Supplementary Information

Acetylacetone-Modified Ubiquitin C-Terminus Enables Specific Capture of Sulfenylated Deubiquitinases

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Table of Contents

1. General Information

1.1 Reagents and materials

Reagents	Company
Perchloric acid($HClO_4$)	J&K Scientific Ltd (Beijing)
Guanidine hydrochloride (Gn·HCl)	Adamas-beta (Shanghai, China)
$NaH_2PO_4 \cdot 12H_2O$	Ouhe Technology Ltd (Beijing)
$Na_2HPO_4 \cdot 12H_2O$	Ouhe Technology Ltd (Beijing)
4-mercaptophenylacetic acid (MPAA)	Alfa Aesar (China) Chemical Co., Ltd
Sodium nitrite ($NaNO_2$)	Beijing Chemical Works Co., Ltd (Beijing)
Tris (2-carboxyethyl) phosphine hydrochloride (TCEP HCl)	Adamas-beta, Titan Scientific Co., Ltd (Shanghai)
NaOH, NaCl, HCl	Sinopharm Chemical Reagent Co., Ltd (Shanghai)
Tris (hydroxymethyl) aminomethane (Tris)	Titan Scientific Co., Ltd (Shanghai)
Sodium 2-mercaptoethanesulfonate (MesNa)	Adamas-beta, Titan Scientific Co., Ltd (Shanghai)
hydrazine dihydrochloride	J&K Scientific Ltd (Beijing)
HEPES, sodium salt	Titan Scientific Co., Ltd (Shanghai)
D/L-methionine	GL Bio chem (Shanghai) Ltd (Shanghai, China)
Yeast extract, Tryptone, Kanamycin	Sigma-Aldrich
Isopropyl-Dthiogalactopyranoside (IPTG)	Energy Chemical
Chemically competent BL21 (DE3) cells	TransGen Biotech ltd (Beijing)
Anti-OTUB1 (ab175200)	Abcam
Anti-β-Actin antibody (4970)	Cell Signaling Technology
Anti-His6 antibody (D191001)	Sangon Biotech.
goat anti mouse IgG (HRP) antibody (D110087)	Sangon Biotech.
goat anti rabbit IgG (HRP) antibody	Sangon Biotech.

1.2 RP-HPLC, FPLC and MS

The SHIMADZU Prominence HPLC system was utilized for the analysis and purification of peptides and proteins. Analytical separation was performed on Welch XB-C18 (4.6 × 250 mm, 5 μm, 120 Å) and XB-C4 (4.6 × 250 mm, 5 μm, 300 Å) columns at 1.0 mL min^{-1}; Semi-preparative chromatography was performed on Welch XB-C4 (250 × 10 mm, 5 μm, 300 Å) and XB-C18 (250 × 10 mm, 5 μm, 120 Å) columns at 4.0 mL min^{-1}; UV detection was carried out at 214 and 254 nm for both stages, employing a binary solvent system of 0.08 % TFA in acetonitrile (A) and 0.1 % TFA in water (B). Purification by FPLC was conducted on a Cytiva AKTA purifier system, with UV absorbance followed at 280 nm. ESI-MS were acquired on a Shimadzu LC/MS-2020 platform, while proteomic profiling was carried out with a Bruker nano-Elute 2–timsTOF Pro 2 configuration.

1.3 Immunoblotting and Antibodies

Following SDS-PAGE, proteins were electro-transferred to PVDF membranes, blocked with 5 % skim milk in TBS (4 °C, 12 h), and sequentially probed with primary (37 °C, 2 h) and HRP-conjugated secondary (37 °C, 2 h) antibodies.

2. Protein expression and purification

2.1 Protein Sequence:

UbG75C: The G75C point mutation in Ub75C was cloned into pET-22b vector by site-directed mutagenesis kit (Takara).

Amino acid sequence:

MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTL SDYNIQKESTLHLVLRLRC

OTUB1: OTUB1 gene was codon optimized and synthesized by Genscript (Nanjing) and cloned into pET-28a vector with *NdeI* and *XhoI* as restriction site.

Amino Acid sequence:

MAAEEPQQQKQEPLGSDSEGVNCLAYDEAIMAQQDRIQQEIAVQNPLVSERL

ELSVLYKEYAEDDNIYQQKIKDLHKKYSYIRKTRPDGNCFYRAFGFSHLEALLD
DSKELQRFKAVSAKSKEDLVSQGFTEFTIEDFHNTFMDLIEQVEKQTSVADLLAS
FNDQSTSDYLVVYLRLLTSGYLQRESKFFEHFIEGGRTVKEFCQQEVEPMCKESD
HIHIIALAQALSVSIQVEYMDRGEGGTTNPHIFPEGSEPKVYLLYRPGHYDILYK

USP7CD: USP7catalytic domain (208-560) gene was codon optimized and synthesized by Genscript (Nanjing) and cloned into pET-28a vector with *NdeI* and *XhoI* as restriction site.

Amino Acid sequence:

KKHTGYVGLKNQGATCYMNSLLQTLFFTNQLRKAVYMMPTEGDDSSKSVPL
ALQRVFYELQHSDKPVGTKKLTKSFGWETLDSFMQHDVQELCRVLLDNVENM
KGTCVEGTIPKLFRGKMVSYIQCKEVDYRSDRREDYYDIQLSIKGKKNIFESFVD
YVAVEQLDGDNKYDAGEHGLQEAEKGVKFLTLPPVLHLQLMRFMYDPQTDQN
IKINDRFEFPEQLPLDEFLQKTDPKDPANYILHAVLVHSGDNHGGHYVVYLNPK
GDGKWCKFDDDVVSRCTKEEAIEHNYGGHDDDLSVRHCTNAYMLVYIRESKLS
EVLQAVTDHDIPQQLVERLQEEKRIEAQKRKERQE

A20: A20 gene was codon optimized and synthesized by Genscript (Nanjing) and cloned into pET-28a vector with *NdeI* and *XhoI* as restriction site.

Amino Acid sequence:

MAEQVLPQALYLSNMRKAVKIRERTPEDIFKPTNGIIHHFKTMHRYTLEMFRT
CQFCPQFREIIHKALIDRNIQATLESQKKLNWCREVRKLVALKTNGDGNCLMHA
TSQYMWGVQDTDLVLRKALFSTLKETDTRNFKFRWQLESLKSQEFVETGLCYD
TRNWNDEWDNLIKMASTDTPMARSGLQYNSLEEIHIFVLCNILRRPIIVISDKMLR
SLESGSNFAPLKVGGIYLPLHWPAQECYRYPIVLGYDSHHFVPLVTLKDSGPEIR
AVPLVNRDRGRFEDLKVHFLTDPENEMKEKLLKEYLMVIEIPVQGWDHGTTHLI
NAAKLDEANLPKEINLVDDYFELVQHEYKKWQENSEQGRRE

OTUB1 C91A: OTUB1 C91A gene was codon optimized and synthesized by Genscript (Nanjing) and cloned into pET-28a vector with *NdeI* and *XhoI* as restriction site.

Amino Acid sequence:

MAAEEPQQQKQEPLGSDSEGVNCLAYDEAIMAQQDRIQQEIAVQNPLVSERL
ELSVLYKEYAEDDNIYQQKIKDLHKKYSYIRKTRPDGNCFYRAFGFSHLEALLD

DSKELQRFKAVSAKSKEDLVSQGFTEFTIEDFHNTFMDLIEQVEKQTSVADLLAS
FNDQSTSDYLVVYLRLLTSGYLQRESKFFEHFIEGGRTVKEFCQQEVEPMCKESD
HIHIIALAQALSVSIQVEYMDRGEGGTTNPHIFPEGSEPKVYLLYRPGHYDILYK

A20 C103A: A20 C103A gene was codon optimized and synthesized by Genscript (Nanjing) and cloned into pET-28a vector with *NdeI* and *XhoI* as restriction site.

Amino Acid sequence:

MAEQVLPQALYLSNMRKAVKIRERTPEDIFKPTNGIIHHFKTMHRYTLEMFRT
CQFCPQFREIIHKALIDRNIQATLESQKKLNWCREVRKLVALKTNGDGNALMHA
TSQYMWGVQDTDLVLRKALFSTLKETDTRNFKFRWQLESLKSQEFVETGLCYD
TRNWNDEWDNLIKMASTDTPMARSGLQYNSLEEIHIFVLCNILRRPIIVISDKMLR
SLESGSNFAPLKVGGIYLPLHWPAQECYRYPIVLGYDSHHFVPLVTLKDSGPEIR
AVPLVNRDRGRFEDLKVHFLTDPENEMKEKLLKEYLMVIEIPVQGWDHGTTHLI
NAAKLDEANLPKEINLVDDYFELVQHEYKKWQENSEQGRRE

2.2 Expression and purification of DUBs.

OTUB1, A20, OTUB1C91A, A20C103A plasmids were transformed into E. coli BL21(DE3), and cultures were grown at 37 °C in LB–kanamycin (0.1 mg mL^{-1}) to OD_{600} ≈ 0.8. Expression was induced with 0.4 mM IPTG at 20 °C for 8 h. Cells were harvested, resuspended in ice-cold lysis buffer A (50 mM Tris, 100 mM NaCl, pH 7.5), and lysed by sonication on ice. The clarified lysate was batch-bound to Ni-NTA resin (4 °C, 2 h), washed with lysis buffer B (20 mM Tris, 150 mM NaCl, 40 mM imidazole, pH 7.5), and eluted with the same buffer containing 250 mM imidazole. Final purification was achieved by FPLC on a Superdex 75 10/300 GL column.

2.3 Expression and purification of UbG75C.

UbG75C was introduced into E. coli BL21(DE3) for expression. The culture was grown at 37 °C in LB–Ampicillin (0.1 mg mL^{-1}) to OD_{600} ≈ 0.8. Expression was induced with 0.4 mM IPTG at 37 °C for 5 h. Cells were harvested, resuspended in ice-cold lysis buffer (25 mM Tris, 150 mM NaCl, pH 7.5), and lysed by sonication on ice. After adjusting to 1 % (v/v) perchloric acid under vigorous agitation, the sample was clarified by centrifugation and dialyzed against 0.1 % aqueous TFA to remove perchloric acid.

3. General procedures for the preparation of Probe 1, Probe 2 and Ub-DYn-2

3.1 General procedure for the preparation of the acetylacetone derivative.

The details for the preparation of the acetylacetone derivative (1-bromo-2,4-pentanedione) were described in the ref. Tetrahedron Lett. 2007, 48, 3767–3769.[1]

3.2 General procedure for the preparation of Ub-DYn-2.

The synthetic protocol for Ub-DYn-2 was essentially as described.[4]

3.3 General procedure for the preparation of Ub(1-74)-$NHNH_2$.

The synthetic protocol for Ub(1-74)-$NHNH_2$ (**b**) was essentially as described.[2] In detail, the Ub mutant UbG75C was first concentrated to 10 mg/mL. Following this step, a hydrazinolysis reagent containing MesNa (100 mg/mL), $NH_2NH_2 \cdot HCl$ (50 mg/mL), and tri(2-carboxyethyl)phosphine (TCEP, 5 mg/mL) was added.[2] After adjusting the reaction pH to 7.0, the reaction proceeded at 50 °C for 48 h with N_2 as the protective gas. Separation of the reaction mixture was achieved by means of semi-preparative HPLC (C18 column), with a gradient elution of 20%-70% A applied for 30 min (isolated yield 41%).

3.4 General procedure for the preparation of Biotin-Ub(1-74)-$NHNH_2$.

The preparation of the 2-Cl-(Trt)-$NHNH_2$ resin was performed essentially as described previously.[3] Fmoc-based solid-phase peptide synthesis was conducted following a previously described protocol, with detailed procedures for chemical protein synthesis provided in the Supporting Information (SI, Page 4)[1]. It should be noted that we used two dipeptide analogues (pseudoproline dipeptide Leu56-Ser57 and dimethoxybenzyl Asp52-Gly53) to improve the synthesis efficiency. Notably, two dipeptide analogues—specifically the pseudoproline dipeptide Leu56-Ser57 and dimethoxybenzyl-modified Asp52-Gly53—were employed to enhance the efficiency of the synthetic process. Peptides were cleaved from the resin support via a standard trifluoroacetic acid (TFA)-based cleavage cocktail (TFA:phenol:H_2O:thioanisole:1,2-ethanedithiol (EDT) = 82.5:5:5:5:2.5, 2-3 mL per 100 mg resin) for a duration of 3 hours.

3.5 The ligation between b and cysteamine was achieved via hydrazide-based native chemical ligation.

8.4 mg of **b** (1 μmol, 1 equiv.) was dissolved in liagtion buffer (0.2 M PBS, 6 M Gn-HCl, pH 3.0), followed by addition of $NaNO_2$ (0.48 mg, 7 equiv.). The reaction mixture was incubated at -10 °C for 20 min to convert the peptide b to the corresponding acyl azide. Next, 4-mercaptophenylacetic (MPAA, 10.1 mg, 60.0 equiv.)[2] and cysteamine (0.39 mg, 5.0 equiv.) were added to the above solution, and the mixture was then stirred at pH 6.5 and

37 °C for 3 h to obtain **c**. Finally, separation of the reaction mixture was achieved by means of semi-preparative HPLC (C18 column), with a gradient elution of 20%-70% A applied for 30 min (isolated yield 37%).

3.6 The ligation between d and cysteamine was achieved via hydrazide-based native chemical ligation.

Like above, 8.9 mg of **d** (1 μmol, 1 equiv.) was dissolved in liagtion buffer (0.2 M PBS, 6 M Gn-HCl, pH 3.0), followed by addition of $NaNO_2$ (0.48 mg, 7 equiv.). The reaction mixture was incubated at -10 °C for 20 min to convert the peptide **d** to the corresponding acyl azide. Next, 4-mercaptophenylacetic (MPAA, 10.1 mg, 60.0 equiv.)[2] and cysteamine (0.39 mg, 5.0 equiv.) were added to the above solution, and the mixture was then stirred at pH 6.5 and 37 °C for 3 h to obtain **e**. Finally, separation of the reaction mixture was achieved by means of semi-preparative HPLC (C18 column), with a gradient elution of 20%-70% A applied for 30 min (isolated yield 32%).

3.7 The ligation between c and acetylacetone derivative was achieved via cysteine-aminoethylation.

8.5 mg of **c** (1 μmol, 1 equiv.) was dissolved in HEPES buffer (6 M Gn-HCl, 0.5 M HEPES, 10 mM D/L-methionine, 10 mM TCEP, pH 8.5), after which the acetylacetone derivative (4.8 mg, 25 equiv.) was added and the mixture was stirred 37 °C for 4 h to obtain **Probe 1**. Finally, separation of the reaction mixture was achieved by means of semi-preparative HPLC (C18 column), with a gradient elution of 20%-70% A applied for 30 min (isolated yield 23%).

3.8 The ligation between e and acetylacetone derivative was achieved via cysteine-aminoethylation.

9.0 mg of **e** (1 μmol, 1 equiv.) was dissolved in HEPES buffer (6 M Gn-HCl, 0.5 M HEPES, 10 mM D/L-methionine, 10 mM TCEP, pH 8.5), after which the acetylacetone derivative (4.8mg, 25 equiv.) was added and the mixture was stirred 37 °C for 4 h to obtain **Probe 2**. Finally, separation of the reaction mixture was achieved by means of semi-preparative HPLC (C18 column), with a gradient elution of 20%-70% A applied for 30 min (isolated yield 36%).

3.9 Probes refolding

Briefly, 1 mg of the probe was solubilized in 1 mL of refolding buffer (6 M Gn-HCl, 0.2 M Na_2HPO_4, pH 7.0). The resulting solution was subsequently subjected to four rounds of

buffer exchange into Tris buffer (20 mM Tris-base, 150 mM NaCl, pH 7.0) at 4 °C, with each exchange step lasting 4 hours and yielding an overall recovery of 82%.

4 Labeling of DUBs

4.4 Labeling of OTUB1 S-sulfenylation using Probe 1 in Vitro.

To evaluate the effect of H_2O_2 concentration on the efficiency of probe 1 labelling OTUB1, 3 μM OTUB1 and 5 μM **Probe 1** were incubated at 37 °C in labeling buffer (50 mM Tris-HCl, 100 mM NaCl, pH 7.5) for 30 min with H_2O_2 of different concentrations (1-1000 μM). To evaluate the kinetics of OTUB1 labeling by **Probe 1**, 3 μM OTUB1 was incubated with 5 μM **Probe 1** at 37 °C in labeling buffer (50 mM Tris-HCl, 100 mM NaCl, pH 7.5) supplemented with 10 μM H_2O_2 for 5, 10, 20, 30, 60, and 120 min. All samples were analyzed by SDS-PAGE.

4.5 Labeling of A20 or USP7-CD S-sulfenylation using Probe 1 in Vitro.

A20 or USP7-CD cross-linked with **Probe 1** under the same conditions as above. 3 μM A20 or USP7-CD and 5 μM **Probe 1** were incubated at 37 °C in labeling buffer (50 mM Tris-HCl, 100 mM NaCl, pH 7.5) for 30 min with 10 μM H_2O_2. All samples were analyzed by SDS-PAGE.

4.6 Labeling of S-Sulfenylated USP7-CD with Probe 1 and Ub-DYn-2 in Vitro

To compare the labeling efficiency of Ub-DYn-2 and **Probe 1**. 3 μM USP7-CD and 5 μM **Probe 1** or **Ub-DYn-2** were incubated at 37 °C in labeling buffer (50 mM Tris-HCl, 100 mM NaCl, pH 7.5) with 10 μM H_2O_2 for 2min, 5min, 10min, 15min. All samples were analyzed by SDS-PAGE.

4.7 Labeling of OTUB1 C91A or A20 C103A using Probe 1 in Vitro.

OTUB1 C91A or A20 C103A cross-linked with **Probe 1** under the same conditions as above. 3 μM OTUB1 C91A or A20 C103A and 5 μM **Probe 1** were incubated at 37 °C in labeling buffer (50 mM Tris-HCl, 100 mM NaCl, pH 7.5) for 30 min with 10 μM H_2O_2. All samples were analyzed by SDS-PAGE.

4.8 Labeling of OTUB1 in cell lysate.

To assess the efficiency of OTUB1 S-sulfenylation cross-linking to **Probe 1** under physiologically complex conditions. 3 μM OTUB1 and 5 μM **Probe 1** were added to 100μg of Hela cell lysate and incubated at 37°C for 30min. The samples were treated with 10 μM

H_2O_2, with untreated samples serving as negative controls. Immunoblotting analysis was performed using an anti-His_6 antibody. Subsequently, To assess whether **Probe 1** can selectively label endogenous OTUB1 S-sulfenylation, like above, 5 μM **Probe 1** was added to 100μg of Hela cell lysate and incubated at 37°C for 30min, The samples were treated with 10 μM H_2O_2, with untreated samples serving as negative controls. Immunoblotting analysis was performed using an anti-OTUB1 antibody.

4.9 Immunoblotting analysis

Briefly, 5 μM **Probe 2** was added to 100 μg HeLa cell lysate and incubated at 37°C for 30min. The samples were treated with 10 μM H_2O_2, with untreated samples serving as negative controls. Immunoblotting analysis was performed using an anti-Biotin antibody.

5 Supplementary figures

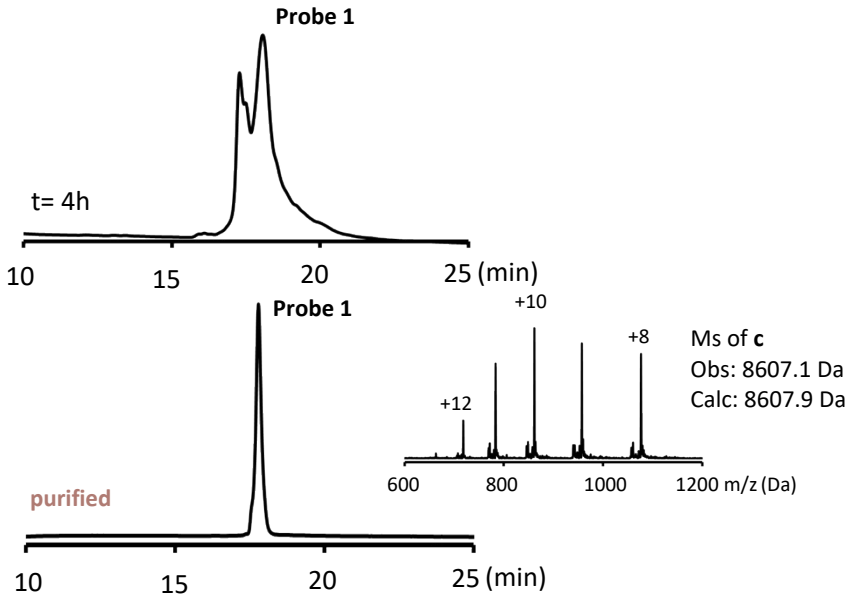


Figure S1. RP-HPLC and ESI-MS of Probe 1.

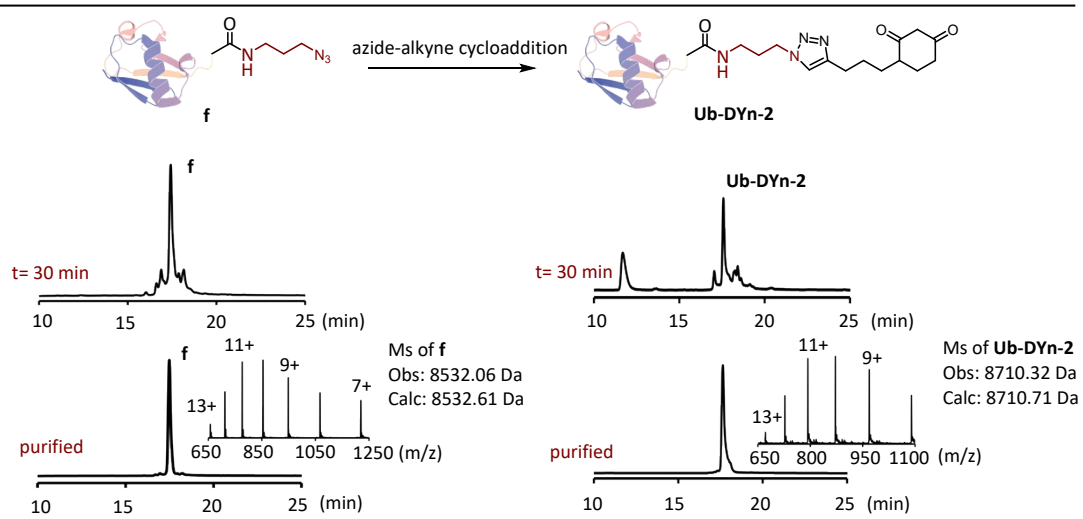


Figure S2. Chemical synthesis and characterization of **Ub-DYn-2**.

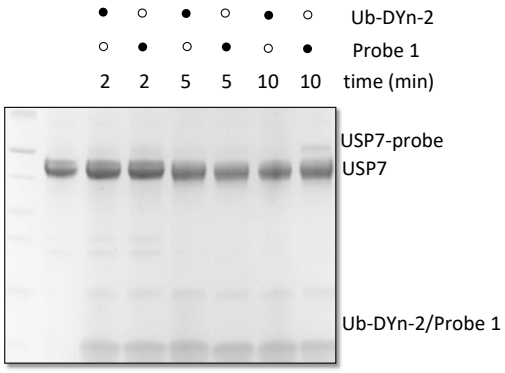


Figure S3. Comparison of Labeling Efficiency Between **Ub-DYn-2** and **Probe 1** for S-Sulfenylated USP7-CD.

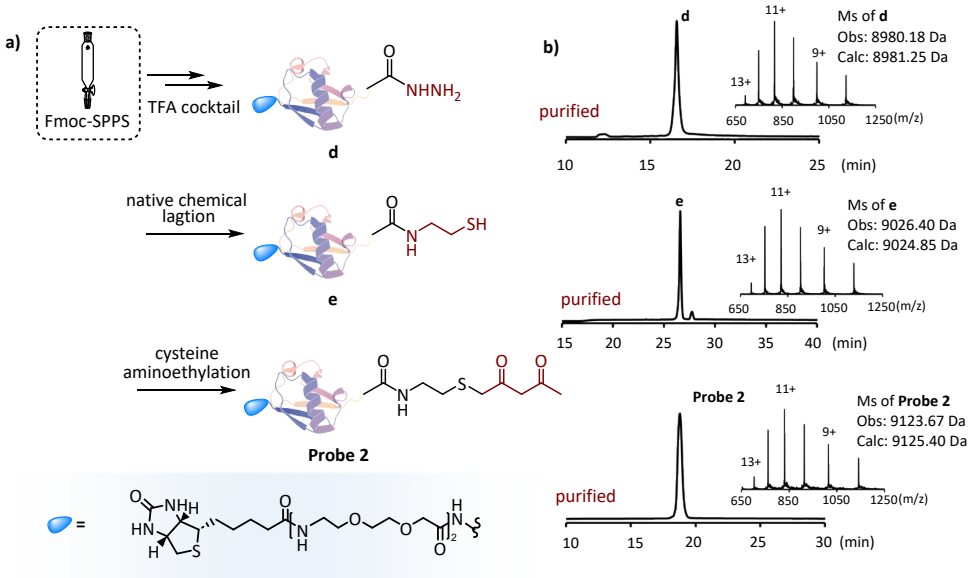
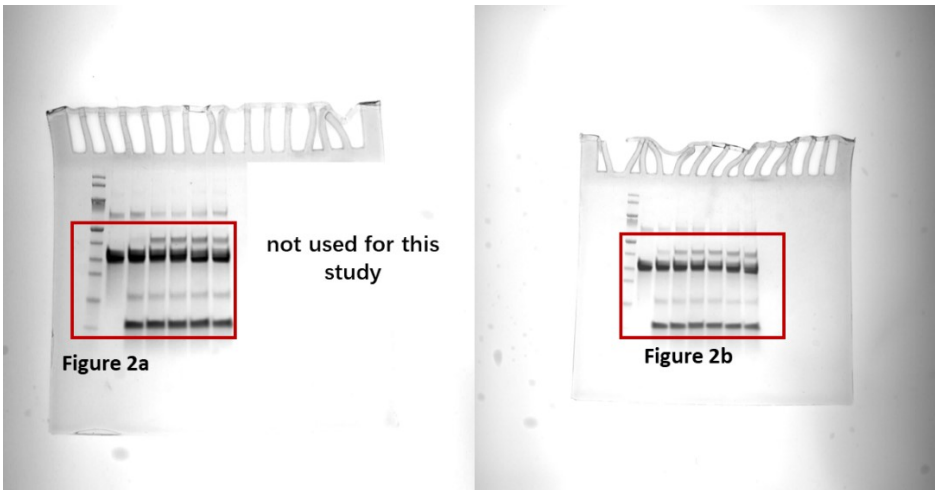
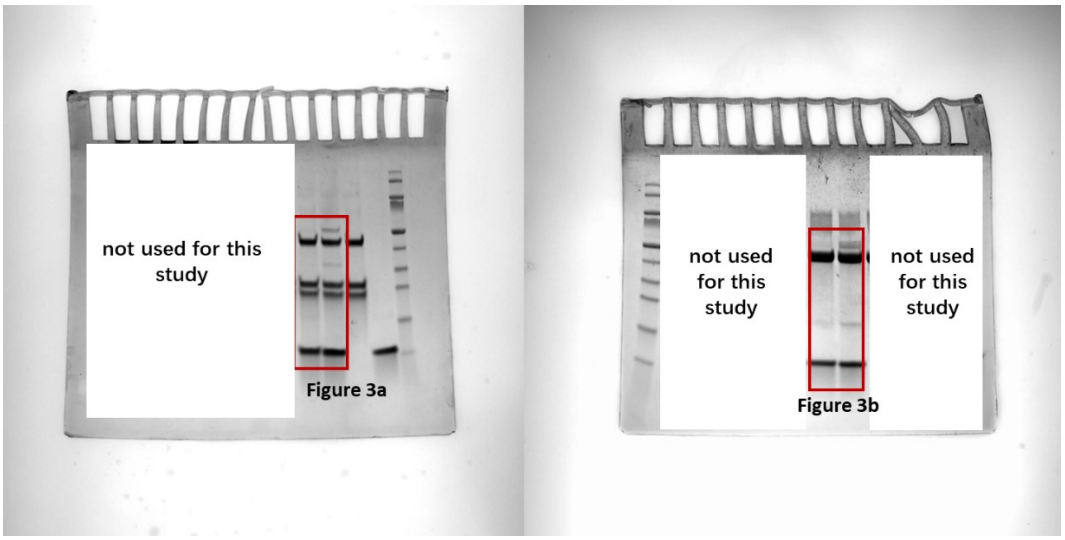
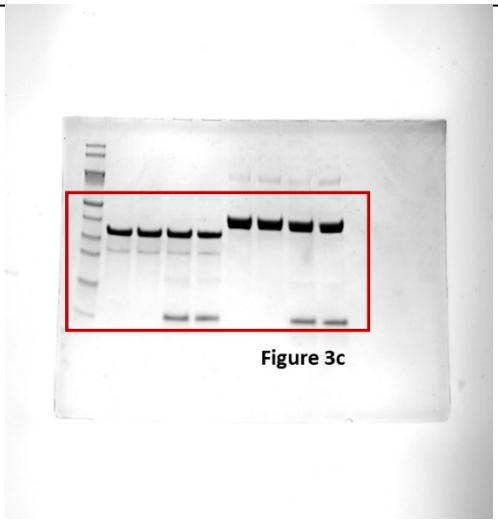


Figure S4. a) Chemical synthesis and characterization of acetylacetone-modified Biotin-Ub probe (**Probe 2**). a) **Synthetic route of Probe 2**. b) RP-HPLC and ESI-MS of d, e and **Probe 2**.

6. Original SDS-PAGE analysis data







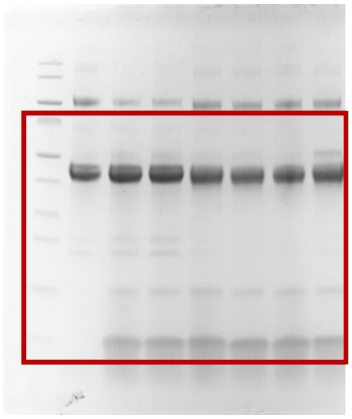
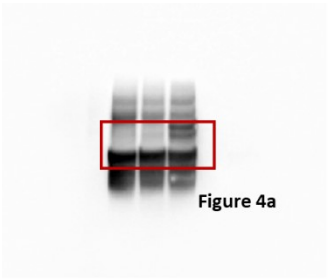
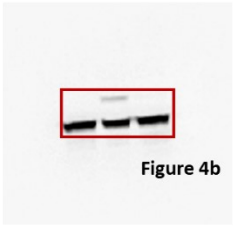
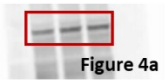


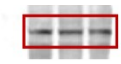
Figure S3

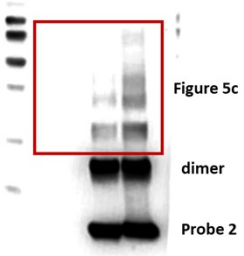
7. Original Western Blots analysis data

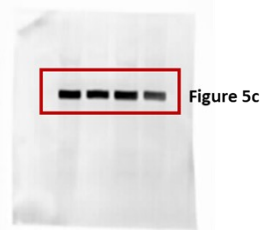












8 References

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(2) (a) Wang, Y.; Xia, Q.; Hua, X.; Guo, Y.; Shi, J.; Li, Y.-M. Semi-synthesis of biotin-bearing activity-based ubiquitin probes through sequential enzymatic ligation, N-S acyl transfer and aminolysis reaction. *Chin. Chem. Lett.* **2023**, 34 (6), 108010. (b) Xu, L.; Huang, J.-F.; Chen, C.-C.; Qu, Q.; Shi, J.; Pan, M.; Li, Y.-M. Chemical synthesis of natural polyubiquitin chains through auxiliary-mediated ligation of an expressed ubiquitin isomer. *Org. Lett.* **2018**, 20 (2), 329-332.

(3) Xia, Q.; Meng, X. B.; Wang, Y.; Yuan, R. J.; Li, P. C.; Liu, L. W.; Li, Y.-M. A cell-permeable Ub-Dha probe for profiling E1–E2–E3 enzymes in live cells. *Chem.Comm.* **2024** 60, 4342-4345.

(4) Chen, Z.; Li, G.; Zhang, J.; Xu, X.; Yan, Q.; Wang, Q.; Zhang, Q.; Huang, J. Activity-Based Ubiquitin Probes Capture the Sulfenylated State of Deubiquitinases. *Angew. Chem. Int. Ed.* **2025**, *64* (43), e202512311.