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#### **1. General Information**

#### a. Materials

All peptide or chemical reagents and solvents were purchased from CS Bio Co. (shanghai), GL Biochem (shanghai), Aladdin-reagent Co. (shanghai), Sinopharm Chemical Reagent Co. Ltd., Alfa Aesar China Co. Ltd., J&K Chemical Co. Ltd. TLC was executed on plates pre-coated with silica gel 60 F254 (250 layer thickness). Visualization was achieved using UV light, iodine vapors, permanganate solution. Column chromatographic purification of products was achieved using forced-flow chromatography on Silica Gel (200-300 mesh on small-scale or 300-400 mesh on large-scale). Manual peptide-synthesis apparatus was using peptide synthesis vessel and in a constant-temperature shaker at 30°C. Automated peptide-synthesis apparatus was using a CS Bio 136XT automated synthesizer conducting with a 0.25 mmol resin scale.

#### **b. HPLC**

Analytical HPLC was conducted on a SHIMADZU (Prominence LC-20AT) instrument utilizing an analytical column (Grace Vydac "Peptide C18 or C8",  $150 \times 4.6$  mm, flow rate 1.2 mL/min, RT). Analytical samples were monitored at 214 and 254 nm. Semi-preparative HPLC was conducted on a SHIMADZU (Prominence LC-20AT) instrument utilizing a semi-preparative column (Grace Vydac "Peptide C18",  $250 \times 10$  mm, flow rate 4.0 mL/min, rt). Solvent A was 0.08% trifluoroacetic acid in acetonitrile, and solvent B was 0.1% trifluoroacetic acid in water. Both solvents were leached through 0.22 µm filter paper and ultrasonicated for 30 min before use.

#### 2. Experimental Section

#### a. Synthesis of thioacid from H-LYRAG-NHNH<sub>2</sub>



Figure S1: The chromatogram of the synthesis of thioacid from H-LYRAG-NHNH<sub>2</sub> under different conditions.

#### b. Compared the synthesis of thioacid from H-LYRAG-MESNa with or without thiol



**Figure S2:** HPLC ( $\lambda$  = 214 nm) analysis of the conversion of peptide thioacids from thioesters with or without added MESNa (1 mM, pH 7.0, 20°C).

#### c. Compared the synthesis of thioacids with different residues





Figure S3: The chromatogram of the synthesis of thioacid from H-LYRAX-NHNH<sub>2</sub>.

Table S1: Synthesis of thioacid from H-LYRAX-NHNH<sub>2</sub>

	1) NaNO <sub>2</sub> , pH3.0 Thiols , pH5-6.0	
5 mM	2) Na <sub>2</sub> S	LINAG SH

Entry	Х	Time(h) <sup>a</sup>	Isolated yield[%] <sup>b</sup>
1	Ser	1.5	51 (1.6 mg)
2	Phe	1.5	50 (1.7 mg)
3	Ala	2.5	46 (1.4 mg)
4	Leu	3.0	47 (1.5 mg)
5	Val	7.0	32 (1.0 mg)
6	Val (pH 9.0)	5.0	42 (1.3 mg)
7	Pro	>12	35 (1.1 mg)
8	Pro(pH 9.0)	>12	32 (1.0 mg)

a: began with thioester; b: 5 mM peptide hydrazides

#### d. Racemization test



Figure S4: The chromatogram of racemization test.

#### e. Fluorescence labeling of protein by thioacid based ligation

<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>):  $\delta$ (ppm) 8.49 (d, J = 8.4 Hz, 1H), 8.17 (d, J = 7.2 Hz, 1H), 8.07 (d, J = 8.4 Hz, 1H), 7.46 (dd,  $J_1 = J_2 = 8.4$  Hz, 1H), 7.39 (dd,  $J_1 = J_2 = 7.2$  Hz, 1H), 7.06 (d, J = 7.2 Hz, 1H), 2.71 (s, 6H).



<sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>): δ(ppm) 152.1, 133.6, 132.7, 130.0, 129.9, 129.6, 129.2, 122.9, 118.6, 115.8, 45.3.



Figure S5: The NMR spectra and Mass Spectrometry of Dansyl-N<sub>3</sub>. (ESI found [M+H]<sup>+</sup>: 277.3, calc for: 276.0).

#### Ub sequence:

## MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYNIQKESTLHLVLR LRG

Ub-NHNH<sub>2</sub> (2.4 mg, 1 mM) was dissolved in 200  $\mu$ L buffer (6 M Gn-Cl, 100 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 3.0) and cooled in an ice bath (-10 °C). 36  $\mu$ L of aqueous 50 mM NaNO<sub>2</sub> solution was added and the reaction was incubated for 20 min. MESNa (1.5 mg) was added and the pH was adjusted to 5.0-6.0. Reaction was incubated for 20 min and adjusted the pH to about 1.0. 60  $\mu$ L of aqueous 1 M Na<sub>2</sub>S was added and then adjusted the pH to 7.0. The reaction was detected by analytical RP-HPLC and isolated by semi-preparative RP-HPLC.

Ub-SH (0.4 mg) was dissolved in 100  $\mu$ L buffer (6 M Gn-Cl, 100 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.0) and 100  $\mu$ L DMSO. 15  $\mu$ L of 0.6 M Dansyl-N<sub>3</sub> and 0.5  $\mu$ L 2, 6-lutidine were added. The reaction was detected by analytical RP-HPLC.



Figure S6: The Mass Spectrometry of Ub-NHNH<sub>2</sub> (ESI found 8522.3, calc for 8521.8), Ub-SH (ESI found 8524.1, calc for 8523.8) and Ub-Dansyl (ESI found 8740.6, calc for 8739.8).

LC3 sequence:

PSEKTFKQRRSFEQRVEDVRLIREQHPTKIPVIIERYKGEKQLPVLDKTKFLVPDHVNMSELIKIIRRRLQLNANQAFFLLVNGHSMVSVSTPISEVYESERDEDGFLYMVYASQETFG

# **3. Spectra**1) LYRAG-NHNH<sub>2</sub>



ESI found [M+H]<sup>+</sup> 593.32, calc for 592.6



ESI found [M+H]<sup>+</sup> 703.25, calc for 701.6

#### 3) LYRAG-MPAA



ESI found [M+H]<sup>+</sup> 729.34, calc for 728.6





ESI found  $[M+H]^+$  655.30, calc for 654.6

#### 5) LYRAG-NH<sub>2</sub>



ESI found [M+H]<sup>+</sup> 578.32, calc for 577.6





ESI found [M+H]<sup>+</sup> 595.28, calc for 594.6

7) LYRAA-NHNH<sub>2</sub>



ESI found 606.6, calc for 606.7



#### 8) LYRAA-MESNa





ESI found 608.3, calc for 608.7







## 11) LYRAS-MESNa



ESI found [M+H]<sup>+</sup>733.3, calc for 731.8

12) LYRAS-SH



ESI found 624.4, calc for 624.7

#### 13) LYRAF-NHNH<sub>2</sub>



ESI found 682.4, calc for 682.8



ESI found [M+H]<sup>+</sup> 793.38, calc for 791.9

15) LYRAF-SH









ESI found 648.5, calc for 648.8

## 17) LYRAL-MESNa



ESI found [M+H]+759.4, calc for 757.9

## 18) LYRAL-SH







ESI found 634.5, calc for 634.7

## 20) LYRAV-MESNa





## 21) LYRAV-SH





#### 22) LYRAP-NHNH<sub>2</sub>





#### 23) LYRAP-MESNa



ESI found 742.4, calc for 741.9





ESI found 634.4, calc for 634.8

#### 25) Bn-LY(Bn)F(L)-NHNH2



ESI found [M+H]<sup>+</sup> 636.24, calc for 635.3

#### 26) Bn-LY(Bn)F(D)-NHNH<sub>2</sub>



ESI found [M+H]+636.28, calc for 635.3

#### 27) Bn-LY(Bn)F(L)-SH



ESI found [M+H]+ 638.20, calc for 637.3





ESI found  $[M+H]^+$  638.18, calc for 637.3

29) Ub-NHNH<sub>2</sub>



ESI found 8522.3, calc for 8521.8



30) Ub-MESNa







32) Ub-Dansyl



ESI found 8740.6, calc for 8739.8

#### 33) LC3-NHNH<sub>2</sub>



34) LC3-SH



ESI found 14029.2, calc for 14028.2







ESI found 2420.1, calc for 2420.8

#### 37) H-M1-A22-SH



ESI found 2422.36, calc for 2422.8

38) Npys-C23-A42-OH (the cys 40 was modified by Acm)



ESI found 2274.01, calc for 2274.5

39) H-M1- A42-OH (the cys 40 was modified by Acm)



ESI found 4509.2, calc for 4509.3





ESI found 4437.48, calc for 4438.3