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PII: S0040-4020(16)30440-9

DOI: 10.1016/j.tet.2016.05.041

Reference: TET 27768

To appear in: *Tetrahedron*

Received Date: 12 April 2016

Revised Date: 13 May 2016

Accepted Date: 16 May 2016

Please cite this article as: Li Y-T, Huang Y-C, Xu Y, Pan M, Li Y-M, Ubiquitin 7-amino-4-carbamoylmethylcoumarin as an improved fluorogenic substrate for deubiquitinating enzymes, *Tetrahedron* (2016), doi: 10.1016/j.tet.2016.05.041.

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Ubiquitin 7-amino-4-carbamoylmethylcoumarin as an improved fluorogenic substrate for deubiquitinating enzymes

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ARTICLE INFO

Article history:
Received
Received in revised form
Accepted
Available online

Keywords:
Ub-ACC
Deubiquitinating enzyme
Ub-AMC
peptide hydrazides
protease assay

ABSTRACT

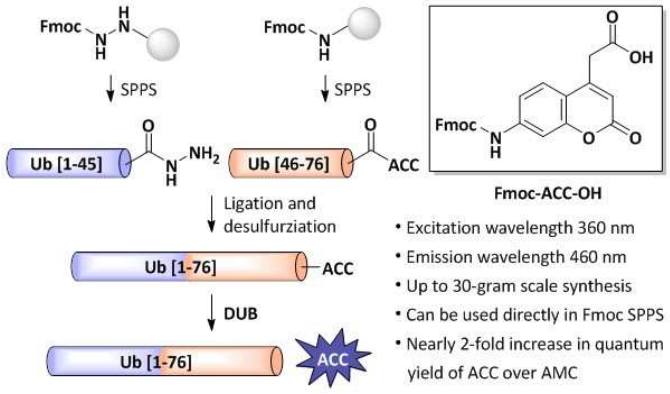
A new fluorogenic substrate Ub-ACC (ubiquitin C-terminal 7-amino-4-carbamoylmethyl-coumarin) was developed for DUB (deubiquitinating enzyme) activity assays. This substrate can be synthesized with higher efficiency than the classical DUB substrate, Ub-AMC (ubiquitin C-terminal 7-amino-4-methylcoumarin). DUB assays using UCH-L3, OTUD2 and USP30 demonstrated that Ub-ACC shows nearly 2-fold higher sensitivity than Ub-AMC.

1. Introduction

As a prevalent posttranslational modification in eukaryotic cells, ubiquitination regulates the architecture, location and stability of many proteins to maintain the homeostasis of organisms.[1] Ubiquitination involves a covalent isopeptide bond linkage between the C-terminal carboxyl group of one ubiquitin (Ub) and a lysine side-chain/N-terminal amino group of the substrate protein or another Ub.[1] The reversible ubiquitination process is controlled by "Ub code writers", i.e. stepwise acting Ub ligases (E1, E2 and E3) and "Ub code erasers", i.e. deubiquitinating enzymes (DUBs).[2] DUBs are classified into five classes including the JAMM domain superfamily metalloprotease and four families of cysteine proteases.[3] Dysregulated DUBs are implicated in many disease-related cellular processes.[4] Although more than 100 human DUBs have been found, substrate and linkage specificities of many DUBs remain elusive. To study these versatile DUBs, it is necessary to develop high-throughput and sensitive enzyme assays. Current biochemical assays of DUB use either activity-based Ub/DiUb suicide probes[5] or artificial Ub/Ub-peptide/DiUb substrates[6] containing reporting groups which can provide fluorogenicity[7], luminescence[8], fluorescence polarization[9] or FRET read-outs[10] upon DUB treatment.

Among the DUB assay reagents, fluorogenicUb-AMC (ubiquitin C-terminal 7-amino-4-methylcoumarin)[7a] and Ub-Rhodamine[7b] have been extensively used in activity profiling of different DUBs and inhibition screening of compound libraries in a high-throughput manner. Earlier methods to prepare these compounds usually relied on intein technology[11] to express the Ub[1-76]-thioester. Direct aminolysis with excess amino-functionalized fluorophore could generate the desired fluorescence-quenched substrates. However this method suffers from significant hydrolysis and low yield. Recently Liu group reported a new semisynthetic strategy towards Ub-AMC through auxiliary-mediated[12] hydrazide-based[13] native chemical ligation (NCL)[14] between the Ub[1-75]-hydrazide and the AMC-auxiliary conjugate.[7c] The main drawbacks of this method are the relatively low expression yield of Ub[1-75]-intein and tedious synthesis of the fluorophore derivative. Alternatively, Ub-AMC can also be generated through total chemical synthesis[15,16] with good efficiency as reported by Ovaa[7d] and Liu groups[7e]. To further streamline these total synthetic protocols, we proposed to reduce in-solution operations. Our plan is to first anchor the AMC fluorophore on solid-phase and then perform automatic solid-phase peptide synthesis (SPPS) and ordinary NCL (Scheme 1).

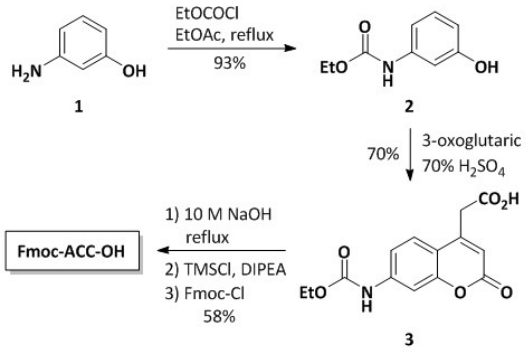
Herein, we report the design and synthesis of a new *in vitro*fluorogenic substrate, Ub-ACC (ubiquitin C-terminal 7-amino-4-carbamoylmethyl-coumarin), for DUB activity assays (Scheme 1). Importantly, the streamlined synthetic strategy for Ub-ACC (1.5-gram scale) is cost-effective and robust, which holds promise for further scaling-up. Furthermore, in our DUB assays using UCH-L3, OTUD2 and USP30, synthetic Ub-ACC shows about 2-fold higher sensitivity than Ub-AMC. This allows lower consumption of both enzyme and Ub-ACC which makes the new fluorogenic molecule favorable for applications in high-throughput screening of DUB inhibitors.



Scheme 1 Synthetic strategy of Ub-ACC.

2. Results and discussion

To make the preparation of fluorogenic DUB substrates more general and efficient, we aimed to employ the AMC derivative as a building block during SPPS to avoid additional post-SPPS and post-NCL operations. Inspired by an elegant report by Ellman and Craik[17a] who used a bifunctional fluorescent compound ACC in place of AMC for the generation of combinatorial fluorogenic substrate libraries[17], we expected equally facile synthesis of Ub-ACC to replace the classical Ub-AMC. By using a slightly modified documented protocol[18], we synthesized Fmoc-ACC-OH on a 1.5-gram scale (Scheme 2) which is suitable for 0.1 mmol-scale Fmoc SPPS. Note that large-scale preparation of this compound has been achieved (up to 30-gram final product)[18], which makes it potentially available commercially.



Scheme 2 Synthesis of Fmoc-ACC-OH.

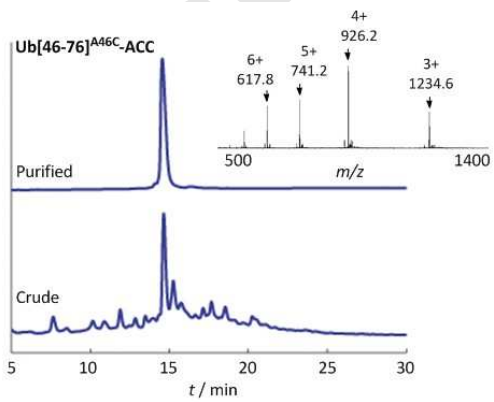


Fig. 1 Automatic microwave-assisted high-temperature SPPS of Ub[46-76]^{A46C}-ACC. HPLC traces and ESI-MS (observed: 3700.8 ± 0.2 Da; calculated: 3701.1 Da) of Ub[46-76]^{A46C}-ACC are shown.

With Fmoc-ACC-OH in hand, we initiated SPPS of Ub[46-76]^{A46C}-ACC conjugate and Ub[1-45] hydrazide for the following NCL. Fmoc-ACC-OH was anchored onto the solid-phase resin and routine peptide elongation was conducted. Both segments were prepared using automatic microwave-assisted high-temperature SPPS starting from Fmoc-hydrazine 2-chlorotrityl chloride resin[19] or Fmoc Rink amide resin and characterized by analytical high performance liquid chromatography (HPLC) and electrospray ionization mass spectrometry (ESI-MS) (Fig. 1 and Fig. S6). Ub[1-45] hydrazide was pre-converted into Ub[1-45]-MESNa (sodium 2-sulfanylethanesulfonate) peptide thioester. Next Ub[1-45]-MESNa (3 mM) was reacted with N-terminal cysteine functionalized peptide, Ub[46-76]^{A46C}-ACC (3 mM), in the aqueous ligation buffer (6 M Guanidine hydrochloride, 0.1 M Na_2HPO_4, 150 mM 4-mercapto-phenylacetic acid (MPAA), pH 6.5)[20] at room temperature. The reaction was monitored by analytical HPLC. Almost quantitative conversion was observed when the ligation was carried out for 2 hr (Fig. 2). The ligated product Ub[1-76]^{A46C}-ACC (8.8 mg, 63% isolated yield) was then purified and treated with the desulfurization buffer (6 M Guanidine hydrochloride, 0.1 M Na_2HPO_4, 25 mM VA-044, 100 mM 2-methyl-2-propanethiol, 250 mM tris(2-carboxyethyl)phosphine (TCEP), pH 6.9) at 37 °C for 12 hr. Ub-ACC was purified through semi-preparative HPLC (5.5 mg, 61% isolated yield). Meanwhile, we examined a one-pot ligation-desulfurization protocol using our recently found alkyl thiol catalyst, methyl thioglycolate[21], which also gave satisfactory yield and efficiency.

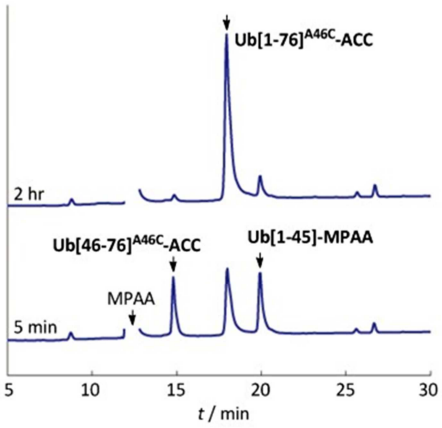


Fig. 2 NCL between Ub[1-45]-MPAA and Ub[46-76]^{A46C}-ACC

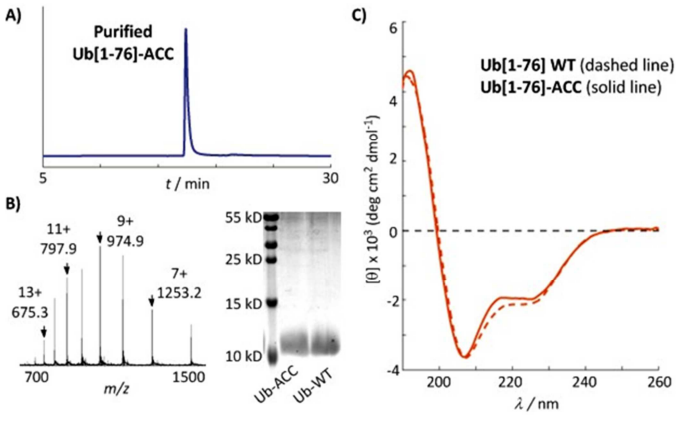


Fig. 3 A) HPLC trace of purified Ub-ACC. B) Direct-injection ESI-MS (observed: 8765.4 ± 0.6 Da; calculated: 8765.2 Da) and SDS-PAGE analysis of Ub-ACC. C) CD spectra of folded Ub and Ub-ACC

Next we characterized full-length Ub-ACC through analytical HPLC (Fig. 3A). Direct-injection electrospray ionization-mass spectrum (ESI-MS) and SDS-PAGE verified the correct molecular weight (Fig. 3B). Then we carried out folding of Ub-ACC through direct dissolution in ddH_2O[22] (for circular dichroism (CD) assay) or aqueous buffer. CD spectra of Ub-ACC and wild-type Ub were almost identical, which confirmed the correct secondary structure of synthetic Ub-ACC (Fig. 3C).

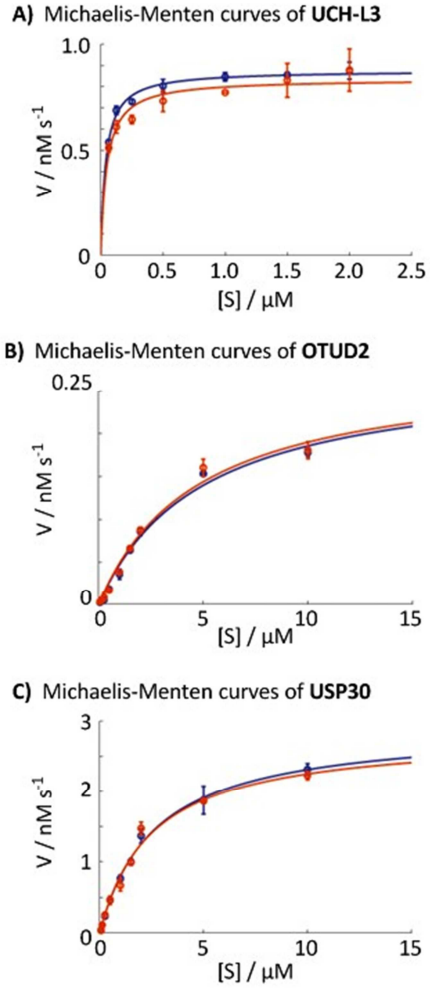


Fig. 4 Michaelis-Menten kinetics of UCH-L3, OTUD2 and USP30 treated with Ub-AMC (blue line) or Ub-ACC (red line). Nonlinear fit model of "v versus [S]": $v = v_{max}\cdot[S] / ([S] + K_m)$, $k_{cat} = v_{max} / [E_0]$.

Table 1 Kinetic parameters using Ub-ACC and Ub-AMC as substrates of UCH-L3, OTUD2 and USP30.

Probe	DUB	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} M^{-1}$)
Ub-AMC	UCH-L3	0.040 ± 0.003	8.8 ± 0.1	(2.23 ± 0.19) x 10^8
	OTUD2	4.91 ±0.98	0.028 ±0.003	(5.64 ± 1.26) x 10^3
	USP30	2.68 ±0.21	0.29 ±0.01	(1.09 ± 0.09) x 10^5
Ub-ACC	UCH-L3	0.047 ± 0.010	8.4 ± 0.3	(1.77 ± 0.38) x 10^8
	OTUD2	4.73 ±1.01	0.028 ±0.003	(5.92 ± 1.40) x 10^3
	USP30	2.50 ± 0.39	0.28 ±0.02	(1.12 ± 0.19) x 10^5

To investigate the application of Ub-ACC as the *in vitro* DUB substrate, we set out to establish fluorogenic assays using three representative enzymes, UCH-L3, OTUD2 and USP30, respectively. These DUBs come from three cysteine protease super-families, i.e. UCH (ubiquitin C-terminal hydrolase), OTU (ovarian tumor) and USP (ubiquitin-specific protease) super-families. Enzymatic assays were conducted by diluting both enzyme and substrate in the working buffer (50 mM Tris, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, pH 7.5). Hydrolysis reactions were all performed in 96-well plates at 30 °C. Concentrations of enzymes were fixed (UCH-L3: 100 pM; OTUD2: 10 nM; USP30: 10 nM) and concentrations of Ub-ACC and Ub-AMC substrates were set in the range from 50 nM to 10 μM. By plotting the initial enzymatic velocity (from fluorescence read-outs) versus starting concentrations of substrates, we calculated the k_{cat} and K_m constants of Ub-ACC and Ub-AMC against three DUBs by using the nonlinear fit model corresponding to the Michaelis-Menten equation. We found that Ub-ACC and Ub-AMC almost gave identical Michaelis-Menten curves (Fig. 4) and similar kinetic constants (Table 1). These data for UCH-L3 were also comparable to those determined in previous reports[7a,7b].

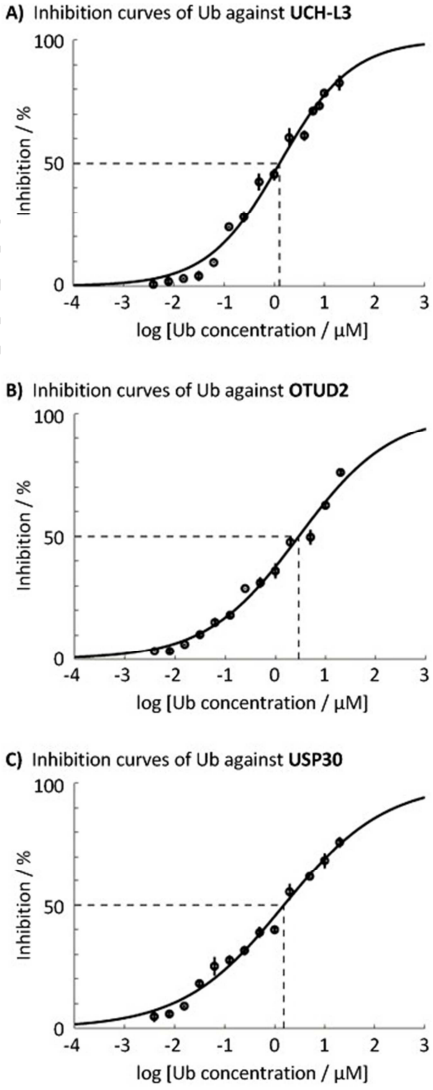


Fig. 5 Inhibition assays of ubiquitin against UCH-L3, OTUD2 and USP30 treated with Ub-ACC. Nonlinear longistic fit model of "Inhibition% versus c": Inhibition% = $1 / (1 + e^{-k(c - c^*)})$, $IC_{50} = 10^{c^*}$, $K_i = IC_{50} / (1 + [S] / K_m)$.

Finally, we further demonstrated the practical utility of Ub-ACC based fluorogenic assay by using free Ub as a noncovalent inhibitor of DUBs (Fig. 5). IC_{50} values of Ub against UCH-L3 (100 pM enzyme, 62.5 nM Ub-ACC), OTUD2 (10 nM enzyme, 500 nM Ub-ACC) and USP30 (10 nM enzyme, 500 nM Ub-ACC) were determined to be 1.28 ± 0.09 μM, 2.92 ± 0.12 μM

and 1.53 ± 0.11 μM, respectively. K_i values were then determined according to the Cheng-Prusoff equation as follows. K_i (UCH-L3) = 0.551 ± 0.039 μM; K_i (OTUD2) = 2.64 ± 0.11 μM; K_i (USP30) = 1.53 ± 0.09 μM. These results illustrated that Ub-ACC might become a robust alternative assay reagent to Ub-AMC for DUB libraries.

3. Summary

In conclusion, we presented an expedient method for the synthesis of a new fluorogenic DUB substrate Ub-ACC for *in vitro* enzymatic assays. According to the DUB assays, Ub-ACC is nearly 2-fold more sensitive to Ub-AMC owing to the higher fluorescent yield of ACC. This permits reduction of the DUB enzyme and Ub-ACC concentration which may save costs for large-scale DUB inhibitor screening assays. Other features of our streamlined protocol include: 1) Synthesis of Fmoc-ACC-OH is feasible and well documented (up to 30-gram scale). 2) Solid-phase peptide synthesis and Ub segment ligation have proved to be robust procedures for scaling-up. 3) The Ub-ACC synthetic protocol is expected to be suitable to other *in vitro* DUB substrates like Ub-Rhodamine as well as reagents derived from ubiquitin-like proteins such as SUMO-ACC. We hope that our cost-competitive synthetic route of Ub-ACC might find broad applications in the identification of novel selective DUB inhibitors.

4. Experimental

4.1. Materials

All reagents and solvents were purchased from Sinopharm Chemical Reagent Co., Ltd., Alfa Aesar China Co., Ltd., J&K Chemical Co., Ltd. THF was distilled from sodium/diphenyl ketone immediately prior to use. DMF was distilled under reduced pressure from sodium sulfate and stored over 4 Å molecular sieves. CH_2Cl_2, pyridine and Et_3N were distilled from calcium hydride immediately prior to use. All other commercially available reagents and solvents were used as received without further purification unless otherwise indicated. All organic extracts were dried over sodium sulfate or magnesium sulfate. TLC was carried out on plates pre-coated with silica gel 60 F254 (250 layer thickness). Visualization was accomplished using UV light, iodine vapors, ninhydrin solution, permanganate solution and/or phosphomolybdic acid (PMA) solution. Flash column chromatographic purification of products was accomplished using forced-flow chromatography on Silica Gel (300-400 mesh on large-scale or 200-300 mesh on small-scale). Fmoc-protected amino acids were purchased from GL Biochem (Shanghai) Co., Ltd. Rink amide AM polystyrene resins (1% DVB, 100-200 mesh, loading = 0.34 mmol/g) were purchased from Tianjian Nankai HECHENG S&T Co., Ltd.

4.2. HPLC analysis

Analytical HPLC was run on a SHIMADZU (Prominence LC-20AT) instrument using an analytical column (Grace Vydac "Peptide C18", 250 × 4.6 mm, 5 μm particle size, flow rate 1.2 mL/min, rt). Analytical injections were monitored at 214 nm and 254 nm. Semi preparative HPLC was run on a SHIMADZU (Prominence LC-20AT) instrument using a semi preparative column (Grace Vydac "Peptide C18", 250 × 10 mm, 10 μm particle size, flow rate 4.0 mL/min). Solvent A was 0.1% TFA in acetonitrile, and solvent B was 0.1% TFA in water. Both solvents were filtered through 0.22 μm filter paper and sonicated for 20 min before use.A linear gradient (20-60%) of solvent B in solvent A over 30 min is used.

4.3. CD spectroscopy

CD spectra were measured on a Pistar π-180 spectrometer from 260 nm to 190 nm at 25 °C in a quartz cell with 0.2 cm path length. Each protein sample was dissolved to a final concentration of 0.2 mg/mL in ultrapure water. The spectra were performed in triplicate, averaged, subtracted from blank and smoothed.

4.4. Synthesis of Fmoc-ACC-OH

Ethyl (3-hydroxyphenyl)carbamate (2). 3-Aminophenol **1** (3.27 g, 30 mmol) and EtOAc (200 mL) was added to a 500 mL round-bottom flask fitted with a stirring bar. After heating to reflux for 10 min, ethyl chloroformate (1.62 g, 15 mmol) was added slowly. The mixture was stirred for 1 h and cooled. Then the precipitate was removed. The combined filtrate was concentrated to affordethyl (3-hydroxyphenyl)carbamate (2.50 g, 14 mmol, 93%) as a white solid. The crude product **2** could be used in the next step without further purification. NMR and mass spectra of **2** were consistent with results reported in the literature.[18] ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.39 (s, 1H), 7.12 (t, *J* = 8.1 Hz, 1H), 6.93 (d, *J* = 56.4 Hz, 1H), 6.78 (s, 1H), 6.67 – 6.54 (m, 2H), 4.23 (q, *J* = 7.1 Hz, 2H), 1.39 – 1.23 (m, 3H).

2-(7-((ethoxycarbonyl)amino)-2-oxo-2H-chromen-4-yl)aceticacid (3). Compound **2** (2.50 g, 14 mmol) and 70% H_2SO_4 (120 mL) was stirred and kept in an ice-bath. Then 1,3-acetonedi-carboxylic acid (2.45 g, 16.8 mmol) was added slowly and the mixture was stirred at room temperature for 12 h. The resulting mixture was quenched with water, and extracted with THF: EtOAc (5:1) three times. The organic phase was concentrated to afford the crude product, (2-(7-((ethoxycarbonyl)amino)-2-oxo-2H-chromen-4-yl)acetic acid (2.85 g, 9.8 mmol, 70%) as a white solid. The crude product could be used in the next step without further purification. NMR and mass spectra of **3** were consistent with results reported in the literature.[18] ^{1}H NMR (400 MHz, DMSO) δ 10.17 (d, *J* = 6.4 Hz, 1H), 7.63 (d, *J* = 8.8 Hz, 1H), 7.57 (d, *J* = 1.9 Hz, 1H), 7.39 (dd, *J* = 8.7, 2.1 Hz, 1H), 6.34 (s, 1H), 4.17 (q, *J* = 7.1 Hz, 2H), 3.86 (s, 2H), 1.27 (t, *J* = 7.1 Hz, 3H).

Fmoc-ACC-OH. Compound **3** (1.60 g, 5.6 mmol), NaOH (2.24 g, 56 mmol), and H_2O (80 mL) was added to a 250 mL round-bottom flask. The mixture was heated to reflux overnight. Then the reaction was quenched with water. Next, pH of the reaction mixture was adjusted to 2 with 4 M HCl, and extracted with THF: EtOAc (5:1) three times. The organic phase was concentrated to afford the crude product, 2-(7-amino-2-oxo-2H-chromen-4-yl)acetic acid (0.96 g, 4.38 mmol). The crude product could be used in the next step without further purification. 2-(7-amino-2-oxo-2H-chromen-4-yl)acetic acid (1.00 g , 4.5 mmol) was dissolved in CH_2Cl_2. Then TMSCl (1.45 g, 13.5 mmol) and DIPEA (1.74 g, 13.5 mmol) were added. The mixture was heated to reflux for 3 h. Then the mixture was cooled in an ice bath. Fmoc-Cl (1.4 g, 5.4 mmol) was added and the reaction was stirred overnight at room temperature. The reaction was quenched with 2 M HCl (pH adjusted to 2), and extracted with THF: EtOAc (5:1) three times. The organic phase was concentrated. The crude product was purified by chromatography (THF: PE = 2:1, 1.5% AcOH, R_f = 0.4) to afford Fmoc-ACC-OH (1.43 g, 3.24 mmol, 58% for two steps) as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 12.83 (s, 1H), 10.22 (s, 1H), 7.92 (d, *J* = 7.4 Hz, 2H), 7.76 (d, *J* = 7.4 Hz, 2H), 7.62 (d, *J* = 8.8 Hz, 1H), 7.55 (s, 1H), 7.45 – 7.34 (m, 5H), 6.34 (s, 1H), 4.56 (d, *J* = 6.4 Hz, 2H), 4.34 (t, *J* = 6.4 Hz, 1H), 3.86 (s, 2H).^{13}C NMR (101 MHz, DMSO-d_6): δ (ppm) 170.5, 159.9, 153.9, 153.2, 149.8, 143.6, 142.6, 140.8, 127.7, 127.1, 126.1, 125.1, 120.2, 114.4, 113.8, 113.7, 104.7, 65.9, 46.5, 37.0. HRMS (positive): 442.13 (observed, M+H), 441.12 (calculated, M).

4.5. SPPS of Ub[1-45]-$NHNH_2$ and Ub[46-76]^{A46C}-ACC

Peptides segments (0.1 mmol scale) were obtained through Fmoc SPPS with the microwave peptide synthesizer (Liberty Blue; CEM Corporation, USA). All Fmoc amino acids were dissolved in DMF, and the coupling reagent DIC (diisopropylcarbodiimide) was

dissolved in NMP containing 0.1 M Oxyma (ethyl cyanoglyoxylate-2-oxime). Ub[1-45]-$NHNH_2$ was synthesized from the Fmoc-hydrazine-2-chlorotrityl chloride resin. Ub[46-76]-ACC was synthesized from the Fmoc Rink amide resin. Fmoc-(Dmb)Gly-OH (2,4-dimethoxybenzyl) was used at the coupling site of Gly53. Ac_2O was used to cap free amino groups after the coupling of ACC. Peptides were elongated using four-fold excess of Fmoc-protected amino acids and four-fold excess of coupling reagent. All coupling reactions were performed at 50-75 °C for 10 min except arginine. Fmoc-Arg(Pbf)-OH was coupled at room temperature for 10 min followed by 5 min at 50 °C. Double coupling was used after the first 30 coupling cycles. Fmoc protecting group was removed with 20% (v/v) piperidine in DMF containing 0.1 M Oxyma.

After chain assembly, the resin was washed by DMF and CH_2Cl_2 three times, respectively. To the dry resin was added 10 mL cleavage reagent [TFA/water/thioanisole/ethanedithiol = 85/5/5/3 (v/v/v/v)] containing 0.5 g phenol. After 3 h, the filtrate were concentrated by blowing with N_2 and precipitated with cold ether. The crude peptides was dissolved in acetonitrile/water (1/1), analyzed by HPLC and ESI-MS. Purification was carried out through semi-preparative HPLC and follow-up lyophilization.

4.6. NCL and desulfurization

Ub[1-45]-$NHNH_2$ (1.0 equiv) was dissolved in the oxidation buffer (6 M Gn·HCl, 100 mM NaH_2PO_4, pH 3.0). Then, 1 M $NaNO_2$ (5.0 equiv) was added dropwise and the reaction was stirred for 20 min at -10 °C. MESNa (50 equiv) was then added and pH was slowly adjusted to 5.0 with 2 M NaOH. The reaction mixture was stirred at room temperature for 1 h to enable quantitative conversion of Ub[1-45]-$NHNH_2$ to Ub[1-45]-MESNa quantitatively.

For the synthesis of full-length Ub-ACC, Ub[1-45]-MESNa (8.4 mg, 1.6 μmol, 1.0 equiv) and Ub[46-76]-ACC (6 mg, 1.6 μmol, 1.0 equiv) were dissolved in 500 μL ligation buffer (6 M Gn·HCl, 100 mM Na_2HPO_4, pH 7.0). MPAA (50 equiv) was added and pH was slowly adjusted to 6.5 with 2 M NaOH. The reaction mixture was stirred at room temperature for 2 h and monitored by RP-HPLC. The product Ub[1-76]^{A46C}-ACC was isolated by semi-preparative HPLC and lyophilized (8.8 mg, 63% isolated yield). Ub[1-76]^{A46C}-ACC (8.8 mg, 1 μmol) was dissolved in 1 mL desulfurization buffer (6 M Gn·HCl and 100 mM Na_2HPO_4, 25 mM VA-044, 100 mM 2-methyl-2-propanethiol, 250 mM tris(2-carboxyethyl)phosphine (TCEP), pH 6.9). The reaction was incubated at room temperature. After 12 h, the reaction was analyzed by RP-HPLC. The product Ub[1-76]-ACC was isolated by semi-preparative HPLC and lyophilized (5.5 mg, 61% isolated yield).

4.7. Expression and purification of UCHL3, OTUD2, and USP30

UCHL3 (full-length), OTUD2 (132-348) and USP30 (65-517) were expressed in BL21 (DE3) cells using a pGEX-6P-1 vector containing an N-terminal GST tag. Cells were grown in LB medium containing ampicillin (100 μg/mL) with shaking overnight at 37 °C. After 1:100 dilution in LB medium containing ampicillin (100 μg/mL), the culture was grown at 37 °C to an OD 600: 0.6-0.8. Then, protein expression was induced by the addition of isopropyl 1-β-D-galactopyranoside (IPTG) to the final concentration of 0.2 mM. After expression for 16 h at 16 °C, cells were harvested by centrifugation (6000 rpm, 10 min), and re-suspended in lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM DTT, pH 8.0). After cell lysis, GST-fused UCHL3 (full-length), OTUD2 (132-348) and USP30 (65-517) were purified using a glutathione-Sepharose 4B affinity chromatography. Glutathione-Sepharose 4B resin and the cell lysate were incubated at 4 °C for 1 h. The resin was washed with 20 mM Tris-HCl, 500 mM NaCl, 1mM DTT, pH 8.0. Then, the PreScission protease was added to the resin. The mixtures were incubated at 4 °C for 4 h and GST tag was excised by PreScission protease. UCHL3 (full-length), OTUD2 (132-348) and USP30 (65-517) were further purified by a Mono Q column followed by a Superdex 200 column.

4.8. Determination of Michaelis-Menten constants

Enzymatic assays of DUBs were conducted on the microplate reader (BioTek Corporation, Synergy HT, USA). The fluorescence signals were recorded at an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Enzymatic reactions were performed in the assay buffer (50 mM Tris, 100 mM NaCl, 1 mM EDTA, 1mM DTT, pH 7.5). Total assay volume was 200 μL. Reaction mixtures were incubated for 1 h at 30 °C and fluorescence signals were recorded every 30 sec. Kinetic assays of UCH-L3 (100 pM) was performed with Ub-ACC or Ub-AMC concentrations of 2, 1.5, 1, 0.5, 0.25, 0.125, 0.0625 and 0 μM. Kinetic assays of OTUD2 (10 nM) was performed with Ub-ACC or Ub-AMC concentrations of 10, 5, 2, 1.5, 1, 0.5, 0.25, 0.125, 0.0625 and 0 μM. Kinetic assays of USP30 (10 nM) was performed with Ub-ACC or Ub-AMC concentrations of 10, 5, 2, 1.5, 1, 0.5, 0.25, 0.125, 0.0625 and 0 μM. Average reaction rates were measured based on three independent reactions. Nonlinear fit model of "v versus [S]": $v = v_{max} \cdot [S] / ([S] + K_m)$, $k_{cat} = v_{max} / [E_0]$.

4.9. Determination of IC_{50} and K_i

All assays were performed at 30 °C in 96-well plates using microplate reader (BioTek Corporation, Synergy HT, USA). The fluorescence signals were recorded at an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Enzymatic reactions were performed in the assay buffer (50 mM Tris, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, pH 7.5). Total assay volume was 200 μL. For the inhibition assay against UCH-L3, UCH-L3 (100 pM) was mixed with Ub-ACC (62.5 nM) in the presence of free ubiquitin at various concentrations (0-20 μM). For the inhibition assay against OTUD2, OTUD2 (10 nM) was mixed with Ub-ACC (500 nM) in the presence of free ubiquitin at various concentrations (0-20 μM). For the inhibition assay against USP30, USP30 (10 nM) was mixed with Ub-ACC (500 nM) in the presence of free ubiquitin at various concentrations (0-20 μM). All DUBs were incubated with free ubiquitin for 1 hour at 30 ℃ before adding Ub-ACC. Nonlinear longistic fit model of "Inhibition% versus c": Inhibition% = $1 / (1 + e^{-k(c - c^*)})$, $IC_{50} = 10^{c^*}$, $K_i = IC_{50} / (1 + [S] / K_m)$.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (Nos. 21372058, 21572043).

References

1 D. Komander and M. Rape, *Annu. Rev. Biochem.*, 2012, **81**, 203.
2 J.-F. Trempe, *Curr. Opin. Struct. Biol.*, 2011, **21**, 792.
3 D. Komander, M. J. Clague and S. Urbe, *Nat. Rev. Mol. Cell Biol.*, 2009, **10**, 550.
4 J. Heideker and I. E. Wertz, *Biochem. J.*, 2015, **465**, 1.
5 R. Ekkebus, D. Flierman, P. P. Geurink and H. Ovaa, *Curr. Opin. Chem. Biol.*, 2014, **23**, 63.
6 (a) P. Gopinath, S. Ohayon, M. Nawatha and A. Brik, *Chem. Soc. Rev.*, DOI: 10.1039/c6cs00083e; (b) R. E. Morgan, V. Chudasama, P. Moody, M. E. B. Smith, S. Caddick, *Org. Biomol. Chem.*, 2015, **13**, 4165.
7 (a) L. C. Dang, F. D. Melandri and R. L. Stein, *Biochemistry*, 1998, **37**, 1868; (b) U. Hassiepen, U. Eidhoff, G. Meder, J.-F. Bulber, A. Hein, U. Bodendorf, E. Lorthiois and B. Martoglio, *Anal. Biochem.*, 2007, **371**, 201; (c) Y. T. Li, J. Liang, J. B. Li, G. M. Fang, Y. Huang and L. Liu, *J. Pept. Sci.*, 2014, **20**, 102; (d) F. El Oualid, R. Merkx, R. Ekkebus, D. S. Hameed, J. J. Smit, A. de Jong, H. Hilkmann, T. K. Sixma and H. Ovaa, *Angew. Chem. Int. Ed.*, 2010, **49**, 10149; (e) J. Liang, G. M. Fang, X. L. Huang, Z. Q. Mei, J. Li, C. L. Tian and L. Liu, *Sci. China Chem.*, 2013, **56**, 1301.
8 S. J. Orcutt, J. Wu, M. J. Eddins, C. A. Leach and J. E. Strickler, *Biochim. Biophys. Acta.*, 2012, **1823**, 2079.

9 (a) A. Tirat, A. Schilb, V. Riou, L. Leder, B. Gerhartz, J. Zimmermann, S. Worpenberg, U. Eidhoff, F. Freuler, T. Settler, L. Mayr, J. Ottl, B. Leuenberger and I. Filipuzzi, *Anal. Biochem.*, 2005, **343**, 244; (b) P. P. Geurink, F. El Oualid, A. Jonker, D. S. Hameed and H. Ovaa, *ChemBioChem*, 2012, **13**, 293; (c) D. Flierman, G. J. van der Heden van Noort, R. Ekkebus, P. P. Geurink, T. E. T. Mevissen, M. K. Hospenthal, D. Komander and H. Ovaa, *Cell Chem. Biol.*, DOI: 10.1016/j.chembiol.2016.03.009.

10 (a) R. A. Horton, E. A. Strachan, K. M. Vogel and S. M. Riddle, *Anal. Biochem.*, 2007, **360**, 138; (b) S. Ohayon, L. Spasser, A. Aharoni and A. Brik, *J. Am. Chem. Soc*., 2012, **134**, 3281; (c) P. P. Geurink, B. D. M. van Tol, D. van Dalen, P. J. G. Brundel, T. E. T. Mevissen, J. N. Pruneda, P. R. Elliott, G. B. A. van Tilburg, D. Komander and H. Ovaa, *ChemBioChem*, DOI: 10.1002/cbic.201600017.

11 T. W. Muir, D. Sondhi and P. A. Cole, *Proc. Natl. Acad. Sci. USA*, 1998, **95**, 6705.

12 (a) P. Botti, M. R. Carrasco and S. B. H. Kent, *Tetrahedron Lett.*, 2001, **42**, 1831; (b) D. Macmillan and D. W. Anderson, *Org. Lett*., 2004, **6**, 4659.

13 (a) G. M. Fang, Y. M. Li, F. Shen, Y. C. Huang, J. B. Li, Y. Lin, H. K. Cui and L. Liu, *Angew. Chem. Int. Ed.*, 2011, **50**, 7645; (b) G. M. Fang, J. X. Wang, L. Liu, *Angew. Chem. Int. Ed*. 2012, **51**, 10347; (c) J. S. Zheng, S. Tang, Y. K. Qi, Z. P. Wang and L. Liu, *Nat. Protoc*., 2013, **8**, 2483; (d) J. Li, Y. Li, Q. He, Y. Li, H. Li, L. Liu, *Org. Biomol. Chem.*, 2014, **12**, 5435; (e) Zheng, J.-S.; Yu, M.; Qi, Y.-K.; Tang, S.; Shen, F.; Wang, Z.-P.; Xiao, L.; Zhang, L.; Tian, C.-L.; Liu, L. *J. Am. Chem. Soc.* **2014**, *136*, 3695-3704; (f) Wang, J.-X.; Fang, G.-M.; He, Y.; Qu, D.-L.; Yu, M.; Hong, Z.-Y.; Liu, L. *Angew. Chem. Int. Ed.* **2015**, *54*, 2194-2198; (g) S. Tang, Y.-Y. Si, Z.-P. Wang, K.-R. Mei, X. Chen, J.-Y. Cheng, J.-S. Zheng, L. Liu, *Angew. Chem. Int. Ed.* 2015, **54**, 5713.

14 P. E. Dawson, T. W. Muir, I. Clark-Lewis and S. B. H. Kent, *Science*, 1996, **118**, 5891.

15 For recent reviews of chemical protein synthesis, see: (a) S. B. H. Kent, *Chem. Soc. Rev.*, 2009, **38**, 338; (b) L. Raibaut, N. Ollivier and O. Melnyk, *Chem. Soc. Rev.*, 2012, **41**, 7001; (c) J.-S. Zheng, S. Tang, Y.-C. Huang and L. Liu, *Acc. Chem. Res.*, 2013, **46**, 2475; (d) L. R. Malins and R. J. Payne, *Top. Curr. Chem.*, 2015, **362**, 27; (e) H. Liu, X. C. Li, *Org. Biomol. Chem.*, 2014, **12**, 3768; (f) F. Rohrbacher, T. G. Wucherpfennig, J. W. Bode, *Top. Curr. Chem.*, 2015, **363**, 1; (g) Y. Huang and L. Liu, *Sci. China Chem.*, 2015, **58**, 1779; (h) A. Fernandez-Tejada, J. Brailsford, Q. Zhang, J.-H. Shieh, M. A. S. Moore and S. J. Danishefsky, *Top. Curr. Chem.*, 2015, **362**, 1; (i) A. Yang, L. Zhao and Y. W. Wu, *Top. Curr. Chem.*, 2015, **362**, 137; (j) R. Yang and C. F. Liu, *Top. Curr. Chem.*, 2015, **362**, 89; (k) C. J. Howard, R. R. Yu, M. L. Gardner, J. C. Shimko and J. J. Ottesen, *Top. Curr. Chem.*, 2015, **363**, 193; (l) Y.-C. Huang, G.-M. Fang and L. Liu, *Natl. Sci. Rev.*, 2016, **3**, 107; (m) R. Meledin, S. M. Mali and A. Brik, *Chem. Rec*., 2016, **16**, 509.

16 For recent reports of total synthesis of native ubiquitin chains and ubiquitinated proteins, see: (a) K. J. Ajish Kumar, L. Spasser, L. A. Erlich, S. N. Bavikar and A. Brik, *Angew. Chem. Int. Ed*., 2010, **49**, 9126; (b) K. J. Ajish Kumar, S. N. Bavikar, L. Spasser, T. Moyal, S. Ohayon and A. Brik, *Angew. Chem. Int. Ed.*, 2011, **50**, 6137; (c) T. Moyal, S. N. Bavikar, S. V. Karthikeyan, H. P. Hemantha and A. Brik, *J. Am. Chem. Soc*., 2012, **134**, 16085; (d) P. Siman, S. V. Karthikeyan, M. Nikolov, W. Fischle and A. Brik, *Angew. Chem. Int. Ed.*, 2013, **52**, 8059; (e) M. Haj-Yahya, B. Fauvet, Y. Herman-Bachinsky, M. Hejjaoui, S. N. Bavikar, S. V. Karthikeyan, A. Ciechanover, H. A. Lashuel and A. Brik, *Proc. Natl. Acad. Sci.*, 2013, **110**, 17726; f) S. Bondalapati, W. Mansour, M. A. Nakasone,S. K. Maity, M. H. Glickman and A. Brik, *Chem. Eur. J*., 2015, **21**, 7360.

17 (a) J. L. Harris, B. J. Backes, F. Leonetti, S. Mahrus, J. A. Ellman and C. S. Craik, *Proc. Natl. Acad. Sci. USA*, 2000, **97**, 7754; (b) M. Drag, J. Mikolajczyk, M. Bekes, F. E. Reyes-Turcu, J. A. Ellman, K. D. Wilkinson and G. S. Salvesen, *Biochem. J.* 2008, **415**, 367; (c) P. Kasperkiewicz, M. Poreba, S. J. Snipas, H. Parker, C. C. Winterbourn, G. S. Salvesen and M. Drag, *Proc. Natl. Acad. Sci. USA*, 2014, **111**, 2518; (d) M. Poreba, A. Szalek, P. Kasperkiewicz and M. Drag, *Methods Mol. Biol.* 2014, **1133**, 41.

18 D. J. Maly, F. Leonetti, B. J. Backes, D. S. Dauber, J. L. Harris, C. S. Carik and J. A. Ellman, *J. Org. Chem.*, 2002, **67**, 910.

19 (a) Y.-C. Huang, C.-C. Chen, S.-J. Li, S. Gao, J. Shi and Y.-M. Li, *Tetrahedron*, 2014, **70**, 2951; (b) Y.-C. Huang, C.-J. Guan, X.-L. Tan, C.-C. Chen, Q.-X. Guo and Y.-M. Li, *Org. Biomol. Chem.*, 2015, **13**, 1500.

20 E. C. B. Johnson and S. B. H. Kent, *J. Am. Chem. Soc*., 2006, **128**, 6640.

21 Y.-C. Huang, C.-C. Chen, S. Gao, Y.-H. Wang, H. Xiao, F. Wang, C.-L. Tian and Y.-M. Li, *Chem. Eur. J.*, DOI: 10.1002/chem.201600101.

22 D. Bang, G. I. Makhatadze, V. Tereshko, A. A. Kossiakoff and S. B. Kent, *Angew. Chem. Int. Ed.*, 2005, **44**, 3852.

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