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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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Chenchen Chen,[a,b‡] Yichao Huang,[c‡] Ling Xu,[b] Yong Zheng,[b] Huajian Xu,[a] Qingxiang Guo,[b] Changlin Tian,[b] Yiming Li,[a,c*] Jing Shi,[b*]

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 successfully used for protein chemical synthesis and site-specific labelling.

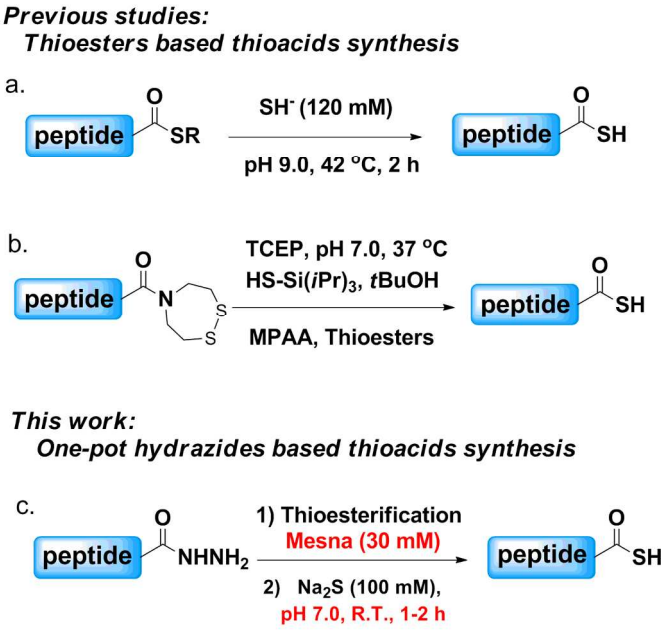
†Electronic Supplementary Information (ESI) available: Experimental details. See DOI: 10.1039/b000000x/

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, even 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 which utilized intein thioesters to make protein thioacids need relative high pH and very long time (16 h).[4] More 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 total, semi-synthesis and site-specific labeling.[6] However, despite the high yields of the thioacids formation from thioesters, relative high pH (more than 8) and temperature (42 °C) was necessary. In additional, the preparing 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 long time for achieving product in high yields is still needs to be optimized.

Since peptide thioacids can be obtained from thioesters, it make possible to be achieved from hydrazides precursor in one-pot approach yet. As easy prepared equivalents of thioesters, peptide/protein hydrazides have been verified for protein chemical synthesis and modification.[9] Herein, we first time report 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 previous reported.[10]

[a] School of Medical Engineering, School of Chemistry and Chemical Engineering, Hefei University of Technology, Hefei, Anhui 230009, China, E-mail: lym2007@mail.ustc.edu.cn, ymli@hfut.edu.cn
[b] Department of Chemistry, University of Science and Technology of China, Email: shijing@ustc.edu.cn
[c] Department of Chemistry, Tsinghua University, Beijing 100084, China
‡These authors contributed equally to this work



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(*i*Pr)$_3$, *t*BuOH, MPAA 60 mM; c): Na_2S 100 mM, pH 7.0, 20 °C.

Results and Discussion

We start with hydrazides based thioacids synthesis of the model peptide Leu-Tyr-Arg-Ala-Gly-$NHNH_2$ (LYRAG-$NHNH_2$). It was first convert 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 subsequent in-situ turn 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 with previously reported.[5] The yield of Leu-Tyr-Arg-Ala-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, Figure 1b indicated that the conversion yield was very low when Na_2S (100 mM) was only 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 obtained thioacids in quantitative yield at similar condition. Decrease in the loading of MESNa to 1 mM obtained the nearly same HPLC conversion rate (Figure S2). The results demonstrated that the added thiol groups facilitate the hydrothiolysis of thioesters.

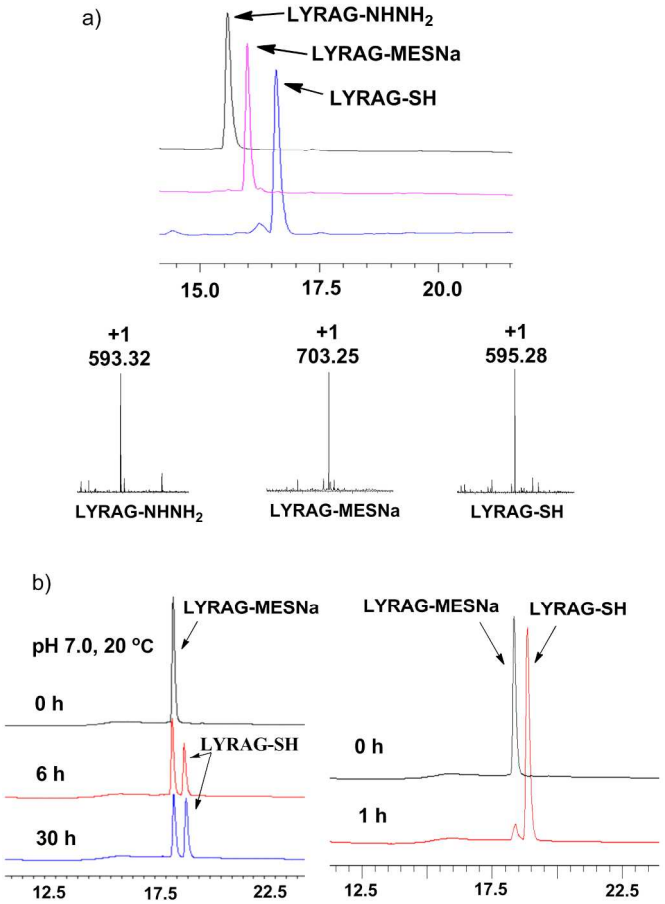


Fig. 1. a) HPLC (λ = 214 nm) analysis of the conversion of peptide thioacids from hydrazides precursors. ESI-MS mass of LYRAG-$NHNH_2$ is 593.3 (calc.592.6); LYRAG-MESNa is 703.3 (calc.701.6); LYRAG-SH is 595.3 (calc.594.6); b) HPLC (λ = 214 nm) analysis of the conversion of peptide thioacids from thioesters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

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 synthesisis possibly beacause of the formation of disulfide intermediate. As shown in Figure 2, when TCEP (Tris(2-carboxyethyl)phosphine) was added, which could reduced the disulfide, decreased the conversion from thiol to thioacids (about 40%) in one hour. As compared, thiol can almost completely converted into thioacids without added TCEP. Therefore, we hypothesized that the thiol group should generate disulfide intermediate and thereby, increase the efficiency of reaction. To collectively, our conclusion indicated that hydrazides based one-pot synthesis of thioacids is a novel thiol assisted strategy with high efficiency.

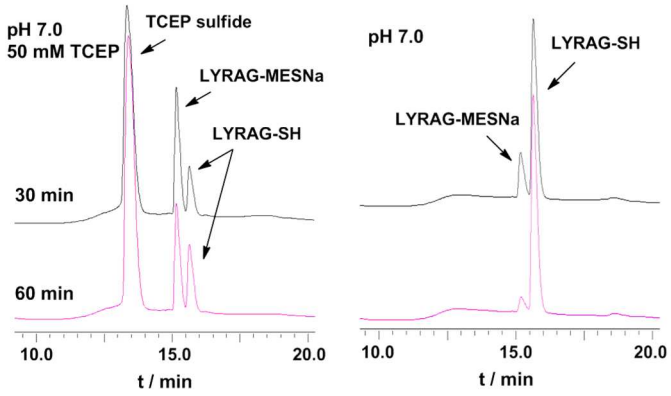


Fig. 2. HPLC (λ = 214 nm) analysis of the conversion of peptide thioacids from thioesters with (L) or without (R) TCEP (50 mM). All the reactions were performed at MESNa 30 mM, Na_2S 100 mM, pH 7.0, 20 ^oC.

This one-pot hydrazides based thioacids synthesis was further explored by using different thiols, including MPAA (4-mercaptophenylacetic Acid) and EDT (1,2-dithioethane). We found that treating with MPAA as ester group in 1 h also lead to near quantitative conversion to the thioacids, while relative long time (3 h) was need for EDT. Moreover, different pH conditions were compared for the synthesis of thioacids. At pH 6.0, R.T., the conversion rate was decreased accordingly, while prolonging the reaction to 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 similar as previous results (Table1, Figure S1). Therefore for practical reasons, the one-pot reactions were all performed at pH 7.0, R.T. in subsequent experiments.

Table 1 Synthesis of thioacids under different pH and thiols

Entry	pH	Thiols	Time(h)	HPLC/Isolated yield[%]*
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

* 5 mM peptide hydrazides Leu-Tyr-Arg-Ala-Gly-$NHNH_2$

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 indicated that C-terminal Ser, Phe, Ala and Leu hydrazides can transform to thioacids within 3 h in 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 relative long time (Figure S3).

Table 2 Synthesis of thioacids with different C-terminal residues

Entry	X	Time(h)	HPLC/Isolated yield[%]*
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

* 5 mM peptide hydrazides Leu-Tyr-Arg-Ala-X-$NHNH_2$

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 (Figure S4).

To verify the application of above in-situ formed thioacids, we selected a 42 amino acid antimicrobial peptide trifolitoxin [Ala23Cys] for chemical synthesis (Figure 3). The ligation site was chosen to be Ala22–Cys23. We first synthesized peptide hydrazide **1** (M1-A22-$NHNH_2$) and Npys modified peptide **2** (C23-A42). After one-pot thioesterification and hydrothiolysis, we got peptide thioacids **3** (M1-A22)-SH (43% isolated yield). Then, by using previous 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 got a full-length trifolitxin[Ala23Cys] with the highly pure and homogeneous.

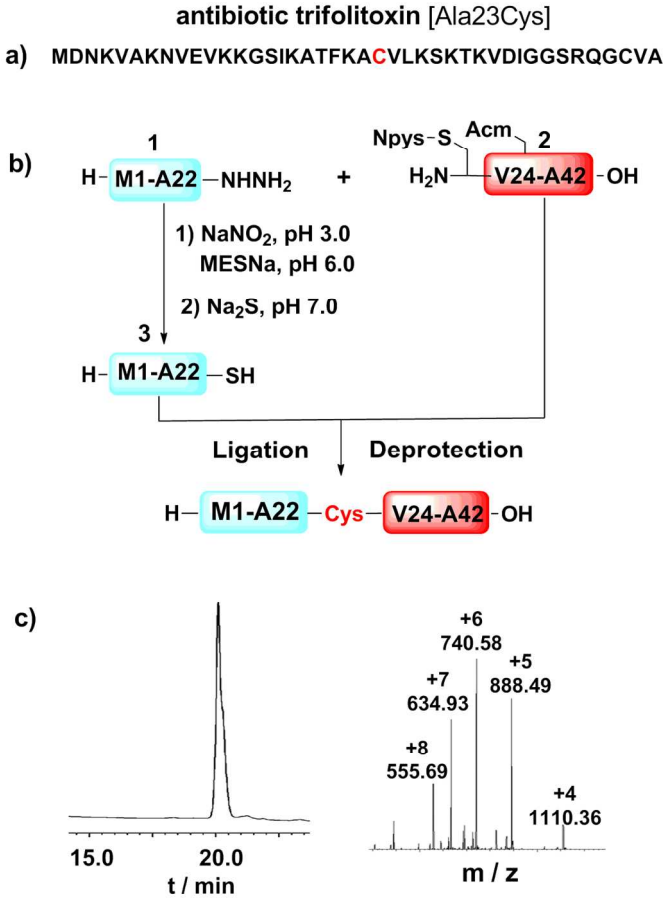


Fig. 3. Synthesis of trifolitoxin using thioacids capture ligation. a) Sequence of trifolitoxin[Ala23Cys]. b) Procedure for ligation. c) HPLC (λ=214 nm) and ESI-MS for the final purified trifolitoxin were performed. Observed mass is 4437.5 Da (calc. 4438.3 Da). The MS data was taken across the whole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

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) was reacted with 10 folds Dansylazide molecules overnight at room temperature, pH 7.0. The final labeled product was purified by using desalting column for two times. As shown in Figure 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 convert to C-terminal thioacids and labeled with Dansylazide (Figure S6).

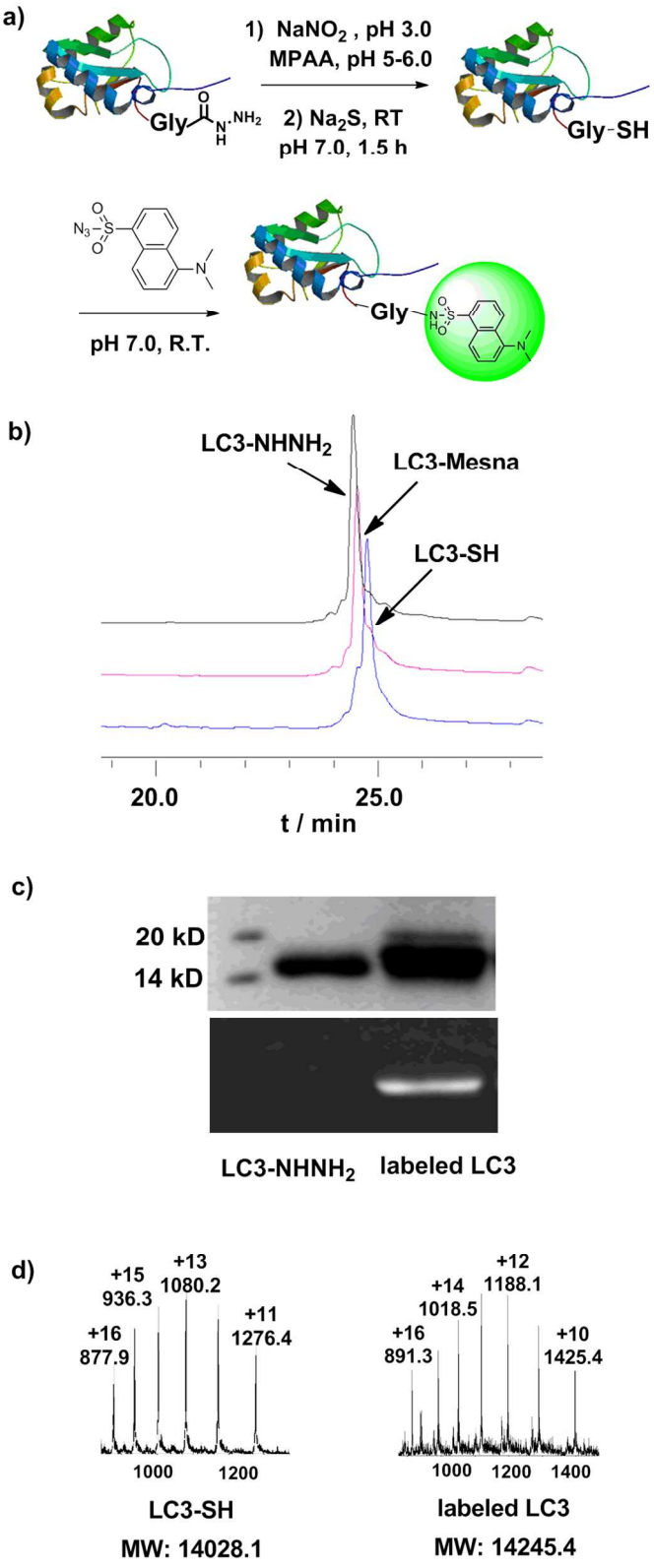


Fig. 4. Fluorescence labeling of LC3 by thioacids based ligation. a) Generation of C terminal labeling of LC3 through thioacid-sulfonazides liagtion; b) HPLC (λ = 214 nm) analysis of the conversion of LC3 thioacids from hydrazides precursors; c) SDS-PAGE of fluorescent labeling of LC3-SH; d) ESI-MS for purified LC3-SH is 14028.1 Da (calc.14029.2) and labeled LC3 is 14245.4 Da (calc.14244.2).

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 existed thiol group was essential for the in-situ formed 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) (2 g) was swelled in CH_2Cl_2/DMF (15/15 mL) at 0℃. $NH_2NH_2{\cdot}H_2O$ (1 mL, 10 eq.) and DIEA (3.3 mL, 20 eq.) were added. The reaction was conducted from 0℃ 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, Ethylether and kept under high vacuum for 3 h. All done, 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 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 is 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 adjusted the pH to about 1.0. 100 μL of aqueous 1 M Na_2S was added and then adjusted the pH 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.

Compared 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 (reaction A not added) and adjusted the pH 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.

Compared 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. Reaction was incubated for 20 min and adjusted the pH to about 1.0. 100 μL of aqueous 1 M Na_2S was added and then adjusted the pH 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 purified by HPLC and lyophilized, we got 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 adjusted the pH to about 1.0. 100 μL of aqueous 1 M Na_2S was added and then adjusted the pH 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 thioacidscapture 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 adjusted the pH to about 1.0. 100 μL of aqueous 1 M Na_2S 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. 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 thioacidsbased 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 adjusted the pH to about 1.0. 20 μL of aqueous 1 M Na_2S 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.

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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