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Thiol-assisted one-pot synthesis of peptide/protein C-terminal thioacids from peptide/protein hydrazides at neutral conditions†

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An efficient thiol-assisted one-pot synthesis of peptide/protein C-terminal thioacids was achieved by using peptide/protein hydrazides precursors at neutral pH and room temperature (about 20 °C). The transformation from hydrazides to thioacids was shown to be efficient for different C-terminal amino acids and was racemization-free. The *in situ* formed peptide-thioacids were further used for protein chemical synthesis and site-specific labelling successfully.

Introduction

Peptide C-terminal thioacids were considered as important functional groups for protein chemoselective reactions.[1] It has been widely used for protein site-specific modification and labeling, also converted to the peptide-thioesters for protein chemical synthesis.[2] Most early strategies reported for preparing peptide thioacids were mainly based on Boc solid phase peptide synthesis (SPPS).[3] Another progress that utilized intein thioesters to make protein thioacids need relatively high pH and very long time (16 h).[4] Recently, C. F. Liu *et al.* developed a simple and efficient method to produce peptide thioacids through hydrothiolysis of thioesters.[5] This method has been successfully used for protein chemical synthesis and site-specific labeling.[6] However, despite the high yields of the thioacids formation from thioesters, relatively high pH (more than 8) and temperature (42 °C) was necessary. In addition, the preparation of peptide thioesters through Fmoc based SPPS for subsequent hydrothiolysis remain challenging till now.[7] To further acquire peptide thioacids by mild methods, bis(2-sulfanylethyl) amido (SEA) peptides were developed to react with triisopropylsilylthiol in water at neutral pH.[8] Nevertheless, the reagent, such as triisopropylsilylthiol, used for the synthesis of SEA peptide is expensive and the preparing of SEA resin is time-consuming. Moreover, the temperature (37 °C) and the long time required for achieving considerable yields of product still needs to be optimized.

Because peptide thioacids can be obtained from thioesters, it is possible to be produced from hydrazides precursor in one-pot approach. As easy prepared equivalents of thioesters, peptide/protein hydrazides have been verified for protein chemical synthesis and modification.[9] Herein, we report for first time an efficient and facile one-pot synthesis of both peptide and recombinant protein C-terminal thioacids by using its hydrazides as precursors at neutral pH and room temperature (about 20 °C) (Scheme 1). The interesting point of this *in situ* transformation is the catalysis of disulfide intermediate, which was possibly generated from thiol groups. Then, the peptide thioacids can be formed after the thioesterification of peptide hydrazides within 2 h. Another important advantage of this method is that peptide or protein hydrazides can be easily obtained through standard protocol as reported previously.[10]

Results and discussion

We started with hydrazides based thioacids synthesis of the model peptide Leu-Tyr-Arg-Ala-Gly-$NHNH_2$ (LYRAG-$NHNH_2$). It was first converted to C terminal thioesters by treating with MESNa (2-mercaptoethane sulfonic acid sodium salt) as thiol using standard protocol ($NaNO_2$, pH 3.0; MESNa 30 mM, pH 5.0–6.0), and subsequently *in situ* turned to thioacids through hydrothiolysis of thioesters. It is surprising to find that thioacids can be easily achieved in high yield under very mild condition (pH 7.0, 20 °C, 1 h), which was quite different to previously reported methods.[5] The yield of Leu-Tyr-Arg-Ala-

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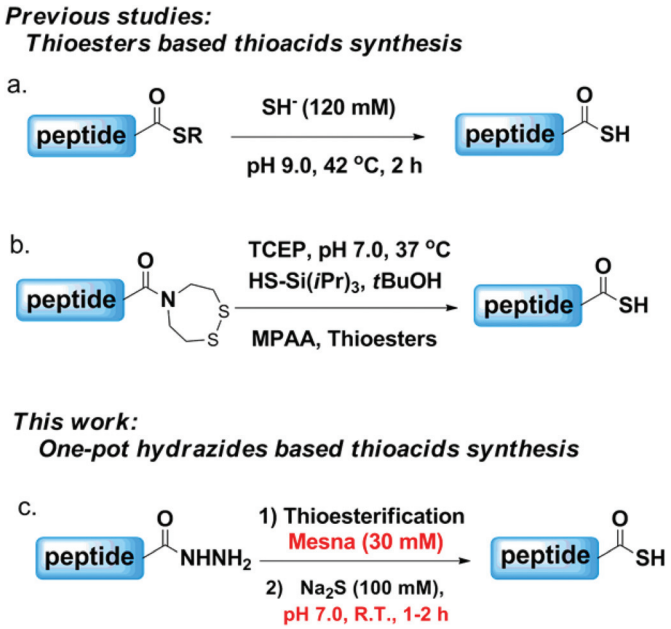

Scheme 1 One-pot synthesis of peptide C-terminal thioacids by using hydrazides as precursors. (a): $NaNO_2$, pH 3.0; MESNa 30 mM, pH 5.0–6.0; (b): TCEP, pH 7.0, 37 °C, HS-Si(iPr)$_3$, *t*BuOH, MPAA 60 mM; (c): Na_2S 100 mM, pH 7.0, 20 °C.

Gly-SH, as determined by HPLC, was very high (48% isolated yield). To determine whether this result was because of the excess of thiol groups (MESNa) existed in thioesterification reaction system, we first purified the peptide thioesters and hydrothiolysis of thioesters in two independent procedures. Similar as C. F. Liu *et al.* reported, Fig. 1b indicated that the conversion yield was very low when Na_2S (100 mM) only was added for hydrothiolysis of thioesters at neutral condition (pH 7.0, 20 °C). As compared, when MESNa (30 mM) was added with Na_2S, it was very easy to obtain thioacids in quantitative yield at similar condition. Decrease in the loading of MESNa to 1 mM produced nearly the same HPLC conversion rate (Fig. S2†). The results demonstrated that the added thiol groups facilitated the hydrothiolysis of thioesters.

It has been reported that thiol group, including GSH and Mesna, can generate disulfide or persulfide (RSSH).[11] Thus, it is worthy to speculate whether the high efficiency of hydrazides based thioacids synthesis was possible because of the formation of disulfide intermediate. As shown in Fig. 2, when TCEP (tris(2-carboxyethyl)phosphine) was added, which could reduce the disulfide, decreased the conversion from thiol to thioacids (about 40%) in one hour. As compared, thiol can be converted into thioacids almost completely without added TCEP. Therefore, we hypothesized that the thiol group should generate disulfide intermediate and thereby, increase the efficiency of the reaction. Hence collectively, our conclusion indicated that hydrazides based one-pot synthesis of thioacids is a novel thiol assisted strategy with high efficiency.

This one-pot hydrazides based thioacids synthesis was further explored by using different thiols, including MPAA

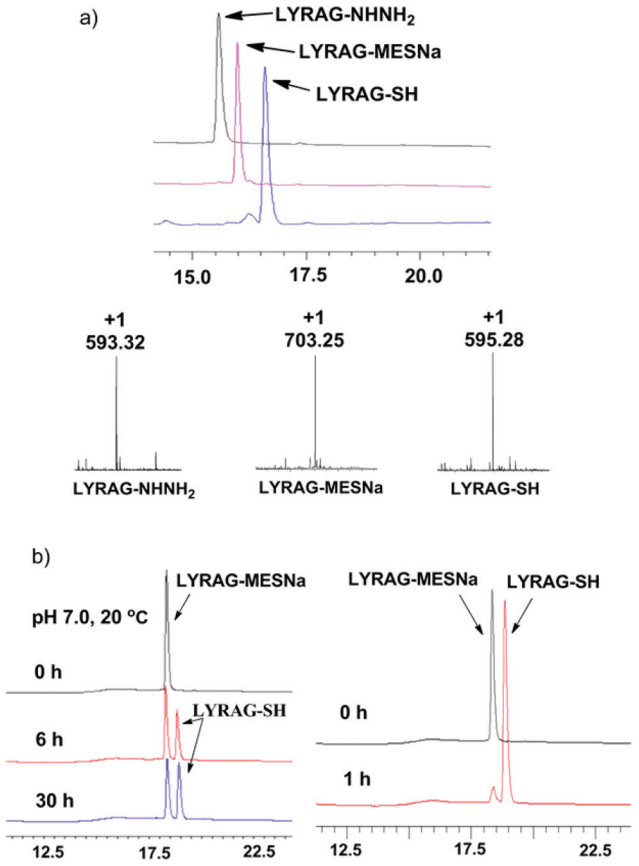

Fig. 1 (a) HPLC (λ = 214 nm) analysis of the conversion of peptide thioacids from hydrazides precursors. ESI-MS mass of LYRAG-$NHNH_2$ is 593.3 (calc. 592.6); LYRAG-MESNa is 703.3 (calc. 701.6); LYRAG-SH is 595.3 (calc. 594.6); (b) HPLC (λ = 214 nm) analysis of the conversion of peptide thioacids from thioesters with (R) or without (L) added MESNa (30 mM, pH 7.0, 20 °C).

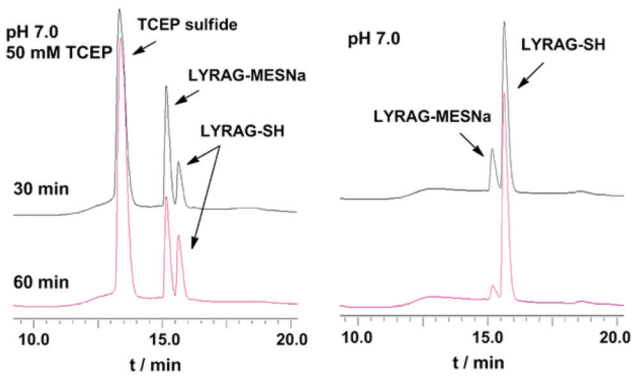

Fig. 2 HPLC (λ = 214 nm) analysis of the conversion of peptide thioacids from thioesters with (L) or without (R) TCEP (50 mM). All the reactions were performed at MESNa 30 mM, Na_2S 100 mM, pH 7.0, 20 °C.

(4-mercaptophenylacetic Acid) and EDT (1,2-dithioethane). We found that treating with MPAA as ester group for 1 h also leads to near quantitative conversion to the thioacids, whereas relative long time (3 h) was needed for EDT. Moreover, different pH conditions were compared for the synthesis of thioacids.

Table 1 Synthesis of thioacids under different pH and thiols

Entry	pH	Thiols	Time(h)	HPLC/Isolated yield[a] [%]
1	7.0	MESNa	1.0	94/48
2	7.0	MPAA	1.0	93/44
3	7.0	EDT	3.0	96/41
4	7.0	No	1.0	21/13
5	6.0	MESNa	5.0	90/34
6	8.0	MESNa	0.75	95/51
7	9.0	MESNa	0.75	92/44

[a] 5 mM peptide hydrazides Leu-Tyr-Arg-Ala-Gly-$NHNH_2$.

At pH 6.0, R.T., the conversion rate was decreased accordingly, whereas prolonging the reaction for 5 h led to near complete conversion of the thioesters to thioacids. Increasing the pH (from pH 8.0 to 9.0) increased the reaction rate in similar way as the previous results (Table 1, Fig. S1†). Therefore for practical reasons, the one-pot reactions were all performed at pH 7.0, R.T. in subsequent experiments.

To demonstrate the general utility of this method, a number of peptide hydrazides with different C terminal residues were tested (Leu-Tyr-Arg-Ala-X-$NHNH_2$). We chose six different amino acids to test the conversion rate of thioacids. All reactions were conducted under conditions of pH 7.0, R.T. and 100 mM aqueous Na_2S. Table 2 shows that C-terminal Ser, Phe, Ala and Leu hydrazides can transform to thioacids within 3 h to nearly 100% HPLC yield and high isolated yield. The large steric hindrance residues, such as Pro and Val, can also achieve better conversion rates in a relatively longer time (Fig. S3†).

Racemization of the C-terminal amino acid is another important issue for the synthesis of the polypeptide-thioacids. To test the possibility of racemization of the synthetic thioacids converted form hydrazides, we compared double-benzyl-protected tripeptide Bn-Leu-Tyr(Bn)-Phe(L)-SH and Bn-Leu-Tyr-(Bn)-Phe(D)-SH. Through RP-HPLC analysis with either L- or D-Phe at the C-terminus, we confirmed that the formation of thioacids was epimerization-free (Fig. S4†).

To verify the application of the above *in situ* formed thioacids, we selected a 42 amino acid antimicrobial peptide trifolitoxin [Ala23Cys] for chemical synthesis (Fig. 3). The ligation site was chosen to be Ala22-Cys23. We first synthesized peptide hydrazide **1** (M1-A22-$NHNH_2$) and Npys modified

Table 2 Synthesis of thioacids with different C-terminal residues

Entry	X	Time (h)	HPLC/Isolated yield[a] [%]
1	Ser	1.5	97/51
2	Phe	1.5	93/50
3	Ala	2.5	92/46
4	Leu	3.0	95/47
5	Val	7.0	88/32
6	Pro	>12	90/35

[a] 5 mM peptide hydrazides Leu-Tyr-Arg-Ala-X-$NHNH_2$.

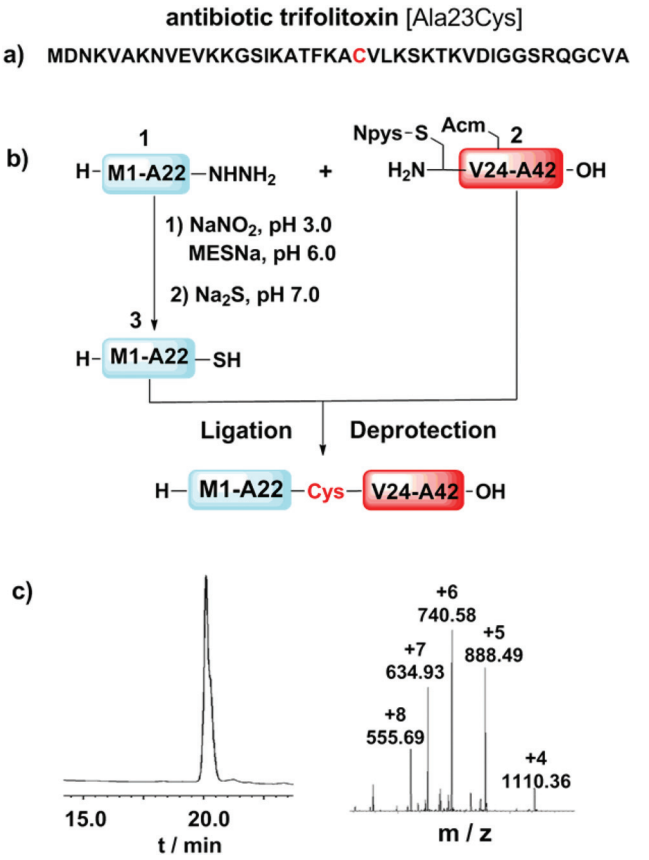

Fig. 3 Synthesis of trifolitoxin using thioacids captured ligation. (a) Sequence of trifolitoxin[Ala23Cys]. (b) Procedure for ligation. (c) HPLC (λ = 214 nm) and ESI-MS for the final purified trifolitoxin were performed. Observed mass is 4437.5 Da (calc. 4438.3 Da). The MS data was taken across the whole UV peak.

peptide **2** (C23-A42). After one-pot thioesterification and hydrothiolysis, we obtained peptide thioacids **3** (M1-A22)-SH (43% isolated yield). Then, by using previously reported thioacid capture ligation,[5] we found that the ligation proceeds provide the desired product in a good HPLC yields. Finally, after de-Acm protecting group, we obtained a full-length trifolitoxin [Ala23Cys] with high purity and homogeneity.

Finally, the functionality of synthetic thioacids was further expanded to recombinant protein labeling. We first obtained LC3-$NHNH_2$ (microtubule-associated protein 1A/1B-light chain 3), an autophagosomal marker protein, from recombinant LC3-intein-CBD.[12] LC3-SH was easily obtained as aforementioned in high yields, because the C terminal residue of this model protein is Gly. Then, LC3 thioacids (0.34 mM) reacted with 10 folds dansyl azide molecules overnight at room temperature, having pH 7.0. The final labeled product was purified by using desalting column for two times. As shown in Fig. 4, ESI-MS and gel electrophoresis experiments proved that the fluorescent molecules were site-specific labeled with LC3 protein. Another important protein Ub-$NHNH_2$ was also proved to be converted to C-terminal thioacids and labeled with dansyl azide (Fig. S6†).

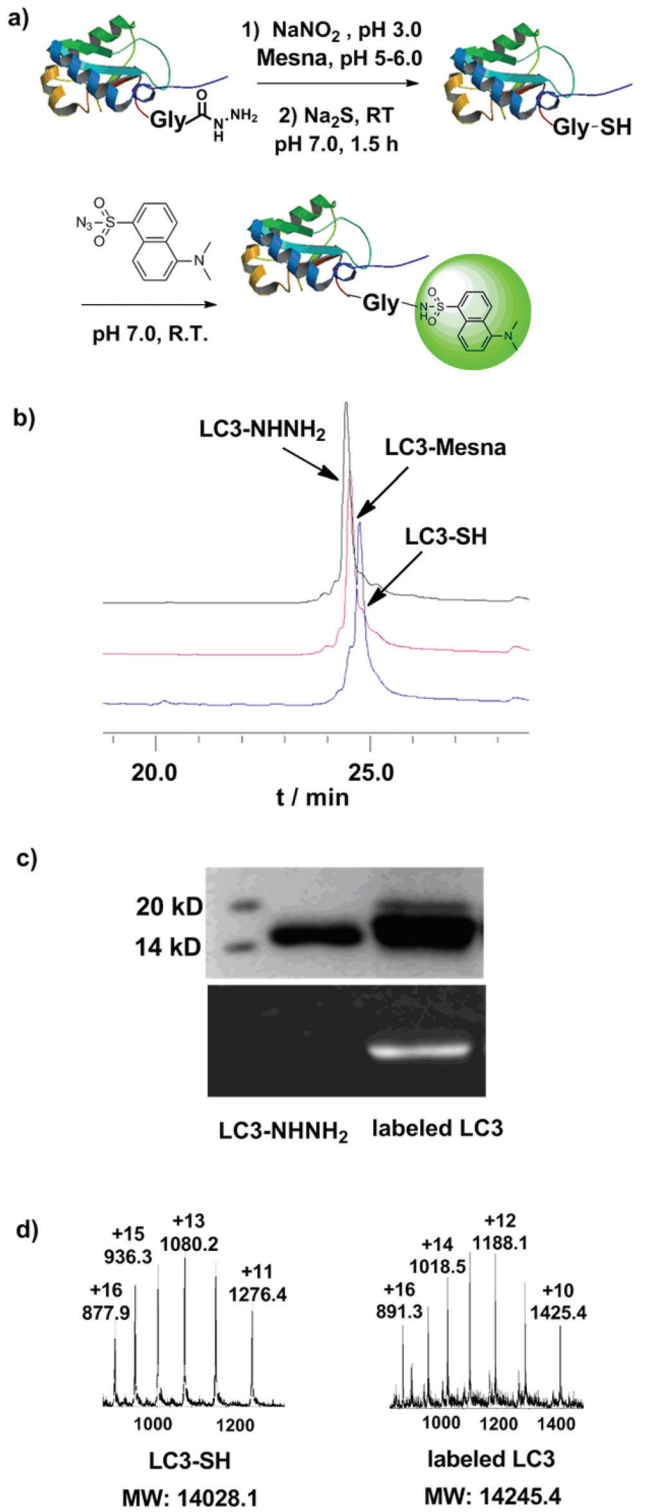

Fig. 4 Fluorescence labeling of LC3 by thioacids based ligation. (a) Generation of C terminal labeling of LC3 through thioacid-sulfonazides ligation; (b) HPLC (λ = 214 nm) analysis of the conversion of LC3 thioacids from hydrazides precursors; (c) SDS-PAGE of fluorescent labeling of LC3-SH; (d) ESI-MS for purified LC3-SH is 14 028.1 Da (calc. 14 029.2) and labeled LC3 is 14 245.4 Da (calc. 14 244.2).

Conclusions

To summarize, we devised a facile and efficient one-pot synthesis of peptide thioacids approach at neutral conditions by using peptide hydrazides precursors. It was demonstrated that existing thiol group was essential for the *in situ* formation of thioacids in high yields. This method was further proved to be efficient for different C-terminal amino acids and racemization free. Finally, the synthetic thioacids was successfully used for the chemical synthesis of antimicrobial peptide trifolitoxin and recombinant protein labeling.

Experimental section

Synthesis of hydrazine 2CTC resin

2-Chlorotrityl chloride resin (loading = 1.0 mmol g^{-1}) (2 g) was swelled in CH_2Cl_2–DMF (15/15 mL) at 0 °C. $NH_2NH_2{\cdot}H_2O$ (1 mL, 10 eq.) and DIEA (3.3 mL, 20 eq.) were then added. The reaction was conducted from 0 °C to room temperature, overnight. Methanol (2 mL) was added to quench the remaining 2-chlorotrityl chloride resin. After reaction, the resin was washed with DMF, H_2O, methanol, ethyl ether and kept under high vacuum for 3 h. Then, the resin was stored at 4 °C.

Synthesis of peptide hydrazine

Hydrazine 2CTC and 2CTC resins were first swelled in DMF–DCM (1/1) for 10 min before use. The coupling step was carried out at 30 °C by standard Fmoc chemistry (4 eq. protected amino acid; 3.6 eq. HBTU or HCTU, 8 eq. DIEA). Each coupling step required 45 min and the resin was washed with DMF, DCM and DMF. If the reaction required two coupling, the time was changed to 30 min × 2. For 2CTC resin, the first amino acid was coupled with 4 eq. protected amino acid, 8 eq. DIEA in DMF–DCM (1 : 1) in 2 h and the resin was capped with methanol. Deprotection reagent was 20% piperidine/DMF (5 min, 10 min). The resin was washed with DMF, DCM and DMF. Cleavage reagent selected reagent B: TFA–phenol–water–TIPS (88/5/5/2). Reaction time was about 2–3 hours. Peptide purification: The crude peptides was dissolved in acetonitrile (0.08% TFA)–water (0.1% TFA), analyzed by analytical HPLC and purified by semi-preparative HPLC and lyophilized immediately.

Synthesis of thioacids from H-Leu-Tyr-Arg-Ala-Gly-$NHNH_2$

H-Leu-Tyr-Arg-Ala-Gly-$NHNH_2$ (0.5 mg, 1 mM) was dissolved in 800 μL buffer (6 M Gn-Cl, 100 mM Na_2HPO_4, pH 3.0) and cooled in an ice bath (−10 °C). 100 μL of aqueous 50 mM $NaNO_2$ solution was added and the reaction was incubated for 20 min. MESNa (5 mg) or MPAA (5 mg) or EDT (2.5 μL) was added and the pH was adjusted to 5.0–6.0. Reaction was incubated for 20 min and the pH was adjusted to about 1.0. 100 μL of aqueous 1 M Na_2S was added and then the pH was adjusted to 6.0 or 7.0 or 8.0 or 9.0 at room temperature (20 °C). The reaction was detected by analytical RP-HPLC.

Comparison of the synthesis of thioacids from H-Leu-Tyr-Arg-Ala-Gly-MESNa with or without thiol

H-Leu-Tyr-Arg-Ala-Gly-MESNa was converted from hydrazides as standard protocol and further purified by HPLC. Then, it (0.7 mg, 1 mM) was dissolved in 900 μL buffer (6 M Gn-Cl, 100 mM Na_2HPO_4). MESNa (5 mg, 1 or 30 mM) was added in reaction B (not added in reaction A) and the pH was adjusted to about 1.0. 100 μL of aqueous 1 M Na_2S was added and then adjusted the pH to 7.0 at 20 °C. The reaction was detected by analytical RP-HPLC.

Comparison of the synthesis of thioacids with different residues

H-Leu-Tyr-Arg-Ala-X-$NHNH_2$ (1 mM) was dissolved in 800 μL buffer (6 M Gn-Cl, 100 mM Na_2HPO_4, pH 3.0) and cooled in an ice bath (−10 °C). 100 μL of aqueous 50 mM $NaNO_2$ solution was added and the reaction was incubated for 20 min. MESNa (5 mg) was added and the pH was adjusted to 5.0–6.0. The reaction was incubated for 20 min and the pH was adjusted to about 1.0. 100 μL of aqueous 1 M Na_2S was added and then the pH was adjusted to 7.0. The reaction was detected by analytical RP-HPLC. All HPLC gradients were conducted at 1%–91% (ACN)/30 min/1.2 mL/C18.

Racemization test

Peptide Leu-Tyr-Phe(L/D)-$NHNH_2$ was obtained by standard Fmoc-SPPS (0.25 mmol resin), after assembly of the peptide chain, 10 mmol benzyl bromide and 20 mmol DIEA were added. The reaction was conducted for overnight and cleaved from resin by reagent B. After it was purified by HPLC and lyophilized, we obtained the Bn-Leu-Tyr(Bn)-Phe(L/D)-$NHNH_2$·Bn-Leu-Tyr(Bn)-Phe(L/D)-$NHNH_2$ (0.6 mg, 1 mM) was dissolved in 800 μL buffer (6 M Gn-Cl, 100 mM Na_2HPO_4, pH 3.0) and cooled in an ice bath (−10 °C). 100 μL of aqueous 50 mM $NaNO_2$ solution was added and the reaction was incubated for 20 min. MESNa (5 mg) was added and the pH was adjusted to 5.0–6.0. Reaction was incubated for 20 min and the pH was adjusted to about 1.0. 100 μL of aqueous 1 M Na_2S was added and then the pH was adjusted to 7.0. The reaction was detected by analytical RP-HPLC. All HPLC gradients were conducted at 1%–91% (ACN)/30 min/1.2 mL/C18.

Synthesis of trifolitoxin using thioacids capture ligation

Antibiotic trifolitoxin [Ala23Cys] was divided into two fragments: H-M1-A22-$NHNH_2$ and H-C23-A42-OH. H-M1-A22-$NHNH_2$ (2.4 mg, 1 mM) was dissolved in 800 μL buffer (6 M Gn-Cl, 100 mM Na_2HPO_4, pH 3.0) and cooled in an ice bath (−10 °C). 100 μL of aqueous 50 mM $NaNO_2$ solution was added and the reaction was incubated for 20 min. MESNa (5 mg) was added and the pH was adjusted to 5.0–6.0. Reaction was incubated for 20 min and the pH was adjusted to about 1.0. 100 μL of aqueous 1 M Na_2S was added and then the pH was adjusted to 7.0. The reaction was detected by analytical RP-HPLC and isolated by semi-preparative RP-HPLC. Peptide H-C23-A42-OH (Cys 40 was modified by Acm) was obtained by standard Fmoc chemistry and cleaved for 2 h in the presence of 4 eq. of 2,2′-dithio-bis-(5-nitropyridine) which was to add the Npys group to Cys thiol. H-M1-A22-SH (2.4 mg, 1 mM) and Npys-C23-A42-OH (the Cys 40 was modified by Acm) (3.3 mg, 1.5 mM) were dissolved in 1 mL 10% ACN–H_2O and the pH was adjusted to 5.0–6.0. Reaction was incubated for 30 min and then AgOAc (8.3 mg) was added for overnight. 1 mL of aqueous 1 M DTT was added to reduce the reaction. The reaction was detected by analytical RP-HPLC.

Fluorescence labeling of protein by thioacids-based ligation

Dansyl chloride (270 mg, 1 mmol, 1 eq.) was dissolved in acetone (6 mL). NaN_3 (97.5 mg, 1.5 mmol, 1.5 eq.) in water (2 mL) was then added. The reaction was kept at R.T. for 4 h. The resulting solution was diluted with NH_4Cl (15 mL) and extracted by using EtOAc (30 mL). The organic phase was washed with sat. NH_4Cl, brine, dried over Na_2SO_4. Concentration *in vacuo* afforded dansyl azide (270 mg, 0.97 mmol, 97%). LC3-$NHNH_2$ (3 mg, 1 mM) was dissolved in 160 μL buffer (6 M Gn-Cl, 100 mM Na_2HPO_4, pH 3.0) and cooled in an ice bath (−10 °C). 25 μL of aqueous 50 mM $NaNO_2$ solution was added and the reaction was incubated for 20 min. MESNa (5 mg) was added and the pH was adjusted to 5.0–6.0. Reaction was incubated for 20 min and the pH was adjusted to about 1.0. 20 μL of aqueous 1 M Na_2S was added and then the pH was adjusted to 7.0. The reaction was detected by analytical RP-HPLC and isolated by semi-preparative RP-HPLC.

LC3-SH (1 mg) was dissolved in 100 μL buffer (6 M Gn-Cl, 100 mM Na_2HPO_4, pH 7.0) and 100 μL DMSO. 4.2 μL of 0.6 M Dansyl-N_3 and 0.2 μL 2, 6-lutidine were added. The reaction was detected by analytical RP-HPLC.

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