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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℃. 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 × 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 × 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-NHNH₂

Figure S1: The chromatogram of the synthesis of thioacid from H-LYRAG-NHNH₂ under different conditions.

b. Compared the synthesis of thioacid from H-LYRAG-MESNa with or without thiol
Figure S2: HPLC (λ = 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$_2$.

Table S1: Synthesis of thioacid from H-LYRAX-NHNH$_2$

<table>
<thead>
<tr>
<th>Entry</th>
<th>X</th>
<th>Time(h)$^a$</th>
<th>Isolated yield[%]$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ser</td>
<td>1.5</td>
<td>51 (1.6 mg)</td>
</tr>
<tr>
<td>2</td>
<td>Phe</td>
<td>1.5</td>
<td>50 (1.7 mg)</td>
</tr>
<tr>
<td>3</td>
<td>Ala</td>
<td>2.5</td>
<td>46 (1.4 mg)</td>
</tr>
<tr>
<td>4</td>
<td>Leu</td>
<td>3.0</td>
<td>47 (1.5 mg)</td>
</tr>
<tr>
<td>5</td>
<td>Val</td>
<td>7.0</td>
<td>32 (1.0 mg)</td>
</tr>
<tr>
<td>6</td>
<td>Val (pH 9.0)</td>
<td>&gt;12</td>
<td>42 (1.3 mg)</td>
</tr>
<tr>
<td>7</td>
<td>Pro</td>
<td>&gt;12</td>
<td>35 (1.1 mg)</td>
</tr>
<tr>
<td>8</td>
<td>Pro(pH 9.0)</td>
<td>&gt;12</td>
<td>32 (1.0 mg)</td>
</tr>
</tbody>
</table>

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

**d. Racemization test**
e. Fluorescence labeling of protein by thioacid based ligation

$^1$H NMR (400 MHz, CDCl$_3$): $\delta$(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).

$^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$(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$_3$. (ESI found [M+H]$^+$: 277.3, calc for: 276.0).

Ub sequence:
MQIFVKTITLGEPSDIENVKAIKGDKEGWPDQQLFAAGQLEDGLSLDYNQKESTHLVLR

Ub-NHNH$_2$ (2.4 mg, 1 mM) was dissolved in 200 μL buffer (6 M Gn-Cl, 100 mM NaH$_2$PO$_4$, pH 3.0) and cooled in an ice bath (-10 °C). 36 μL of aqueous 50 mM NaNO$_2$ 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 μL of aqueous 1 M Na$_2$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 μL buffer (6 M Gn-Cl, 100 mM NaH$_2$PO$_4$, pH 7.0) and 100 μL DMSO. 15 μL of 0.6 M Dansyl-N$_3$ and 0.5 μL 2, 6-lutidine were added. The reaction was detected by analytical RP-HPLC.
Figure S6: The Mass Spectrometry of Ub-NHNH$_2$ (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:
PSEKTFQRRSFEQRVEDVRLIREQHPKQIREKLYGKQPVLQDKFIQVPMHVNMSLKIIRQL
NANQAFFLLVNGHSMVSTPISEVYESERDEDFLVMYASQETF
3. Spectra

1) LYRAG-NHN$\textsubscript{2}$

ESI found [M+H]$^+$ 593.32, calc for 592.6

2) LYRAG-MESNa

ESI found [M+H]$^+$ 703.25, calc for 701.6
3) LYRAG-MPAA

ESI found \([\text{M+H}]^+\) 729.34, calc for 728.6

4) LYRAG-EDT

ESI found \([\text{M+H}]^+\) 655.30, calc for 654.6
5) LYRAG-NH₂

ESI found [M+H]⁺ 578.32, calc for 577.6

6) LYRAG-SH

ESI found [M+H]⁺ 595.28, calc for 594.6
7) LYRAA-NHNH$_2$

ESI found 606.6, calc for 606.7

8) LYRAA-MESNa

ESI found 716.3, calc for 715.8
9) LYRAA-SH

ESI found 608.3, calc for 608.7

10) LYRAS-NNH$_2$

ESI found 622.4, calc for 622.7
11) LYRAS-MESNa

ESI found [M+H]$^+$ 733.3, calc for 731.8

12) LYRAS-SH

ESI found 624.4, calc for 624.7
13) LYRAF-NHNH$_2$

ESI found 682.4, calc for 682.8

14) LYRAF-MESNa

ESI found [M+H]$^+$ 793.38, calc for 791.9
15) LYRAF-SH

ESI found 684.4, calc for 684.8

16) LYRAL-NNH₂

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 650.4, calc for 650.8
19) LYRAV-NHNH$_2$

ESI found 634.5, calc for 634.7

20) LYRAV-MESNa

ESI found [M+H]$^+$ 745.4, calc for 743.9
21) LYRAV-SH

ESI found 636.4, calc for 636.8

22) LYRAP-NHNH₂

ESI found [M+H]⁺ 633.4, calc for 632.7
23) LYRAP-MESNa

ESI found 742.4, calc for 741.9

24) LYRAP-SH

ESI found 634.4, calc for 634.8
25) Bn-LY(Bn)F(L)-NHNH$_2$

ESI found [M+H]$^+$ 636.24, calc for 635.3

26) Bn-LY(Bn)F(D)-NHNH$_2$

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

28) Bn-LY(Bn)F(D)-SH

ESI found [M+H]+ 638.18, calc for 637.3
29) Ub-NHNH₂

ESI found 8522.3, calc for 8521.8

30) Ub-MESNa

ESI found 8632.4, calc for 8630.8
31) Ub-SH

ESI found 8524.1, calc for 8523.8

32) Ub-Dansyl

ESI found 8740.6, calc for 8739.8
33) LC3-NH₂

ESI found 14026.7, calc for 14026.1

34) LC3-SH

ESI found 14029.2, calc for 14028.2
35) LC3-Dansyl

ESI found 14244.5, calc for 14244.2

36) H-M1-A22-NHNH₂

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

40) H-M1- A42-OH

ESI found 4437.48, calc for 4438.3