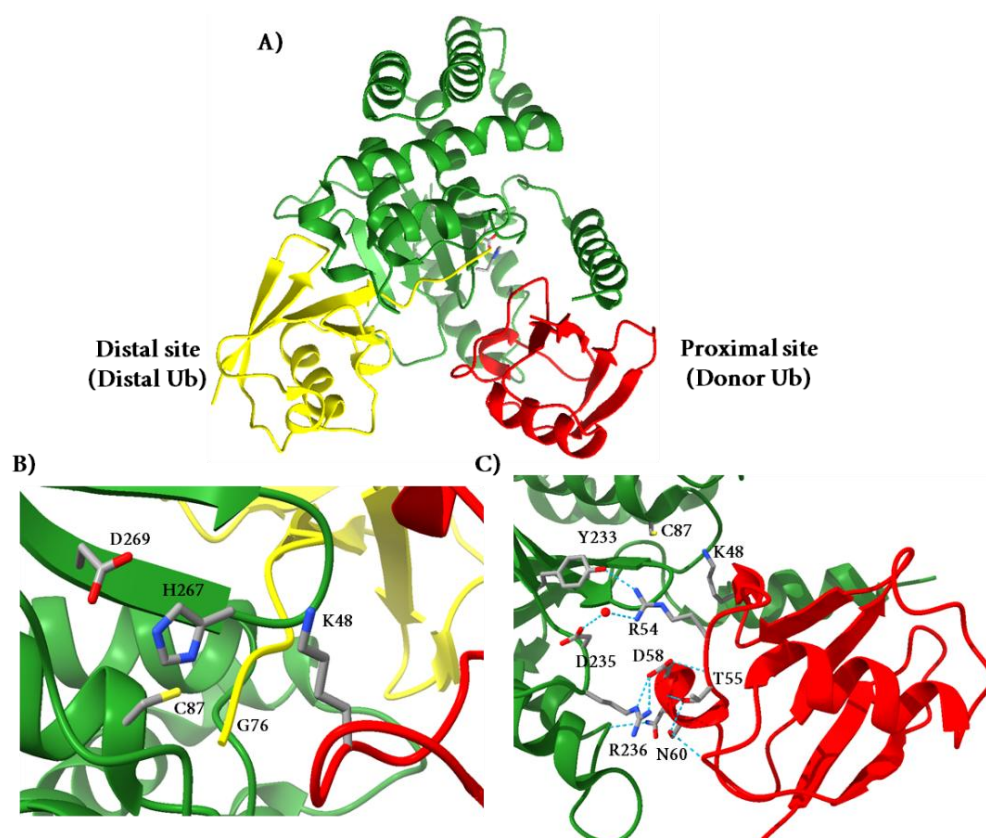
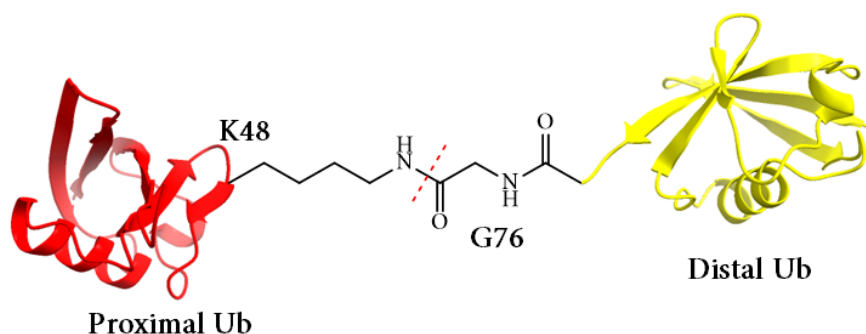


Figure 3.1: A) Crystal structure of a human/worm OTUB1 (green) bound to two ubiquitin moieties (yellow in the distal site and red in the proximal site respectively). B) Detailed view of the active site, showing catalytic triad (Asp, His, Cys), distal Ub C-terminus (Gly76) and proximal Ub Lys 48. C) Close up view of the interactions between 60 proximal Ub residues 54- and OTUB1. PDB structure: 4DHZ.

A similar approach to that outlined in Chapter 2 was employed for the design, synthesis, and testing of a set of ABPs to target OTUB1. To target the catalytic cysteine residue in the active site, a propargyl and a VME were employed as electrophilic moieties to replace the native isopeptide linkage (Fig. 3.2 A). The warheads were incorporated in the side chain of Fmoc-protected UAA 23 and 53 that were used as building blocks for standard SPPS to build the ABPs recognition scaffold (Fig. 3.2 B). A biotin tag was employed for the visualisation and quantification of the labelled OTUB1.

A) Native Scaffold



B) Proposed warheads

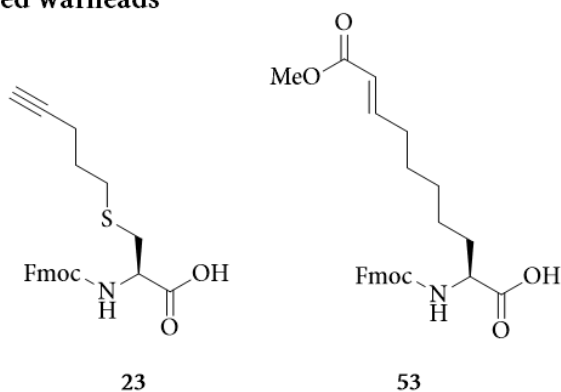


Figure 3.2: A) View of the native di-ubiquitin isopeptide linkage. The amide bond (dotted red line) is targeted by the active site cysteine of OTUB1. B) Structure of the proposed warheads.

3.2 Synthesis of warheads

The synthesis of propargyl UAA 23 was discussed in section 2.2.2. (p. 48).

To synthesise the vinyl methyl ester (VME) Fmoc-protected modified amino acid 53 a retrosynthetic pathway was initially devised (Fig. 3.3).

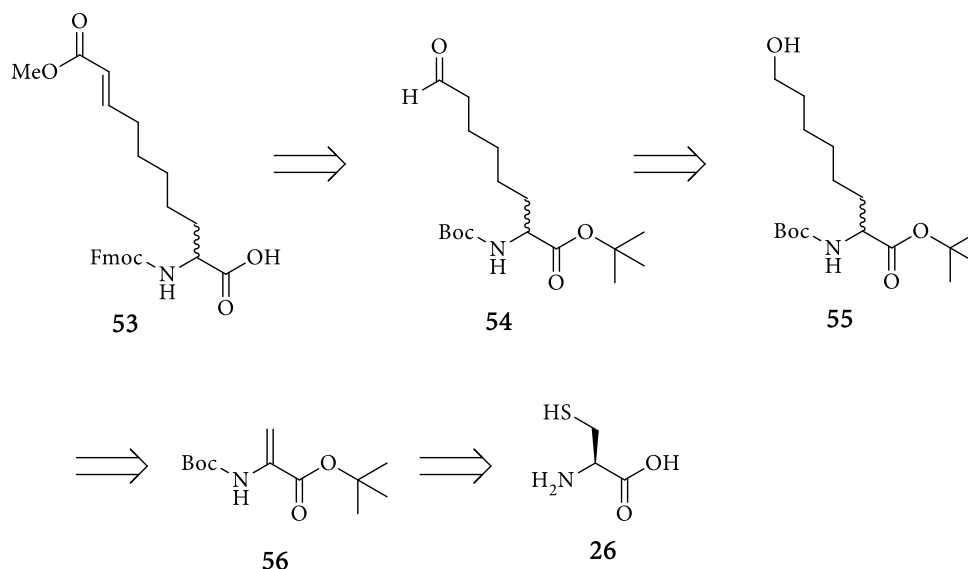


Figure 3.3: Retrosynthetic scheme for the synthesis of Fmoc protected UAA 53.

The target compound 53 could be accessed carrying out a Wittig reaction on an aldehyde derivative 54, which was obtained from the corresponding primary alcohol 55. The 6 carbon units alkyl chain could be attached availing of a well-known copper mediated radical addition on a dehydroalanine intermediate 56 that was easily derived from commercially available cysteine 26.

The synthesis of compound 55 was attempted with the following route (Fig. 3.4).

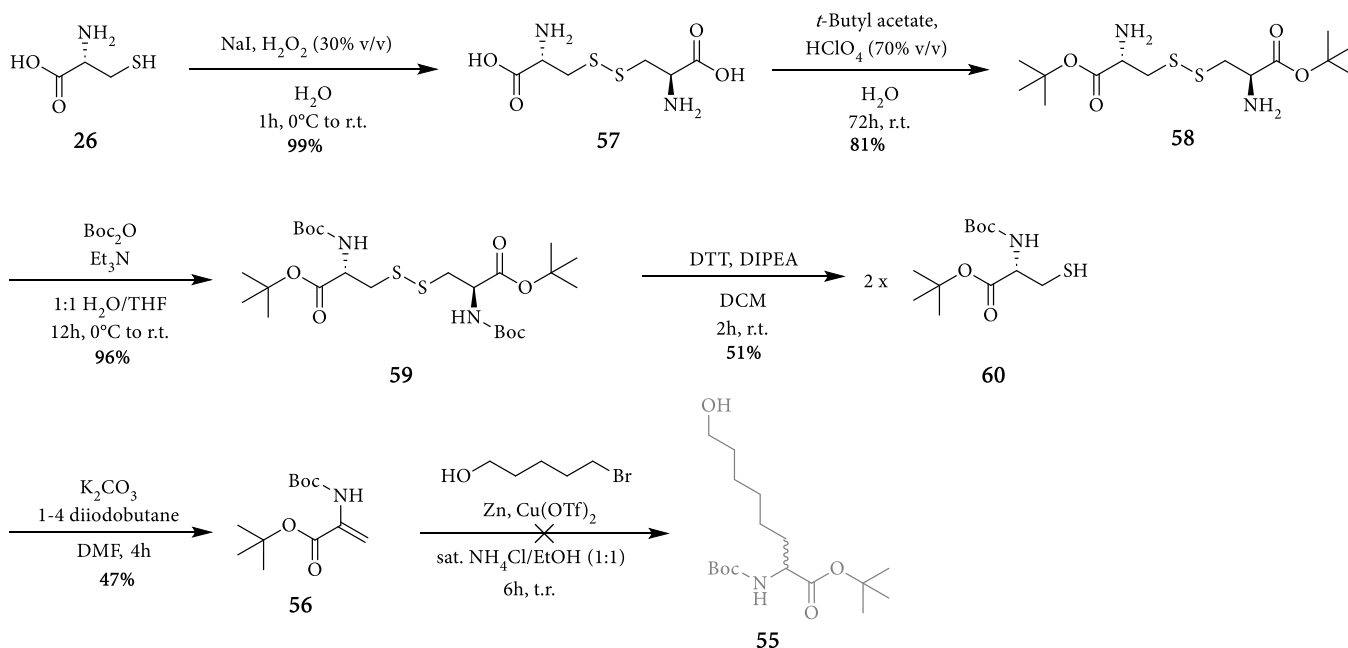


Figure 3.4: Reaction scheme for the synthesis of Fmoc protected UAA 53.

The synthetic procedure featured an initial dimerization of cysteine 26 to cystine 57, that was obtained in quantitative yield.¹⁸⁰ *Tert*-butylation of the carboxylic groups was carried out using perchloric acid and *tert*-butyl acetate and yielded 58 with an excellent yield of 81%.¹⁸¹ This step was followed by N-Boc protection using di-*tert*-butyl dicarbonate which afforded the fully protected cystine 59 in 96% yield.¹⁸² Reduction of 59 with DTT yielded Boc-Cysteine-O^tBu 60 in 51% yield. Following a literature procedure, a bis-alkylation elimination was carried out on compound 60 when reacted with 1,4-diiodobutane and K₂CO₃ to obtain the dehydroalanine derivative 56.¹⁶⁶ Unfortunately, all the attempts to synthesise the alkylated compound 55 from Boc-Dha-O^tBu 56 failed and this strategy was abandoned.

To synthetically access intermediate 55, the retrosynthetic pathway discussed in Fig. 3.3 was modified to access compound 53 with an alternative strategy (Fig. 3.5).

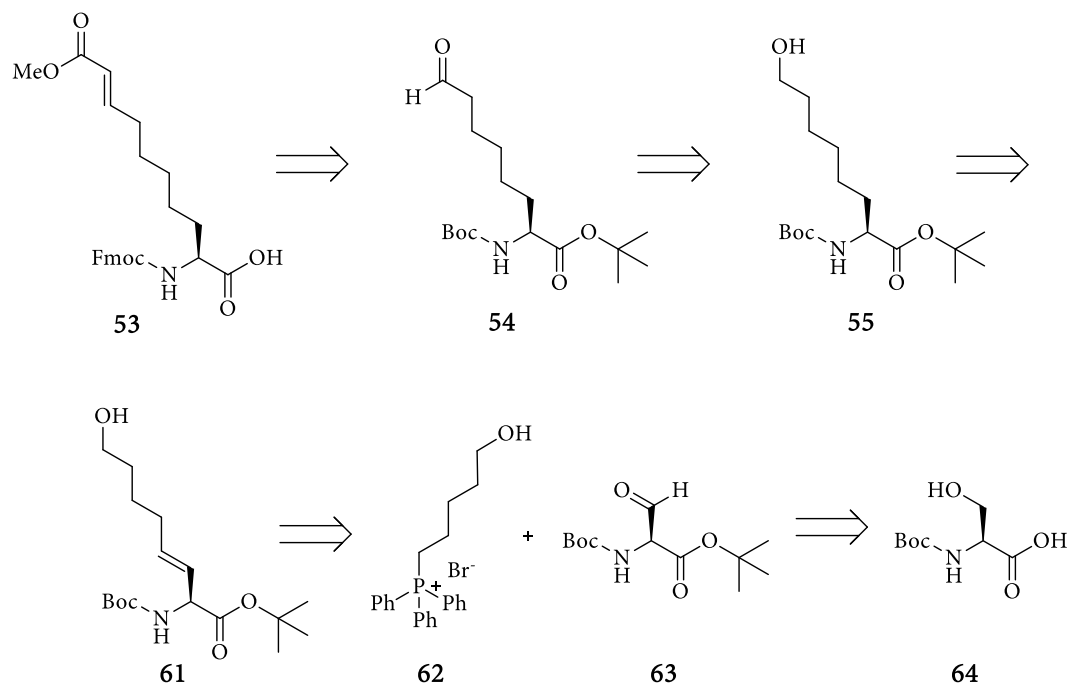
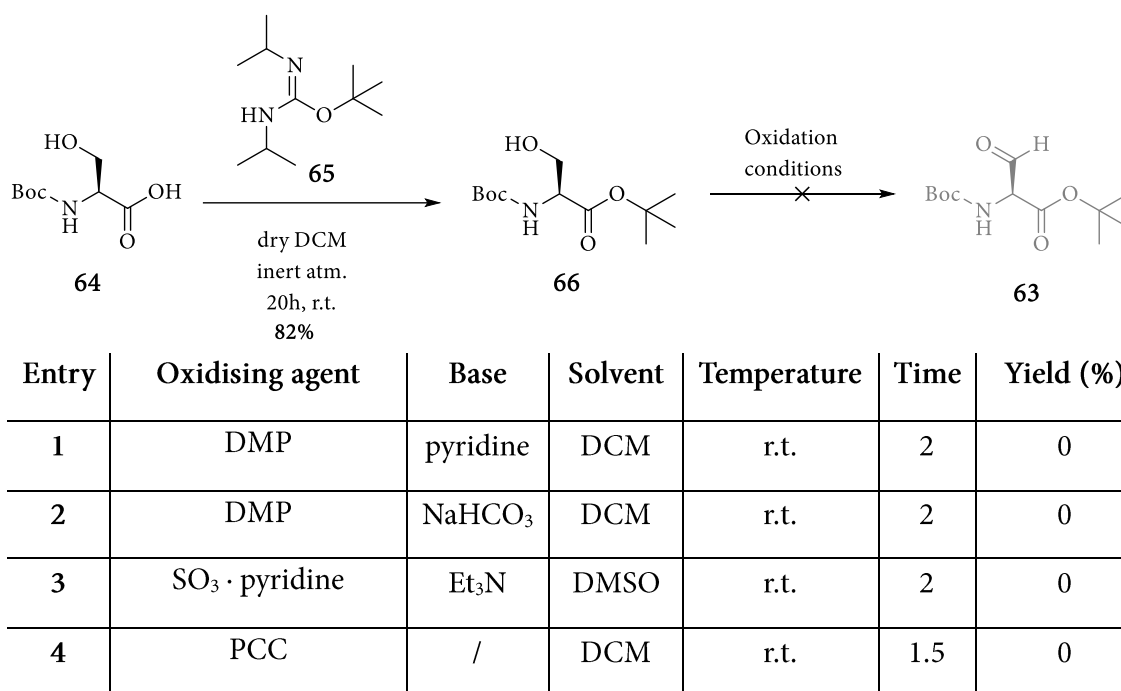


Figure 3.5: Retrosynthesis scheme for the synthesis of Fmoc protected UAA XX.

The target compound 53 could be obtained carrying out a protecting group exchange and a Wittig reaction to install the VME portion of the warhead on the aldehyde derivative 54. Compound 54 could be easily derived from the alcohol 55 via oxidation, which could be obtained from the alkene derivative 61 via reduction of the double bond. At this point, a Schlosser modification of the Wittig reaction could replace the copper mediated alkylation step, and compound 61 could be disconnected in the appropriate triphenyl phosphonium bromide 62 and aldehyde derivative 63. Aldehyde 63 could be derived from commercially available Boc-serine 64. This new retrosynthesis maintained the correct stereochemistry at the α position of the target compound, making it a closer mimic of the native lysine residue taking part in the isopeptide bond.

The synthesis of aldehyde **63** was attempted in different conditions (**Scheme 3.1**).



Scheme 3.1: Synthetic scheme for aldehyde derivative **63**.

Commercially available Boc-Ser-OH **64** was protected with a *tert*-butyl group to obtain Boc-Ser-O^tBu **66** with excellent yields using *tert*-butyl *N,N'*-diisopropylcarbamimidate **65**. Different oxidation conditions were tried to access aldehyde derivative **63**. Firstly, Dess-Martin periodinane (DMP) was employed as a mild oxidising agent, and two different bases were tested (**Scheme 3.1**, entry 1-2). Secondly, a Parikh-Doering oxidation was attempted using DMSO activated by sulphur trioxide pyridine complex in the presence of triethylamine (**Scheme 3.1**, entry 3). Lastly, pyridinium chlorochromate complex (PCC) was used as a more reactive oxidant (**Scheme 3.1**, entry 4). Unfortunately, all the aforementioned attempts to oxidise the primary alcohol of **66** to its aldehyde derivative **63** failed to yield the desired product, therefore this synthetic pathway was abandoned.

In an attempt to synthetically access intermediate 55 without having to recur to a serine derivative, the retrosynthetic pathway discussed in Fig. 3.5 was modified to access intermediate 55 with an alternative strategy (Fig. 3.6).

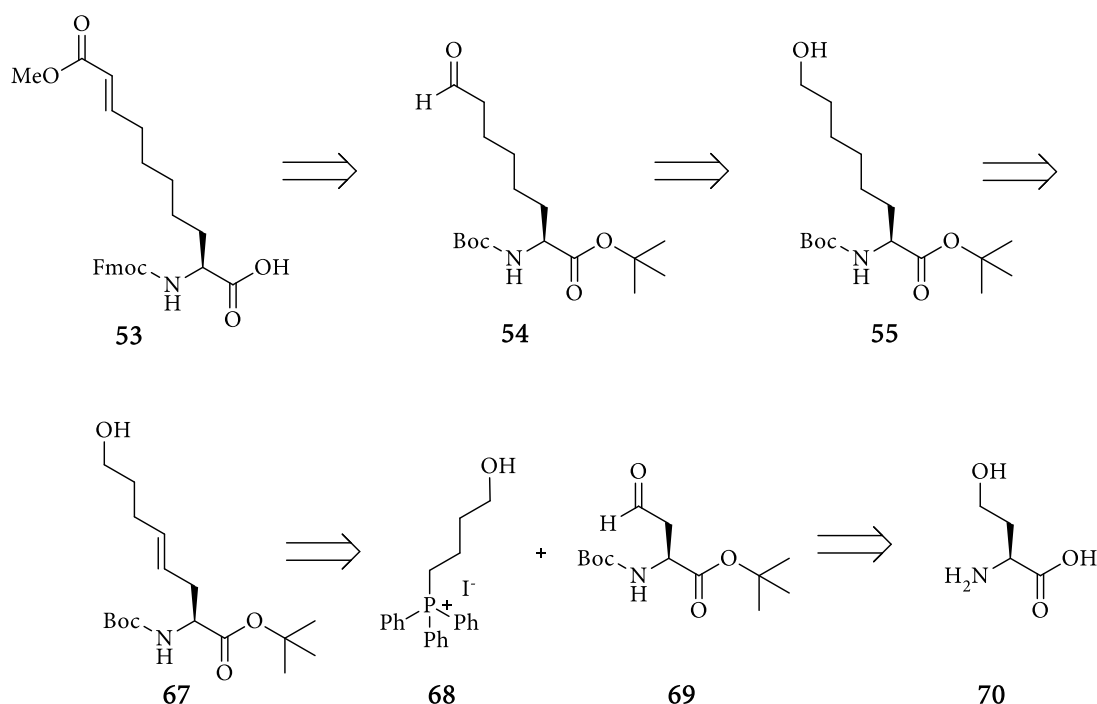


Figure 3.6: Retrosynthesis scheme for the synthesis of Fmoc protected UAA 63.

This retrosynthetic pathway featured a similar strategy to the one adopted in Figure 3.5, availing of two subsequent Wittig-like reactions to elongate the carbon chain and install the VME warhead at the desired position while maintaining the correct stereochemistry at the α position. To facilitate the synthesis of the aldehyde precursor 55 needed for the first Wittig reaction, the serine precursor 63 was replaced by its homoserine derivative 69 which is one carbon unit longer. Consequently, the phosphonium salt 68 used to generate the ylide was one carbon unit shorter than compound 62 used in the previous synthesis.

The first five steps in the synthesis of UAA 53 were carried out to access the aldehyde intermediate 69 needed for the first Wittig transformation (Fig. 3.7).

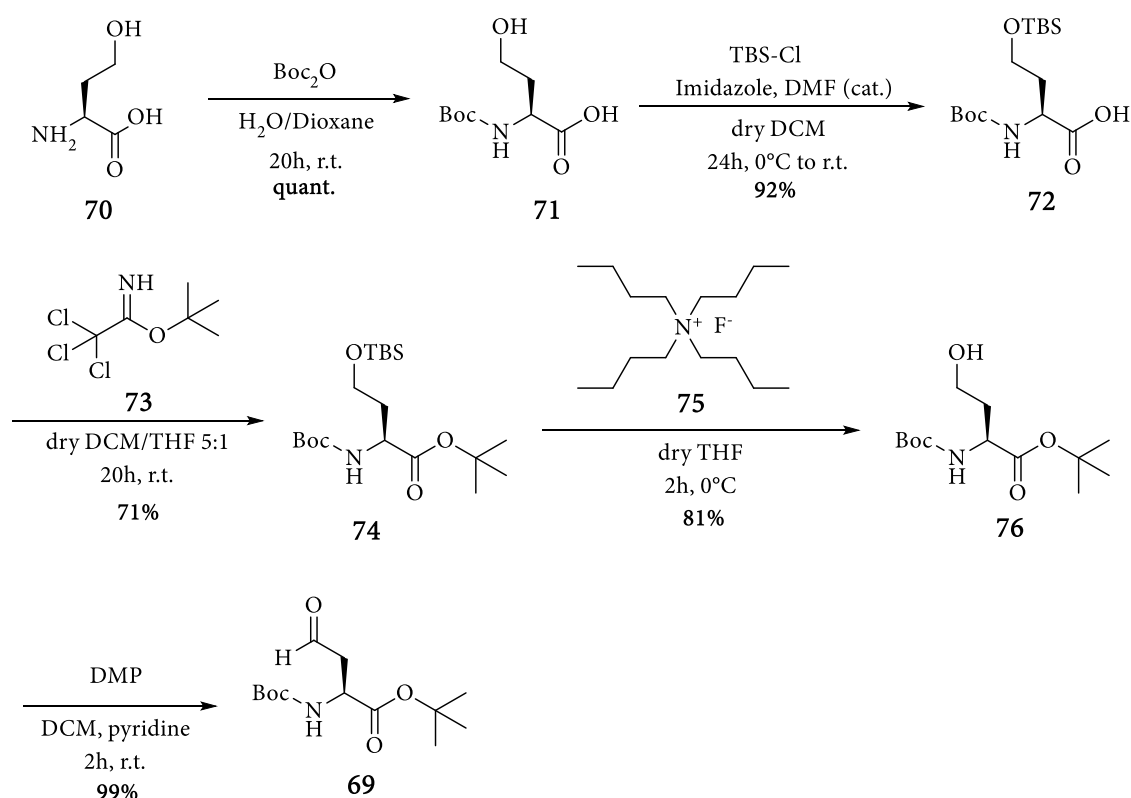
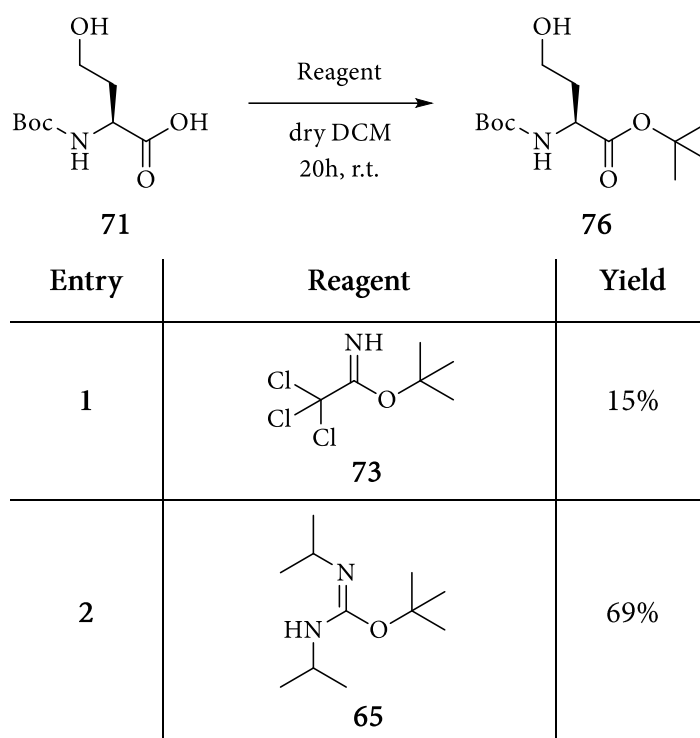


Figure 3.7: Synthetic scheme for aldehyde derivative **69**.

Homoserine **70** was Boc protected with di-*tert*-butyl dicarbonate which furnished **71** in quantitative yield, and the hydroxy group was subsequently protected using TBSCl in an excellent 92% yield. Protection of the carboxylic acid was carried out using *tert*-butyl 2,2,2-trichloroacetimidate **73** which converted compound **72** to its respective *tert*-butyl ester **74** in 71% yield. The TBS group in compound **74** was then efficiently removed in presence of tetra-*n*-butyl ammonium fluoride **75** (TBAF), which afforded the desired alcohol **76** with 81% yield. In the last step, DMP was employed to oxidise alcohol **76** to its aldehyde derivative **69** in excellent yield.

In an attempt to shorten the synthesis of aldehyde **69**, two different *tert*-butylating reagents were tested with the aim of directly installing the protecting group on the carboxylic acid without the need of protecting the side chain alcohol (**Scheme 3.2**).



Scheme 3.2: Reagent screening for direct *tert*-butylation of **71**.

When *tert*-butyl 2,2,2-trichloroacetimidate **73** was used directly on compound **71**, a mixture of products was observed, and the desired compound **76** was isolated with poor yields due to the presence of the reactive alcohol (**Scheme 3.2**, entry 1). Gratifyingly, when the milder reagent *tert*-butyl *N,N'*-diisopropylcarbamimidate **65** was used to carry out the reaction, the desired monoprotected compound **76** was isolated as the major product with a yield of 69% (**Scheme 3.2**, entry 2). Therefore, TBS protection/deprotection of the alcohol was considered unnecessary and the aldehyde precursor **69** was directly obtained from the corresponding carboxylic acid **76** using *tert*-butyl *N,N'*-diisopropylcarbamimidate **65** as the *tert*-butylating agent.

Having successfully achieved the synthesis of compound **69**, efforts were directed towards the synthesis of the phosphonium salt that would be used to generate the ylide **68** for the first Wittig reaction (**Fig. 3.8**).

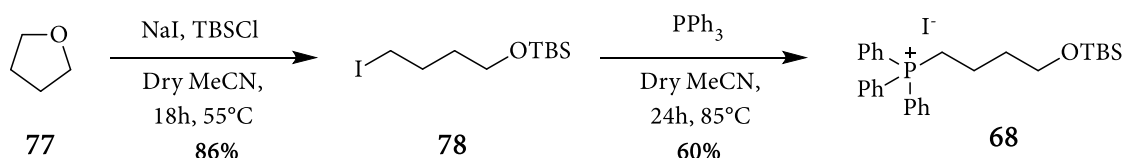
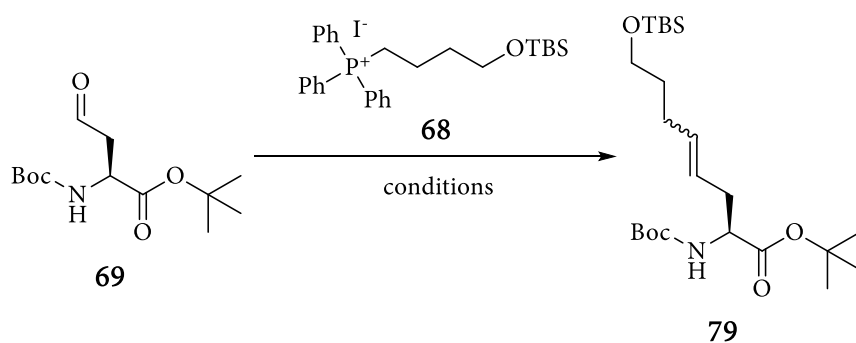


Figure 3.8: Synthetic scheme for triphenyl phosphonium iodide **68**.

A ring opening reaction was performed by iodine on tetrahydrofuran **77**, followed by a simultaneous TBS protection in the presence of TBS-Cl which afforded the desired compound **78** in excellent yields.¹⁸³ The very hygroscopic triphenyl phosphonium iodide **68** was obtained with a modest yield of 60% after refluxing in the presence of triphenyl phosphine.

Once the compounds **68-69** were obtained, conditions for the Schlosser modification of the first Wittig reaction to obtain the desired compound **79** were optimised (Scheme 3.3).



Entry	68 pretreatment	Base	69:68:base	Temperature	Time	Yield
1	No pretreatment	BuLi	1:1.05:1.05	-20 °C to r.t.	2	5%
2	No pretreatment	BuLi	1:1.05:1.05	-30 °C to r.t.	2	13%
3	No pretreatment	BuLi	1:1.05:3	-30 °C to r.t.	2	20%
4	No pretreatment	BuLi	1:1.05:2	-73 °C to r.t.	2	25%
5	No pretreatment	BuLi	1:1.05:3	-73 °C to r.t.	2	30%
6	No pretreatment	NaH	1:1.05:3	-73 °C to r.t.	2	0%
7	Dried	BuLi	1:1.05:2	-73 °C to r.t.	2	60%

Scheme 3.3: Conditions screening for Wittig reaction (Schlosser modification) to obtain compound **79**.

The reaction was first tested by deprotonating the protected phosphonium salt **68** at -20 °C in dry THF for 30 minutes followed by addition of a precooled solution of the aldehyde **69** (**Scheme 3.3**, entry 1). The yield was slightly improved when the ylide was generated at lower temperatures (**Scheme 3.3**, entry 2) and when an excess of BuLi was used (**Scheme 3.3**, entry 3). The best conditions for an efficient ylide formation were found when using 3 equivalents of BuLi at -73 °C (**Scheme 3.3**, entry 5). In an attempt to attenuate decomposition of THF by organolithium compounds, the deprotonation of phosphonium salt **68** was carried out with sodium hydride, but the reaction did not yield any product (**Scheme 3.3**, entry 6). The yield of the Wittig reaction was remarkably improved to 60% when triphenyl phosphonium iodide **68** was thoroughly dried before carrying out the reaction. The amount of BuLi was also reduced to 2 equivalents as it was understood that the higher excess needed in previous entries was quenched by the residual water contaminating compound **68** (**Scheme 3.3**, entry 7).

Once the first Wittig reaction was carried out and the conditions to synthetically access **79** were optimised, the rest of the synthetic steps were performed and VME modified Fmoc-protected UAA **53** was obtained in 6 steps (**Fig. 3.9**).

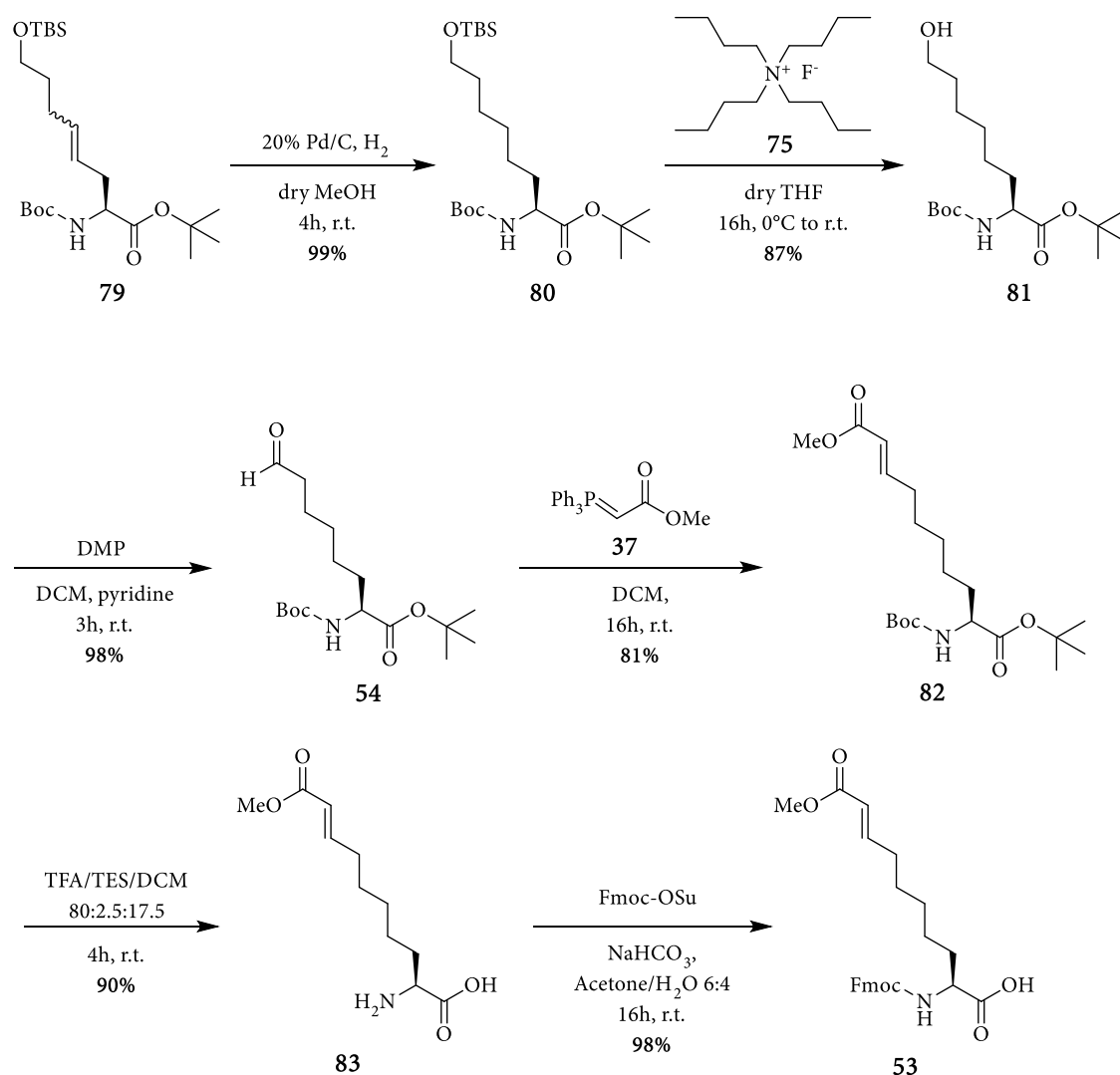


Figure 3.9: Synthetic scheme for UAA 53.

Hydrogenation of **79** was carried out using palladium on activated carbon and the double bond was reduced in quantitative yield. In the following step, the TBS protecting group was efficiently removed with TBAF under inert atmosphere in 87% yield to obtain compound **81**. The primary alcohol of **81** was readily oxidised to its aldehyde derivative **54** with DMP in almost quantitative yield of 98%. To install the VME portion of the warhead on the amino acid side chain, a Wittig reaction was carried out using methyl triphenyl-phosphoranylidene acetate **37** as the stable ylide and compound **82** was obtained with an excellent yield of 81%. A mixture of TFA/TES/DCM was used to simultaneously remove the Boc protecting group and the *tert*-butyl group without hydrolyzing the α - β unsaturated methyl ester. TES was used to scavenge any cation generated in the protecting group removal. The target compound **83**

was isolated as a TFA salt with 90% yield and used without further purification. In the final step, compound **83** was protected with Fmoc succinimide in basic environment and this afforded the desired target compound **53** which was suitable to be used in SPPS as UAA.

3.3 Design and synthesis of peptidic probes

To generate a set of ABPs to target OTUB1, two peptidic portions of the Ub sequence were chosen as the recognition scaffold. The first ubiquitin sequence covers residues Gly35-Leu50 which are part of a β -sheet structured region (Fig. 3.10 A), whereas the second ubiquitin sequence selected covers residues Ala46-Asp60 which fall in an unstructured region (Fig. 3.10 B). Both the sequences include the Lys48 that is involved in the reaction at the active site of OTUB1. The Ala46-Asp60 sequence was postulated to have enhanced recognition capabilities compared to the Gly35-Leu50 sequence due to residues 56-60 directly interacting with OTUB1 upon binding of ubiquitin.⁴² Biotin was again chosen as the tag, allowing visualisation of the labelled OTUB1 via anti-biotin western blots and quantification via proteomic analysis.

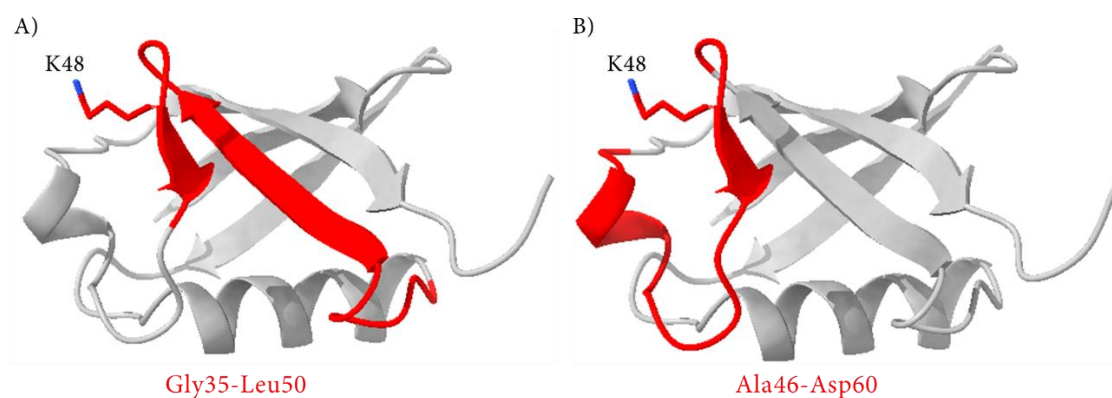


Figure 3.10: Recognition sequences (red) selected as ABPs scaffold in the ubiquitin sequence (grey). **A)** Gly35-Leu50 sequence. **B)** Ala46-Asp60 sequence.

Using standard Fmoc-based SPPS and the biotinylation conditions optimised in section 2.4 (p. 52), the native 15-mer and 16-mer biotinylated peptides **84** and **85** were efficiently synthesised (Fig. 3.11).

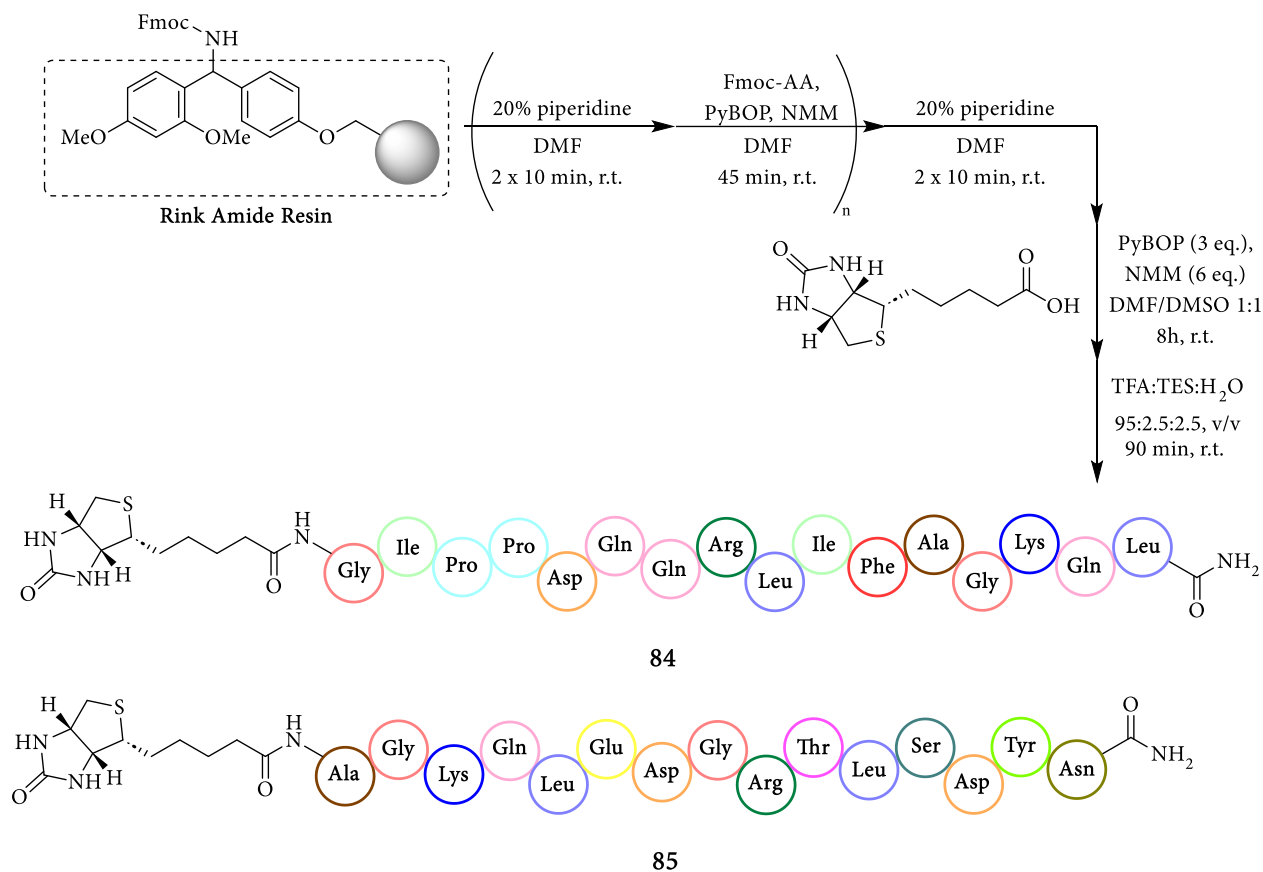


Figure 3.11: SPPS scheme of native biotinylated sequences **84** and **85**.

The same strategy was adopted when UAA 23 and 53 were used to replace the native Lys48 generating probes **86-87** bearing a propargyl warhead and probes **88-89** bearing a VME warhead (Fig. 3.12).

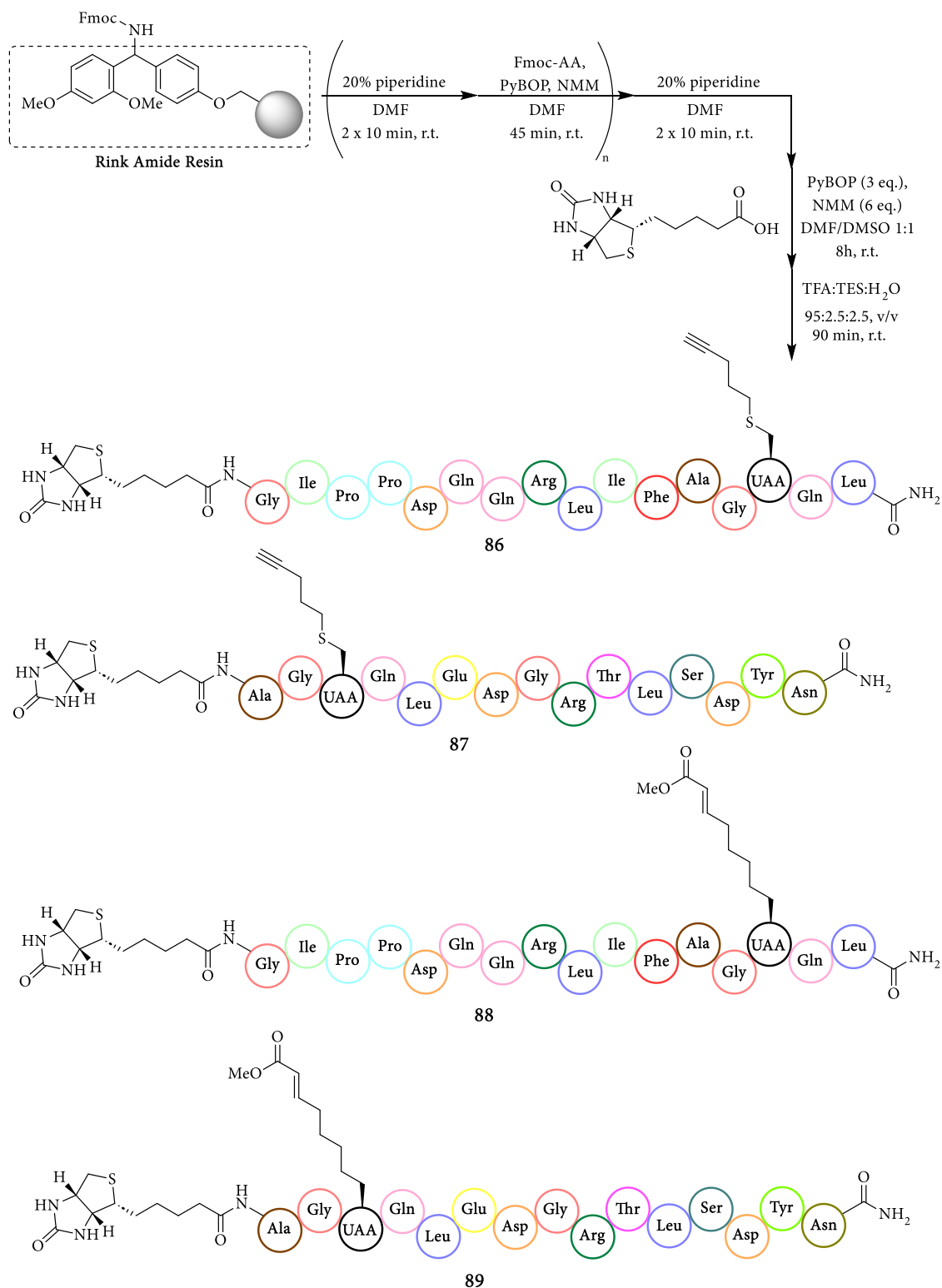


Figure 3.12: SPPS scheme of propargyl probes **86** and **87** and VME probes **88** and **89**. Structure of the modified side chain is shown. (UAA = Unnatural Amino Acid).

As discussed in section 2.3 (p. 50), standard SPPS coupling reactions were carried out using the appropriate Fmoc protected amino acid building blocks to assemble the peptides **84-89** on resin. Biotin was efficiently coupled at the N-terminus of the growing peptides followed by global deprotection and cleavage from the resin. Crude biotinylated peptides **84-89** were precipitated with Et₂O and purified by HPLC prior to their use in biology assays. Biotinylated peptides **84-85** feature the native Lys48 and will act as a negative control, whereas the probes **86-89** feature reactive groups that will covalently label OTUB1 in its active site.

3.4. Activity based profiling using peptide probes

3.4.1 Labelling of recombinant OTUB1

To assess if the set of synthesised ABPs **86-89** was capable of labelling OTUB1, the recombinant enzyme was employed in preliminary labelling assays. An equal amount of recombinant OTUB1 was titrated against increasing amounts of the alkyne probes **86** and **87** along with the corresponding native controls **84** and **85**. Following separation by gel electrophoresis, the labelled OTUB1 was imaged with an anti-biotin western blot (Fig. 3.13).

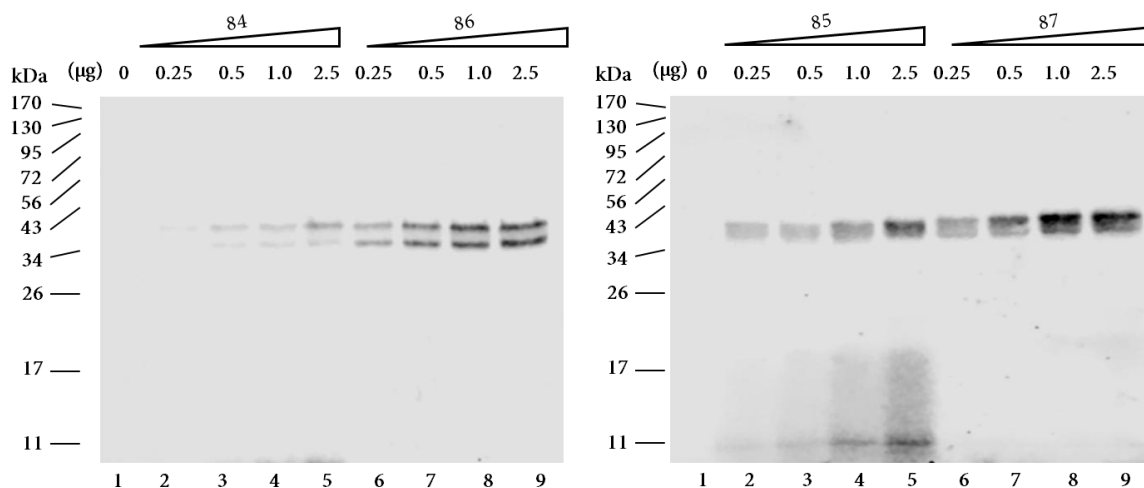


Figure 3.13: Western blot showing recombinant OTUB1 (1 µg) incubated for 1.5h at 37°C with increasing concentrations of alkyne ABPs **86** (left, lanes 6-9) and **87** (right, lanes 6-9). Biotinylated native sequence **84** (left, lanes 2-5) and **85** (right, lanes 2-5) were used as negative controls.

Gratifyingly, a progressive increase in labelling was observed when recombinant OTUB1 (0.95 μ M) was titrated against both the alkyne probes **86** and **87** (Fig. 3.13, lanes 6-9). However, a similar pattern could also be observed for the native control sequences **84** and **85** that were not designed to label OTUB1 due to the absence of a reactive warhead (Fig. 3.13, lanes 2-5). This undesired phenomenon was postulated to be caused by a non-specific adhesion of the peptides when used in excess compared to the recombinant OTUB1. Therefore, the titration experiment was repeated and the ABPs concentrations were reduced from a range of 4.2-42 μ M to a range of 0.17-3.4 μ M. The previous ABPs to OTUB1 range screened the labelling when the ABPs were present in large excess compared to the recombinant enzyme, whereas the optimised range allowed to test both submolar and equimolar ABPs to OTUB1 ratios (reduced from a range of 5:1-50:1 to a range of 0.2:1-4:1, Fig. 3.14).

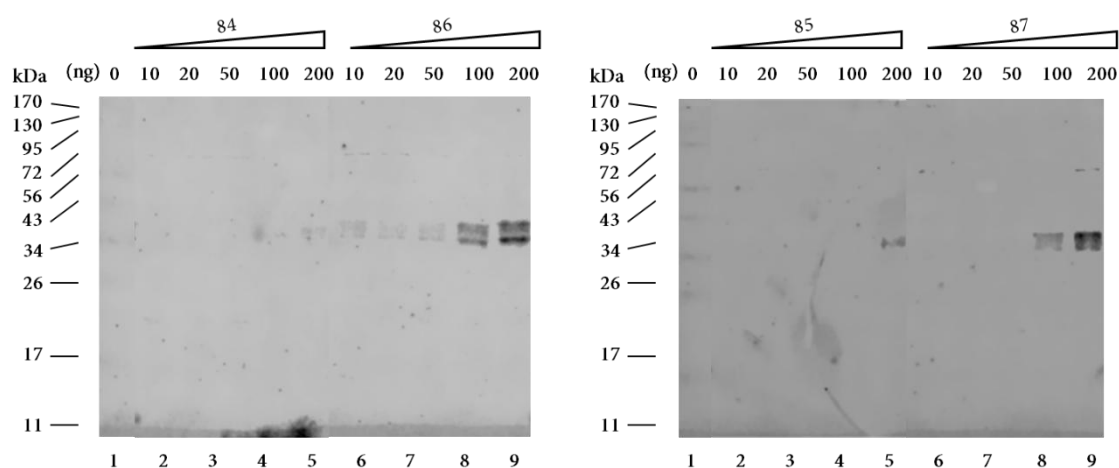


Figure 3.14: Western blot showing recombinant OTUB1 (1 μ g) incubated for 1.5h at 37°C with increasing concentrations of alkyne ABPs **86** (left, lanes 2-5) and **87** (right, lanes 2-5). Biotinylated native sequence **84** (left, lanes 6-9) and **85** (right, lanes 6-9) were used as negative controls.

Following optimisation of the ABPs to OTUB1 ratio, the undesired non-specific labelling from the native control sequences was not observed (Fig. 3.14, lanes 6-9). Moreover, labelling of recombinant OTUB1 by the alkyne ABPs **86** and **87** was observed when 100 ng and 200 ng of the probes were incubated with 1 μ g of OTUB1, corresponding to ABP:OTUB1 molar

ratios of 2:1 and 4:1 respectively (Fig. 57, lanes 4-5). These optimised ratios were used for all the subsequent biology assays.

Once a suitable ABP to OTUB1 ratio was found, the selectivity of ABPs **86** and **87** were tested in a preliminary competition assay. The alkyne probes **86** and **87** were incubated with OTUB1 in the presence or absence of Bovine Serum Albumin (BSA), a well-established model protein with a free thiol group at Cys34 that could compete with the active site cysteine of OTUB1 upon labelling of the ABPs (Fig. 3.15).¹⁸⁴

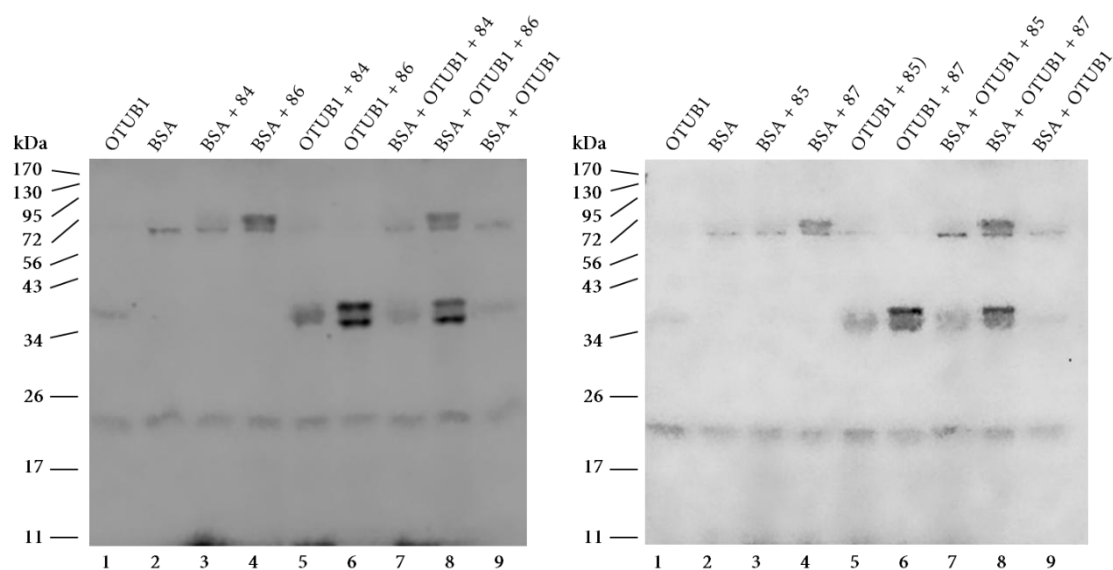


Figure 3.15: Western blot showing competition assays between recombinant OTUB1 (1 µg) and BSA (1 µg) incubated for 1.5h at 37°C with 0.1 µg of alkyne ABPs **86** (left, lanes 4, 6 and 8) and **87** (right, lanes 4, 6 and 8). 0.1 µg of biotinylated native sequence **84** (left, lanes 3, 5 and 7) and **85** (right, lanes 3, 5 and 7) were used as negative controls.

Notably, when the alkyne probes **86** and **87** were incubated with OTUB1 a band around 35kDa was observed (Fig. 3.15, lanes 6 and 8). However, several issues were observed upon visualisation of the preliminary competition assays via anti-biotin western blot. Specifically, a band at approximately 22 kDa appeared consistently across all the lanes, indicating contamination of the homogenate buffer used during incubation. To overcome this issue, homogenate buffer was freshly prepared before every labelling experiment. Moreover, the characteristic OTUB1 band appeared as two distinct bands (Fig. 3.15, lanes 6 and 8). This phenomenon was also observed over all the previously shown western blots (Fig. 3.13 and

3.14). This suggested the recombinant enzyme was degrading and therefore was unsuitable for use in labelling assays. To address this issue, the recombinant enzyme was re-expressed in BL21(DE3) cells transfected with a pET28a-LIC vector expressing a N-terminal His6 tagged OTUB1. Additionally, OTUB1 and BSA appeared to be labelled when incubated with the native control probes **84** and **85**, which should not result in any labelling (Fig. 3.15, lanes 3, 5 and 7). Furthermore, OTUB1 and BSA were detected on the gel even when incubated in the absence of any probe (Fig. 3.15, lanes 1, 2 and 9), which should also not result in any band. These findings suggested that the issue was not related to non-specific probe adherence to either OTUB1 or BSA, but rather caused by non-specific labelling of the streptavidin IR dye used for visualizing labelled adducts on the nitrocellulose membrane, resulting in artefact signals. To overcome this difficulty, the anti-biotin western blot protocol was modified to reduce non-specific labelling of the streptavidin IR dye. Prior to visualisation of the nitrocellulose membrane, the incubation with blocking buffer was extended from 1 h at room temperature to 3 h at 4 °C to increase the efficiency of the membrane blocking. In addition, the IR dye was diluted from 1:5000 to 1:8500 and SDS was added to the imaging solution to a final concentration of 0.1% which was postulated to reduce non-specific labelling. These optimised conditions were employed in all the following western blots.

The competition assays were repeated carrying out the labelling with both the alkyne probes **86** and **87** (Fig. 3.16) and both the VME probes **88** and **89** (Fig. 3.17).

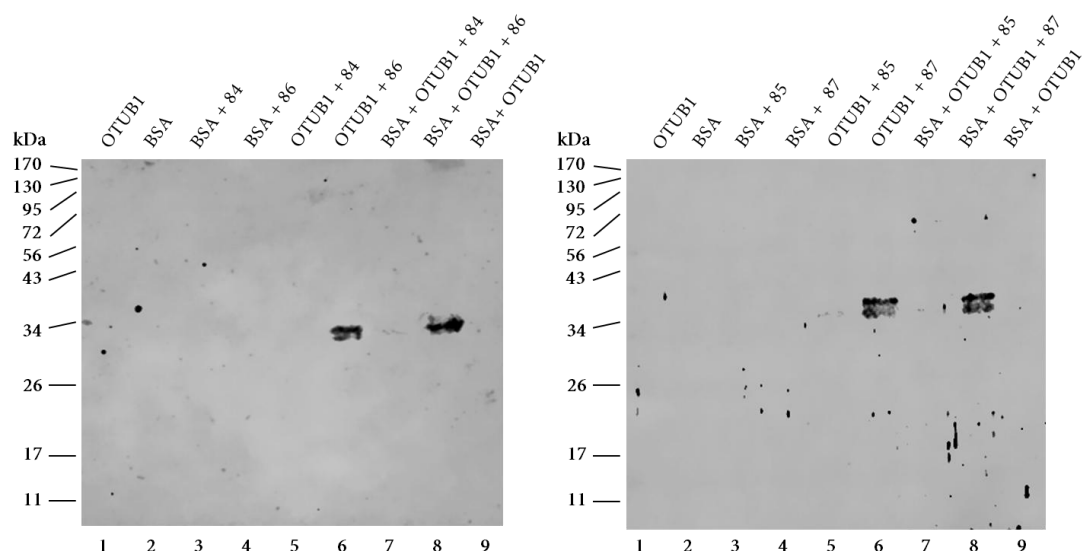


Figure 3.16 Western blot showing optimised competition assays between recombinant OTUB1 (1 μ g) and BSA (1 μ g) incubated for 1.5h at 37°C with 0.1 μ g of alkyne ABPs 86 (left, lanes 4, 6 and 8) and 87 (right, lanes 4, 6 and 8). 0.1 μ g of biotinylated native sequence 84 (left, lanes 3, 5 and 7) and 85 (right, lanes 3, 5 and 7) were used as negative controls.

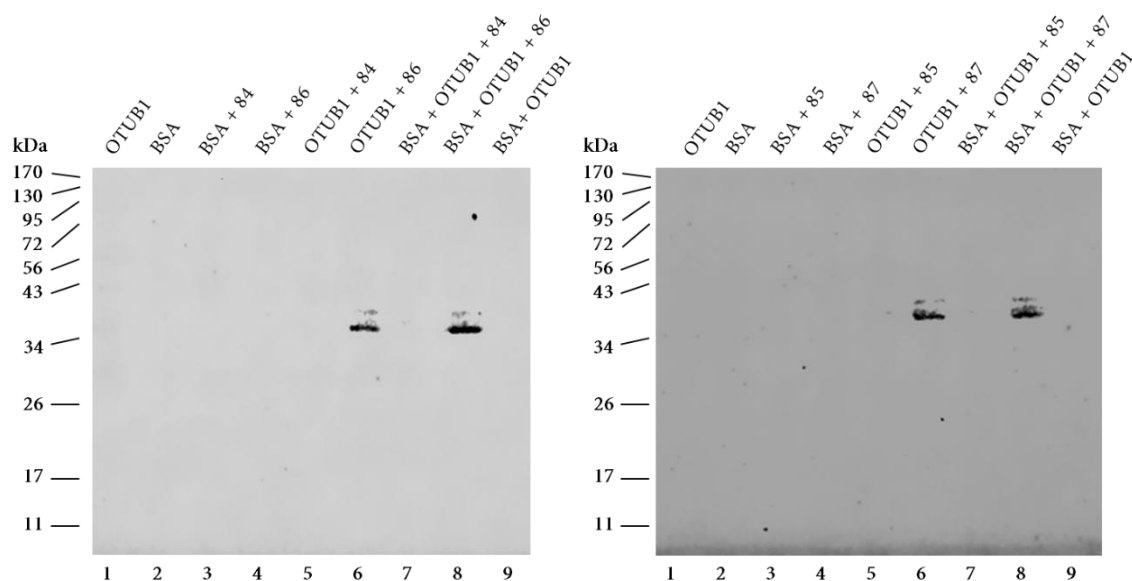


Figure 3.17: Western blot showing optimised competition assays between recombinant OTUB1 (1 μ g) and BSA (1 μ g) incubated for 1.5h at 37°C with 0.1 μ g of alkyne ABPs 88 (left, lanes 4, 6 and 8) and 89 (right, lanes 4, 6 and 8). 0.1 μ g of biotinylated native sequence 84 (left, lanes 3, 5 and 7) and 85 (right, lanes 3, 5 and 7) were used as negative controls.

Gratifyingly, non-specific labelling was efficiently eliminated when the optimised protocol for anti-biotin western blot was carried out. Undesired artefact bands related to non-specific labelling of the IR dye to OTUB1/BSA were not detected during membrane imaging (Fig. 3.16 and 3.17, lanes 1, 2 and 9). Moreover, OTUB1 and BSA were not labelled by the native control sequences 84 and 85 (Fig. 3.16 and 3.17, lanes 3, 5 and 7). Interestingly, OTUB1 was labelled by both the alkyne and VME probes 86-89 even when co-incubated with BSA (Fig. 3.16 and 3.17, lanes 6 and 8) and no labelling was observed when the probes 86-89 were incubated with BSA (Fig. 3.16 and 3.17, lane 4). Overall, these findings demonstrated that the probes 86-89 were capable of labelling OTUB1 even in the presence of other thiol containing proteins. Therefore, probes 86-89 were tested in more complex environments such as the one of cell lysates to fully assess their selectivity.

3.4.2 Labelling of OTUB1 in cell lysates

Human Colorectal Tumour 116 (HCT116) cell lysate was employed to evaluate the labelling of the probes 86-89 in a preliminary assay. HCT116 cells were selected as they are known to express a wild type OTUB1.¹⁸⁵ An equal amount of HCT116 cell lysate was titrated against increasing amounts of alkyne probes 86 and 87 (Fig. 3.18 A-B) and VME probes 88 and 89 (Fig. 3.18 C-D). Following incubation with the ABP/control, proteins were separated with gel electrophoresis and biotinylated adducts were imaged with an anti-biotin western blot.

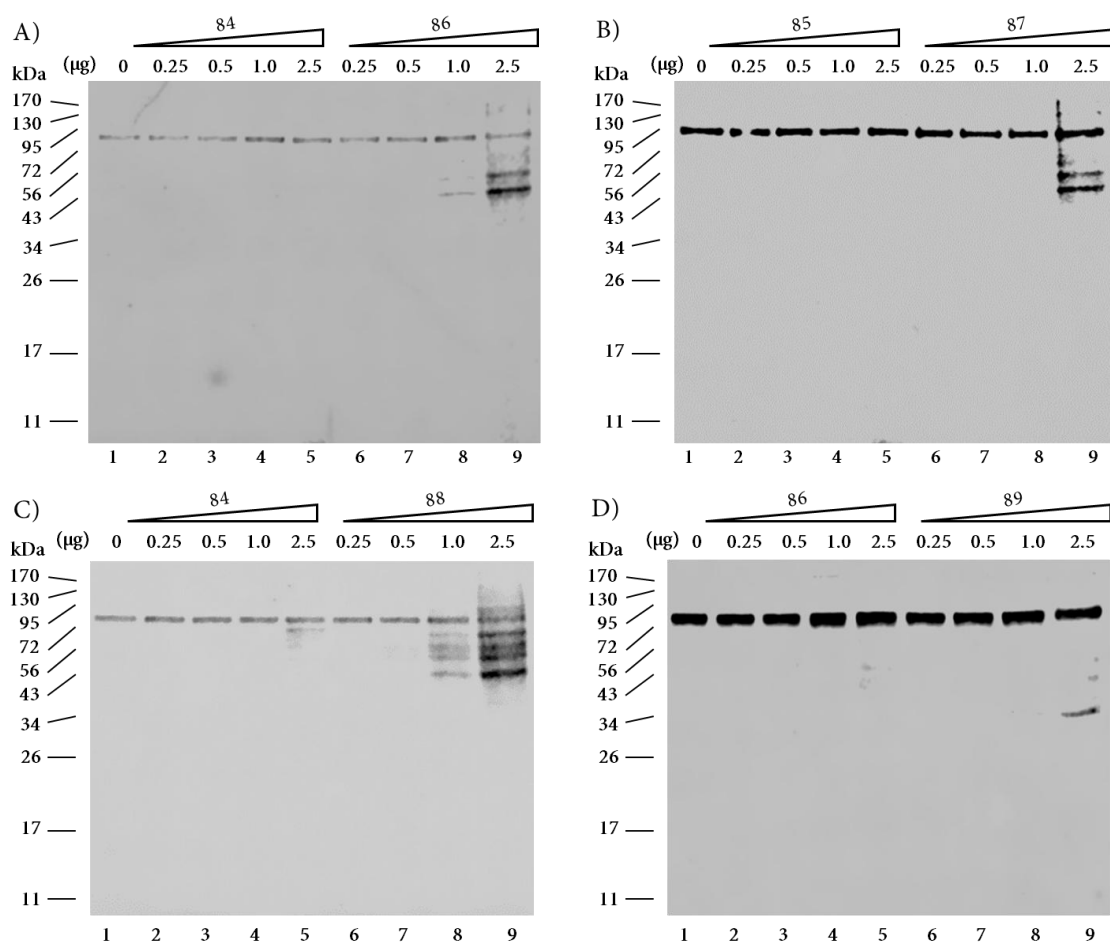


Figure 3.18: Western blot of HCT116 cell lysate (25μg) incubated for 1h at 37°C with increasing quantities of: **A)** alkyne ABP **86** (lanes 6-9), **B)** alkyne ABP **87** (lanes 6-9), **C)** vinyl methyl ester ABP **88** (lanes 6-9), and **D)** vinyl methyl ester ABP **89** (lanes 6-9). Native sequences **84** and **85** (A-D, lanes 2-5) were used as a negative control.

Following membrane visualisation, a band around 75 kDa was observed across all gel lanes. This was postulated to be due to the presence of two naturally occurring biotinylated proteins involved in metabolism of CoA: 3-methylcrotonyl CoA carboxylase and propionyl CoA carboxylase.¹⁸⁶ As anticipated, no covalent labelling occurred when HCT116 cell lysate was titrated against the native control peptides **84** and **85** (Fig. 3.18 A-D, lanes 2-5), confirming that the warhead was essential to covalently label enzymes in the lysate. Notably, when 2.5 μg of probes **86-89** were incubated with 25 μg of HCT116 lysate, distinct bands were observed (Fig. 3.18 A-D, lane 9). The alkyne probes **86** and **87** exhibited a similar labelling pattern. However, they were not capable of labelling OTUB1 in HCT116 lysate, as the characteristic

band at 35 kDa was not detected (Fig. 3.18 A-B, lane 9). Remarkably, the VME probes **88** and **89** showed very different labelling patterns when incubated with HCT116 lysate (Fig. 3.18 C-D, lane 9). Probe **88**, in which the VME warhead was installed on the Gly35-Leu50 scaffold, labelled more proteins in HCT116 lysate compared to probe **86** that featured the same scaffold but with an alkyne warhead (Fig. 3.18 A and C, lane 9). This observation is consistent with the fact that the VME warhead exhibits higher reactivity compared to the propargyl warhead. Unfortunately, probe **88** failed to label OTUB1 when incubated with HCT116 cell lysate. Surprisingly, when 2.5 μ g of probe **89** were incubated with 25 μ g of HCT116 cell lysate, the characteristic band relative to labelled OTUB1 was observed (Fig. 3.18 D, lane 9). This result demonstrates that VME probe **89** is capable of labelling OTUB1 even within complex cellular environments.

To further evaluate the labelling of OTUB1 by the VME probes **88** and **89**, the labelling was repeated with and without spiking the HTC116 cell lysate with recombinant OTUB1. The rationale behind this experiment was to simulate an overexpression of OTUB1 and assess whether the VME probes **88** and **89** could label the enzyme in such conditions (Fig. 3.19).

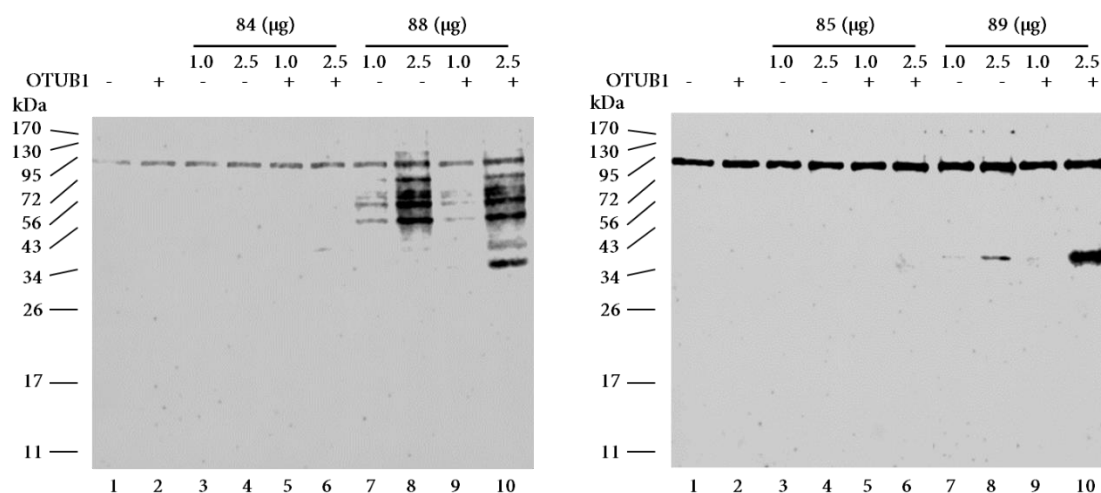


Figure 3.19: Western blot of showing HCT116 cell lysate (25 μ g) incubated for 1h at 37°C with 1 μ g or 2.5 μ g of VME probe **88** (left, lanes 7-10) and **89** (right, lanes 7-10). Biotinylated native sequence **84** (left, lanes 3-6) and **85** (right, lanes 3-6) were used as negative controls. Recombinant OTUB1 (1 μ g) was added to the cell lysate (lanes 2, 5-6, 9-10) prior to incubation.

The spike experiment was carried out similarly to a titration experiment, and 25 µg of HCT116 lysate were incubated with either 1 µg or 2.5 µg of VME probes **88** and **89**. Interestingly, VME probe **88** was able to label OTUB1 when the recombinant enzyme was spiked in the lysate (Fig. 3.19 left, lane 10). This result indicated that the VME probe could label OTUB1 when the concentration of the enzyme was increased. Moreover, VME probe **89** confirmed its ability to label OTUB1 with or without spiking the recombinant enzyme in the lysate (Fig. 3.19 right, lane 8 and 10). Taken together, these results revealed that VME probes **88** and **89** successfully functioned as ABPs capable of covalently labelling OTUB1.

3.5 Conclusion

Two set of ABPs to target OTUB1 were successfully designed, synthesised, and purified by HPLC. Probes **86** and **87** featured a propargyl warhead whereas probes **88** and **89** featured a VME warhead. The probes were designed in a way that both the warhead and the biotin tag could be efficiently installed while the recognition scaffold was being assembled via SPPS. This approach simplified the synthesis of the ABPs and offered considerable versatility. Notably, the Fmoc-protected UAAs **23** and **53** containing the warhead were compatible with solid phase peptide synthesis (SPPS) and could be incorporated into any peptide-based recognition scaffold. Moreover, the synthesis of Fmoc-protected UAA **53** has never been reported in the literature.

Upon optimisation of the labelling of OTUB1, the probes **86-89** showed ability to label the recombinant enzyme. This result was corroborated when the probes **86-89** showed to be able to label OTUB1 in the presence of a thiol containing protein such as BSA. Moreover, the VME probe **89** was able to label wild type OTUB1 expressed in HCT116 cell lysate, suggesting that this probe could possess a higher specificity compared to probes **86-89**. Interestingly, VME probe **88** could label OTUB1 only when the recombinant enzyme was spiked into HCT116 cell lysate, suggesting that the ABP is capable of detecting the enzyme at higher concentrations.

Following this qualitative assessment of OTUB1 labelling by probes **86-89**, quantitative analysis of the labelled OTUB1 could be carried out through proteomic analysis. This would allow for the identification of the proteins labelled by the ABPs **86-89** when incubated in HCT116 lysate and their enrichment compared to control samples.

Chapter 4

Conclusion and future outlook

The work described in this dissertation aimed to design peptidic Activity Based Probes to profile Deubiquitinating enzymes. In the first study, covalent peptidic probes 51-52 were designed and synthesised to target the DUB/s interacting with p53 at the newly discovered K291-K292 sites. In the second study, two sets of covalent probes 86-87 and 88-89 were designed and synthesised to profile OTUB1. In both the studies, the activity of the ABPs were evaluated via labelling assays in cell lysates and proteomic analysis was performed to quantify covalently labelled targets.

Chapter 2 described the development of ABPs to target p53 deubiquitination and focused on the synthesis and evaluation of two probes 51-52. The probes consisted of a biotinylated p53-based recognition scaffold in which the native lysines K291/292 were replaced by propargyl warheads. The activities of such probes were validated in competition with the native control 50 which did not bear the covalent warhead and was therefore incapable of labelling. Following optimization of a biotin enrichment protocol, proteomic analysis was carried out and revealed USP5 as a potential deubiquitinating enzyme that could remove ubiquitin from p53 at its K291/292 sites.

There could be multiple opportunities to expand the findings of this study. The Fmoc-protected UAA 19, 22 and 53 which featured a terminal alkene and a VME warhead have been synthesised but not yet installed on the p53-based peptide recognition scaffold. ABPs featuring an alkene warhead have recently been demonstrated to be a valuable tool to profile DUBs,⁸³ whereas VME warheads are well-known in the literature.⁶³ Therefore, a set of alkene and VME probes 90-92 (Fig. 4.1) could be synthesised and their activity could be tested in labelling assays availing of the protocol already optimized for the propargyl probes 51-52.

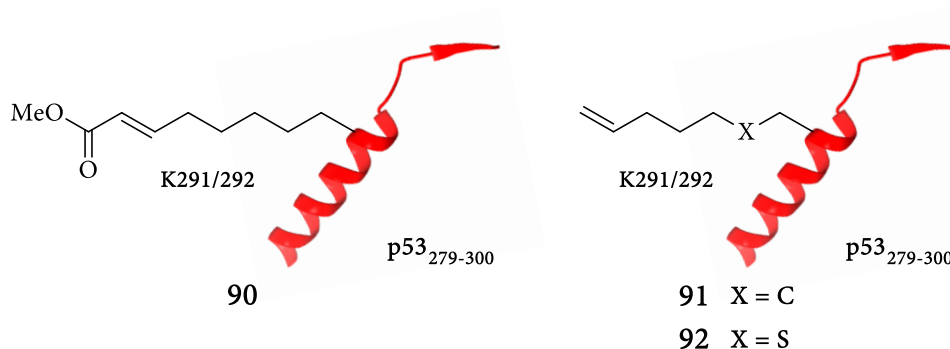


Figure 4.1: Proposed structure of a new set of ABPs to target the DUBs interacting with p53 at its K291/292 sites.

Another opportunity to expand this work regards the recognition scaffold of the proposed ABPs. There remains a need for a study aimed to understand the secondary structure of probes 51-52. As discussed in section 2.3 (p. 49), the secondary structure of p53 at residues 277-294 is an α -helix. Circular Dichroism (CD) would be a useful technique to elucidate the structural arrangement of ABPs 51-52 and would provide further understanding on whether the secondary structure could influence the binding of the ABPs to their targets.

Moreover, an alternative strategy for the warhead installment focused on the selective cysteine modification of p53-based peptide scaffolds could be investigated. Such attractive strategy consists in the transformation of a cysteine derivative installed on the recognition scaffold into dehydroalanine, which could be later functionalised to incorporate a suitable warhead (Fig. 4.2). Rather than performing a synthesis for each of the probes, this “tag and modify” strategy would allow the synthesis of a general scaffold that can be diversified into different probes based on the alkylating reagent used.^{168, 187, 188} Moreover, this strategy would prove useful to install warheads that are incompatible with solid phase peptide synthesis.

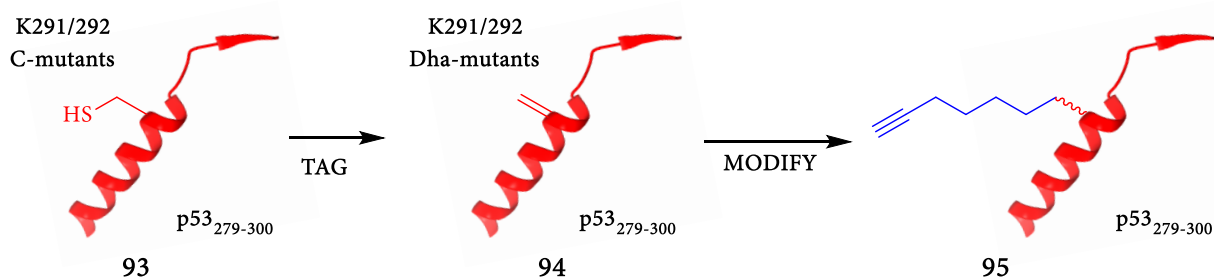


Figure 4.2: Depiction of the proposed “tag and modify” strategy that would allow to access modified peptides bearing different warheads (in blue) from their Dha precursor (in red).

The development of peptidic covalent tools to target the deubiquitinating enzyme OTUB1 were discussed in Chapter 3. Two sets of ABPs **86-87** and **88-89** were designed and synthesised availing of a similar strategy to the one used for the design of ABPs to probe the DUB/s interacting with ubiquitinated p53. These probes featured a biotinylated Ub-based recognition scaffold, where the native lysine K48 side chain was substituted with either a propargyl warhead or a VME warhead.

Following optimization of the labelling experiments, all the biotinylated peptides **86-89** were found capable of working as ABPs, labelling recombinant OTUB1. However, only the more reactive VME probes **88-89** were reactive and selective enough to capture OTUB1 in cell lysates. To further extend the set of probes to target OTUB1, different other warheads could be installed in place of the native lysine side chain. For example, a bromide or chloride alternative warheads could be easily installed in the recognition scaffold if the corresponding Fmoc-protected UAA are accessed by synthesis. However, such probes are reported in the literature to be highly reactive and can therefore yield to undesired non-specific labelling.⁶³

Future studies regarding this work could involve proteomic analysis to quantify the OTUB1 enrichment of each probe **86-89** compared to its appropriate negative control **84** or **85**. Upon choosing an appropriate cell line in which OTUB1 is expressed (or overexpressing the enzyme in a suitable cell line), the biotinylated adducts could be enriched using streptavidin beads. Following an on-bead trypsin digestion protocol, the OTUB1 captured by the ABPs could be efficiently detected and quantified by LC-MS/MS proteomic analysis.

In summary, peptidic ABPs were developed and applied to study DUBs. The driving force of this work was to combine the versatility and accessibility of SPPS with the development of

selective ABPs to target elusive enzymes such as DUBs. Overall, novel methods for the synthesis of UAA and for their instalment into peptidic recognition scaffolds were successfully developed. Moreover, significant process has been made in the development of peptidic ABPs employed as covalent tools to target DUBs. Finally, this study outlined a useful and versatile approach to probe enzymatic activity and could be further expanded to enrich our current knowledge on poorly understood fields of research.

Chapter 5

Experimental

5.1 Standard chemical methods

NMR spectra were recorded on either an Agilent MR 400 spectrometer with operating frequencies of 399.84 MHz for ^1H and 100.55 MHz for ^{13}C , a Bruker Avance III 400 spectrometer with operating frequencies of 400.23 MHz for ^1H and 100.63 MHz for ^{13}C , or a Bruker Avance II 600 spectrometer with operating frequencies of 600.13 MHz for ^1H and 150.90 MHz for ^{13}C . NMR spectra were processed using MestReNova. ^1H NMR chemical shifts were referenced to residual non-deuterated solvent peaks within the NMR solvent; CHCl_3 ($d_{\text{H}} = 7.26$ ppm) and DMSO ($d_{\text{H}} = 2.50$ ppm). The chemical shifts (δ) are reported in parts per million (ppm) and signal multiplicity as s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, m = multiplet and combinations thereof. Coupling constant (J) are quoted in Hertz (Hz) and reported to the nearest 0.1 Hz. ^{13}C NMR spectra are proton-decoupled with chemical shifts referenced to residual deuterated solvent peaks in the NMR solvent; CDCl_3 ($d_{\text{C}} = 77.16$ ppm) and DMSO- d_6 ($d_{\text{C}} = 39.52$ ppm). Product identification and peak assignments were carried out using additional 1D experiments (TOCSY), 2D experiments (HSQC, HMBC) and pulse experiments (DEPT-135) where appropriate. Mass spectrometry measurements were carried out on a Bruker ESI or APCI HRMS. Flash column chromatography was carried out using silica gel, particle size 0.04 - 0.063 mm, purchased from Sigma Aldrich or VWR. Room temperature is classified in this work as 19.5 – 20.5 °C. TLC analysis was performed on TLC Silica gel 60 F₂₅₄ plates purchased from Merck and visualised by UV irradiation (254 nm), potassium permanganate stain (3 g KMnO_4 , 20 g K_2CO_3 , 300 mL H_2O), ammonium molybdate staining (0.26 M ammonium molybdate in 1M aq. H_2SO_4), ninhydrin stain (1.5 g ninhydrin, 5 mL AcOH , 500 mL 95% EtOH) and anisaldehyde stain (9.2 mL *p*-methoxybenzaldehyde, 3.75 mL AcOH , 338 mL 95% EtOH , 12.5 mL conc. H_2SO_4). All solvents were obtained from commercial sources and used as received. Anhydrous DCM, THF, Et_2O and MeCN were obtained from a PureSolv solvent purification system, DMF was used dry from SureSeal bottles. Petroleum ether refers to the fraction of petroleum ether that boils at 40-60 °C.

5.2 Standard procedure for Solid Phase Peptide Synthesis

General procedure 1: First amino acid coupling to Rink Amide resin

Rink amide AM resin (0.78 mmol/g, 100 - 200 mesh, 1.0 equiv.) was added to a SPPS syringe (Carl Roth GmbH, Karlsruhe, Germany) followed by DMF (4 mL). The syringe was agitated for 20 min and then drained. The resin was treated with 20% (v/v) piperidine in DMF (4 mL) for 2 x 10 min. The deprotected resin was then washed with DMF (3 x 5 mL), DCM (3 x 5 mL) and DMF (3 x 5 mL). PyBOP (4.0 equiv.) and NMM (8.0 equiv.) were added to a solution of Fmoc protected amino acid (4.0 equiv., 0.2 M) in DMF. This solution was transferred to the resin in the syringe and agitated for 45 min. Excess reagents were drained from the syringe and the resin was washed with DMF (3 x 5 mL), DCM (3 x 5 mL) and DMF (3 x 5 mL).

General procedure 2: Bromophenol blue monitoring of coupling and deprotection reactions

The extent of each coupling reaction was qualitatively monitored by treatment of 5-10 resin beads with a solution of bromophenol blue (BPB) in DCM (0.15 mM, 0.1 mL). The following amino acid was coupled only when the beads turned blue upon treatment with BPB.

General procedure 3: Second and subsequent amino acid Fmoc deprotection and coupling

The following amino acid coupling cycles consisted of (I) Fmoc deprotection by the addition of 20% (v/v) piperidine in DMF (4 mL) to the resin for 2 x 10 min. (II) Resin washes with DMF (3 x 5 mL), DCM (3 x 5 mL) and DMF (3 x 5 mL). (III) Peptide coupling with addition of PyBOP (3.0 equiv.), NMM (6.0 equiv.) and N-protected amino acid (3.0 equiv., 0.2 M) in DMF to the peptide resin for 45 min. (IV) Resin washes with DMF (3 x 5 mL), DCM (3 x 5 mL) and DMF (3 x 5 mL). (V) Qualitative BPB test as per General Procedure 2. Following the final coupling, the resin was treated with 20% (v/v) piperidine in DMF (2 x 10 min, 5 mL) and the resin was washed with 2 rounds of DMF (3 x 5 mL), DCM (3 x 5 mL), followed by drying of the resin under reduced pressure.

General procedure 4: Resin cleavage and global deprotection

DCM (4 mL) was added to the dry peptide resin. The syringe was agitated for 20 min, then drained. The cleavage cocktail (TFA:TES:H₂O; 95:2.5:2.5 v/v/v; 5 mL) was transferred to the syringe which was agitated for 90 min. The syringe was drained, and the filtrate was collected. The resin was washed with cleavage cocktail (2 x 3 mL) and the washings were combined with the first filtrate. The combined solution was concentrated under a flow of N₂ into a viscous oil, followed by precipitation of the crude peptide with Et₂O (7 mL). The peptide was washed with Et₂O (2 x 10 mL) and the crude solid was dried under reduced pressure.

General procedure 5: On resin biotinylation

DMF (4 mL) was added to the dry peptide resin. The syringe was agitated for 20 min, then drained. D-Biotin (146 mg, 0.6 mmol), PyBOP (312 mg, 0.6 mmol) and NMM (132 μ L, 0.6 mmol) were dissolved in 4 mL of a 1:1 DMF:DMSO mixture. The coupling cocktail was transferred to the syringe which was agitated at room temperature for 24 h. Following the final coupling, the resin was washed with 2 rounds of DMF (3 x 5 mL), DCM (3 x 5 mL), followed by drying of the resin under reduced pressure.

5.3 General biological methods

5.3.1 Buffers

2X Reducing sample buffer (Laemmli buffer): 0.2 M Tris-Cl pH 6.8, 30% glycerol, 0.4% β -mercaptoethanol, 9% SDS, 0.1 % bromophenol blue.

PBS: 8 mM Na₂HPO₄, 150 mM NaCl, 2 mM KH₂PO₄, 3 mM KCl.

PBS-T: 8 mM Na₂HPO₄, 150 mM NaCl, 2 mM KH₂PO₄, 3 mM KCl, 0.1% Tween 20.

Column buffer: 50 mM HEPES pH 6.8, 100 mM NaOAc.

Homogenate buffer: 50 mM Tris-Cl pH 7.4, 5 mM MgCl₂, 250 mM sucrose, 1 mM dithiothreitol (DTT) or 1 mM (tris (2-carboxyethyl) phosphine) (TCEP).

Fixative solution: 40% EtOH, 10% AcOH.

Developer solution: (3% K₂CO₃, 0.05% formaldehyde).

Resolving gel: 1.3 mL 1.5 M Tris-Cl pH 6.8, 1.5 mL 40% acrylamide/bis-acrylamide (29:1), 2 mL H₂O, 50 µL 10% SDS, 50 µL 10% ammonium persulfate (APS), 5 µL tetramethylethylenediamine (TEMED).

Stacking gel: 630 µL 0.5 M Tris-Cl pH 6.8, 300 µL 40% acrylamide/bis-acrylamide (29:1), 1.3 mL H₂O, 25 µL 10% SDS, 25 µL 10% ammonium persulfate (APS), 2.5 µL tetramethylethylenediamine (TEMED).

Running buffer: 25 mM Tris, 190 mM Glycine, 1% SDS.

Blotting transfer buffer: 25 mM Tris, 190 mM Glycine, 20% MeOH.

Anti-biotin WB blocking solution: 5% BSA in PBS-T.

Anti-biotin WB imaging solution: 2.5% BSA in PBS-T, 0.1% SDS, 0.2 µg/mL of IRDye 800CW-Streptavidin.

Urea buffer (UA): 8M urea in 0.1 M Tris-Cl pH 8.5.

Iodoacetamide solution: 50 mM iodoacetamide in UA buffer.

DTT solution: 10 mM in UA buffer.

DTT stock solution: 1M solution of DTT in distilled water.

Trypsin solution: ratio is 50:1 enzyme to protein in 50 mM ammonium bicarbonate.

Ammonium bicarbonate (NH₄HCO₃): 50 mM NH₄HCO₃ in distilled water, pH 7.8.

MS Buffer A: 98% water, 2% MeCN, 0.1% in TFA.

MS Buffer B (for peptides): 20% water, 80% MeCN, 0.1% in TFA.

LB medium: 20 g of Luria-Bertani broth in 1 L water.

Ni wash buffer: 50 mM sodium phosphate pH 8.0, 300 mM NaCl, 10 mM imidazole.

Ni elution buffer A: 50 mM sodium phosphate pH 8.0, 300 mM NaCl, 150 mM imidazole.

Ni elution buffer B: 50 mM sodium phosphate pH 8.0, 300 mM NaCl, 300 mM imidazole.

5.3.2 SDS-PAGE

Proteins were separated on a 12% acrylamide gel (resolving gel: 4.9 mL; stacking gel: 1.5 mL). Samples were prepared for separation by adding 2X reducing sample buffer followed by heating at 95 °C for 5 min. The proteins were loaded along with Fisher's EZ-Run™ Pre-Stained Rec Protein Ladder. Separation was achieved at 150 V for 1 h and 20 min in running buffer (600 mL) and visualised *via* silver staining or western blotting.

5.3.3 Silver staining

Gels were treated with fixative solution at room temperature for 1 h or at 4 °C for 16 h. Gels were washed in 20% EtOH (2 x 10 min), then in H₂O (2 x 10 min). Gels were sensitised in aq. Na₂S₂O₃ (0.02%) for 1 min and then immediately washed with H₂O (2 x 1 min). Gels were incubated in a solution of AgNO₃ (12 mM) with formaldehyde (0.02%) at 4 °C for a minimum of 20 min and up to 2 h. Following this, gels were washed in H₂O (2 x 30 s) and transferred to developer solution. Development was stopped using 5% AcOH. All gels were imaged using a Typhoon FLA9500 (GE Healthcare, Illinois USA).

5.3.4 Anti-biotin Western Blotting

A nitrocellulose membrane (GE Healthcare, Illinois USA) was soaked in blotting transfer buffer along with the filter papers and sponges that will form the transfer sandwich. The sandwich was assembled starting from a black plate on top of which two wetted sponges and a filter paper were positioned. The acrylamide gel was transferred to the top of the filter paper and the nitrocellulose membrane was placed on top of it, followed by filter paper, two sponges and a red plate. Proteins were transferred onto the nitrocellulose membrane in blotting transfer buffer overnight at 15 V and 4 °C. The membrane was incubated in 20 mL of BSA blocking buffer for 1 h at 4 °C with 140 rpm shaking. The membrane was incubated in 12 mL of BSA imaging solution for 1 h at 4 °C with 140 rpm shaking. The membrane was washed

with PBS-T (4 x 5 min) and imaged using an Odyssey XF Infrared Imaging System (LI-COR Biosciences, Nebraska, USA).

5.3.5 Cells lysis through mechanical method

A cell pellet (100-200 μ L) was combined with an equivalent amount of glass beads (500-750 μ m, Acros Organics, New Jersey, USA) in an Eppendorf, and 200-400 μ L of homogenization buffer was added. The Eppendorf was vortexed for 20 seconds, and immediately cooled down for 90 seconds on ice. Lysing and cooling steps were repeated for 20 times. The resulting cell contents were centrifuged 13,200 rpm for 5 minutes. The supernatant was separated and stored on ice immediately. The supernatant was aliquoted into 10 μ L aliquots for storage at -73°C. Concentration was analyzed by Nanodrop 1000 (Protein A280).

5.3.6 Labelling experiment general procedure

Probe (0.25 μ g to 2.5 μ g) was added to HCT116 or HEK293T cell lysate (25 μ g) in homogenate buffer, for a total incubation volume of 30 μ L. Upon preparation of samples incubation was started. Samples were incubated for 30 min, 1 h or 1.5 h at 37°C with 150 rpm shaking. After incubation, samples can be treated with Laemmli buffer (30 μ L) as per General Biological Method 2.

5.3.7 Desalting step for MS/MS sample preparation (Zip-Tip)

A 10 μ L zip-tip with 0.6 μ L C18 resin (Merk Millipore, Massachusetts USA) was equilibrated by aspirating MS buffer B four times and further four times with MS buffer A. 10 μ L were aspirated and dispensed in the protein sample for a total of ten times. Buffer A (10 μ L) was used to wash the sample by aspirating and dispensing four times. The protein or peptides were then eluted in buffer B (2 x 10 μ L) and dried using a vacuum centrifuge. The sample was analysed by LC-MS/MS.

5.3.8 Filter Aided Sample Preparation (FASP)

FASP was carried out using Vivaspin 500 centrifugal concentrators (10 kDa or 30 kDa MW cut-off). The standard protocol is suitable for 10 to 200 µg of total protein. 60 µL of sample was transferred to the filter and UA buffer (200 µL) was added to the filter and centrifuged at 11,800 rpm for 15 min. 200 µL of UA buffer was added and the filter was centrifuged at 11,800 rpm for 15 min. DTT solution (100 µL, 10 mM in UA buffer) was added and the solution was incubated for 30 minutes. After that time, solution was centrifuged at 11800 rpm for 15 minutes. IAA solution (100 µL, 50 mM in UA buffer) was added, and the solution was incubated for 30 minutes. After that time, solution was centrifuged at 11800 rpm for 15 minutes. Washes were performed using UA buffer (2 x 100 µL) followed by NH_4HCO_3 solution (2 x 100 µL, 50 mM). Material was centrifuged until 5% of the initial solution remained in the concentrator. Trypsin solution (80 µL, 50 mM NH_4HCO_3 solution, 1:50 enzyme:protein) was added and the concentrator was incubated overnight at 37 °C. After that time, the concentrator was centrifuged at 11,800 rpm for 10 min or until all the solution had passed the filter. 50 mM NH_4HCO_3 solution (20 µL) was added, and it was centrifuged at 11,800 rpm until all the of the solution had passed the filter.

5.3.9 Biotin enrichment using streptavidin beads

Labelling experiments were performed on HCT-116 cell lysate or HEK293T cell lysate as per General Biological Method scaled up to a total volume of 100 µL. 20% of the material was retained at key steps through the protocol as a reference. 100 µL of resin (50 µL of settled beads) was added to an Eppendorf and centrifuged at 2800 rpm for 2 minutes. Solvent was aspirated. Beads were equilibrated with 250 µL of PBS solution, centrifuged at 2800 rpm for 2 minutes, and the supernatant aspirated. This equilibration step was performed twice. 20 µL of master labelling solution were retained a reference. The remaining of the labelling solution (80 µL) was added in the eppendorf containing the beads. Solution was incubated overnight at 4 °C. After that time, the solution was centrifuged at 2800 rpm for 2 minutes. 16 µL of the supernatant was aspirated and used as a reference. Beads were washed with 100 µL of PBS

solution and vortexed for 20 seconds, centrifuged at 2800 rpm for 2 minutes and supernatant was aspirated. The washing step was repeated for a total of four times in different Eppendorfs. Beads were treated with 2X reducing buffer (100 μ L) and heated at 95°C for 5 minutes. Beads were centrifuged at 2800 rpm for 2 minutes and supernatant was aspirated. The eluting steps were repeated for a total of two times in different Eppendorfs.

5.3.10 Expression and purification of OTUB1

The expression and purification of His tagged OTUB1 was carried out according to literature procedures.^{63, 189} BL21 (DE3) cells transfected with a pET28a-LIC vector containing an N-terminal His6 tagged OTUB1 was transferred from a glycerol stock into LB medium (8 mL) containing kanamycin (100 μ g/mL) and grown for 18 h at 37 °C at 180 rpm. The cells were transferred into fresh LB medium (300 mL) containing kanamycin (100 μ g/mL) and grown at 37 °C at 180 rpm until an OD₆₀₀ of 0.6 to 0.9 was reached. IPTG was added at a final concentration of 0.4 mM and the bacteria were incubated at 18 °C for 16 h with vigorous shaking. The cells were centrifuged at 6,000 rpm for 15 min. The resulting pellet was re-suspended in homogenate buffer (25 mL) containing PMSF (20 μ M) and lysed *via* sonication. The lysate was centrifuged at 13,000 rpm for 45 min. Ni NTA agarose resin (Sigma-Aldrich, Missouri, USA) (1.5 mL, 1:1 suspension in 20% EtOH) was centrifuged at 2,200 rpm for 5 min and the supernatant was discarded. H₂O (2 x 0.7 mL) was added to the beads which were gently inverted until the resin was fully resuspended. The resulting solution was centrifuged at 2,200 rpm and the supernatant was discarded. Ni wash buffer (1.4 mL) was added to the resin which was transferred to the clarified supernatant and incubated overnight at 4 °C with rolling. The resin was centrifuged at 2,200 rpm and the supernatant was discarded. Ni wash buffer (1 mL) was added to the resin which was fully resuspended by gentle inversion and centrifuged at 2,200 rpm for 5 min. This wash step was repeated four times. The supernatant was discarded after each washing step. Ni elution buffer A (4 x 0.7 mL) was added to the resin that was resuspended by gentle inversion. The solution was centrifuged at 2,200 rpm for 5 min and the supernatants from these washes were pooled in clean microcentrifuge tubes. A final wash step was carried out with Ni elution buffer B (1 mL) at 2,200 rpm for 5 min. The

supernatant was discarded, and the beads were stored in 20% EtOH. The pooled supernatants were concentrated in a 10 kDa MW cut-off Vivaspin 500 centrifugal concentrators in a centrifuge at 9,000 rpm to a final concentration of 50 μ L. Ni wash buffer (450 μ L) was added to the tubes and concentrated to 50 μ L in a centrifuge at 9,000 rpm. This wash step was repeated, and the solution was resuspended in storage buffer (150 μ L, 20 mM Tris-Cl pH 8.0, 1 mM DTT, 10% glycerol, 50 mM NaCl). The concentration was measured by nanodrop.

5.3.11 Proteomic analysis and data processing

Configuration of HPLC-MS/MS apparatus and data acquisition was performed by Dr. Kieran Wynne, University College Dublin, School of Medicine, Conway Institute.

Samples were run on a Bruker timsTof Pro mass spectrometer connected to an Evosep One liquid chromatography system. Tryptic peptides were resuspended in 0.1% formic acid and each sample was loaded on to an Evosep tip. The Evosep tips were placed in position on the Evosep One, in a 96-tip box. The autosampler is configured to pick up each tip, elute and separate the peptides using a set chromatography method (30 samples a day). The mass spectrometer was operated in positive ion mode with a capillary voltage of 1200-1600 V, dry gas flow of 3 l/min and a dry temperature of 180 °C. All data was acquired with the instrument operating in trapped ion mobility spectrometry (TIMS) mode. Trapped ions were selected for MS/MS parallel accumulation serial fragmentation (PASEF). A scan range of (100-1700 m/z) was performed at a rate of 5 PASEF MS/MS frames to 1 MS scan with a cycle time of 1.03s/.

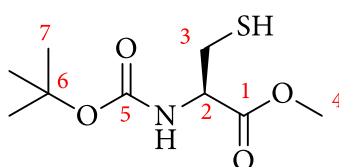
Data processing was performed using MaxQuant software (version 2.2.0.0) for identification and quantification of peptides using a custom FASTA file based on the *homo sapiens* proteome from the Uniprot database (UPID: UP000005640, downloaded April 2023). Each proteome was supplemented with the inclusion of streptavidin from *Streptomyces Avidinii* (UPID: P22629) and trypsin from *Bos Taurus* (UPID: P00760).

The following adjustments to MaxQuant standard settings were applied. The digestion enzyme was set to Trypsin with maximum missed cleavages set to 2. LFQ was chosen with a minimum ratio count of 1. The minimum number of peptides required for protein

identification was set to 2. The peptide length was set to be between 8 and 30 with a maximum peptide mass of 4600 Da. Carbamidomethylation (C) was set as a fixed modification, whereas Oxidation (O) and N terminal acetylation (A) were set as variable peptide modifications. Perseus software for Windows (version 1.6.15.0) was used for the analysis of identified peptides. Data sets were filtered to remove contaminants and $\log_2(x)$ and p-values were determined for remaining proteins. Standard volcano plots settings were applied with a false discovery rate of 0.05.

5.4 Experimental procedures

Methyl (*tert*-butoxycarbonyl)-L-cysteinate (**30**)



30

Methyl (*tert*-butoxycarbonyl)-L-cysteinate **30** was synthesized following a modified literature procedure.¹⁹⁰ Methanol (100 mL) was cooled to 0 °C, and acetyl chloride (17.6 mL, 248 mmol) was added dropwise over 5 minutes. The solution was stirred an additional 10 minutes at 0 °C to give a concentrated solution of HCl. L-cysteine (2.00 g, 16.51 mmol) was then added, and the resulting mixture was stirred at rt for 24 h under inert atmosphere (N₂). The solvent was then removed under reduced pressure to give the crude cysteine methyl ester hydrochloride as a pale-yellow solid. The crude ester was resuspended in DCM (100 mL) and cooled to 0 °C. Triethylamine (5.06 mL, 36.3 mmol) was added dropwise followed by di-*tert*-butyl dicarbonate (4.32 g, 19.8 mmol). The reaction was stirred at rt for 3.25 h after which time TLC analysis (EtOAc:Hex, 3:7) revealed complete consumption of starting material ($R_f = 0.5$) the desired product ($R_f = 0.6$) and its corresponding disulfide ($R_f = 0.3$). The solvent was removed under reduced pressure and the resulting residue was redissolved in a 5:1 MeOH:H₂O mixture (48 mL). Tributylphosphine (2.0 mL, 8.1 mmol) was added dropwise to the stirred solution. After 15 minutes, TLC analysis (EtOAc:Hex, 3:7) revealed complete consumption of the

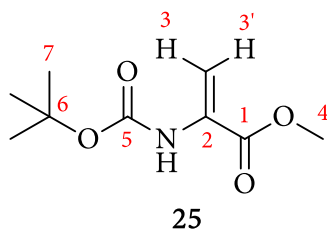
disulfide. The reaction was then diluted with Et₂O (100 mL) and H₂O (50 mL). The organic phase was separated, and the aqueous layer was extracted with Et₂O (2 x 50 mL). The combined organics were washed with brine (100 mL), dried over MgSO₄, and filtered. The solvent was removed under reduced pressure and the residue purified by column chromatography (EtOAc:Hex 1:20 to 1:4). Methyl (*tert*-butoxycarbonyl)-L-cysteinate **XX** was isolated as a colourless oil (2.47 g, 10.59 mmol, 64% yield). Spectral data is in agreement with the literature.¹⁹⁰

¹H-NMR (400 MHz, CDCl₃): δ 5.42 (s, 1H, NH), 4.61-4.55 (m, 1H, H2), 3.76 (s, 3H, H4), 3.00-2.90 (m, 2H, H3), 1.43 (s, 9H, H7) ppm.

¹³C-NMR (101 MHz, CDCl₃): δ 170.9 (C1), 155.2 (C5), 80.4 (C6), 54.9 (C2), 52.8 (C4), 28.4 (C7), 27.4 (C3).

HRMS (ESI⁺): (*m/z*) C₉H₁₇NNaO₄S [M+Na]⁺, calculated: 258.0771, found: 258.0776.

Methyl 2-((*tert*-butoxycarbonyl)amino)acrylate (**25**)



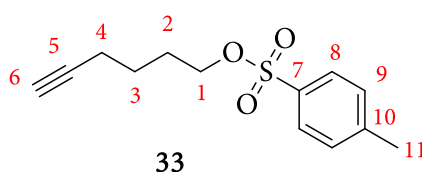
Dehydroalanine **25** was synthesized following a literature procedure.¹⁶⁶ 1,4-diiodobutane (1.2 mL, 9.26 mmol) and K₂CO₃ (1.70 g, 12.34 mmol) were added to a solution of methyl (*tert*-butoxycarbonyl)-L-cysteinate **30** (1.45 g, 6.17 mmol) in DMF (50 mL). The reaction mixture was stirred at rt for 4 h. After this time, TLC analysis (EtOAc:Hex 1:20) showed formation of product (*R_f* = 0.4) and consumption of starting material (*R_f* = 0.15). The solution was diluted with H₂O and subsequently extracted with EtOAc (3 x 100 mL). The organic layers were combined and washed with brine (3 x 100 mL), dried over MgSO₄, filtered and the solvent was evaporated under reduced pressure. Purification by silica plug (EtOAc:Hex 1:50 to 1:20) afforded 2-((*tert*-butoxycarbonyl)amino)acrylate **25** as a colourless oil (587 mg, 2.90 mmol, 47% yield). Spectral data is in agreement with the literature.¹⁶⁵

¹H-NMR (400 MHz, CDCl₃): δ 7.01 (s, 1H, NH), 6.15 (s, 1H, H3), 5.72 (d, J_{H3H3'} = 1.2 Hz, 1H, H3'), 3.82 (s, 3H, H4), 1.48 (s, 9H, H7) ppm.

¹³C-NMR (101 MHz, CDCl₃): δ 164.6 (C1), 152.7 (C5), 131.4 (C2), 105.3 (C3), 80.8 (C6), 53.0 (C4), 28.4 (C7) ppm.

HRMS (APCI): (m/z) C₉H₁₄NO₄ [M-H]⁻, calculated: 200.0928, found: 200.0931.

Hex-5-yn-1-yl 4-methylbenzenesulfonate (33)



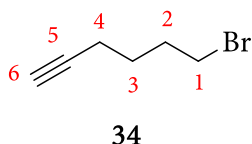
Hex-5-yn-1-yl 4-methylbenzenesulfonate **33** was synthesized following a literature procedure.¹⁷⁰ *p*-toluene sulfonyl chloride (1.53 g, 8.02 mmol) was added in three portions to a solution of hex-5-yn-1-ol **32** (100 μL, 0.9 mmol), triethylamine (150 μL, 1.08 mmol), and 4-(dimethylamino)pyridine (DMAP, 2.2 mg, 0.018 mmol) in DCM (25 mL) at 0 °C. The reaction mixture was brought to rt and stirred for 15 h. After that time, TLC analysis (EtOAc:Hex, 1:7) revealed complete consumption of starting material (*R*_f = 0.1) and formation of the desired product (*R*_f = 0.3). Aqueous NaOH (1 M, 5 mL) was added, and the mixture was vigorously stirred for 15 min at rt. The aqueous phase was extracted with DCM (3 x 10 mL). The combined organic fractions were washed with H₂O, followed by brine, and then dried over Na₂SO₄, filtered, and concentrated under reduced pressure. The crude residue was purified by column chromatography (EtOAc:Hex 1:5) to give Hex-5-yn-1-yl 4-methylbenzenesulfonate **33** as a yellow oil (174 mg, 0.72 mmol, 80% yield). Spectral data is in agreement with the literature.¹⁷⁰

¹H-NMR (400 MHz, CDCl₃): δ 7.79 (d, J_{H8H9} = 8.2 Hz, 2H, H8), 7.34 (d, J_{H8H9} = 8.2 Hz, 2H, H9), 4.05 (t, J_{H1H2} = 6.3 Hz, 2H, H1), 2.45 (s, 3H, H11), 2.16 (td, J_{H4H3} = 6.9 Hz, J_{H4H6} = 2.6 Hz, 2H, H4), 1.92 (t, J_{H4H6} = 2.6 Hz, 1H, H6), 1.82-1.73 (m, 1H, H2), 1.56 (dt, J_{H2H3} = 7.1 Hz, J_{H4H3} = 6.9 Hz, 2H, H3) ppm.

^{13}C NMR (101 MHz, CDCl_3): δ 144.8 (C7), 133.1 (C10), 129.9 (C9), 127.90 (C8), 83.4 (C5), 69.9 (C1), 69.0 (C6), 27.8 (C2), 24.2 (C3), 21.7 (C11), 17.7 (C4) ppm.

HRMS (ESI⁺): (m/z) $\text{C}_{13}\text{H}_{16}\text{NaO}_3\text{S}$ $[\text{M}+\text{Na}]^+$, calculated: 275.0712, found: 275.0710.

6-Bromo-1-hexyne (34)

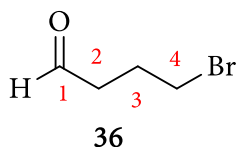


6-Bromo-1-hexyne **34** was synthesized following a literature procedure.¹⁷⁰ LiBr (1.930 g, 22.2 mmol) was added to a stirred solution of tosylate **33** (1.87 g, 7.4 mmol) in dry DMF (22 mL). The mixture was stirred for 24 h at rt. After that time, TLC analysis (EtOAc:Hex, 1:7) revealed complete consumption of starting material ($R_f = 0.3$) and formation of the desired product ($R_f = 0.8$). H_2O (50 mL) was added, and the aqueous phase extracted with Et_2O (3 x 100 mL). The collected organic fractions were washed with brine (2 x 50 mL) dried over Na_2SO_4 , filtered, and concentrated under reduced pressure. 6-bromo-1-hexyne **34** was obtained as a colourless oil (708 mg, 60%). Spectral data is in agreement with the literature.¹⁷⁰ Compound **34** was found to be volatile and was used immediately after purification.

^1H -NMR (400 MHz, CDCl_3): δ 3.46 (t, $J_{\text{H}_2\text{H}_1} = 6.7$ Hz, 2H, H1), 2.27 (td, $J_{\text{H}_3\text{H}_4} = 7.0$ Hz, $J_{\text{H}_6\text{H}_4} = 2.7$ Hz, 2H, H4), 2.08-1.96 (m, 3H, H2-H6), 1.72-1.62 (m, 2H, H3) ppm.

^{13}C -NMR (101 MHz, CDCl_3): δ 83.6 (C5), 68.9 (C6), 33.1 (C1), 31.5 (C2), 26.8 (C3), 17.6 (C4) ppm.

4-Bromo-1-butanal (36)



Method A:

DMSO (128 μ L, 1.76 mmol) in DCM (2 mL) was added to a solution of oxalyl chloride (108 μ L, 1.24 mmol) in DCM (2 mL) at -78 $^{\circ}$ C. After 5 minutes, a solution of 4-Bromo-1-butanol (172 μ L, 1.20 mmol) in DCM (4 mL) was added to the mixture and after 10 minutes, DIPEA (680 μ L, 3.88 mmol) was added. The solution was stirred at -65 $^{\circ}$ C for 15 minutes and for an additional 15 minutes at room temperature. After this time, TLC analysis (DCM:Hex 1:1) showed formation of a new product (R_f = 0.3) and complete consumption of starting material (R_f = 0.1). The solution was poured into 10% citric acid (15 mL) and extracted with DCM (3 x 15 mL). The organic layer was washed with saturated NaHCO_3 (15 mL) and dried over MgSO_4 . The solution was evaporated to dryness and the residue purified by silica column chromatography (1:1 DCM:Hex) to yield 4-Bromo-1-Butanal **36** as a colourless oil (153 mg, 1.12 mmol, 91% yield). Compound **36** was found to be prone to decomposition and was used immediately after purification.

Method B:

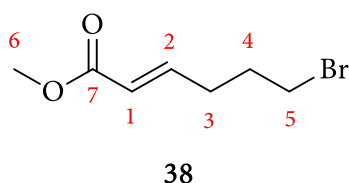
4-Bromo-1-butanol **36** was synthesized following a literature procedure.¹⁹¹ Pyridinium chlorochromate (233 mg, 1.08 mmol) was added in portions to a solution of 4-Bromo-1-butanol (145 mg, 0.980 mmol) in DCM (5 mL) at rt. After the addition was complete, the mixture was stirred at rt for 1.5 h. After this time, TLC analysis (DCM:Hex 1:1) showed formation of a new product (R_f = 0.3) and complete consumption of starting material (R_f = 0.1). Silica gel (1.25 g) was added to the reaction mixture. The solvent was removed under reduced pressure, and Et_2O (10 mL) was then added. The resulting mixture was filtered, and the filter cake was washed with additional Et_2O (10 mL). The filtrate was dried under reduced pressure and purified by column chromatography (DCM:Hex 1:1) to give 4-Bromo-1-butanol **36** as colourless oil (105 mg, 0.76 mmol, 70% yield). Spectral data is in agreement

with the literature.¹⁹¹ Compound **36** was found to be prone to decomposition and was used immediately after purification.

¹H-NMR (400 MHz, CDCl₃): δ 9.81 (s, 1H, H1), 3.45 (t, J_{H3H4} = 6.4 Hz, 2H, H4), 2.71 (dt, J_{H2H3} = 7.0 Hz, J_{H1H2} = 0.9 Hz, 2H, H2), 2.21 (ap, J = 6.7 Hz, 2H, H3) ppm.

¹³C-NMR (101 MHz, CDCl₃): δ 200.9 (C1), 42.3 (C2), 32.9 (C4), 25.1 (C3) ppm.

Methyl (E)-6-bromohex-2-enoate (**38**)

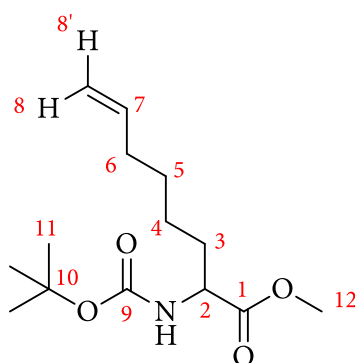


Methyl (E)-6-bromohex-2-enoate **38** was synthesized following a literature procedure.¹⁹¹ A solution of methyl(triphenylphosphoranylidene)acetate (5.5 g, 16.6 mmol) in DCM (50 mL) was added dropwise to a solution of aldehyde **36** (2.1 g, 14.1 mmol) in DCM (30 mL). After the addition was complete, reaction was stirred at rt for 16 h. After this time, TLC analysis (EtOAc:Hex 1:9) showed complete consumption of starting material (R_f = 0.2) and formation of a new product (R_f = 0.3). Silica gel (20 g) was added to the reaction mixture, and solvent was removed under reduced pressure. The filter cake was purified by column chromatography (EtOAc:Hex 1:9) to give compound **38** as colourless oil (2.0 g, 9.63 mmol, 68% yield). Spectral data is in agreement with the literature.¹⁹¹ Compound **38** was found to be prone to decomposition and was used immediately after purification.

¹H-NMR (400 MHz, CDCl₃): δ 6.95 (dt, J_{H2H1} = 15.7 Hz, J_{H2H3} = 7.1 Hz, 1H, H2), 5.91 (d, J_{H2H1} = 15.7 Hz, 1H, H1), 3.76 (s, 3H, H6), 3.44 (t, J_{H5H4} = 6.5 Hz, 2H, H5), 2.41 (aq, J = 7.0 Hz, 2H, H3), 2.05 (ap, J = 6.9 Hz, 2H, H4) ppm.

¹³C-NMR (101 MHz, CDCl₃): δ 166.8 (C7), 147.1 (C2), 122.2 (C1), 51.5 (C6), 32.5 (C5), 30.8 (C4), 30.4 (C3) ppm.

Methyl 2-((*tert*-butoxycarbonyl)amino)oct-7-enoate (41**)**



41

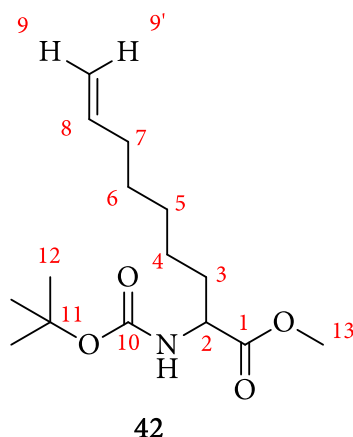
Methyl 2-((*tert*-butoxycarbonyl)amino)oct-7-enoate **41** was synthesized following a modified literature procedure.¹⁶⁵ Cu(OTf)₂ (16 mg, 0.044 mmol), Zn (86 mg, 1.26 mmol), and 5-Br-1-pentene (157 μ L, 1.26 mmol) were added to dehydroalanine **25** (88.5 mg, 0.44 mmol) in sat. NH₄Cl/EtOH (1:1 v/v, 1.5 mL) at rt. After 2.5 h, additional Zn (29 mg, 0.44 mmol) and 5-Br-1-pentene (52 μ L, 0.44 mmol) were added. 1 h later, additional Zn (29 mg, 0.44 mmol) and 5-Br-1-pentene (52 μ L, 0.44 mmol) were added. The reaction mixture was stirred for further 2 h at rt. After this time, TLC analysis (EtOAc:Hex 1:20) showed formation of a new product (R_f = 0.4) and consumption of starting material (R_f = 0.8). The reaction mixture was poured into H₂O and extracted with EtOAc (3 x 10 mL). The combined organic phases were dried over MgSO₄, filtered, and concentrated under reduced pressure. The crude mixture was purified by column chromatography on silica gel (EtOAc:Hex 1:20 to 1:10) to give methyl 2-((*tert*-butoxycarbonyl)amino)oct-7-enoate **41** as colourless oil (79.9 mg, 0.29 mmol, 67% yield).

¹H-NMR (400 MHz, CDCl₃): δ 5.77 (ddt, J_{H8H7} = 17.0 Hz, $J_{H8'H7}$ = 10.2 Hz, J_{H7H6} = 6.9 Hz, 1H, H7), 4.99 (dd, J_{H8H7} = 17.0 Hz, $J_{H8H8'}$ = 1.8 Hz, 1H, H8), 4.94 (dd, $J_{H8'H7}$ = 10.2 Hz, $J_{H8H8'}$ = 1.8 Hz, 1H, H8'), 4.34-4.24 (m, 1H, H2), 3.73 (s, 3H, H12), 2.04 (q, J_{H7H6} = 6.9 Hz, 2H, H6), 1.84-1.74 (m, 1H, H3), 1.68-1.55 (m, 1H, H3'), 1.44 (s, 9H, H11), 1.42-1.25 (m, 4H, H4-H5) ppm.

¹³C-NMR (101 MHz, CDCl₃): δ 170.0 (C1), 159.8 (C9) 138.7 (C7), 114.7 (C8), 79.8 (C10), 53.5 (C2), 52.3 (12), 33.6 (C6), 32.7 (C3), 28.5 (C5), 28.5 (C11), 24.8 (C4) ppm.

HRMS (ESI⁺): (m/z) C₁₄H₂₅NNaO₄ [M+Na]⁺, calculated: 294.1676, found: 294.1671.

Methyl 2-((*tert*-butoxycarbonyl)amino)non-8-enoate (42)



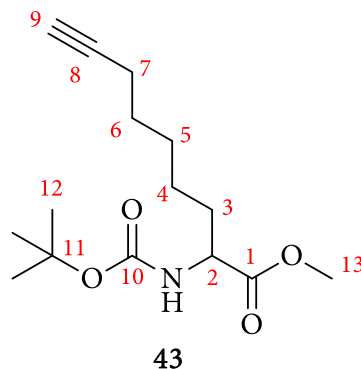
Methyl 2-((*tert*-butoxycarbonyl)amino)non-8-enoate **42** was synthesized following a modified literature procedure.¹⁶⁵ Cu(OTf)₂ (160 mg, 0.44 mmol), Zn (867 mg, 13.3 mmol), and 6-Br-1-hexene (1.77 mL, 13.3 mmol) were added to dehydroalanine **25** (887.4 mg, 4.41 mmol) in sat. NH₄Cl/EtOH (1:1 v/v, 30 mL) at rt. After 2.5 h, additional Zn (289 mg, 4.4 mmol) and 6-Br-1-hexene (591 μ L, 4.4 mmol) were added, and 1 h later, additional Zn (289 mg, 4.4 mmol) and 6-Br-1-hexene (591 μ L, 4.4 mmol) were added. The reaction mixture was stirred for 2 h at rt. After that time, TLC analysis (EtOAc:Hex 1:20) showed formation of product (R_f = 0.46) and consumption of starting material (R_f = 0.8). The reaction mixture was poured into H₂O (30 mL) and extracted with EtOAc (3 x 70 mL). The combined organic phases were dried over MgSO₄, filtered, and concentrated under reduced pressure. The crude mixture was purified by column chromatography on silica gel (EtOAc:Hex 1:20 to 1:34) to give methyl 2-((*tert*-butoxycarbonyl)amino)non-8-enoate **42** as colourless oil (1.0 g, 3.50 mmol, 80% yield).

¹H-NMR (400 MHz, CDCl₃): δ 5.78 (ddt, $J_{H8H9'} = 16.9$ Hz, $J_{H8H9} = 10.2$ Hz, $J_{H8H7} = 6.8$ Hz, 1H, H8), 4.98 (dd, $J_{H8H9'} = 16.9$ Hz, $J_{H9H9'} = 1.9$ Hz, 1H, H9'), 4.93 (dd, $J_{H8H9} = 10.2$ Hz, $J_{H9H9'} = 1.9$ Hz, 1H, H9), 4.32-4.24 (m, 1H, H2), 3.73 (s, 3H, H13), 2.07 (aq, $J = 6.8$ Hz, 2H, H7), 1.84-1.71 (m, 2H, H3), 1.66-1.55 (m, 1H, H3'), 1.44 (s, 9H, H12), 1.40-1.21 (m, 6H, H4-H5-H6) ppm.

¹³C-NMR (101 MHz, CDCl₃): δ 173.5 (C1), 155.4 (C10), 138.9 (C8), 114.4 (C7), 79.8 (C11), 53.4 (C2), 52.2 (C13), 33.5 (C7), 32.7 (C3), 31.6 (C5), 28.6 (C6), 28.3 (C12), 25.1 (C4) ppm.

HRMS (ESI⁺): (*m/z*) C₁₅H₂₇NNaO₄ [M+Na]⁺, calculated: 308.1832, found: 308.1837.

Methyl 2-(*tert*-butylamino)non-8-ynoate (43)



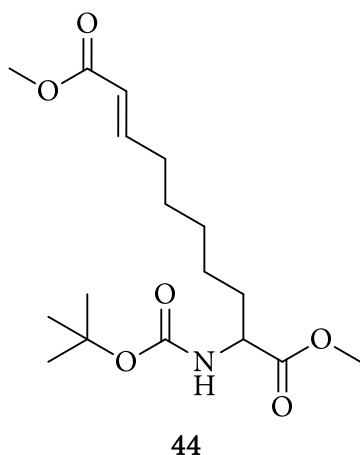
Methyl 2-(*tert*-butylamino)non-8-ynoate **43** was synthesized following a modified literature procedure.¹⁶⁵ Cu(OTf)₂ (23 mg, 0.062 mmol), Zn (123 mg, 1.86 mmol), and 6-Bromohex-1-yne **34** (300 mg, 1.86 mmol) were added to dehydroalanine **25** (125 mg, 0.62 mmol) in sat. NH₄Cl/EtOH (1:1, 4 mL) and the reaction mixture was stirred at room temperature for 2.5 h. After that time, additional Zn (40 mg, 0.62 mmol) and 6-Bromohex-1-yne **34** (100 mg, 0.62 mmol) were added, and 1 h later, additional Zn (40 mg, 0.62 mmol) and 6-Bromohex-1-yne **34** (100 mg, 0.62 mmol) were added. The reaction mixture was stirred for 2 h at room temperature. After this time, TLC analysis (EtOAc:Hex 1:9) showed formation of a new product (*R*_f = 0.3) and consumption of starting material (*R*_f = 0.7). The reaction mixture was poured into H₂O and extracted with EtOAc (3 x 10 mL). The combined organic phases were dried over MgSO₄, filtered, and concentrated under reduced pressure. The crude mixture was purified by column chromatography on silica gel (EtOAc:Hex 1:10) to give compound **43** as colourless oil (96 mg, 0.34 mmol, 55% yield).

¹H-NMR (400 MHz, CDCl₃): δ 7.23 (d, *J*_{H_NH₂} = 7.8 Hz, 1H, NH), 3.93 (td, *J*_{H_NH₁} = 7.8 Hz, *J*_{H₂H₃} = 5.5 Hz, 1H, H₂), 3.62 (s, 3H, H₁₃), 2.73 (t, *J*_{H₉H₇} = 2.6 Hz, 1H, H₉), 2.14 (td, *J*_{H₇H₆} = 6.9 Hz, *J*_{H₇H₉} = 2.6 Hz, 2H, H₇), 1.67-1.50 (m, 2H, H₃), 1.46-1.22 (m, 15H, H₁₂-H₄-H₅-H₆) ppm.

¹³C-NMR (101 MHz, CDCl₃): δ 173.7 (C₁), 156.1 (C₁₀), 84.9 (C₈), 78.6 (C₁₁), 71.6 (C₉), 53.9 (C₂), 52.1 (C₁₃), 31.0 (C₃), 28.7 (C₁₂), 28.2 (C₆), 28.1 (C₄), 25.4 (C₅), 18.0 (C₇) ppm.

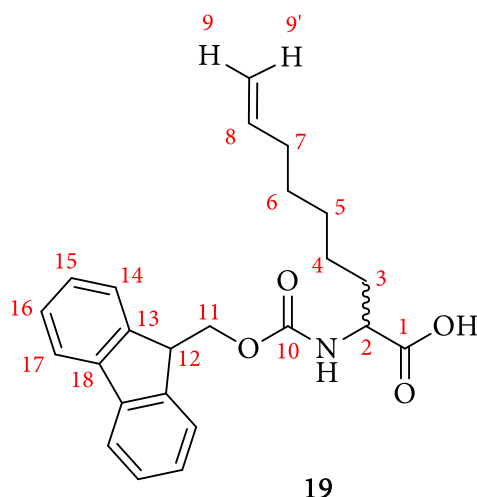
HRMS (ESI⁺): (*m/z*) C₁₅H₂₅NNaO₄ [M+Na]⁺, calculated: 306.1676, found: 306.1678.

Dimethyl (E)-9-((*tert*-butoxycarbonyl)amino)dec-2-enedioate (44**, attempted)**



The synthesis of methyl 2-((*tert*-butoxycarbonyl)amino)non-8-enoate **44** was attempted following a modified literature procedure.¹⁶⁵ Cu(OTf)₂ (160 mg, 0.44 mmol), Zn (867 mg, 13.3 mmol), and methyl (E)-6-bromohex-2-enoate **38** (1.77 mL, 13.3 mmol) were added to dehydroalanine **25** (887.4 mg, 4.41 mmol) in sat. NH₄Cl/EtOH (1:1 v/v, 30 mL) at rt. After 2.5 h, additional Zn (289 mg, 4.4 mmol) and methyl (E)-6-bromohex-2-enoate **38** (591 μL, 4.4 mmol) were added, and 1 h later, additional Zn (289 mg, 4.4 mmol) and methyl (E)-6-bromohex-2-enoate **38** (591 μL, 4.4 mmol) were added. The reaction mixture was stirred for 2 h at rt. After that time, TLC analysis (EtOAc:Hex 1:20) showed formation of a complex mixture products and consumption of starting material (*R*_f = 0.8). The reaction mixture was poured into H₂O (30 mL) and extracted with EtOAc (3 x 70 mL). The combined organic phases were dried over MgSO₄, filtered, and concentrated under reduced pressure. The crude mixture was analysed by ¹H-NMR and HRMS which did not indicate formation of the desired compound **44**.

2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)non-8-enoic acid (**19**)



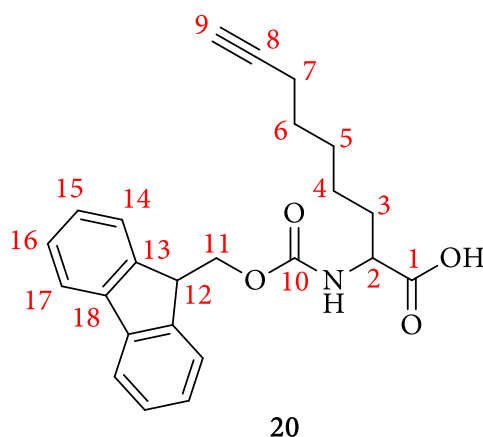
2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)non-8-enoic acid **19** was synthesized following a modified literature procedure.¹⁷¹ Boc protected methyl ester **42** (100 mg, 0.35 mmol) was dissolved in a 20% (v/v) mixture of TFA:DCM (1.5 mL). Reaction mixture was stirred for 1 h at rt. After this time, TLC analysis (EtOAc:Hex 3:7) revealed complete consumption of starting material ($R_f = 0.3$) and formation of a new product ($R_f = 0.1$). The mixture was concentrated under reduced pressure. The resulting solid was dissolved in MeOH (2.5 mL) and the solution was heated to 30 °C. 2.8 mL of 1M NaOH was added dropwise, and the mixture was stirred overnight at 30 °C. After this time, TLC analysis (DCM:MeOH 4:1) revealed complete consumption of starting material ($R_f = 0.1$) and formation of a new product ($R_f = 0.3$). The resulting solution was dissolved in 4 mL of H₂O, and NaHCO₃ (88 mg, 1.05 mmol) was added. A solution of Fmoc-OSu (130 mg, 0.39 mmol) in acetone (6 mL) was added after 5 minutes. pH was adjusted to 8-9 with 1M aq. NaOH and the reaction mixture was stirred for 20 h at room temperature. After that time, TLC analysis (DCM:MeOH 13:1) showed formation of product ($R_f = 0.3$) and complete consumption of starting material ($R_f = 0.1$). The organics were concentrated under reduced pressure. The aqueous phase was acidified with aq. 1M HCl to pH 2, extracted with EtOAc (3 x 15 mL) and washed with brine (2 x 20 mL). The combined organic phases were dried over MgSO₄ and concentrated under reduced pressure. Purification by column chromatography (DCM:MeOH 13:1) afforded **19** as a clear oil (129 mg, 0.33 mmol, 93% yield). Spectral data is in agreement with the literature.¹⁹²

¹H-NMR (600 MHz, DMSO-*d*₆): δ 12.58 (br. s, 1H, COOH), 7.90 (d, J_{H16H17} = 7.5 Hz, 2H, H17), 7.74 (d, J_{H14H15} = 4.6 Hz, 2H, H14), 7.60 (s, 1H, NH), 7.43 (at, J = 7.4 Hz, 2H, H16), 7.33 (at, J = 7.5 Hz, 2H, H15), 5.80 (ddt, J_{H8H9'} = 16.8 Hz, J_{H8H9} = 9.8 Hz, J_{H8H7} = 6.4 Hz, 1H, H8), 5.01 (dd, J_{H8H9'} = 16.8 Hz, J_{H9H9'} = 1.9 Hz, 1H, H9'), 4.95 (dt, J_{H8H9} = 9.8 Hz, J_{H9H9'} = 1.9 Hz, 1H, H9), 4.36-4.17 (m, 3H, H11-H12), 3.96 – 3.88 (m, 1H, H1), 2.02 (t, J_{H8H7} = 6.4 Hz, 2H, H7), 1.75-1.69 (m, 2H, H3), 1.63-1.54 (m, 2H, H4), 1.41-1.21 (m, 4H, H5-H6) ppm.

¹³C-NMR (101 MHz, DMSO-*d*₆): δ 174.5 (C1), 156.6 (C10), 144.3 (C13), 141.2 (C18), 139.5 (C8), 128.1 (C15), 127.5 (C16), 125.8 (C14), 120.6 (C17), 115.2 (C9), 66.0 (C11), 54.3 (C2), 47.2 (C12), 33.3 (C7), 31.2 (C3), 28.5 (C6), 25.7 (C5), 25.2 (C4) ppm.

HRMS (ESI⁺): (*m/z*) C₂₄H₂₈NNaO₄ [M+Na]⁺, calculated: 416.183229, found: 434.181719.

2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)non-8-ynoic acid (20)



2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)non-8-ynoic acid **20** was synthesized following a modified literature procedure.¹⁷¹ Boc protected methyl ester **43** (100 mg, 0.35 mmol) was dissolved in a 20% (v/v) mixture of TFA:DCM (1.5 mL). Reaction mixture was stirred for 1 h at room temperature. After this time, TLC analysis (EtOAc:Hex 3:7) revealed complete consumption of starting material (*R*_f = 0.3) and formation of a new product (*R*_f = 0.1). The mixture was concentrated under reduced pressure. The resulting solid was dissolved in MeOH (2.5 mL) and the solution was heated to 30 °C. Then, 2.8 mL of 1M NaOH was added dropwise, and the mixture was stirred overnight at 30°C. After this time, TLC

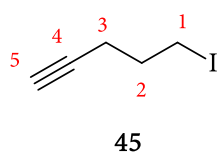
analysis (DCM:MeOH 4:1) revealed complete consumption of starting material ($R_f = 0.1$) and formation of a new product ($R_f = 0.3$). The resulting solution was dissolved in 4 mL of H_2O , and $NaHCO_3$ (88 mg, 1.05 mmol) was added. A solution of Fmoc-OSu (130 mg, 0.39 mmol) in acetone (6 mL) was added after 5 minutes. pH was adjusted to 8-9 and the reaction mixture was stirred for 20h at room temperature. After that time, TLC analysis (DCM:MeOH 13:1) showed formation of product ($R_f = 0.3$) and complete consumption of starting material ($R_f = 0.1$). The organics were concentrated under reduced pressure. The aqueous phase was acidified with aq. 1M HCl to pH 2. This was then extracted with EtOAc (3 x 15 mL) and washed with brine (2 x 20 mL). The combined organic phases were dried over $MgSO_4$ and concentrated under reduced pressure. Purification by column chromatography (DCM:MeOH 13:1) afforded **20** as a clear oil (78 mg, 0.20 mmol, 57% yield). Spectral data is in agreement with the literature.¹⁹³

1H -NMR (400 MHz, $CDCl_3$): δ 12.84 (br. s, 1H, COOH), 7.90 (d, $J_{H17H16} = 7.5$ Hz, 2H, H17), 7.74 (d, $J_{H14H15} = 7.5$ Hz, 2H, H14), 7.42 (at, $J = 7.5$ Hz, 2H, H16), 7.33 (at, $J = 7.5$ Hz, 2H, H15), 4.36-4.18 (m, 3H, H12-H11), 3.90 – 3.80 (m, 1H, H2), 2.74 (t, $J_{H7H9} = 2.4$ Hz, 1H, H9), 2.14 (td, $J_{H6H7} = 6.8$ Hz, $J_{H7H9} = 2.4$ Hz, 2H, H7), 1.75-1.65 (m, 1H, H3), 1.63-1.53 (m, 1H, H3'), 1.47-1.38 (m, 2H, H4), 1.36-1.24 (m, 4H, H5-H6) ppm.

^{13}C -NMR (101 MHz, $CDCl_3$): δ 144.3 (C13), 141.2 (C18), 128.1 (C16), 127.8 (C15), 125.7 (C14), 120.6 (C17), 85.0 (C8), 71.6 (C9), 65.9 (C11), 54.3 (C2), 47.2 (C12), 32.7 (C3), 28.5 (C4), 28.4 (C6), 25.3 (C5), 18.1 (C7).

HRMS (APCI⁺): (m/z) $C_{24}H_{26}NO_4$ $[M+H]^+$, calculated: 392.18563, found: 392.18570.

5-I-1-pentyne (45)

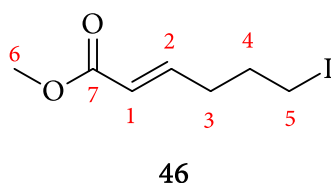


5-I-1-pentyne **45** was synthesized following a literature procedure.¹⁹⁴ 5-Cl-1-pentyne (3.0 mL, 28.3 mmol) was dissolved in 200 mL of distilled acetone. After a few minutes, NaI (2.9 g, 141.6 mmol) was added to the reaction mixture. The reaction mixture was stirred under reflux for 48 h until TLC analysis (neat Hex) showed complete consumption of starting material ($R_f = 1$) and formation of a new product ($R_f = 0.3$). After cooling to room temperature, 150 mL of H₂O was added to the reaction mixture. The solution was extracted with Hex (3 x 50 mL). The combined organic layers were washed with brine and dried over Na₂SO₄. The solvent was evaporated under reduced pressure to give compound **45** as a colourless oil (2.2 g, 25.47 mmol, 90% yield). Spectral data is in agreement with the literature.¹⁹⁴

¹H-NMR (400 MHz, CDCl₃): δ 3.34 (t, $J_{H1H2} = 6.7$ Hz, 2H, H1), 2.37 (dt, $J_{H3H2} = 6.7$ Hz, $J_{H3H5} = 2.6$ Hz, 2H, H3), 2.02 (ap, $J = 6.7$ Hz, 2H, H2), 2.00 (t, $J_{H5H3} = 6.7$ Hz, 2H, H5) ppm.

¹³C-NMR (101 MHz, CDCl₃): δ 82.3 (C4), 69.5 (C5), 31.8 (C2), 19.5 (C3), 5.0 (C1) ppm.

methyl (E)-6-iodohex-2-enoate (46)



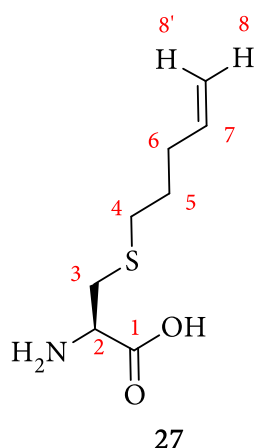
Methyl (E)-6-iodohex-2-enoate **46** was synthesized following a modified literature procedure.¹⁹⁴ methyl (E)-6-bromohex-2-enoate **38** (2.0 g, 9.63 mmol) was dissolved in 200 mL of distilled acetone. After a few minutes, NaI (3.6 g, 24.1 mmol) was added to the reaction mixture. The reaction mixture was stirred under reflux for 7 h until TLC analysis (EtOAc:Hex 1:9) showed complete consumption of starting material ($R_f = 0.2$) and formation of a new product ($R_f = 0.3$). After cooling to room temperature, the precipitate was dissolved in 150 mL of distilled H₂O. The reaction solution was extracted with Hex (4 x 100 mL). The

combined organic layers were washed with brine and dried over Na₂SO₄. The solvent was evaporated under reduced pressure to give compound **46** as a colourless oil (2.2 g, 8.67 mmol, 90% yield). Spectral data is in agreement with the literature.¹⁹⁵ Compound **XX** was found to be prone to decomposition and was used immediately after purification.

¹H-NMR (400 MHz, CDCl₃): δ 6.93 (dt, J_{H₂H₁} = 15.6 Hz, J_{H₂H₃} = 7.0 Hz, 1H, H₂), 5.92 (dt, J_{H₂H₁} = 15.6 Hz, J_{H₁H₃} = 1.5 Hz, 1H, H₁), 3.76 (s, 3H, H₆), 3.22 (t, J_{H₅H₄} = 6.8 Hz, 2H, H₅), 2.36 (qd, J_{H₂H₃} = 7.0 Hz, J_{H₁H₃} = 1.5 Hz, 2H, H₃), 2.00 (ap, J = 7.0 Hz, 2H, H₄) ppm.

¹³C-NMR (101 MHz, CDCl₃): δ 166.8 (C₇), 146.9 (C₂), 122.2 (C₁), 51.5 (C₆), 32.7 (C₃), 31.4 (C₄), 5.4 (C₅) ppm.

S-(pent-4-en-1-yl)-L-cysteine (**27**)



S-(pent-4-en-1-yl)-L-cysteine **27** was synthesized following a modified literature procedure.¹⁹⁶ To a solution of L-cysteine (1.05 g, 8.66 mmol) in 2 M NH₄OH:EtOH (5:4, v/v, 27 mL) was added 5-Br-1-pentene (1.55 mL, 13.4 mmol) and the mixture was stirred for 20 h at rt. After this time, TLC analysis (DCM:MeOH 4:1) showed formation of product (*R_f* = 0.4) and complete consumption of starting material (*R_f* = 0.1). Reaction mixture was then concentrated under reduced pressure, and the resulting mixture was filtrated to obtain S-(pent-4-en-1-yl)-L-cysteine **27** as a white solid (1.4 g, 7.36 mmol, 85% yield). Spectral data is in agreement with the literature.¹⁶⁷

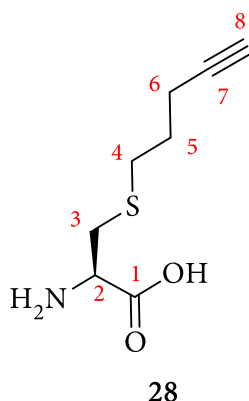
¹H-NMR (400 MHz, D₂O): δ 5.81 (ddt, J_{H7H8'} = 17.0 Hz, J_{H7H8} = 10.2 Hz, J_{H6H7} = 6.8 Hz, 1H, H7), 5.02 (dd, J_{H7H8'} = 17.0 Hz, J_{H8H8'} = 1.8 Hz, 1H, H8), 4.95 (dd, J_{H7H8} = 10.2 Hz, J_{H8H8'} = 1.8 Hz, 1H, H8'), 3.83 (dd, J_{H3'H2} = 7.4 Hz, J_{H3H2} = 4.3 Hz, 1H, H2), 3.05 (dd, J_{H3H3'} = 14.8 Hz, J_{H3H2} = 4.3 Hz, 1H, H3), 2.94 (dd, J_{H3H3'} = 14.8 Hz, J_{H3'H2} = 7.4 Hz, 1H, H3'), 2.55 (t, J_{H4H5} = 7.2 Hz, 2H, H4), 2.09 (aq, J = 7.2 Hz, 2H, H6), 1.63 (ap, J = 7.2 Hz, 2H, H5) ppm.

¹³C-NMR (101 MHz, D₂O): δ 173.0 (C1), 138.6 (C7), 115.0 (C8), 53.6 (C2), 32.0 (C6), 31.9 (C3), 30.7 (C4), 27.8 (C5) ppm.

HRMS (ESI): (m/z) C₈H₁₄NO₂S [M-H]⁻, calculated: 188.0751, found: 188.0749.

m.p.: 182-185 °C.

S-(pent-4-yn-1-yl)-L-cysteine (28)



Method A:

S-(pent-4-yn-1-yl)-L-cysteine **28** was synthesized following a modified literature procedure.¹⁹⁶ To a solution of L-cysteine (812 mg, 6.7 mmol) in 2 M NH₄OH:EtOH (5:4, v/v, 18 mL) was added 5-Cl-1-pentyne (1.1 mL, 10.4 mmol) and the mixture was stirred for 20 h at rt. After this time, TLC analysis (2:1 DCM/MeOH) showed formation of product (R_f = 0.3) and complete consumption of starting material (R_f = 0.1). The reaction mixture was then concentrated under reduced pressure, and the resulting solid was filtrated and washed with EtOH (200 mL). S-(pent-4-yn-1-yl)-L-cysteine **28** was isolated as a white solid (806 mg, 4.29 mmol, 64% yield). Spectral data is in agreement with the literature.¹⁶⁷

Method B:

S-(pent-4-yn-1-yl)-L-cysteine **28** was synthesized following a modified literature procedure.¹⁹⁶ To a solution of L-cysteine (2.04 g, 16.9 mmol) in 2 M NH₄OH:EtOH (5:4, v/v, 162 mL) was added 5-I-1-pentyne **45** (4.92 g, 25.36 mmol) and the mixture was stirred for 20 h at rt. After this time, TLC analysis (2:1 DCM/MeOH) showed formation of product (R_f = 0.3) and complete consumption of starting material (R_f = 0.1). The reaction mixture was then concentrated under reduced pressure, and the resulting solid was filtrated and washed with EtOH (200 mL). S-(pent-4-yn-1-yl)-L-cysteine **28** was isolated as a white solid (2.75 g, 14.70 mmol, 87% yield). Spectral data is in agreement with the literature.¹⁶⁷

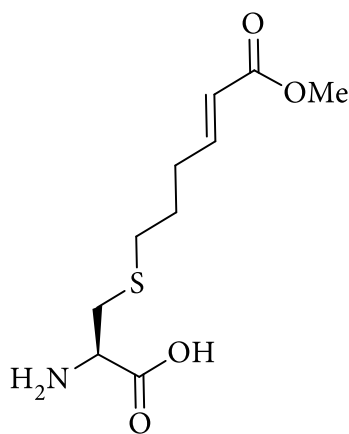
¹H-NMR (400 MHz, CDCl₃): δ 4.04 (m, 1H, H₂), 3.10-2.80 (m, 3H, H₈, H₃), 2.58 (t, $J_{H_4H_5}$ = 7.2 Hz, 2H, H₄), 2.22 (m, 2H, H₆), 1.68 (ap, J = 7.1 Hz, 2H, H₅) ppm.

¹³C-NMR (101 MHz, CDCl₃): δ 173.8 (C₁), 84.4 (C₇), 65.9 (C₈), 55.0 (C₂), 47.2 (C₁₁), 30.8 (C₃), 28.4 (C₅), 25.5 (C₄), 17.6 (C₆) ppm.

HRMS (ESI): (m/z) C₈H₁₂NO₂S [M-H]⁻, calculated: 186.059423, found: 186.059702.

m.p.: 176-179 °C.

(E)-S-(6-methoxy-6-oxohex-4-en-1-yl)-L-cysteine (**29**, attempted)

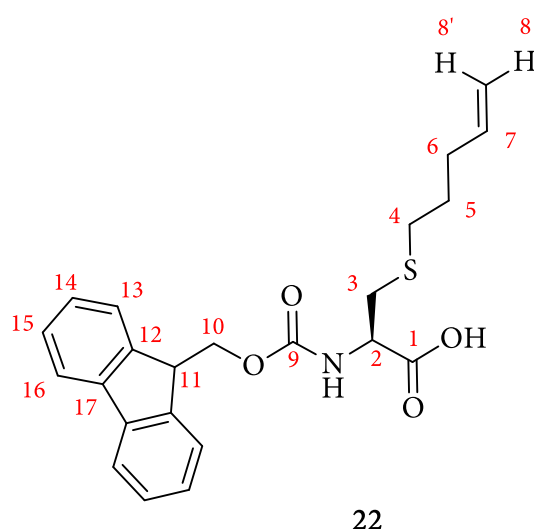


29

(E)-S-(6-methoxy-6-oxohex-4-en-1-yl)-L-cysteine **29** was synthesized following a modified literature procedure.¹⁹⁶ To a solution of L-cysteine (121 mg, 0.31 mmol) in 2 M NH₄OH:EtOH

(5:4, v/v, 3 mL) was added methyl (E)-6-iodohex-2-enoate **46** (100 mg, 0.40 mmol) and the mixture was stirred for 30 h at rt. After this time, TLC analysis (DCM:MeOH 4:1) showed complete consumption of starting material ($R_f = 0.1$) and formation of a complex mixture of products. Reaction mixture was then concentrated under reduced pressure, and the resulting mixture was filtrated. The crude mixture was analysed by ^1H -NMR and HRMS which did not indicate formation of the desired compound **29**.

N-(((9H-fluoren-9-yl)methoxy)carbonyl)-S-(pent-4-en-1-yl)-L-cysteine (**22**)



N-(((9H-fluoren-9-yl)methoxy)carbonyl)-S-(pent-4-en-1-yl)-L-cysteine **22** was synthesized following a modified literature procedure.¹⁷¹ NaHCO_3 (1.87 g, 22.2 mmol) was added to a solution of S-(pent-4-en-1-yl)-L-cysteine **27** (1.4 g, 7.40 mmol) in H_2O (30 mL). A solution of Fmoc-OSu (2.75 g, 8.13 mmol) in acetone (45 mL) was added after 5 minutes. The pH was adjusted to 8-9 with 1M aq. NaOH and the reaction mixture was stirred for 20 h at rt. After that time, TLC analysis (DCM:MeOH 13:1) showed formation of product ($R_f = 0.3$) and complete consumption of starting material ($R_f = 0.1$). The organics were concentrated under reduced pressure. The aqueous phase was washed with Et_2O (2 x 80 mL) then acidified with aq. 1M HCl to pH 2. This was then extracted with EtOAc (5 x 80 mL). The combined organic phases were dried over MgSO_4 and concentrated under reduced pressure. Purification by column chromatography (DCM:MeOH 13:1) afforded N-(((9H-fluoren-9-

yl)methoxy)carbonyl)-S-(pent-4-en-1-yl)-L-cysteine **22** as a white solid (2.40 g, 5.83 mmol, 79% yield). Spectral data is in agreement with the literature.¹⁹⁶

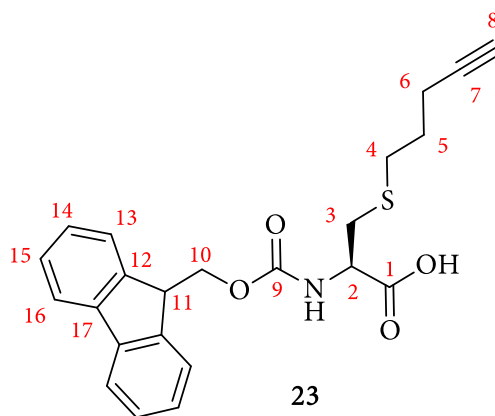
¹H-NMR (400 MHz, CDCl₃): δ 12.32 (br. s, 1H, COOH), 7.89 (d, $J_{\text{H16H15}} = 7.5$ Hz, 2H, H16), 7.74 (d, $J_{\text{H13H14}} = 7.5$ Hz, 2H, H13), 7.42 (at, $J = 7.4$ Hz, 2H, H15), 7.33 (at, $J = 7.4$ Hz, 2H, H14), 5.78 (ddt, $J_{\text{H7H8}'} = 16.9$ Hz, $J_{\text{H7H8}} = 10.2$ Hz, $J_{\text{H6H7}} = 6.9$ Hz, 1H, H7), 5.01 (dd, $J_{\text{H7H8}'} = 16.9$ Hz, $J_{\text{H8H8}'} = 1.7$ Hz, 1H, H8'), 4.96 (d, $J_{\text{H7H8}} = 10.2$ Hz, 1H, H8), 4.35 - 4.28 (m, 2H, H10), 4.25 (t, $J_{\text{H10H11}} = 6.5$ Hz, 1H, H11), 4.14 (td, $J_{\text{H2H3}'} = 9.0$, $J_{\text{H3H2}} = 4.6$ Hz, 1H, H2), 2.92 (dd, $J_{\text{H3H3}'} = 13.7$, $J_{\text{H3H2}} = 4.6$ Hz, 1H, H3), 2.76 (dd, $J_{\text{H3H3}'} = 13.7$, $J_{\text{H2H3}'} = 9.0$ Hz, 1H, H3'), 2.57-2.49 (m, 2H, H4), 2.09 (q, $J_{\text{H6H7}} = 6.9$ Hz, 2H, H6), 1.60 (ap, $J = 7.4$ Hz, 2H, H5) ppm.

¹³C-NMR (101 MHz, CDCl₃): δ 172.8 (C1), 156.5 (C9), 144.3 (C12), 141.2 (C17), 138.4 (C7), 128.1 (C15), 127.5 (C14), 125.8 (C13), 120.6 (C16), 115.7 (C8), 66.2 (C10), 54.6 (C2), 47.1 (C11), 33.2 (C3), 32.6 (C6), 31.3 (C4), 28.7 (C5) ppm.

HRMS (ESI⁺): (m/z) C₂₃H₂₅NNaO₄S [M+Na]⁺, calculated: 434.1397, found: 434.1398.

m.p.: 126-127 °C.

N-(((9H-fluoren-9-yl)methoxy)carbonyl)-S-(pent-4-yn-1-yl)-L-cysteine (**23**)



N-(((9H-fluoren-9-yl)methoxy)carbonyl)-S-(pent-4-yn-1-yl)-L-cysteine **23** was synthesized following a modified literature procedure.¹⁷¹ NaHCO₃ (1.07 g, 12.78 mmol) was added to a solution of S-(pent-4-yn-1-yl)-L-cysteine **28** (806 mg, 4.26 mmol) in H₂O (24 mL). A solution of Fmoc-OSu (1.58 g, 4.68 mmol) in acetone (36 mL) was added after 5 minutes. The pH was adjusted to 8-9 with 1M NaOH and the reaction mixture was stirred for 20 h at rt. After that

time, TLC analysis (13:1 DCM:MeOH) showed formation of product ($R_f = 0.3$) and complete consumption of starting material ($R_f = 0.1$). The organics were concentrated under reduced pressure. The aqueous phase was washed with Et₂O (2 x 60 mL) then acidified with aq. 1M HCl to pH 2. This was then extracted with EtOAc (3 x 100 mL). The combined organic phases were washed with brine (2 x 100 mL), dried over MgSO₄ and concentrated under reduced pressure. Purification by column chromatography (DCM:MeOH 13:1) afforded N-(((9H-fluoren-9-yl)methoxy)carbonyl)-S-(pent-4-yn-1-yl)-L-cysteine **23** as a white solid (508 mg, 1.24 mmol, 29% yield). Spectral data are in agreement with the literature.¹⁹⁷

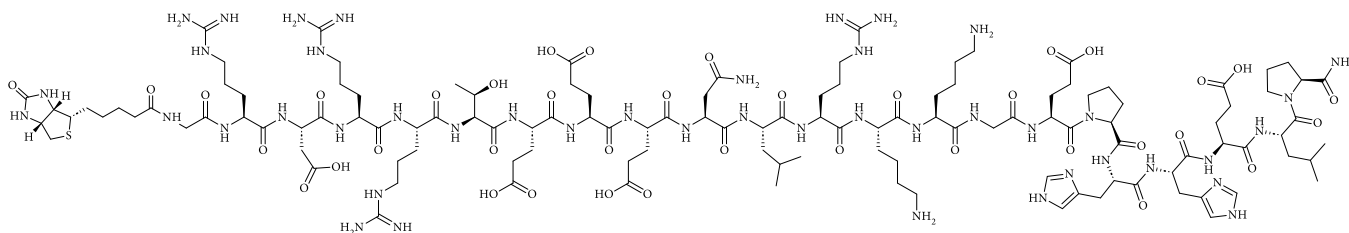
¹H-NMR (400 MHz, CDCl₃): δ 12.84 (br. s, 1H, COOH), 7.87 (d, $J_{H16H15} = 7.5$ Hz, 2H, H16), 7.71 (d, $J_{H13H14} = 7.5$ Hz, 2H, H13), 7.40 (at, $J = 7.4$ Hz, 2H, H15), 7.31 (at, $J = 7.4$ Hz, 2H, H14), 4.36-4.20 (m, 3H, H10-H11), 4.10 (td, $J_{H2H3'} = 9.0$ Hz, $J_{H3H2} = 4.5$ Hz, 1H, H2), 2.89 (dd, $J_{H3H3'} = 13.7$ Hz, $J_{H3H2} = 4.5$ Hz, 1H, H3), 2.78 (t, $J_{H6H8} = 2.7$ Hz, 1H, H8), 2.75 (dd, $J_{H3H3'} = 13.7$ Hz, $J_{H2H3'} = 9.0$ Hz, 1H, H3'), 2.58 (t, $J_{H4H5} = 7.2$ Hz, 2H, H4), 2.22 (td, $J_{H6H5} = 7.2$ Hz, $J_{H6H8} = 2.7$ Hz, 2H, H6), 1.69 (p, $J_{H6H5} = J_{H4H5} = 7.2$ Hz, 2H, H5) ppm.

¹³C-NMR (101 MHz, CDCl₃): δ 173.4 (C1), 156.5 (C9), 144.3 (C12), 141.2 (C17), 128.1 (C15), 127.6 (C14), 125.8 (C13), 120.6 (C16), 84.2 (C7), 72.1 (C8) 66.1 (C10), 55.2 (C2), 47.1 (C11), 33.6 (C3), 30.9 (C4), 28.5 (C5), 17.2 (C6) ppm.

HRMS (ESI⁺): (m/z) C₂₃H₂₄NO₄S [M+H]⁺, calculated: 410.1421, found: 410.1419.

m.p.: 160-161 °C.

p53 279-300 native sequence biotinylated (50)



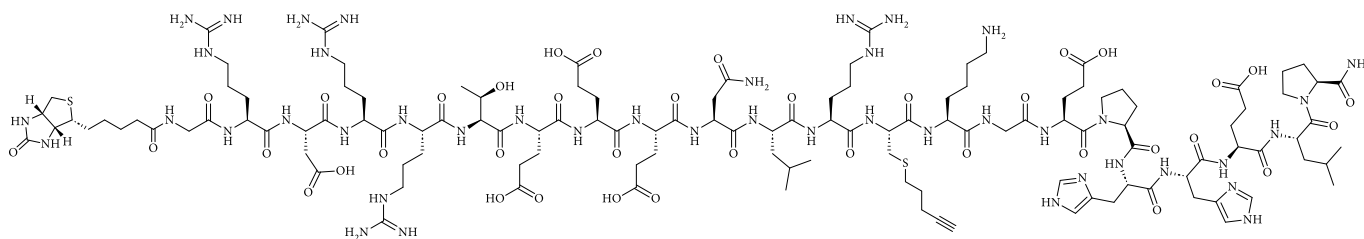
50

Fmoc-Pro-OH (270 mg, 0.8 mmol) was loaded to Rink amide AM resin (256 mg, 0.2 mmol) as per General Procedure 1. Fmoc-Gly-OH (178 mg, 0.6 mmol), Fmoc-Arg(Pbf)-OH (390 mg, 0.6 mmol), Fmoc-Asp(*t*Bu)-OH (247 mg, 0.6 mmol), Fmoc-Thr(*t*Bu)-OH (239 mg, 0.6 mmol), Fmoc-Glu(*t*Bu)-OH (255 mg, 0.6 mmol), Fmoc-Asn(Trt)-OH (358 mg, 0.6 mmol), Fmoc-Leu-OH (212 mg, 0.6 mmol), Fmoc-Lys(*t*Bu)-OH (281 mg, 0.6 mmol), Fmoc-Pro-OH (203 mg, 0.6 mmol), Fmoc-His(Trt)-OH (372 mg, 0.6 mmol), were coupled as per General Procedure 3. On resin biotinylation was performed according to General Procedure 5. The peptide was cleaved from the resin using General Procedure 4, yielding the crude product as a white solid. The peptide was purified by C₁₈ semi-preparative HPLC with a gradient of 5% MeCN (+ 0.1% TFA) with 95% H₂O (+ 0.1% TFA) to 50% MeCN (+ 0.1% TFA) with 50% H₂O (+ 0.1% TFA).

HRMS (ESI⁺): (*m/z*) C₁₂₁H₂₀₅N₄₄O₃₈S [M+6H]⁶⁺, calculated: 485.7525, found: 485.7508. (*m/z*) C₁₂₁H₂₀₄N₄₄O₃₈S [M+5H]⁵⁺, calculated: 582.7015, found: 582.7011. (*m/z*) C₁₂₁H₂₀₃N₄₄O₃₈S [M+4H]⁴⁺, calculated: 728.1251, found: 708.1223.

HPLC: R_T = 9.62 min (5-95% MeCN in H₂O over 30 min, 0.1% TFA modifier, Ascentis Express C18, 2.7 μm, 10 mm x 4.6 mm).

p53 279-300 sequence K291Alkyne mutant biotinylated (51)



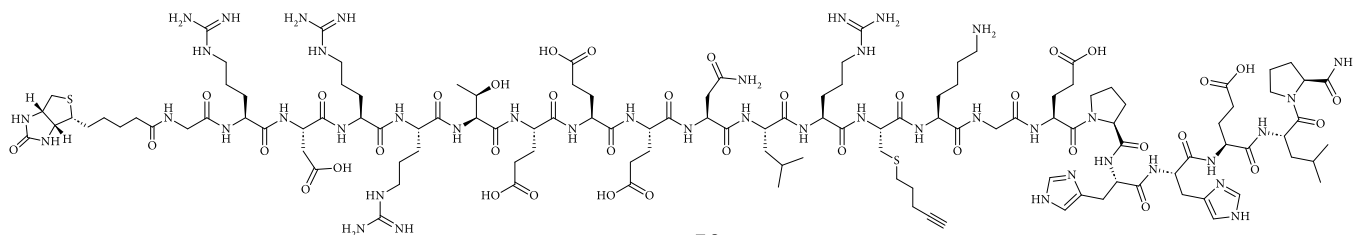
51

Fmoc-Pro-OH (270 mg, 0.8 mmol) was loaded to Rink amide AM resin (256 mg, 0.2 mmol) as per General Procedure 1. Fmoc-Gly-OH (178 mg, 0.6 mmol), Fmoc-Arg(Pbf)-OH (390 mg, 0.6 mmol), Fmoc-Asp(*t*Bu)-OH (247 mg, 0.6 mmol), Fmoc-Thr(*t*Bu)-OH (239 mg, 0.6 mmol), Fmoc-Glu(*t*Bu)-OH (255 mg, 0.06 mmol), Fmoc-Asn(Trt)-OH (358 mg, 0.6 mmol), Fmoc-Leu-OH (212 mg, 0.6 mmol), Fmoc-Lys(*t*Bu)-OH (281 mg, 0.6 mmol), Fmoc-Pro-OH (203 mg, 0.6 mmol), Fmoc-His(Trt)-OH (372 mg, 0.6 mmol), N-(((9H-fluoren-9-yl)methoxy)carbonyl)-S-(pent-4-yn-1-yl)-L-cysteine **23** (246 mg, 0.6 mmol), were coupled as per General Procedure 3. On resin biotinylation was performed according to General Procedure 5. The peptide was cleaved from the resin using General Procedure 4, yielding the crude product as a white solid. The peptide was purified by C₁₈ semi-preparative HPLC with a gradient of 5% MeCN (+ 0.1% TFA) with 95% H₂O (+ 0.1% TFA) to 50% MeCN (+ 0.1% TFA) with 50% H₂O (+ 0.1% TFA).

HRMS (ESI⁺): (*m/z*) C₁₂₃H₂₀₃N₄₃O₃₈S₂ [M+6H]⁶⁺, calculated: 492.4113, found: 492.4102.
(*m/z*) C₁₂₃H₂₀₂N₄₃O₃₈S₂ [M+5H]⁵⁺, calculated: 590.6922, found: 590.6923.

HPLC: R_T = 9.47 min (5-95% MeCN in H₂O over 25 min, 0.1% TFA modifier, Ascentis Express C18, 2.7 μm, 10 mm x 4.6 mm).

p53 279-300 sequence K292Alkyne mutant biotinylated (52)



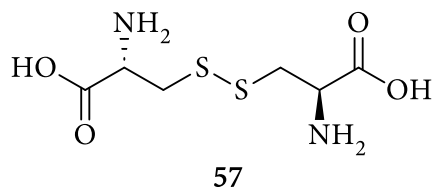
52

Fmoc-Pro-OH (270 mg, 0.8 mmol) was loaded to Rink amide AM resin (256 mg, 0.2 mmol) as per General Procedure 1. Fmoc-Gly-OH (178 mg, 0.6 mmol), Fmoc-Arg(Pbf)-OH (390 mg, 0.6 mmol), Fmoc-Asp(*t*Bu)-OH (247 mg, 0.6 mmol), Fmoc-Thr(*t*Bu)-OH (239 mg, 0.6 mmol), Fmoc-Glu(*t*Bu)-OH (255 mg, 0.6 mmol), Fmoc-Asn(Trt)-OH (358 mg, 0.6 mmol), Fmoc-Leu-OH (212 mg, 0.6 mmol), Fmoc-Lys(*t*Bu)-OH (281 mg, 0.6 mmol), Fmoc-Pro-OH (203 mg, 0.6 mmol), Fmoc-His(Trt)-OH (372 mg, 0.6 mmol), N-(((9H-fluoren-9-yl)methoxy)carbonyl)-S-(pent-4-yn-1-yl)-L-cysteine **23** (246 mg, 0.6 mmol), were coupled as per General Procedure 3. On resin biotinylation was performed according to General Procedure 5. The peptide was cleaved from the resin using General Procedure 4, yielding the crude product as a white solid. The peptide was purified by C₁₈ semi-preparative HPLC with a gradient of 5% MeCN (+ 0.1% TFA) with 95% H₂O (+ 0.1% TFA) to 50% MeCN (+ 0.1% TFA) with 50% H₂O (+ 0.1% TFA).

HRMS (ESI⁺): (*m/z*) C₁₂₃H₂₀₃N₄₃O₃₈S₂ [M+6H]⁶⁺, calculated: 492.4113, found: 492.4097.
(*m/z*) C₁₂₃H₂₀₂N₄₃O₃₈S₂ [M+5H]⁵⁺, calculated: 590.6922, found: 590.6922.

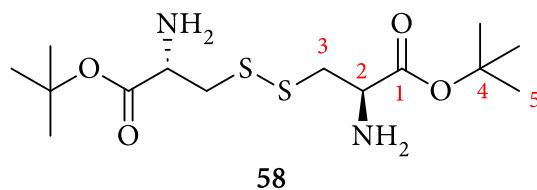
HPLC: R_T = 9.53 min (5-95% MeCN in H₂O over 25 min, 0.1% TFA modifier, Ascentis Express C18, 2.7 μm, 10 mm x 4.6 mm).

L,L-Cystine (XX)



The reaction was performed following a literature procedure.¹⁸⁰ In a conical flask, L-cysteine (3 g, 24.78 mmol) and NaI (186 mg, 1.23 mmol) were dispersed in ice-cold H₂O (30 mL) then aqueous hydrogen peroxide 30% (2.53 mL, 24.78 mmol) was added slowly into the central vortex of the reaction apparatus over the course of 5 minutes. The reaction mixture was allowed to vigorously stir for 1 hour as it approached rt. The yellow slurry suspension was then filtered via Büchner funnel and washed with ice-cold water (60 mL). The solid was dried under reduced pressure until a solid white powder was obtained (2.99 g, 12.23 mmol, 99% yield). As reported in the literature,^{180, 181} disulfide 57 is unstable and was used immediately without further purification or characterization.

L,L-Cystine-O^tBu (58)



The reaction was performed following a literature procedure.¹⁸¹ *Tert*-butyl acetate (25 mL) was added to a stirred solution of L,L-Cystine 57 (2.95 g, 12.29 mmol) in perchloric acid (70 % aq. v/v, 4.8 mL, 49.14 mmol, 4 equiv.) at room temperature resulting in two immiscible layers, which dispersed on stirring after approximately 3 h. The reaction mixture was left to stir for a further 72 h after which it was cooled in an ice bath for 30 min. After that time, TLC analysis (DCM:MeOH 13:1) revealed consumption of starting material ($R_f = 0.1$) and formation of product ($R_f = 0.3$). Sodium hydroxide (2M aq.) was slowly added until the solution reached pH 11. After that, the product was extracted with EtOAc (3 x 50 mL) and the combined layers

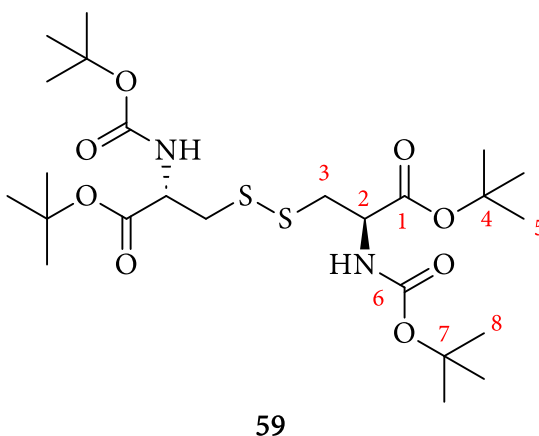
were dried over MgSO₄. Removal of the solvent under reduced pressure afforded compound **58** as a yellow liquid (1.97 g, 5.59 mmol, 46% yield). Spectral data is in agreement with the literature.¹⁸¹

¹H-NMR (400 MHz, CDCl₃): δ 3.68 (dt, J_{H2H3} = 7.8 Hz, J_{HNH2} = 4.4 Hz, 2H, H2), 3.13 (dd, J_{H3H3'} = 13.4 Hz, J_{H3H2} = 4.4 Hz, 1H, H3), 2.88 (dd, J_{H3H3'} = 13.4 Hz, J_{H3'H2} = 7.8 Hz, 1H, H3'), 1.47 (s, 18H, H5) ppm.

¹³C-NMR (101 MHz, CDCl₃): δ 171.2 (C1), 82.0 (C4), 53.0 (C2), 43.2 (C3), 28.0 (C5) ppm.

HRMS (ESI⁺): (*m/z*) C₁₄H₂₉N₂O₄S₂ [M+H]⁺, calculated: 353.156326, found: 353.156745.

Boc-L,L-Cystine-O^tBu (**59**)



The reaction was performed following a literature procedure.¹⁸² L,L-Cystine-O^tBu **58** (1.97 g, 5.59 mmol) was dissolved in 1:1 H₂O/dioxane (50 mL). Sodium bicarbonate (1.88 g, 22.35 mmol) was then added followed by the addition of Boc anhydride (2.56 g, 11.74 mmol). The reaction mixture was allowed to stir at rt for 16h. After that time, TLC analysis showed consumption of the starting material (*R*_f = 0.3 in DCM:MeOH 13:1, *R*_f = 0 in EtOAc:Hex 3:7) and formation of the product (*R*_f = 0.9 in DCM:MeOH 13:1, *R*_f = 0.5 in EtOAc:Hex 3:7). The reaction mixture was concentrated under reduced pressure and the resulting aqueous solution was extracted with EtOAc (3 x 40 mL). The combined organic layers were washed with brine (100 mL), dried over MgSO₄ and concentrated under reduced pressure to obtain compound **59** as a pale-yellow solid (2.89 g, 5.24 mmol, 94% yield). Spectral data is in agreement with the literature.¹⁸²

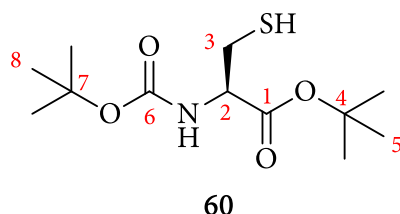
¹H-NMR (400 MHz, CDCl₃): δ = 5.32 (d, J_{H_{NH}H₂} = 5.0 Hz, 2H, NH), 4.47-4.40 (m, 4H, H₂), 3.20 (dd, J_{H₃H₃'} = 13.3 Hz, J_{H₃H₂} = 4.8 Hz, 2H, H₃), 2.88 (dd, J_{H₃H₃'} = 13.4 Hz, J_{H₃'H₂} = 7.8 Hz, 2H, H₃'), 1.52 (s, 18H, H₈), 1.48 (s, 18H, H₅).

¹³C-NMR (101 MHz, CDCl₃): δ 169.9 (C₁), 155.2 (C₆), 82.4 (C₄), 79.7 (C₇), 53.6 (C₂), 43.2 (C₃), 28.3 (C₅), 27.1 (C₈) ppm.

HRMS (ESI⁺): (m/z) C₂₄H₄₄N₂NaO₈S₂ [M+Na]⁺, calculated: 575.243129, found: 575.243162.

m.p.: 95-96 °C.

***tert*-butyl (*tert*-butoxycarbonyl)-L-cysteinate (60)**



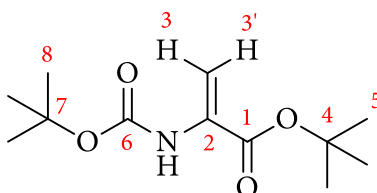
The reaction was performed following a literature procedure.¹⁹⁸ Tri-*n*-butyl phosphine (1.36 mL, 5.5 mmol, 1.05 eq.) was added to a solution of compound 59 (2.89 g, 5.24 mmol) in THF (25 ml). The mixture was stirred at room temperature for 2 minutes and then H₂O (1 ml) was added. After being stirred overnight, TLC analysis (EtOAc:Hex, 3:7) revealed complete consumption of starting material (R_f = 0.5) and formation of product (EtOAc:Hex 1:9, R_f = 0.3). The reaction mixture was concentrated under reduced pressure. The residue was purified with flash chromatography (silica gel, EtOAc:Hex 1:30) to give compound 60 as a colourless oil (2.08g, 7.51 mmol, 72% yield). Spectral data is in agreement with the literature.¹⁹⁸

¹H-NMR (400 MHz, CDCl₃): δ 5.38 (d, J_{H_{NH}H₂} = 4.9 Hz, 2H, NH), 4.51-4.42 (m, 1H, H₂), 2.96 (dd, 2H, J_{H₃'H₂} = 8.9 Hz, J_{H₃H₂} = 4.0 Hz, 2H, H₃), 1.49 (s, 9H, H₅), 1.45 (s, 9H, H₈) ppm.

¹³C-NMR (101 MHz, CDCl₃): δ 169.3 (C₁), 155.2 (C₆), 82.7 (C₄), 80.0 (C₇), 55.1 (C₂), 28.3 (C₅), 28.0 (C₈), 27.6 (C₃).

HRMS (ESI⁺): (*m/z*) C₁₂H₂₃NNaO₄S [M+Na]⁺, calculated: 300.124000, found: 300.124267.

***tert*-butyl 2-((*tert*-butoxycarbonyl)amino)acrylate (56)**



56

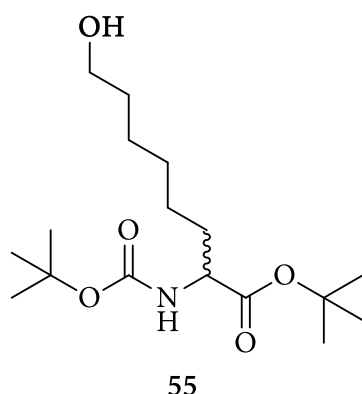
The reaction was performed following a literature procedure.¹⁶⁶ To a solution of Boc-Cys-O^tBu **60** (942 mg, 3.4 mmol) in DMF (30 mL) was added K₂CO₃ (2.35 g, 17 mmol), followed immediately by 1,4-diiodobutane (0.67 mL, 5.1 mmol). The reaction mixture was stirred at room temperature for 4 h. After that time, TLC analysis (EtOAc:Hex 1:50) showed formation of product (*R*_f = 0.3) and consumption of starting material (*R*_f = 0.1). The resulting mixture was diluted with H₂O (50 mL) and extracted with Et₂O (40 mL x 3). The combined organic layers were washed with H₂O (40 mL) and brine (40 mL). The combined organic layer was dried over MgSO₄, filtered, and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel (EtOAc:Hex 1:50) to give compound **56** as a colourless oil (389 mg, 1.6 mmol, 47% yield). Spectral data is in agreement with the literature.¹⁶⁵

¹H-NMR (400 MHz, CDCl₃): δ 7.02 (br. s, 1H, NH), 6.06 (s, 1H, H3'), 5.66 (d, *J*_{H3H3'} = 1.6 Hz, H3), 1.50 (s, 9H, H5), 1.48 (s, 9H, H8) ppm.

¹³C-NMR (101 MHz, CDCl₃): δ 163.0 (C1), 152.6 (C6), 132.4 (C2), 104.0 (C3), 82.6 (C4), 80.4 (C7), 28.2 (C5), 27.8 (C8) ppm.

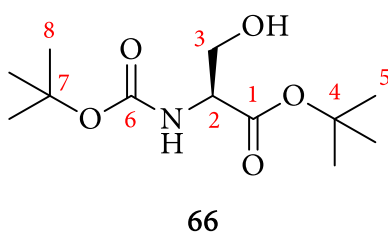
HRMS (APCI): (*m/z*) C₁₂H₂₀NO₄ [M-H]⁻, calculated: 242.139782, found: 242.139874.

***tert*-butyl 2-((*tert*-butoxycarbonyl)amino)-8-hydroxyoctanoate (55, attempted)**



The synthesis of *tert*-butyl 2-((*tert*-butoxycarbonyl)amino)-8-hydroxyoctanoate 55 was attempted following a modified literature procedure.¹⁶⁵ Cu(OTf)₂ (29 mg, 0.08 mmol), Zn (150 mg, 2.28 mmol), and 5-Br-1-pentanol (273 μ L, 2.28 mmol) were added to *tert*-butyl 2-((*tert*-butoxycarbonyl)amino)acrylate 56 (184 mg, 0.76 mmol) in sat. NH₄Cl/EtOH (1:1 v/v, 30 mL) at rt. After 2.5 h, additional Zn (50 mg, 0.76 mmol) and 5-Br-1-pentanol (91 μ L, 0.76 mmol) were added, and 1 h later, additional Zn (50 mg, 0.76 mmol) and 5-Br-1-pentanol (91 μ L, 0.76 mmol) were added. The reaction mixture was stirred for 2 h at rt. After that time, TLC analysis (EtOAc:Hex 1:20) showed no consumption of starting material (R_f = 0.8). The reaction mixture was poured into H₂O (30 mL) and extracted with EtOAc (3 x 70 mL). The combined organic phases were dried over MgSO₄, filtered, and concentrated under reduced pressure. The crude mixture was analysed by ¹H-NMR and HRMS which did not indicate formation of the desired compound 55.

Boc-Serine-O^tBu (66)



The reaction was performed following a literature procedure.¹⁹⁹ *tert*-(butoxycarbonyl)-L-serine (1.25 g, 6 mmol) was dissolved in dry DCM (25 mL) and cooled to 0 °C under inert atmosphere (Ar). *tert*-butyl *N,N'*-diisopropylcarbamimidate 65 (3.66 g, 18 mmol) was added

in three portions (3 x 1.4 mL) over 5 min. Reaction was stirred overnight at rt. After that time, TLC analysis (EtOAc:Hex 1:3) showed formation of product ($R_f = 0.35$) and consumption of starting material ($R_f = 0$). The reaction was stirred with hexanes (50 mL) for 30 min, then filtered through celite and concentrated under reduced pressure. Reaction was purified using a silica plug (eluent EtOAc:Hex 1:3 to 1:1). Product **66** was isolated as a white solid (1.07g, 4.11 mmol, 70% yield). Spectral data is in agreement with the literature.¹⁹⁹

¹H-NMR (400 MHz, CDCl₃): δ 5.43 (s, 1H, NH), 4.28 (br. s, 1H, H2), 3.94-3.89 (m, 2H, H3), 1.50 (s, 9H, H5), 1.48 (m, 9H, H8) ppm.

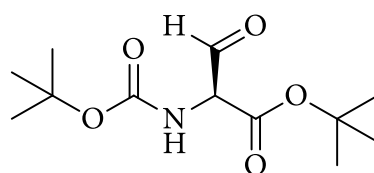
¹³C-NMR (101 MHz, CDCl₃): δ 169.7 (C1), 155.9 (C6), 82.7 (C4), 80.2 (C7), 64.2 (C3), 56.4 (C2), 28.3 (C5), 28.0 (C8) ppm.

¹⁵N-NMR (50.7 MHz, CDCl₃): δ 82.7 (NH) ppm.

HRMS (ESI⁺): (m/z) C₁₂H₂₃NNaO₅ [M+Na]⁺, calculated: 284.146884, found: 284.147663.

m.p.: 75-76 °C.

tert-butyl (S)-2-((*tert*-butoxycarbonyl)amino)-3-oxopropanoate (**66**)



63

Method A:

To a solution of Boc-Serine-O^tBu **66** (50 mg, 0.19 mmol) in DCM (10 mL) was added pyridine (50 μ L, 0.57 mmol) and Dess-Martin Periodinane (122 mg, 0.29 mmol). The reaction was stirred for 3 h at rt. After that time, TLC analysis (EtOAc:Hex 1:3) showed no consumption of the starting material ($R_f = 0.35$) and no formation of a product. The reaction mixture was diluted with EtOAc (20 mL), washed with 10% sodium thiosulphate solution (20 mL), saturated sodium bicarbonate (20 mL), and brine (20 mL). The combined aqueous layers were extracted with EtOAc (20 mL). The combined organic layers were dried over MgSO₄ and

evaporated under reduced pressure. The crude mixture was analysed by ^1H -NMR and HRMS which did not indicate formation of the desired compound **63**.

Method B:

To a solution of Boc-Serine-O^tBu **66** (100 mg, 0.38 mmol) in DCM (20 mL) was added NaHCO_3 (95 mg, 1.14 mmol) and Dess-Martin Periodinane (322 mg, 0.76 mmol). The reaction was stirred for 3 h at rt. After that time, TLC analysis (EtOAc:Hex 1:3) showed no consumption of the starting material ($R_f = 0.35$) and no formation of a product. The reaction mixture was diluted with EtOAc (40 mL), washed with 10% sodium thiosulphate solution (40 mL), saturated sodium bicarbonate (40 mL), and brine (40 mL). The combined aqueous layers were extracted with EtOAc (40 mL). The combined organic layers were dried over MgSO_4 and evaporated under reduced pressure. The crude mixture was analysed by ^1H -NMR and HRMS which did not indicate formation of the desired compound **63**.

Method C:

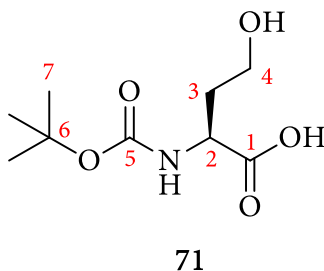
To a solution of Boc-Serine-O^tBu **66** (50 mg, 0.19 mmol) in DCM (10 mL) was added DMSO (30 μL , 0.40 mmol), triethylamine (110 μL , 0.80 mmol) and sulphur trioxide pyridine complex (64 mg, 0.4 mmol). The reaction was stirred for 3 h at rt. After that time, TLC analysis (EtOAc:Hex 1:3) showed no consumption of the starting material ($R_f = 0.35$) and no formation of a product. The reaction mixture was quenched with aqueous sat. NH_4Cl (10 mL) and extracted with DCM (3 x 20 mL). The combined organic layers were washed with water (20 mL), dried over MgSO_4 and evaporated under reduced pressure. The crude mixture was analysed by ^1H -NMR and HRMS which did not indicate formation of the desired compound **63**.

Method D:

To a solution of Boc-Serine-O^tBu **66** (100 mg, 0.38 mmol) in DCM (20 mL) was added PCC (91 mg, 0.42 mmol). The reaction was stirred for 3 h at rt. After that time, TLC analysis (EtOAc:Hex 1:3) showed no consumption of the starting material ($R_f = 0.35$) and no formation of a product. The reaction mixture was quenched with water (20 mL) and extracted with DCM (3 x 20 mL). The combined organic layers were washed with brine (20 mL), dried

over MgSO_4 and evaporated under reduced pressure. The crude mixture was analysed by ^1H -NMR and HRMS which did not indicate formation of the desired compound **63**.

Boc-L-homoserine-OH (**71**)



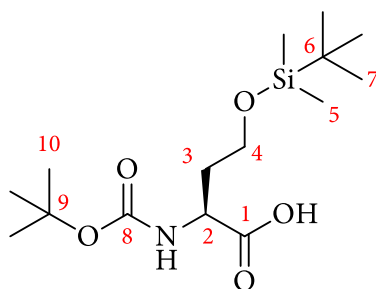
The reaction was performed following a literature procedure.²⁰⁰ L-homoserine (500 mg, 4.20 mmol) was dissolved in aq. NaOH (1 M, 5 mL) and the solution cooled to 0 °C. A solution of di-*tert*-butyl dicarbonate (0.96 g, 4.41 mmol) in 1,4-dioxane (5 mL) was added dropwise. The reaction mixture was warmed to rt and stirred for 20 h. After this time TLC analysis (DCM:MeOH 4:1) showed consumption of the starting material ($R_f = 0.1$) and formation of the product ($R_f = 0.5$). Half of the solvent was evaporated under reduced pressure and the aqueous phase was acidified to pH 1 with aq. HCl (1 M), followed by extraction with EtOAc (3 x 20 mL). The combined organic phases were dried over anhydrous MgSO_4 , filtered, and concentrated under reduced pressure to afford a Boc-L-homoserine **71** as a colourless oil. Spectral data is in agreement with the literature.²⁰⁰

^1H -NMR (400 MHz, CDCl_3): δ 12.37 (br. s, 1H, COOH), 6.90 (br. s, 1H, NH), 4.04-3.96 (m, 1H, H2), 3.49-3.36 (m, 2H, H4), 1.88-1.60 (m, 2H, H3), 1.38 (s, 9H, H7) ppm.

^{13}C -NMR (101 MHz, CDCl_3): δ 174.5 (C1), 155.6 (C5), 77.9 (C6), 57.3 (C4), 50.6 (C2), 33.8 (C3), 28.2 (C7) ppm.

HRMS (ESI⁺): (m/z) $\text{C}_9\text{H}_{17}\text{NNaO}_5$ [$\text{M}+\text{Na}$]⁺, calculated: 242.099893, found: 242.099820.

Boc-L-homoserine(OTBS)-OH (72)



72

The reaction was performed following a literature procedure.²⁰⁰ Boc-Homoserine **71** (3.3 g, 15.05 mmol) and imidazole (7.3 g, 45.15 mmol) were dissolved in dry DCM (20 mL) and catalytic DMF (1.7 mL) then cooled to 0°C. TBSCl (2.9 g, 19.5 mmol) in dry DCM (5 mL) was added dropwise under inert atmosphere (N₂). The reaction mixture was then left to warm up to rt and stirred for 24 h. After this time TLC analysis showed consumption of the starting material (R_f = 0.5 in DCM:MeOH 4:1, R_f = 0 in EtOAc:Hex 1:1) and formation of the product (R_f = 0.9 in DCM:MeOH 4:1, R_f = 0.5 in EtOAc:Hex 1:1). 17 mL of chilled 1M HCl was added, and reaction stirred for 30 minutes at 0°C. The aqueous layer was extracted with DCM (3 x 50 mL). The combined organic layers were dried over anhydrous Na₂SO₄, filtered off and the filtrate concentrated under reduced pressure to a colorless oil residue. The residue was dissolved in methanol (10 mL) and H₂O (16.5 mL) and pH of the solution was adjusted to 2-3 using chilled 1M HCl. The product **72** was collected as a white powder after suction filtration (3.85 g, 11.55 mmol, 77% yield). Spectral data is in agreement with the literature.²⁰¹

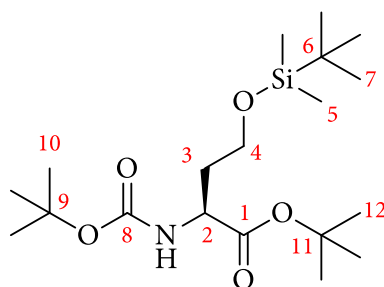
¹H-NMR (400 MHz, CDCl₃): δ 12.40 (br. s, 1H, COOH), 7.01 (d, J_{HNH2} = 8.1 Hz, 1H, NH), 4.03 (ddd, $J_{\text{H3'H3}}$ = 13.9 Hz, $J_{\text{H2H3'}}$ = 10.0 Hz, J_{H3H4} = 4.6 Hz, 1H, H2), 3.67-3.56 (m, 2H, H4), 1.92-1.79 (m, 1H, H3), 1.68 (ddt, $J_{\text{H3'H2}}$ = 10.3 Hz, J_{HNH2} = 8.1 Hz, J_{H3H2} = 4.1 Hz, 1H, H3'), 1.36 (s, 9H, H10), 0.84 (s, 9H, H7), -0.02 (s, 6H, H5) ppm.

¹³C-NMR (101 MHz, CDCl₃): δ 174.5 (C1), 155.5 (C8), 77.8 (C9), 58.9 (C4), 50.1 (C2), 33.4 (C3), 28.2 (C10), 25.8 (C7), 18.3 (C6) -3.3 (C5) ppm.

HRMS (ESI⁻): (m/z) C₁₅H₃₀NO₅Si [M-H]⁻, calculated: 332.18987, found: 332.18952.

m.p.: 87-89 °C.

Boc-L-homoserine(OTBS)-O^tBu (74)



74

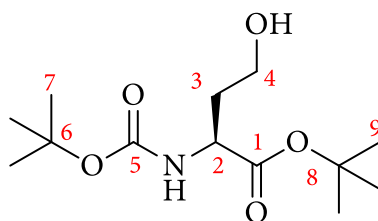
The reaction was performed following a literature procedure.²⁰⁰ N-(*tert*-butoxycarbonyl)-O-(*tert*-butyldimethylsilyl)-L-homoserine 72 (3.85 g, 11.55 mmol) was dissolved in dry DCM (20 mL) and cooled to 0°C. *tert*-Butyl 2,2,2-trichloroacetimidate 73 (2.76 g, 12.62 mmol) was dissolved in dry DCM (15 mL) added dropwise under inert atmosphere (N₂) and the mixture was stirred for 36 h at rt. After that time, TLC analysis (EtOAc:Hex 1:6) showed consumption of the starting material (R_f = 0.05) and formation of the product (R_f = 0.3). Precipitates were filtered out and filtrate concentrated under reduced pressure. The residue was purified using flash silica gel chromatography (eluent Hex to EtOAc:Hex 1:6) to obtain 74 as a colourless oil (2.76 g, 7.1 mmol, 61% yield). Spectral data is in agreement with the literature.²⁰⁰

¹H-NMR (400 MHz, CDCl₃): δ 5.61 (d, J_{NH2} = 7.6 Hz, 1H, NH), 4.21 (s, 1H, H2), 3.71 (dd, J_{H4H3} = 7.1 Hz, J_{H4H3'} = 4.6 Hz, 2H, H4), 2.07-1.96 (m, 1H, H3), 1.91-1.82 (m, 1H, H3'), 1.46 (s, 9H, H12), 1.43 (s, 9H, H10), 0.90 (s, 9H, H7), 0.05 (s, 6H, H5) ppm.

¹³C-NMR (101 MHz, CDCl₃): δ 171.7 (C1), 155.5 (C8), 81.4 (C11), 79.2 (C9), 60.4 (C4), 52.9 (C2), 34.2 (C3), 28.3 (C10), 28.0 (C12), 25.9 (C7), 18.3 (C6) -5.6 (C5) ppm.

HRMS (ESI⁺): (m/z) C₁₉H₄₀NO₅Si [M+H]⁺, calculated: 390.267026, found: 390.266421.

Boc-L-homoserine-O^tBu (76)



76

Method A:

This reaction was performed following a literature procedure.²⁰⁰ Boc-L-homoserine(OTBS)-O^tBu **74** (2.76 g, 7.08 mmol) was dissolved in dry THF (20 mL) and cooled to 0 °C. 1M TBAF in THF (9.2 mL, 9.2 mmol) was added dropwise under inert atmosphere (N₂) and the mixture was stirred for 2 h at 0°C and for 36 h at rt. After this time, TLC analysis showed consumption of the starting material (R_f = 0.3 in EtOAc:Hex 1:6, R_f = 0.9 in EtOAc:Hex 1:1) and formation of the product (R_f = 0.05 in EtOAc:Hex 1:6, R_f = 0.5 in EtOAc:Hex 1:1). Contents were diluted 10 mL EtOAc and washed with saturated NH₄Cl (2 x 15 mL), NaHCO₃ (2 x 15 mL), brine (1 x 15 mL) and H₂O (1 x 15 mL). The organic layer was dried over Na₂SO₄ and filtered. The filtrate was concentrated under reduced pressure and product was purified using flash silica gel chromatography (eluent EtOAc:Hex 1:3 to 6:4) to obtain compound **76** as a colorless oil (1.59 g, 5.77 mmol, 81% yield). Spectral data is in agreement with the literature.²⁰⁰

Method B:

The reaction was performed following a modified literature procedure.¹⁹⁹ Boc-L-homoserine-OH **71** (422 mg, 1.92 mmol) was dissolved in a 5:1 mixture of dry DCM:THF (62.5 mL). *tert*-butyl-2,2,2-trichloroacetimidate **73** (2.55 g, 11.7 mmol) was added to the solution. The mixture was stirred at rt for 20h under inert atmosphere (Ar). At this time TLC analysis (EtOAc:Hex 1:1) revealed consumption of starting material (R_f = 0.1) and formation of product (R_f = 0.5). The reaction was concentrated under reduced pressure. The residue was diluted with EtOAc (50 mL) and washed with sat. aq. NaHCO₃ (50 mL) and brine (50 mL). The organic phase was dried over Na₂SO₄, filtered and concentrated under reduced pressure.

The resulting crude was purified by flash chromatography (EtOAc:Hex 1:2) to afford the desired product **76** as a colourless oil (165 mg, 15% yield).

Method C:

The reaction was performed following a modified literature procedure.¹⁹⁹ Boc-L-homoserine-OH **71** (422 mg, 1.92 mmol) was dissolved in dry DCM (20 mL). *tert*-butyl *N,N'*-diisopropylcarbamimidate **65** (1.38 mL, 5.78 mmol) was added in three portions (3 x 0.46 mL) over 5 min. The mixture was stirred at rt for 20h under inert atmosphere (Ar). At this time TLC analysis (EtOAc:Hex 1:1) revealed consumption of starting material ($R_f = 0.1$) and formation of product ($R_f = 0.5$). The reaction was stirred with hexanes (20 mL) for 30 min, then filtered through celite and concentrated under reduced pressure. The resulting crude was purified by flash chromatography (EtOAc:Hex 1:2) to afford the desired product **76** as a colourless oil (360 mg, 69% yield).

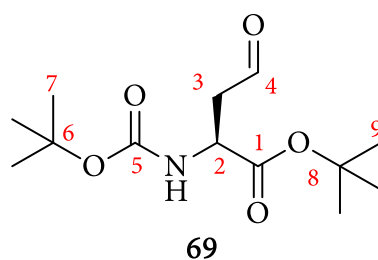
¹H-NMR (400 MHz, CDCl₃): δ 5.35 (d, $J_{\text{HNH}_2} = 7.0$ Hz, 1H, NH), 4.40-4.34 (m, 1H, H2), 3.72 (dtd, $J_{\text{H}_4\text{H}_4'} = 12.0$ Hz, $J_{\text{H}_4\text{H}_3'} = 4.7$ Hz, $J_{\text{H}_4\text{H}_3} = 3.0$ Hz, 1H, H4), 3.65 (dt, $J_{\text{H}_4\text{H}_4'} = 12.0$ Hz, $J_{\text{H}_4'\text{H}_3} = 3.0$ Hz, 2H, H4'), 2.19-2.02 (m, 2H, H3), 1.49 (s, 9H, H9), 1.47 (s, 9H, H7) ppm.

¹³C-NMR (101 MHz, CDCl₃): δ 172.04 (C1), 156.7 (C5), 82.3 (C8), 80.37 (C6), 58.3 (C4), 50.9 (C2), 36.6 (C3), 28.8 (C7), 28.0 (C9) ppm.

¹⁵N-NMR (50.7 MHz, CDCl₃): δ 85.4 (NH) ppm.

HRMS (ESI⁺): (m/z) C₁₃H₂₆NO₅ [M+H]⁺, calculated: 276.180549, found: 276.180414.

tert-butyl (S)-2-((*tert*-butoxycarbonyl)amino)-4-oxobutanoate (**69**)



To a solution of Boc-L-homoserine-O^tBu **76** (250 mg, 0.91 mmol) in DCM (15 mL) was added at rt pyridine (0.22 mL, 2.73 mmol) and Dess-Martin Periodinane (578 mg, 1.36 mmol). After

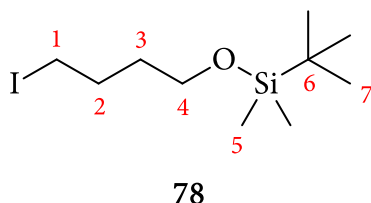
90 minutes, TLC analysis (EtOAc:Hex 1:1) showed consumption of the starting material ($R_f = 0.5$) and formation of the product ($R_f = 0.8$). The reaction mixture was diluted with EtOAc (30 mL), washed with 10% sodium thiosulphate solution (20 mL), saturated sodium bicarbonate (30 mL), and brine (30 mL). The combined aqueous layers were extracted with EtOAc (30 mL), dried over $MgSO_4$, and evaporated under reduced pressure. Compound **69** was isolated as a colorless oil (243 mg, 0.89 mmol, 98% yield) that was used without further purification. Spectral data is in agreement with the literature.²⁰²

1H -NMR (400 MHz, $CDCl_3$): δ 9.76 (s, 1H, H4), 5.37 (d, $J_{HNH2} = 7.9$ Hz, 1H, NH), 4.54-4.47 (m, 1H, H2), 2.97 (ddd, $J_{H3H3} = 17.8$ Hz, $J_{H3H2} = 18.1$ Hz, $J_{H3H4} = 5.2$ Hz, 2H, H3) 1.49 (s, 9H, H9), 1.47 (s, 9H, H7) ppm.

^{13}C -NMR (101 MHz, $CDCl_3$): δ 198.7 (C4), 170.0 (C1), 155.7 (C5), 82.1 (C8), 80.7 (C6), 53.1 (C2), 46.2 (C3), 28.8 (C7), 28.4 (C9) ppm.

HRMS (ESI⁺): (m/z) $C_{13}H_{23}NNaO_5$ $[M+Na]^+$, calculated: 296.146844, found: 296.146997.

tert-Butyl(4-iodobutoxy)dimethylsilane (**78**)



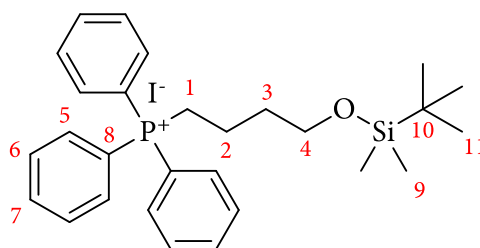
The reaction was performed following a literature procedure.²⁰³ THF (8.2 mL, 100 mmol) was added to dry MeCN (80 mL), followed by addition of sodium iodide (12.0 g, 80.0 mmol) and *tert*-butyldimethylsilyl chloride (6.03 g, 40.0 mmol) and the solution heated at 55 °C for 18 h. H_2O was added (150 mL) and the suspension extracted with Pet. ether: Et_2O (9:1, 3 x 100 mL). The combined organic extracts were washed with saturated sodium hydrogen sulphite solution (100 mL) and brine (100 mL), dried over Na_2SO_4 , filtered and the solvent removed under reduced pressure. The product was purified using flash silica gel chromatography (eluent EtOAc:Hex 1:99 to 1:24) to obtain compound **78** as a colorless oil (10.8 g, 34.3 mmol, 86% yield). Spectral data is in agreement with the literature.²⁰³

^1H -NMR (400 MHz, CDCl_3): δ 3.64 (t, $J_{\text{H4H3}} = 6.2$ Hz, 2H, H4), 3.23 (t, $J_{\text{H1H2}} = 7.02$ Hz, 2H, H1), 1.91 (p, $J_{\text{H4H3}} = J_{\text{H2H3}} = 6.5$ Hz, 2H, H3), 1.66-1.51 (m, 2H, H2), 0.90 (s, 9H, H7), 0.05 (s, 6H, H5) ppm.

^{13}C -NMR (101 MHz, CDCl_3): δ 61.9 (C4), 33.5 (C2), 30.2 (C3), 26.0 (C7), 18.3 (C6), 7.1 (C1), -5.3 (C5) ppm.

HRMS (ESI^+): (m/z) $\text{C}_{10}\text{H}_{24}\text{IO}_5\text{Si}$ $[\text{M}+\text{H}]^+$, calculated: 315.06356, found: 315.06399.

(4-(TBS)oxy)butyl)(triphenyl)phosphonium iodide (68)



68

The reaction was performed following a literature procedure.²⁰⁴ A solution of *tert*-butyl(4-iodobutoxy)dimethyl silane **78** (10.8 g, 34.3 mmol) and triphenylphosphine (9.1 g, 34.6 mmol) were heated together in dry MeCN (60 ml) at 85°C for 24 h. The mixture was evaporated, and the residue was triturated with anhydrous ether to afford the title compound **68** as a white powder (11.71 g, 20.31 mmol, 60% yield). Spectral data is in agreement with the literature.²⁰⁴

^1H NMR (CD_2Cl_2 , 400 MHz) = 7.88–7.87 (m, 9H, H7-H6), 7.74-7.68 (m, 6H, H5), 3.85-3.74 (m, 2H, H1), 3.68 (t, $J_{\text{H4H3}} = 5.5$ Hz, 2H, H4), 1.94 (adt, $J_{\text{H2H3}} = 7.7$ Hz, $J_{\text{H4H3}} = 5.5$ Hz, 2H, H3), 1.69 (ah, $J_{\text{H2H3}} = 7.6$ Hz, $J_{\text{H2H1}} = 7.1$ Hz, 2H, H2), 0.78 (s, 9H, H11), -0.02 (s, H9) ppm.

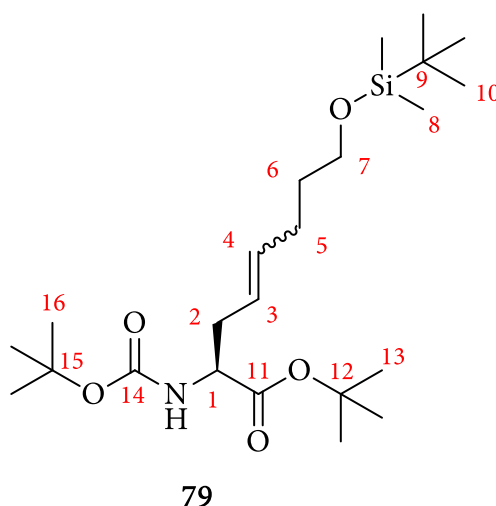
^{13}C -NMR (101 MHz, CDCl_3): δ 135.0 (d, $J_{\text{C7P}} = 3.0$ Hz, C7), 133.8 (d, $J_{\text{C6P}} = 10.0$ Hz, C6), 130.5 (d, $J_{\text{C5P}} = 12.5$ Hz, C5), 118.31 (d, $J_{\text{C8P}} = 85.9$ Hz, C8), 61.7 (C4), 33.5 (C2), 32.7 (d, $J_{\text{C3P}} = 6.2$ Hz, C3), 25.8 (C11), 22.2 (d, $J_{\text{C1P}} = 49.9$ Hz, C1), 19.3 (d, $J_{\text{C2P}} = 4.0$ Hz, C2), 18.3 (C10), and -5.3 (C9) ppm.

^{31}P -NMR (101 MHz, CDCl_3): δ 24.54 ppm.

HRMS (ESI⁺): (*m/z*) C₂₈H₃₈OPSi [M+H]⁺, calculated: 449.242405, found: 449.243371.

m.p.: 216-220 °C.

tert-butyl (S)-2-((*tert*-butoxycarbonyl)amino)-8-((TBS)oxy)oct-4-enoate (**79**)



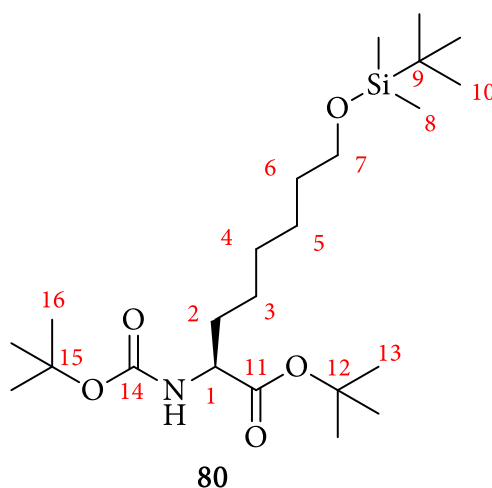
To a suspension of phosphonium iodide **68** (16.4 g, 28.46 mmol) in dry THF (150 mL) at -73°C was added dropwise *n*-BuLi (2.5 M in Hex, 14.2 mL, 35.58 mmol) and the mixture was stirred for 30 min under inert atmosphere (N₂). A solution of **69** (4.86 mg, 17.79 mmol) in THF (50 mL) was then added dropwise at -73°C. The mixture was allowed to warm to rt and stirred for 3h after which TLC analysis (EtOAc:Hex 1:4) revealed complete consumption of the starting material (*R*_f = 0.3) and formation of product (*R*_f = 0.8). The reaction was quenched with sat. aq. NH₄Cl (200 mL) and extracted with DCM (3 x 100 mL). The combined organic phases were washed with brine (150 mL), dried with Na₂SO₄ and concentrated under reduced pressure. The product was purified using flash silica gel chromatography (eluent EtOAc:Hex 1:9) to obtain compound **79** as a colorless oil (4.62 g, mmol, 10.7 mmol, 60% yield).

¹H-NMR (400 MHz, CDCl₃): δ 5.59-5.60 (m, 1H, H₄), 5.34-5.26 (m, 1H, H₃), 5.15-5.01 (m, 1H, NH), 4.28-4.18 (m, 1H, H₁), 3.62 (t, *J*_{H₆H₇} = 6.6 Hz, 2H, H₇), 2.61-2.40 (m, 2H, H₂), 2.13-2.01 (m, 2H, H₅), 1.57 (ap, *J* = 6.7 Hz, 2H, H₆), 1.47 (s, 9H, H₁₃), 1.45 (s, 9H, H₁₆), 0.91 (s, 9H, H₁₀), 0.06 (s, 6H, H₈) ppm.

$^{13}\text{C-NMR}$ (101 MHz, CDCl_3): δ 171.4 (C11), 155.2 (C14), 133.2 (C4), 123.6 (C3), 81.8 (C12), 79.4 (C15), 62.4 (C7), 53.6 (C1), 32.3 (C6), 30.3 (C2), 28.3 (C16), 28.0 (C13), 25.9 (C10), 23.6 (C5), 18.3 (C9), -5.3 (C8) ppm.

HRMS (ESI^+): (m/z) $\text{C}_{23}\text{H}_{46}\text{NO}_5\text{Si}$ [$\text{M}+\text{H}$] $^+$, calculated: 444.313977, found: 444.313455.

***tert*-butyl (S)-2-((*tert*-butoxycarbonyl)amino)-8-((TBS)oxy)octanoate (80)**

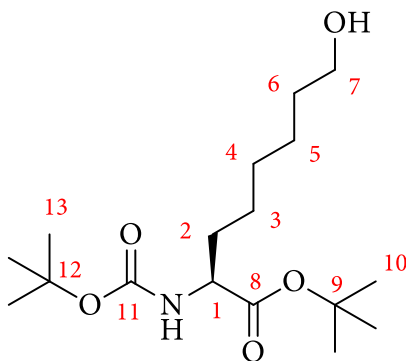


To a solution of *tert*-butyl (S)-2-((*tert*-butoxycarbonyl)amino)-8-((TBS)oxy)oct-4-enoate **79** (100 mg, 0.22 mmol) in dry MeOH (10 mL) at rt was added Pd (20% on charcoal, 10 mg, 10% w/w) and the mixture was purged with nitrogen gas (3 times). Hydrogen gas was charged in the solution at standard pressure for 12h. After that time, TLC analysis (EtOAc:Hex 1:9) revealed complete consumption of the starting material ($R_f = 0.3$) and formation of product ($R_f = 0.5$). The reaction mixture was purged with nitrogen gas (3 times) and filtered on celite. The solvent was evaporated under reduced pressure to yield compound **80** as a colorless oil (96 mg, 0.21 mmol, 97% yield).

$^1\text{H-NMR}$ (400 MHz, CDCl_3): δ 4.99 (d, $J_{\text{HNH1}} = 8.5$ Hz, 1H, NH), 4.20-4.11 (m, 1H, H1), 3.59 (t, $J_{\text{H6H7}} = 6.7$ Hz, 2H, H7), 1.80-1.53 (m, 2H, H2), 1.58-1.54 (m, 2H, H6), 1.46 (m, 9H, H13), 1.44 (s, 9H, H16), 1.35-1.27 (m, 6H, H3-H4-H5), 0.89 (s, 9H, H10), 0.04 (s, 6H, H8) ppm.

$^{13}\text{C-NMR}$ (101 MHz, CDCl_3): δ 171.2 (C11), 155.4 (C14), 81.6 (C12), 79.5 (C14), 63.1 (C7), 54.0 (C1), 32.9 (C2), 32.7 (C6), 29.1 (C3), 28.3 (C16), 28.0 (C13), 26.0 (C10), 25.6 (C5), 25.1 (C4), 18.4 (C9), -5.5 (C8) ppm.

***tert*-butyl (S)-2-((*tert*-butoxycarbonyl)amino)-8-hydroxyoctanoate (81)**

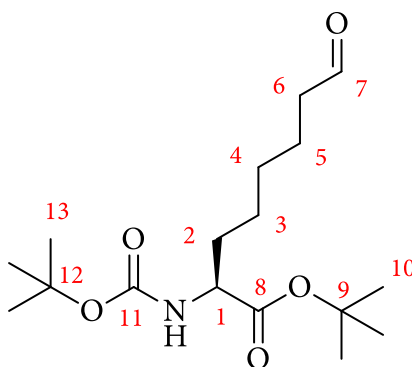


81

¹H-NMR (400 MHz, CDCl₃): δ 5.00 (d, J_{H_{NH}H} = 8.5 Hz, 1H, NH), 4.21-4.10 (m, 1H, H1), 3.64 (t, J_{H₆H₇} = 6.5 Hz, 2H, H7), 1.82–1.51 (m, 2H, H2), 1.58-1.54 (m, 2H, H6), 1.46 (s, 9H, H10), 1.44 (s, 9H, H13), 1.40-1.30 (m, 6H, H3-H4-H5) ppm.

HRMS (ESI⁺): (*m/z*) C₁₇H₃₃NNaO₅ [M+Na]⁺, calculated: 354.225094, found: 354.225503.

tert-butyl (S)-2-((*tert*-butoxycarbonyl)amino)-8-oxooctanoate (**54**)



54

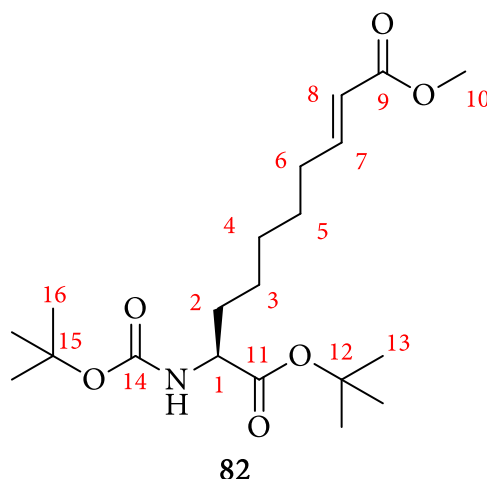
To a solution of *tert*-butyl (S)-2-((*tert*-butoxycarbonyl)amino)-8-hydroxyoctanoate **81** (3 g, 9.04 mmol) in DCM (150 mL) was added at room temperature pyridine (0.630 mL, 13.56 mmol) and Dess-Martin Periodinane (5.75 g, 13.56 mmol). The reaction was stirred for 3 h at room temperature. After that time, TLC analysis (EtOAc:Hex 3:7) showed consumption of the starting material ($R_f = 0.6$) and formation of the product ($R_f = 0.5$). The reaction mixture was diluted with EtOAc (300 mL), washed with 10% sodium thiosulphate solution (200 mL), saturated sodium bicarbonate (200 mL), and brine (200 mL). The combined aqueous layers were extracted with EtOAc (200 mL). The combined organic layers were dried over MgSO_4 and evaporated under reduced pressure. Compound **54** was isolated as a colorless oil (2.91 g, 8.84 mmol, 98% yield) and was used without further purification.

$^1\text{H-NMR}$ (400 MHz, CDCl_3): δ 9.76 (t, $J_{\text{H7H6}} = 1.7$ Hz, 1H, H7), 5.00 (d, $J_{\text{HNH1}} = 8.4$ Hz, 1H, NH), 4.19-4.12 (m, 1H, H1), 2.42 (td, $J_{\text{H7H6}} = 1.7$ Hz, $J_{\text{H5H6}} = 7.3$ Hz, 2H, H6), 1.80-1.70 (m, 1H, H2), 1.66-1.55 (m, 3H, H2', H5), 1.46 (s, 9H, H10), 1.44 (s, 9H, H13), 1.40-1.30 (m, 4H, H3-H4) ppm.

$^{13}\text{C-NMR}$ (101 MHz, CDCl_3): δ 202.7 (C7), 172.1 (C8), 155.4 (C11), 81.9 (C9), 79.8 (C12), 53.8 (C1), 43.7 (C6), 32.8 (C2), 28.8 (C5), 28.3 (C13), 28.0 (C10), 24.9 (C3), 21.9 (C4) ppm.

HRMS (ESI $^+$): (m/z) $\text{C}_{17}\text{H}_{32}\text{NO}_5$ [$\text{M}+\text{H}$] $^+$, calculated: 330.227500 found: 330.227799.

10-(tert-butyl) 1-methyl (S,E)-9-((tert-butoxycarbonyl)amino)dec-2-enedioate (**82**)



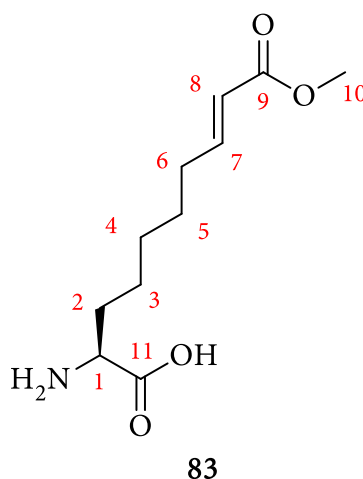
A solution of methyl(triphenylphosphoranylidene)acetate **37** (3.34 g, 10.0 mmol) in DCM (50 mL) was added dropwise to a solution of aldehyde **54** (2.91 g, 8.84 mmol) in DCM (30 mL). The reaction was stirred at rt for 16h. After this time, TLC analysis (EtOAc:Hex 3:7) showed complete consumption of starting material ($R_f = 0.5$) and formation of a new product ($R_f = 0.4$). Silica gel (20 g) was added to the reaction mixture, and solvent was removed under reduced pressure. The powder was purified by column chromatography (EtOAc:Hex 3:7) to give compound **82** as colorless oil (2.00 g, 5.19 mmol, 59% yield).

$^1\text{H-NMR}$ (400 MHz, CDCl_3): δ 6.95 (dt, $J_{\text{H8H7}} = 15.7$ Hz, $J_{\text{H7H8}} = 7.0$ Hz, 1H, H7), 5.81 (dt, $J_{\text{H8H7}} = 15.7$ Hz, $J_{\text{H6H8}} = 1.6$ Hz, 1H, H8), 4.99 (d, $J_{\text{HNH1}} = 8.4$ Hz, 1H, NH), 4.19-4.10 (m, 1H, H1), 3.72 (s, 3H, H10), 2.19 (adq, $J = 7.0$ Hz, $J = 1.6$ Hz, 2H, H6), 1.80–1.70 (m, 1H, H2), 1.62–1.53 (m, 1H, H2'), 1.46 (s, 9H, H13), 1.44 (s, 11H, H16-H5), 1.36-1.31 (m, 4H, H4-H5) ppm.

$^{13}\text{C-NMR}$ (101 MHz, CDCl_3): δ 172.2 (C11), 167.3 (C9), 155.5 (C14), 149.5 (C7), 121.1 (C8), 81.9 (C12), 79.7 (C15), 54.0 (C1), 51.5 (C10), 33.0 (C2), 32.2 (C6), 28.9 (C4), 28.5 (C16), 28.2 (C13), 28.0 (C5), 25.0 (C3) ppm.

HRMS (ESI $^+$): (m/z) $\text{C}_{20}\text{H}_{36}\text{NO}_6$ $[\text{M}+\text{H}]^+$, calculated: 386.253714 found: 386.253564.

(S,E)-2-amino-10-methoxy-10-oxodec-8-enoic acid (83)



(S,E)-2-amino-10-methoxy-10-oxodec-8-enoic acid **82** (1.85 g, 4.80 mmol) was dissolved in a mixture of TFA/TES/DCM (80/2.5/17.5, 5 mL). The reaction was stirred at room temperature for 4 h. After this time, TLC analysis (EtOAc:Hex 3:7) showed complete consumption of starting material ($R_f = 0.4$). The reaction mixture was concentrated under a flow of N_2 gas until a viscous oil was obtained, followed by precipitation with Et_2O (8 mL). The solution was transferred to a falcon tube and centrifuged for 30s at 7500 rpm. The supernatant was discarded, and the white solid was washed with Et_2O (2 x 8 mL). After the final wash, the obtained solid was dried under reduced pressure to obtain compound **83** as a white solid (0.99 g, 4.31 mmol, 90% yield).

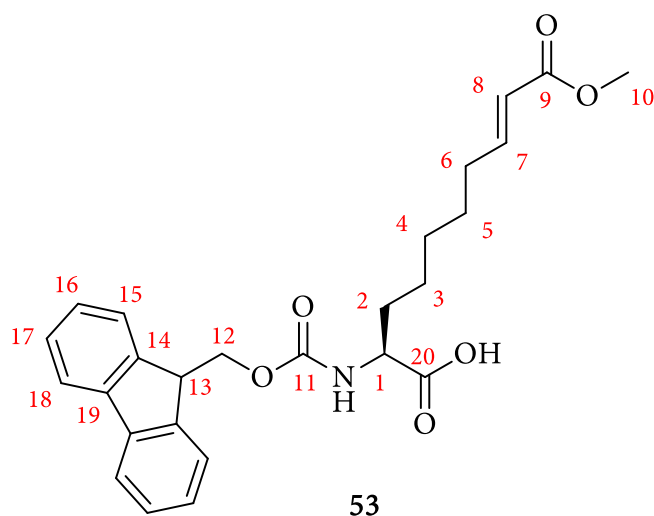
1H -NMR (400 MHz, $DMSO-d_6$): δ 7.50 (br. s, 2H, NH_2), 6.90 (dt, $J_{H8H7} = 15.7$ Hz, $J_{H7H8} = 7.0$ Hz, 1H, H7), 5.87 (dt, $J_{H8H7} = 15.6$ Hz, $J_{H6H8} = 1.6$ Hz, 1H, H8), 3.64 (s, 3H, H10), 3.18-3.10 (m, 1H, H1), 2.19 (aq, $J = 7.3$ Hz, 2H, H6), 1.74–1.64 (m, 1H, H2), 1.60-1.50 (m, 1H, H2'), 1.46-1.24 (m, 6H, H3-H4-H5) ppm.

^{13}C -NMR (101 MHz, $DMSO-d_6$): δ 169.9 (C11), 166.1 (C9), 149.7 (C7), 120.7 (C8), 53.7 (C1), 51.5 (C10), 31.2 (C6), 30.7 (C2), 28.2 (C5), 27.2 (C4), 24.6 (C3) ppm.

HRMS (ESI $^+$): (m/z) $C_{11}H_{20}NO_4$ [$M+H$] $^+$, calculated: 230.138685 found: 230.139175 (m/z).

m.p.: 184-186 $^{\circ}C$.

(S,E)-2-((Fmoc)amino)-10-methoxy-10-oxodec-8-enoic acid (**53**)



(S,E)-2-amino-10-methoxy-10-oxodec-8-enoic acid **83** (915 mg, 4.00 mmol) was dissolved in 40 mL of H₂O, and NaHCO₃ (1 g, 12 mmol) was added. A solution of Fmoc-OSu (1.5 g, 4.40 mmol) in acetone (60 mL) was added after a few minutes. pH was adjusted to 8-9 and the reaction mixture was stirred for 16h at room temperature. After that time, TLC analysis (DCM:MeOH 16:1) showed complete consumption of starting material ($R_f = 0.1$) and formation of a new product ($R_f = 0.3$). The reaction mixture was concentrated at reduced pressure and aqueous layer were washed with Et₂O (100 mL x 2). The pH was brought to 2 with 1M HCl and the aqueous solution was extracted with EtOAc (100 mL x 3), dried on MgSO₄ and evaporated under reduced pressure. Product **53** was isolated as a white solid (1.77 g, 3.91 mmol, 98% yield).

¹H-NMR (400 MHz, DMSO-d₆): δ 7.86 (d, $J_{H18H17} = 7.5$ Hz, 2H, H18), 7.68 (d, $J_{H15H16} = 7.5$ Hz, 2H, H15), 7.38 (at, $J = 7.5$ Hz, 2H, H17), 7.29 (at, $J = 7.5$ Hz, 1H, H16), 7.04 (br. s, 1H, NH), 6.85 (dt, $J_{H8H7} = 14.8$ Hz, $J_{H7H8} = 6.9$ Hz, 1H, H7), 5.81 (d, $J_{H8H7} = 15.6$ Hz, 1H, H8), 4.38-4.14 (m, 3H, H11-H12), 3.84-3.79 (m, 1H, H1), 3.60 (s, 3H, H10), 2.12 (aq, $J = 7.3$ Hz, 2H, H6), 1.76-1.61 (m, 1H, H2), 1.60-1.48 (m, 1H, H2'), 1.42-1.09 (m, 6H, H3-H4-H5) ppm.

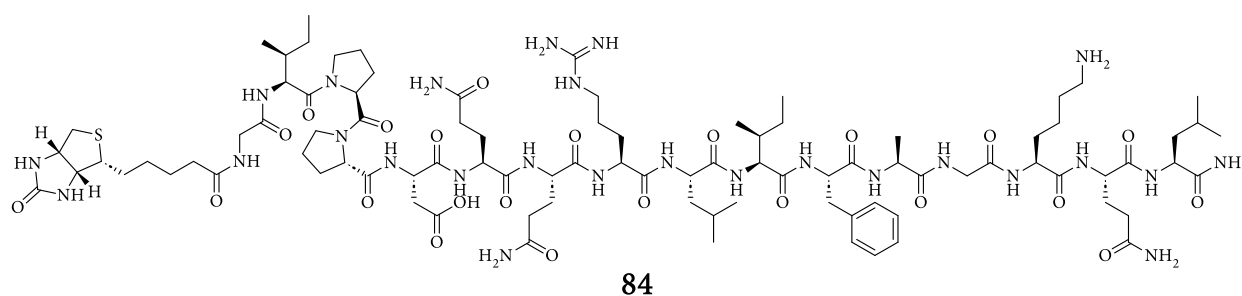
¹³C-NMR (101 MHz, DMSO-d₆): δ 170.0 (COOH), 166.1 (C9), 155.6 (C11), 149.7 (C7), 143.8 (C14), 140.7 (C19), 127.5 (C17), 127.0 (C16), 125.2 (C15), 120.5 (C8), 120.1 (C18),

65.3 (C12), 54.7 (C1), 51.1 (C10), 46.9 (C13), 31.7 (C2), 31.3 (C6), 28.3 (C5), 27.4 (C4), 25.0 (C3) ppm.

HRMS (ESI⁺): (*m/z*) C₂₆H₂₉NNaO₆ [M+Na]⁺, calculated: 474.18878, found: 474.188702.

m.p.: 131-133 °C.

Ubiquitin 35-50 native sequence biotinylated (84)

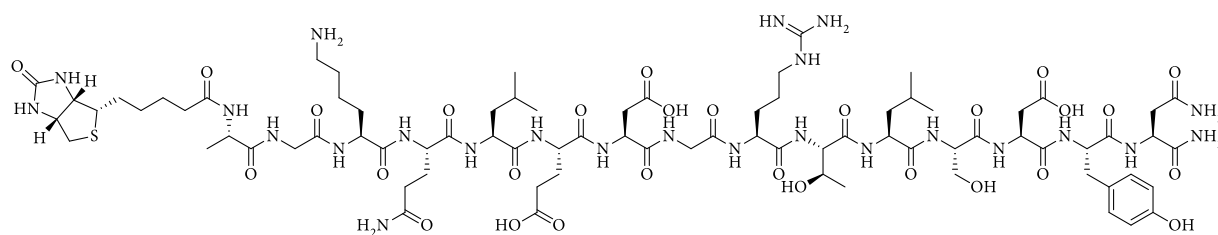


Fmoc-Leu-OH (283 mg, 0.8 mmol) was loaded to Rink amide AM resin (256 mg, 0.2 mmol) as per General Procedure 1. Fmoc-Gly-OH (178 mg, 0.6 mmol), Fmoc-Arg(Pbf)-OH (390 mg, 0.6 mmol), Fmoc-Asp(*t*Bu)-OH (247 mg, 0.6 mmol), Fmoc-Leu-OH (212 mg, 0.6 mmol), Fmoc-Lys(*t*Bu)-OH (281 mg, 0.6 mmol), Fmoc-Pro-OH (203 mg, 0.6 mmol), Fmoc-Ile-OH (212 mg, 0.6 mmol), Fmoc-Gln(Trt)-OH (366 mg, 0.6 mmol), Fmoc-Phe-OH (232 mg, 0.6 mmol) and Fmoc-Ala-OH (187 mg, 0.6 mmol) were coupled as per General Procedure 3. On resin biotinylation was performed according to General Procedure 5. The peptide was cleaved from the resin using General Procedure 4, yielding the crude product as a white solid. The peptide was purified by C₁₈ semi-preparative HPLC with a gradient of 5% MeCN (+ 0.1% TFA) with 95% H₂O (+ 0.1% TFA) to 50% MeCN (+ 0.1% TFA) with 50% H₂O (+ 0.1% TFA).

HRMS (ESI⁺): (*m/z*) C₉₁H₁₅₀N₂₆O₂₃S [M+2H]²⁺, calculated: 1003.553845, found: 1003.553959.

HPLC: R_T = 12.47 min (5-95% MeCN in H₂O over 25 min, 0.1% TFA modifier, Ascentis Express C18, 2.7 μm, 10 mm x 4.6 mm).

Ubiquitin 46-60 native sequence biotinylated (85)



85

Fmoc-Asn(Trt)-OH (477 mg, 0.8 mmol) was loaded to Rink amide AM resin (256 mg, 0.2 mmol) as per General Procedure 1. Fmoc-Gly-OH (178 mg, 0.6 mmol), Fmoc-Arg(Pbf)-OH (390 mg, 0.6 mmol), Fmoc-Asp(*t*Bu)-OH (247 mg, 0.6 mmol), Fmoc-Thr(*t*Bu)-OH (239 mg, 0.6 mmol), Fmoc-Glu(*t*Bu)-OH (255 mg, 0.6 mmol), Fmoc-Leu-OH (212 mg, 0.6 mmol), Fmoc-Lys(*t*Bu)-OH (281 mg, 0.6 mmol), Fmoc-Ala-OH (187 mg, 0.6 mmol), Fmoc-Gln(Trt)-OH (366 mg, 0.6 mmol), Fmoc-Ser(*t*Bu)-OH (230 mg, 0.6 mmol), Fmoc-Tyr(*t*Bu)-OH (276 mg, 0.6 mmol) were coupled as per General Procedure 3. On resin biotinylation was performed according to General Procedure 5. The peptide was cleaved from the resin using General Procedure 4, yielding the crude product as a white solid. The peptide was purified by C₁₈ semi-preparative HPLC with a gradient of 5% MeCN (+ 0.1% TFA) with 95% H₂O (+ 0.1% TFA) to 50% MeCN (+ 0.1% TFA) with 50% H₂O (+ 0.1% TFA).

HRMS (ESI⁺): (*m/z*) C₇₉H₁₂₈N₂₄O₂₈S [M+2H]²⁺, calculated: 946.451982, found: 946.452200.

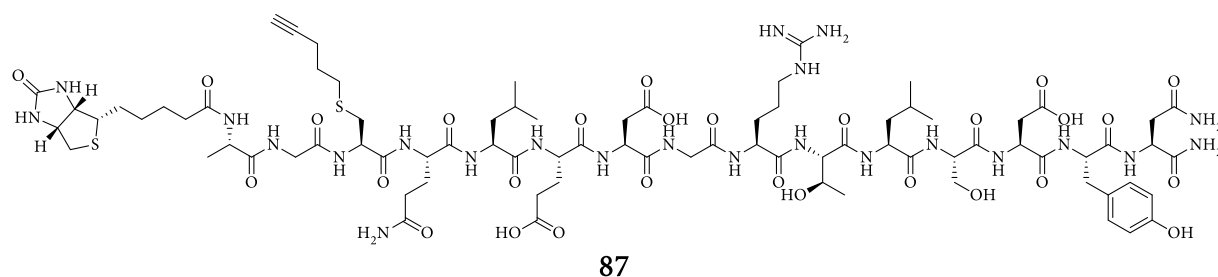
HPLC: R_T = 11.36 min (5-95% MeCN in H₂O over 25 min, 0.1% TFA modifier, Ascentis Express C18, 2.7 μm, 10 mm x 4.6 mm).

Chemical structure of compound 86, a complex peptide derivative. The structure features a central backbone with various side chains, including a thiazolidine ring, a pyrrolidine ring, a guanidino group, a benzyl group, and a propargyl group. The structure is labeled with the number 86.

HRMS (ESI⁺): (m/z) C₉₃H₁₄₉N₂₅O₂₃S₂ [M+2H]²⁺, calculated: 1024.034430, found: 1024.034435.

157

Ubiquitin 46-60 sequence K48Alkyne mutant biotinylated (87)

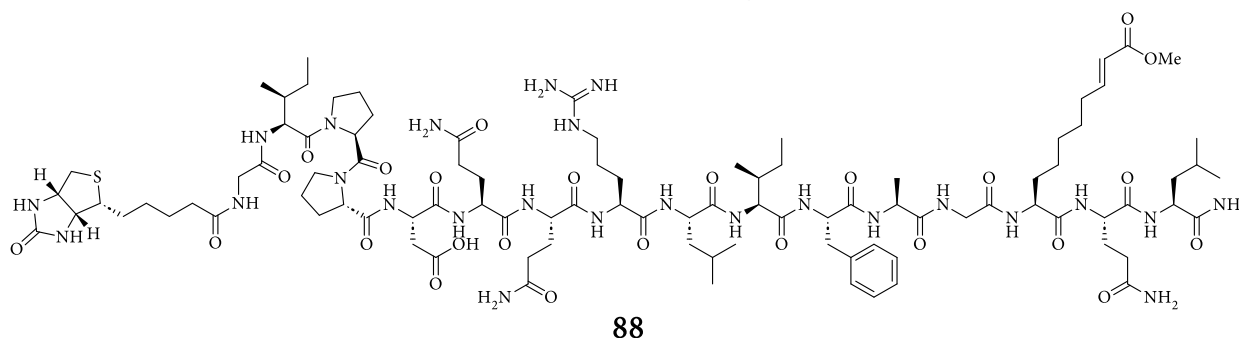


Fmoc-Asn(Trt)-OH (477 mg, 0.8 mmol) was loaded to Rink amide AM resin (256 mg, 0.2 mmol) as per General Procedure 1. Fmoc-Gly-OH (178 mg, 0.6 mmol), Fmoc-Arg(Pbf)-OH (390 mg, 0.6 mmol), Fmoc-Asp(*t*Bu)-OH (247 mg, 0.6 mmol), Fmoc-Thr(*t*Bu)-OH (239 mg, 0.6 mmol), Fmoc-Glu(*t*Bu)-OH (255 mg, 0.6 mmol), Fmoc-Leu-OH (212 mg, 0.6 mmol), N-(((9H-fluoren-9-yl)methoxy)carbonyl)-S-(pent-4-yn-1-yl)-L-cysteine **23** (246 mg, 0.6 mmol), Fmoc-Ala-OH (187 mg, 0.6 mmol), Fmoc-Gln(Trt)-OH (366 mg, 0.6 mmol), Fmoc-Ser(*t*Bu)-OH (230 mg, 0.6 mmol), Fmoc-Tyr(*t*Bu)-OH (276 mg, 0.6 mmol) were coupled as per General Procedure 3. On resin biotinylation was performed according to General Procedure 5. The peptide was cleaved from the resin using General Procedure 4, yielding the crude product as a white solid. The peptide was purified by C₁₈ semi-preparative HPLC with a gradient of 5% MeCN (+ 0.1% TFA) with 95% H₂O (+ 0.1% TFA) to 50% MeCN (+ 0.1% TFA) with 50% H₂O (+ 0.1% TFA).

HRMS (ESI⁺): (*m/z*) C₈₁H₁₂₇N₂₃O₂₈S₂ [M+2H]²⁺, calculated: 967.932568, found: 967.932326.

HPLC: R_T = 11.49 min (5-95% MeCN in H₂O over 25 min, 0.1% TFA modifier, Ascentis Express C18, 2.7 μm, 10 mm x 4.6 mm).

Ubiquitin 35-50 sequence K48VME mutant biotinylated (88)

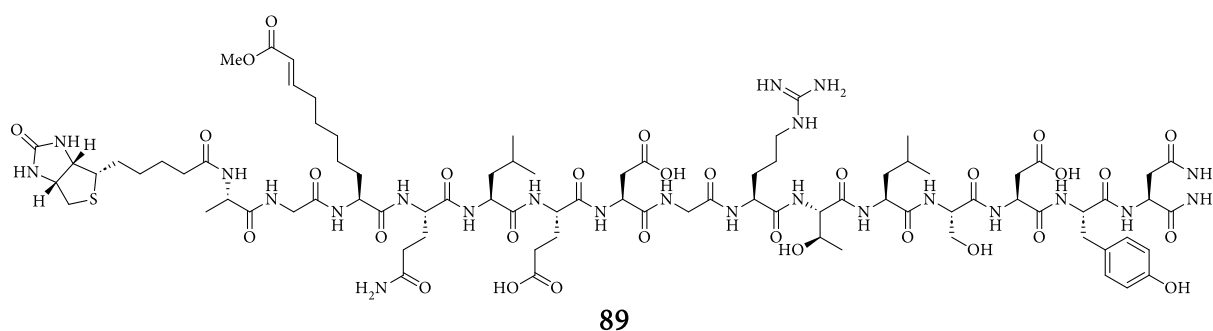


Fmoc-Pro-OH (270 mg, 0.8 mmol) was loaded to Rink amide AM resin (256 mg, 0.2 mmol) as per General Procedure 1. Fmoc-Gly-OH (178 mg, 0.6 mmol), Fmoc-Arg(Pbf)-OH (390 mg, 0.6 mmol), Fmoc-Asp(*t*Bu)-OH (247 mg, 0.6 mmol), Fmoc-Thr(*t*Bu)-OH (239 mg, 0.6 mmol), Fmoc-Glu(*t*Bu)-OH (255 mg, 0.6 mmol), Fmoc-Asn(Trt)-OH (358 mg, 0.6 mmol), Fmoc-Leu-OH (212 mg, 0.6 mmol), Fmoc-Lys(*t*Bu)-OH (281 mg, 0.6 mmol), Fmoc-Pro-OH (203 mg, 0.6 mmol), Fmoc-His(Trt)-OH (372 mg, 0.6 mmol), (S,E)-2-((Fmoc)amino)-10-methoxy-10-oxodec-8-enoic acid **53** (271 mg, 0.6 mmol), were coupled as per General Procedure 3. On resin biotinylation was performed according to General Procedure 5. The peptide was cleaved from the resin using General Procedure 4, yielding the crude product as a white solid. The peptide was purified by C₁₈ semi-preparative HPLC with a gradient of 5% MeCN (+ 0.1% TFA) with 95% H₂O (+ 0.1% TFA) to 50% MeCN (+ 0.1% TFA) with 50% H₂O (+ 0.1% TFA).

HRMS (ESI⁺): (*m/z*) C₉₆H₁₅₅N₂₅O₂₅S [M+2H]²⁺, calculated: 1045.066785, found: 1045.064851.

HPLC: R_T = 13.44 min (5-95% MeCN in H₂O over 25 min, 0.1% TFA modifier, Ascentis Express C18, 2.7 μm, 10 mm x 4.6 mm).

Ubiquitin 46-60 sequence K48VME mutant biotinylated (89)



Fmoc-Asn(Trt)-OH (477 mg, 0.8 mmol) was loaded to Rink amide AM resin (256 mg, 0.2 mmol) as per General Procedure 1. Fmoc-Gly-OH (178 mg, 0.6 mmol), Fmoc-Arg(Pbf)-OH (390 mg, 0.6 mmol), Fmoc-Asp(*t*Bu)-OH (247 mg, 0.6 mmol), Fmoc-Thr(*t*Bu)-OH (239 mg, 0.6 mmol), Fmoc-Glu(*t*Bu)-OH (255 mg, 0.6 mmol), Fmoc-Leu-OH (212 mg, 0.6 mmol), (S,E)-2-((Fmoc)amino)-10-methoxy-10-oxodec-8-enoic acid **53** (271 mg, 0.6 mmol), Fmoc-Ala-OH (187 mg, 0.6 mmol), Fmoc-Gln(Trt)-OH (366 mg, 0.6 mmol), Fmoc-Ser(*t*Bu)-OH (230 mg, 0.6 mmol), Fmoc-Tyr(*t*Bu)-OH (276 mg, 0.6 mmol) were coupled as per General Procedure 3. On resin biotinylation was performed according to General Procedure 5. The peptide was cleaved from the resin using General Procedure 4, yielding the crude product as a white solid. The peptide was purified by C₁₈ semi-preparative HPLC with a gradient of 5% MeCN (+ 0.1% TFA) with 95% H₂O (+ 0.1% TFA) to 50% MeCN (+ 0.1% TFA) with 50% H₂O (+ 0.1% TFA).

HRMS (ESI⁺): (*m/z*) C₈₄H₁₃₃N₂₃O₃₀S [M+2H]²⁺, calculated: 987.964922, found: 987.965549.

HPLC: R_T = 11.44 min (5-95% MeCN in H₂O over 25 min, 0.1% TFA modifier, Ascentis Express C18, 2.7 μm, 10 mm x 4.6 mm).

Chapter 6

References

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