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Chemical synthesis of Ub-AMC via ligation of peptide hydrazides

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The C-terminal conjugate of ubiquitin with 7-amino-4-methylcoumarin (Ub-AMC) is an important probe for fluorescence-based analysis of deubiquitinating enzyme (DUB) activity. It is important to develop more efficient methods for the preparation of Ub-AMC because the currently available technology is still expensive for scaled-up production. In the present work we report an efficient strategy for total chemical synthesis of Ub-AMC through ligation of peptide hydrazides. Three peptide segments are assembled via N-to-C sequential ligation and the resulting product is converted to Ub-AMC via TCEP-mediated desulfurization. The synthetic Ub-AMC is shown to have expected biological functions through the measurement of its DUB activity.

ligation of peptide hydrazides, peptide segment condensation, deubiquitinating enzymes, ubiquitin

1 Introduction

Ubiquitination is one of the most common protein post-translational modifications. It plays an important role in cell biology, because ubiquitination not only can direct the location and degradation of the modified protein, but also may facilitate the transcription and repair of DNA [1–5]. As the key unit of ubiquitination, Ub is a highly conserved small protein of 76 amino acid residues. Ubiquitination occurs through the attachment of Ub's C-terminus to the lysine residue (or less commonly, the N-terminus) of the target protein. This process is mediated by three enzymes named E1, E2 and E3. In the beginning of the process, the C-terminus of Ub is activated with the assistance of E1 and ATP to form an Ub-thioester intermediate. Subsequently, the conjugating E2 enzyme covalently binds to Ub through a thiol-exchange reaction. In the final step, the E3 ligase mediates the transfer of Ub to the Lys side chain of the target protein. Note that the last step necessitates the help of RING-domain E3 family which brings the Ub thioester-E2 and the ε-amino group of a Lys residue close together to form an isopeptide bond regio-specifically.

The detail process of ubiquitination is fairly complex because seven lysine residues in Ub (i.e. K6, K11, K27, K29, K33, K48, K63) can be used to link the consecutive Ub molecules. As a result, the attachment of Ub to a target protein may involve a chain of Ubs with various linkage types and different lengths. Previous studies have mostly focused on Lys48- and Lys63-linked Ub chains due to the poor availability of the E2/E3 enzymes systems for preparation of other Ub linkage types [6]. Moreover, the identification, isolation, and characterization of E2/E3 enzymes for a specific ubiquitination target remain extraordinarily tedious even with the most advanced technologies. Therefore, it is important to develop chemical approaches to prepare Ub

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derivatives required for the biomedical researches.

In this context several studies have been reported for the chemical synthesis of Ub derivatives (Figure 1). Examples include the synthesis of Ub derivatives via native chemical ligaiton [7], total chemical synthesis of ubiquitinated peptides bearing various ubiquitin chains by δ-mercaptolysine assisted ligation [8–11], dual native chemical ligation at lysine for synthesis of ubiquitinated peptides [12], and Fmoc solid-phase peptide synthesis of Ub probes by the use of pseudoproline building blocks and dimethoxybenzyl dipeptides [13]. Despite these advances, there are still technological problems limiting the application of chemical synthesis in the study of ubiquitination. For instance, the preparation of the thioester intermediates via mild Fmoc chemistry remains difficult in the previous synthetic studies. The use of expensive dipeptide building blocks in some of the previous studies also prevents the scaled-up production of Ub derivatives.

Recently, we have developed a new ligation procedure through the chemoselective reaction between a Cys-peptide and a C-terminal peptide hydrazide [14]. In this method, the peptide hydrazide was activated by $NaNO_2$ and an external thiol *in situ* to become the peptide thioester. Compared to the previous methods for the preparation of peptide thioesters, peptide hydrazides can be easily obtained through Fmoc-based solid-phase peptide synthesis and thereby, provide suitable substrates for protein chemical synthesis. Previously we have applied this method to the synthesis of several proteins including trifolitoxin, CssII, circular proteins, and ribosomal protein S25 [15–21]. In the present work we report the use of peptide hydrazide ligation for the total synthesis of an Ub derivative Ub-AMC. The molecule contains 7-amino-4-methylcoumarin at its C-terminus and is a widely used probe for the monitoring of deubiquitinating enzymes (DUBs) as well as for the identification of inhibitors against DUBs.

2 Experimental

2.1 Materials

Fmoc-protected amino acids were purchased from Beijing BoMaiJie Technology Company. HBTU, DIC, 7-amino-4-methylcoumarin, and $POCl_3$ were obtained from Alfa Aesar. Et_2O, THF, DMF, DCM, DIEA, EtOAc, piperidine, and other common reagent were gained from Sinopharm Chemical Reagent Co. Ltd. Purification of crude peptides was carried out by using HPLC (LC-20AT, SHIMADZU) with dual wavelength (215 and 254 nm). Identification of synthetic peptides was performed by using MALDI-TOF and ESI-MS (Agilent).

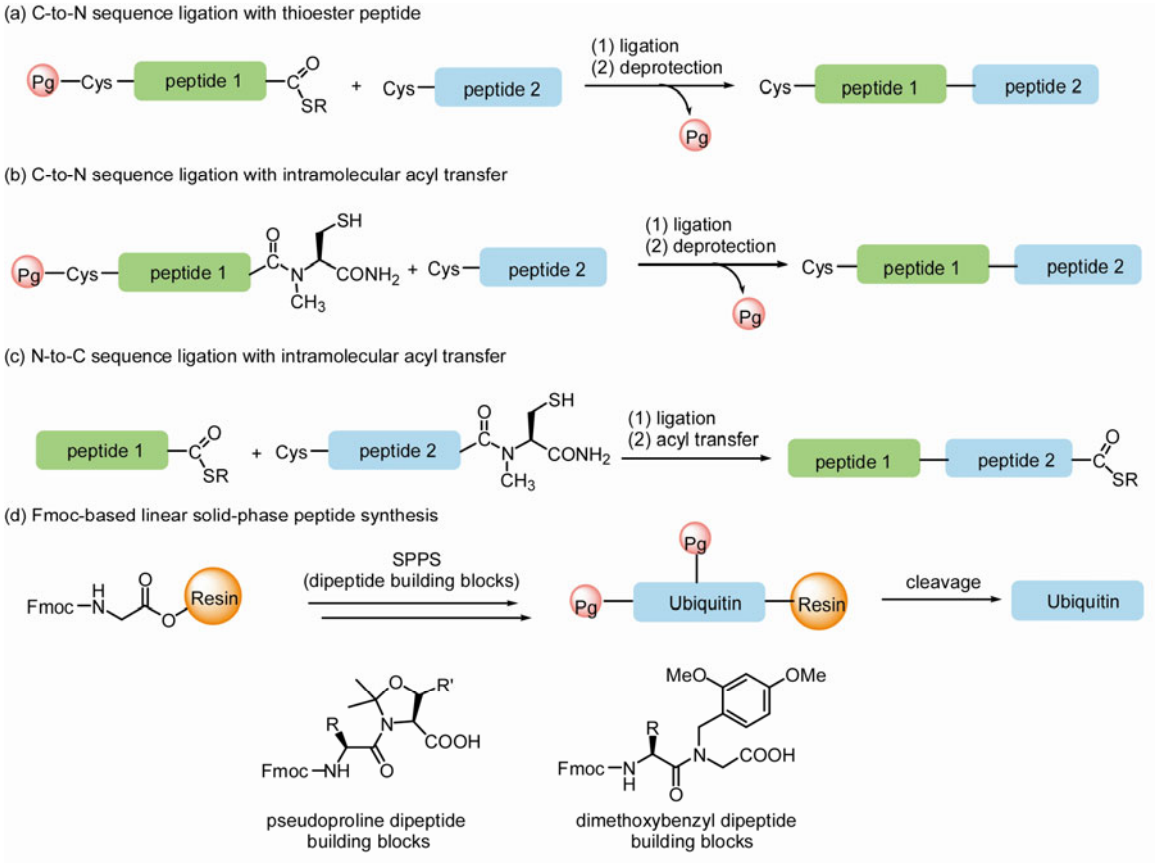
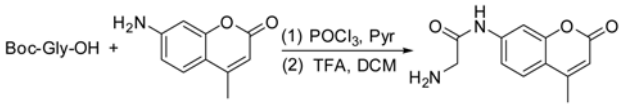


Figure 1 Previous methods for chemical synthesis of Ub derivatives.

2.2 Fmoc solid-phase peptide synthesis (SPPS)

Resin for SPPS was initially swelled with DMF/DCM (1:1) for 2 h in the screw-cap glass peptide synthesis apparatus, followed by washing of the resin with DMF, DCM, and DMF. Fmoc-amino acids were coupled with the DMF solution containing HBTU (3.6 equiv.), HOBt (4.0 equiv.), DIEA (8.0 equiv.), and Fmoc-amino acid (4.0 equiv.). Note that the activation step initiated by DIEA was performed in less than 5 min, and the condensation was performed for 90 min followed by K-Test. The Fmoc group was removed by dual treatment (5 and 10 min) with a solution of piperidine in DMF (20%, *v*/*v*). After the assembly of the target peptide, the resin was washed with DMF and DCM, and dried at room temperature. A cocktail reagent (87.5% TFA, 5% H_2O, 5% PhOH, 2.5% TIPS) was then added to the resin for 2.5 h. After the concentration of the combined cocktail reagent by a N_2 blowing, the crude peptides were obtained by cold ether precipitation and centrifugation. The crude peptides were dissolved in water, identified by ESI-MS and purified by semi-preparative HPLC. Finally, the dried peptides were obtained by lyophilization as white powders.

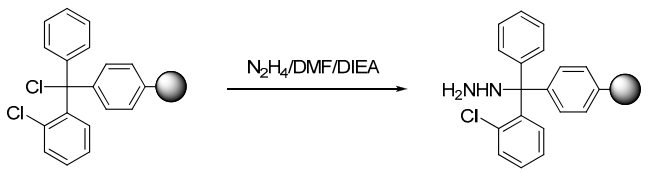
2.3 Synthesis of H-Gly-AMC



Eq. (1)

7-Amino-4-methylcoumarin (0.87 g, 5.0 mM) and Boc-Gly-OH (0. 87 g, 5.0 mM) were dissolved in dried pyridine (15 mL). At −15 °C, 0.51 mL of $POCl_3$ was added to the mixture dropwise. After 2 h, the ice-salt bath was removed, and the mixture was stirred at room temperature. The reaction was stirred overnight and quenched by treatment with water. The organic reagents were removed by evaporation in vacuum. Boc-Gly-AMC was isolated by column chromatography with a yield of 53% (0.87 g, 2.7 mM). Finally, the removal of Boc group was achieved by treatment with a solution of TFA/DCM (4:1) for 1 h. The product H-Gly-AMC was obtained by concentration as solid with a yield of 95% (0.49 g) (Eq. (1)). ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.97 (1H, s), 7.78 (2H, d, J = 8.1 Hz), 7.47 (1H, d, J = 8.6 Hz), 6.31 (1H, s), 3.87 (2H, s), 2.41 (3H, s) ppm. ^{13}C NMR (400 MHz, DMSO-d_6): δ 166.14, 160.37, 154.12, 153.53, 141.83, 126.76, 116.04, 115.64, 113.12, 106.26, 41.73, 18.44 ppm. ESI-MS: *m/z* = 233.2 (M + H), calc.: 232.1.

2.4 Preparation of NH_2-NH-Trt(2-Cl) resin



Eq. (2)

2-Cl-Trt(Cl) resin (2.0 g, 0.5 mol/g) was added to a flask. At 0 °C, a solution of hydrazine in DMF ($N_2H_4 \cdot H_2O$: DIEA : DMF = 0.4 g : 1.33mL : 4.0 mL) was added dropwise to the flask. After 90 min, the reaction was quenched by the addition of methanol (0.2 mL) for 20 min (Eq. (2)). The hydrazine-Trt(2-Cl) resin was washed with DMF, H_2O, DMF, MeOH, and Et_2O in sequence, and dried in a vacuum.

2.5 Preparation of Ub1 and Ub2

The hydrazine-Trt(2-Cl) resin (0.4 g) was swelled with DMF/DCM (1 : 1) for 1 h in the screw-cap glass peptide apparatus. The coupling of Fmoc-amino acids was carried out according to the standard Fmoc SPPS. After dried at room temperature, the peptide bound resin was treated by the cocktail reagent for 2.5 h (87.5% TFA, 5% H_2O, 5% PhOH, 2.5% TIPS). The combined reagent was concentrated by a N_2 blowing. The crude peptide hydrazides were obtained by cold ether precipitation and centrifugation as white powders. The crude peptides were characterized by ESI-MS, and purified by HPLC. They were lyophilized as white powders. ESI-MS: **Ub1** *m/z* = 3048.4 (M + 1), calc.: 3047.6; **Ub2** *m/z* = 2142.0 (M + 1), calc.: 2141.1.

2.6 Two step synthesis of Ub3

The 2-Cl-Trt-Cl resin (0.4 g) was swelled with DMF/DCM (1 : 1) for 15 min in the screw-cap glass peptide apparatus. The first amino acid was coupled with the DMF solution containing DIEA (8.0 equiv.) and Fmoc-Gly-OH (4.0 equiv.). Note that the last amino acid attachment was performed with Boc-Cys-OH. After the assembly of the target peptide, the full protected peptide was cleaved from resin with HFIP/DCM (1 : 4) at room temperature. After 0.5 h, the resin was washed with DCM and then the combined reagent was concentrated in a vacuum. The crude full protected peptide was obtained by cold ether precipitation and centrifugation as a white powder. The fully protected peptide was condensed with H-Gly-AMC in DCM by using PyBOP (5.0 equiv.) initiated with DIEA at room temperature. After the reaction was stirred overnight, the mixture was concentrated in vacuum. The protecting group was removed by using a mixed solution (TFA : H_2O : TIPS = 95: 2.5: 2.5, *v*/*v*) for 3 h. The identification and isolation step was carried out as described above. ESI-MS: **Ub3** *m/z* = 3657.3 (M+H), calc.: 3654.9.

2.7 The ligation of Ub1 with Ub2

Ub1 (6.5 mg, 2.1 μmol) was dissolved in 0.7 mL of ligation buffer (6.0 M Gn · HCl, 0.2 M Na_2HPO_4). At a low pH (3.0) and −10 °C, 70 μL of $NaNO_2$ (0.2M) was added to the mixture. After 20 min, the mixture was treated by a 0.2 M

MPAA solution (0.7 mL) and its pH was slowly adjusted to 7.0 with 2.0 M NaOH solution. Then, the ligation reaction was treated with **Ub2** (6.1 mg, 2.8 μmol) at room temperature. The reaction was monitored by HPLC at different times. The ligation product was isolated by semi-preparative HPLC after reduction of the mixture with TCEP. The Ub[Met^{1}-Phe^{45}] was obtained as a white powder (30%). ESI-MS: *m/z* = 5159.5 (M + H), calc.: 5158.7.

2.8 Ligation with Ub3

Ub[Met^{1}-Phe^{45}] (5.6 mg, 1.1 μmol) was dissolved in 0.35 mL of ligation buffer (6.0 M Gn·HCl, 0.2 M Na_2HPO_4). At a low pH (3.0) and −10 °C, 35 μL of $NaNO_2$ (0.2M) was added to the mixture. After 20 min, the mixture was treated by a 0.2 M MPAA solution (0.35 mL) and its pH was slowly adjusted to 7.0 with 2.0 M NaOH solution. Then, the ligation reaction was triggered by addition of **Ub3** (5.5 mg, 1.5 μmol) at room temperature. The reaction was monitored by HPLC at different times. The ligation product was isolated by semi-preparative HPLC after reduction of the mixture with TCEP. The Ub-AMC[Ala28,46Cys] was obtained as a white powder (35%). ESI-MS: *m/z* = 8780.5 (M + H), calc.: 8779.6.

2.9 Free-radical desulfurization

Ub-AMC[Ala28, 46Cys] (1.5 mg, 0.17 μmol) was dissolved in the buffer solution (6.0 M Gn·HCl，0.2 M Na_2HPO_4, pH 7.0). The mixture was treated by addition of *t*-BuSH (0.15 mL), a 0.1 M VA-044 solution, and a 0.5 M TCEP solution (0.2 mL). Then its pH was adjusted to 7.0 by 2.0 M NaOH solution. The desulfurization reaction was performed for 12 h at 35 °C. The purified Ub-AMC was obtained by HPLC as a white powder with a yield of 80%. ESI-MS: *m/z* = 8716.6 (M + H), calc.: 8715.7.

2.10 Measurement of Ub-AMC activity

1 μL Ubp6 (0.1 mM) was incubated for 15 minutes at 30 °C in 60 μL of 50 mM Tris 7.5 solution containing 1 mM EDTA, 1 mM ovalbumin, 5 mM ATP/$MgCl_2$ and 1 mM DTT. After addition of 4 μL of 0.2 mM Ub-AMC, the fluorescence emission was determined at an excitation wavelength of 355 nm and an emission wavelength of 460 nm. Each experiment included three independent reactions and was repeated three times.

3 Results and discussion

3.1 Design of synthetic route for Ub-AMC

Generally speaking, peptide fragments containing less than 50 residues can be made directly by standard solid-phase peptide synthesis. However, Ub-AMC consists of 76 amino acid residues, so that we cannot reliably prepare it through a single SPPS. Instead, we had to use the method of multiple peptide segment condensation (Figure 2). Because Ub does not have any Cys residue, we mutated Ala located at the positions of 28 and 46 to Cys. After the assembly of three peptide segments, the two Cys residues were converted back to Ala by using the metal-free desulfurization method. It is noteworthy that the three peptide segments (i.e. **Ub1**, **Ub2**, and **Ub3**) contained 27, 18, and 31 amino acid residues, respectively. They are therefore, possible to prepare in large scales. Moreover, **Ub1** and **Ub2** are peptide hydrazides and therefore, can be obtained easily by using the Fmoc SPPS method with the hydrazine-Trt(2-Cl) resin. On the other hand, **Ub3** contains an AMC group at its C-terminus and therefore, cannot be synthesized directly by SPPS. We developed a two-step method to solve the problem. Specifically, we used the 2-Cl-trityl resin to prepare the fully protected peptide that contains the first 30 residues. This intermediate was then condensed with H-Gly-AMC in DCM by using PyBOP assisted by DIEA. Subsequently, the protecting group was removed by using the standard TFA cocktail reagent, and **Ub3** was obtained by cold ether precipitation. The crude product was purified by semi-preparative HPLC.

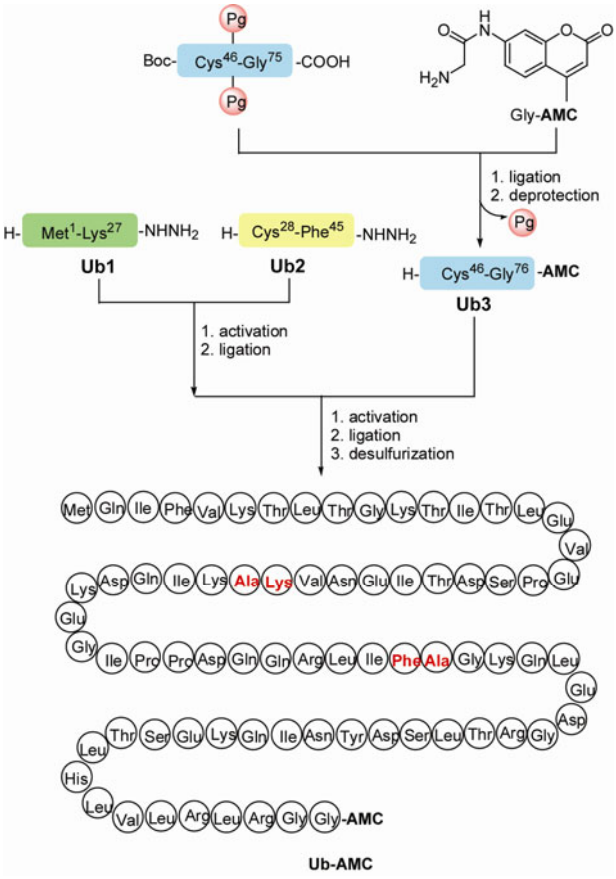


Figure 2 The sequence of Ub-AMC and its synthetic route.

3.2 Total synthesis of Ub-AMC

With **Ub1**, **Ub2**, and **Ub3** in hand, we synthesized Ub-AMC by using the N-to-C sequential ligation of three peptide segments.

Synthesis of Ub-AMC was initiated with the ligation between **Ub1** and **Ub2** (Figure 3). At a low pH (3.0) and −10 °C, **Ub1** was converted into the peptide azide intermediate by the assistance of $NaNO_2$ (6.0 equiv.). After 20 min, MPAA (65.0 equiv.) was added and the pH values was adjusted to 7.0 to facilitate the formation of the thioester intermediate Ub[Met^1-Lys^{27}]-S-aryl. Then, **Ub2** (1.3 equiv.) was added and this step was allowed to proceed until the consumption of the thioester peptide. Before purification by HPLC, TCEP was added to reduce any oxidized thiols. The yield of the isolated product in this step was 30% and its product was Ub[Met^1-Phe^{45}]. Note that Ub[Met^1-Lys^{27}]-S-aryl was found to produce a circular peptide byproduct probably through cyclization reaction between the side chain of Lys^{27} and the thioester moiety. This side reaction, which can be slightly suppressed by reducing the acidity of the mixture, led to the relatively low yield in this step.

Our next step was the second ligation step with **Ub3**, which was carried out in a way similar to the first step as described above (Figure 4). Ub[Met^1-Phe^{45}] was oxidized by $NaNO_2$ to the peptide azide in less than 20 min at pH 3.0. After the addition of MPAA, Ub[Met^1-Phe^{45}]-S-aryl was formed. After adjusting pH of the mixture to 7.0, **Ub3** was added and triggered the final ligation. The ligation product Ub-AMC[Ala28,46Cys] was purified from the mixture with yield of 35%. Fortunately, the impurity contained in the reagent of **Ub3** does not ligate with the peptide thioester as shown in Figure 4. Besides, we found a significant precipitation of the final ligation product. To solve this solubility problem, we added acetonitrile to the ligation media but this method did not improve the synthesis.

The final step was the conversion of two cys residues in the Ub-AMC[Ala28, 46Cys] to two Ala residues to generate Ub-AMC by using the metal-free desulfurization reaction (Figure 5). This reaction was performed at pH 7.0 and 35 °C upon the addition of VA-044, *t*-BuSH, and TCEP. Ub-AMC was obtained by HPLC with a yield of 80%.

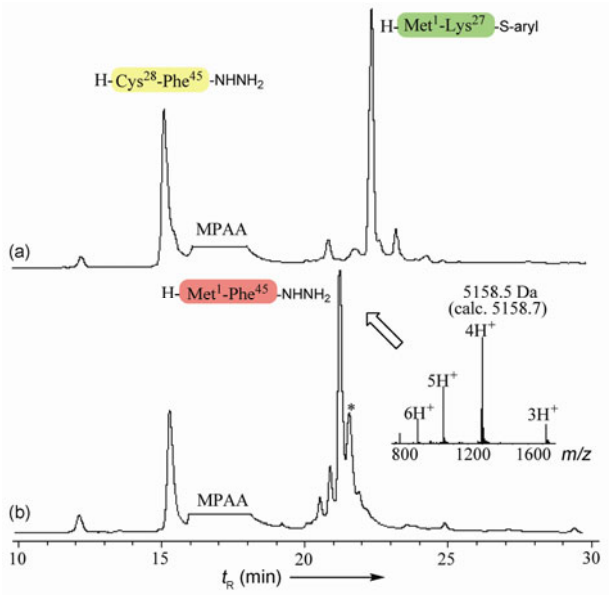


Figure 3 HPLC monitoring of the ligation between **Ub1** and **Ub2**. (a) After the activation of **Ub1** followed by addition of MPAA; (b) after overnight.

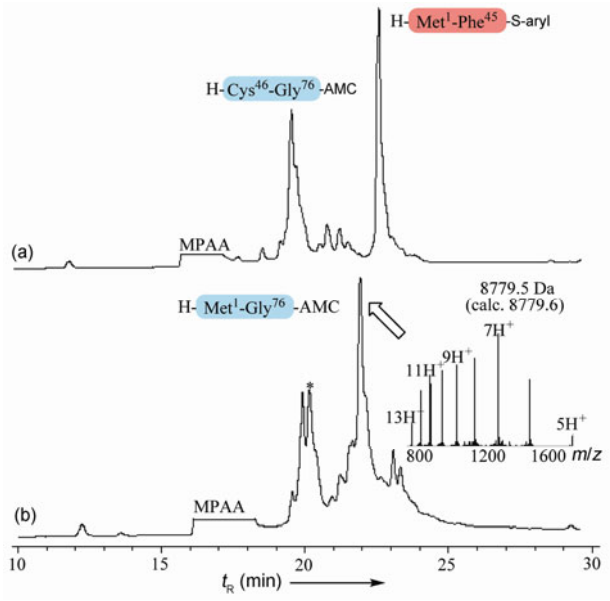


Figure 4 HPLC monitoring of the ligation between Ub[Met^1-Phe^{45}] and **Ub3**. (a) After the activation of Ub[Met^1-Phe^{45}] followed by addition of MPAA; (b) after overnight.

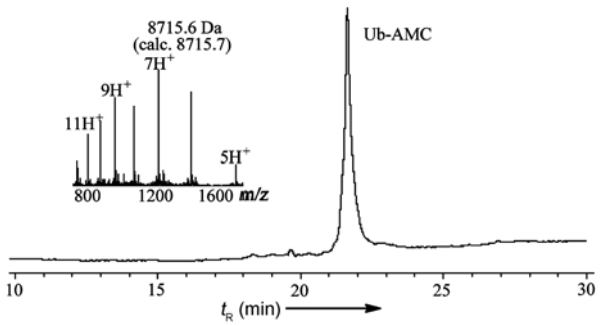


Figure 5 Desulfurization of Ub-AMC[Ala28, 46Cys].

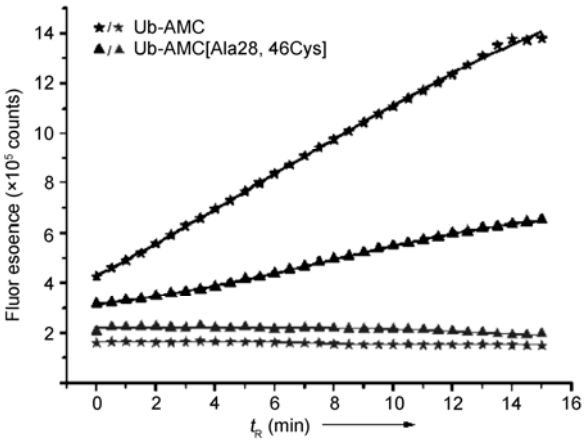


Figure 6 Enzymatic activity with Ub-AMC. The gray spots represent the change of fluorescence in the absence of DUBs. The black spots represent the change of fluorescence in the presence of DUBs.

idues on its sensitivity to DUBs.

The assays of DUBs activity showed that the Ub-AMC produced by our method can be directly used in DUBs detection. The intensity of fluorescence was instantly increased with the presence of DUBs as shown in Figure 8. Interestingly, unlike Ub-AMC, Ub-AMC[Ala28, 46Cys] was not sensitive to DUBs. Based on the fact reported that displacement of Ala^{46} with Cys^{46} in the native Ub has no effect on its sensitivity to DUBs [9], our result indicated that Ala^{28} probably plays an important role in the interaction between Ub and DUBs.

4 Conclusions

In summary, we have developed an efficient method for chemical synthesis of Ub-AMC by ligation of peptide hydrazides. We confirmed that the synthetic Ub-AMC can be used for the detection of DUBs. The peptide hydrazides used in Ub synthesis can be readily prepared by Fmoc-SPPS, and all of the peptide fragments can be prepared readily. Therefore, this method may be extended to the synthesis of other Ub derivatives.

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1 Hershko A, Ciechanover A. The ubiquitin system. *Annu Rev Biochem*, 1998, 67: 425–479
2 Jenuwein T, Allis CD. Translating the histone code. *Science*, 2001, 293: 1074–1080
3 Shabek N, Herman-Bachinsky Y, Buchsbaum S, Lewinson O, Haj-Yahya M, Hejjaoui M, Lashuel HA, Sommer T, Brik A, Ciechanover A. The size of the proteasomal substrate determines whether its degradation will be mediated by mono- or polyubiquitylation. *Mol Cell*, 2012, 48: 87–97
4 Hejjaoui M, Haj-Yahya M, Kumar KSA, Brik A, Lashuel HA. Towards elucidation of the role of ubiquitination in the pathogenesisof parkinson's disease with semisynthetic ubiquitinated alpha-synuclein. *Angew Chem Int Ed*, 2011, 50: 405–409
5 McGinty RK, Kohn M, Chatterjee C, Chiang KP, Pratt MR, Muir TM. Structure–activity analysis of semisynthetic nucleosomes: mechanistic insights into the stimulation of DotL by ubiquitylated histone H_2B. *Chem Biol*, 2009, 4: 958–968
6 Spasser L, Brik A. Chemistry and biology of the ubiquitin signal. *Angew Chem Int Ed*, 2012, 51: 6840–6862
7 Bang D, Makhatadze GI, Tereshko V, Kossiakoff AA, Kent SB. Total chemical synthesis and X-ray crystal structure of a protein diastereomer: [D-Gln35]ubiquitin. *Angew Chem Int Ed*, 2005, 44: 3852–3856
8 Kumar KSA, Spasser L, Erlich LA, Bavikar SN, Brik A. Total chemical synthesis of di-ubiquitin chains. *Angew Chem Int Ed*, 2010, 49: 9126–9131
9 Bavikar SN, Spasser L, Haj-Yahya M, Karthikeyan SV, Moyal T, Kumar KSA, Brik A. Chemical synthesis of ubiquitinated peptides with varying lengths and types of ubiquitin chains to explore the activity of deubiquitinases. *Angew Chem Int Ed*, 2012, 51: 758–763
10 Kumar KSA, Bavikar SN, Spasser L, Moyal T, Ohayon S, Brik A. Total chemical synthesis of a 304 amino acid K48-linked tetraubiquitin protein. *Angew Chem Int Ed*, 2011, 50: 6137–6141
11 Ohayon S, Spasser L, Aharoni A, Brik A. Targeting deubiquitinases enabled by chemical synthesis of proteins. *J Am Chem Soc*, 2012, 134: 3281–3289
12 Yang R, Pasunooti KK, Li F, Liu XW, Liu CF. Dual native chemical ligation at lysine. *J Am Chem Soc*, 2009, 131: 13592–13593
13 Oualid FE, Merkx R, Ekkebus R, Hameed DS, Smit JJ, Jong A, Hilkmann H, Sixma TK, Ovaa H. Chemical synthesis of ubiquitin, ubiquitin-based probes, and diubiquitin. *Angew Chem Int Ed*, 2010, 49: 10149–10153
14 Fang GM, Li YM, Shen F, Huang YC, Li JB, Lin Y, Cui HK, Liu L. Protein chemical synthesis by ligation of peptide hydrazides. *Angew Chem Int Ed*, 2011, 50: 7645–7649
15 Zheng JS, Tang S, Guo Y, Chang HN, Liu L. Synthesis of cyclic peptides and cyclic proteins via ligation of peptide hydrazides. *ChemBioChem*, 2012, 13: 542–546
16 Zheng JS, Chang HN, Wang FL, Liu L. Fmoc synthesis of peptide thioesters without post-chain-assembly manipulation. *J Am Chem Soc*, 2011, 133: 11080–11083
17 Li YM, Yang MY, Huang YC, Li YT, Chen PR, Liu L. Ligation of expressed alpha-hydrazides via genetic incorporation of an alpha-hydroxyl acid. *ACS Chem Biol*, 2012, 7: 1015–1022
18 Fang GM, Wang JX, Liu L. Convergent chemical synthesis of proteins by ligation of peptide hydrazides. *Angew Chem Int Ed*, 2012, 51: 10347–10350
19 Zheng JS, Chang HN, Shi J, Liu L. Chemical synthesis of a cyclotide via intramolecular cyclization of peptide O-esters. *Sci China Chem*, 2012, 55: 64–69
20 Shen F, Tang S, Liu L. Hexafluoro-2-propanol as a potent cosolvent for chemical ligation of membrane proteins. *Sci China Chem*, 2011, 54: 110–116
21 Tang S, Zheng JS, Yang K, Liu L. Synthesis of cyclic tetrapeptides via ligation of peptide hydrazides. *Acta Chim Sinica*, 2012, 70: 1471–1476