Preparation of Peptide Thioesters from Naturally Occurring Sequences Using Reaction Sequence Consisting of Regioselective S-Cyanylation and Hydrazinolysis†

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† This paper is dedicated to Emeritus Professor Haruaki Yajima on the occasion of his 90th birthday.

ABSTRACT

Vital roles of peptide/protein thioesters in protein chemistry, including chemical or semi synthesis of proteins, have encouraged studies on the development of methods for the preparation of such chemical units. Biochemical protocols using intein or sortase have proved to be useful in protein chemistry as methods suitable for naturally occurring sequences, including recombinant proteins. Although chemical protocols are potential options for thioester preparation, only a few are applicable to naturally occurring sequences, because standard chemical protocols require an artificial chemical device for producing thioesters. In this context, the chemical preparation of thioesters based on a reaction sequence consisting of regioselective S-cyanylation and hydrazinolysis was investigated. Regioselective S-cyanylation, which is required for cysteine-containing thioesters, was achieved with the aid of zinc-complex formation of a CCHH-type zinc-finger sequence. Free cysteine residues that are not involved in complex formation were selectively protected with a 6-nitroveratryl group followed by S-cyanylation of the zinc-binding cysteine. Hydrazinolysis of the resulting *S*-cyanopeptide and subsequent photo-removal of the 6-nitroveratryl group yielded the desired peptide hydrazide, which was then converted to the corresponding thioester. The generated thioester was successfully used in N–to–C-directed one-pot/sequential native chemical ligation using an *N*-sulfanylethylanilide peptide to give a 64-residue peptide toxin.

INTRODUCTION

Peptide thioesters have enjoyed increasingly widespread use in native chemical ligation (NCL),[1] this technique enables chemical synthesis of small proteins to be easily achieved. Much effort has therefore been focused on the development of chemical or biochemical methods for the preparation

of peptide thioesters. Biochemical methods typified by the use of engineered intein[2] or sortase[3] enable thioesters to be prepared from expressed proteins. However, such biochemical protocols do not always successfully produce peptide/protein thioesters, therefore other methods, such as chemical methods, need to be developed as alternatives to intein- or sortase-mediated protocols. Generally, chemical protocols require an unnatural structural scaffold as a chemical device for producing thioesters,[4] therefore chemical methods have not been used for naturally occurring peptide sequences such as recombinant proteins. The increasing demand for chemical methods that are applicable to naturally occurring sequences has stimulated studies on the development of expressed-protein-compatible chemical methods for thioester preparation.[5] Recently, innovations based on an acyl-transfer reaction have been made by several research groups, including ours,[6] in which peptide hydrazides have served as indispensable intermediates for thioesters. The contribution by Liu and co-workers[7] rekindled interest in peptide hydrazides, which can be converted to the corresponding peptide thioesters via peptide azides. Herein, we focus on the development of a new chemical method that enables the conversion of natural peptide sequences to peptide hydrazides.

Scheme 1

Initially, we paid attention to the S-cyanylation-induced hydrolysis of Xaa–Cys peptide bonds (Xaa: proteogenic amino acid) of cysteine-containing peptides **1** (Scheme 1A).[8] S-Cyanylation of a cysteine residue followed by nucleophilic attack by a hydroxyl anion (NaOH in H_2O) on the peptide bond at the N-terminal side of the cysteine induces hydrolysis to afford peptide acid **4** and 2-iminothiazolidine-4-carbonyl (Itc)-capped peptide **6**. Nishimura and co-workers reported that replacement of NaOH with NH_3 in the hydrolysis reaction produced peptide amides **5**, with practical applications to non-enzymatic conversion of recombinant proteins to peptide amides.[9] This research prompted us to attempt hydrazinolysis of an *S*-cyanocysteine-containing peptide **2** to produce a peptide hydrazide **7** as a requisite precursor for thioesters **9** (Scheme 1B). In this paper, we report the S-cyanylation-mediated synthesis of peptide thioesters applicable to naturally occurring peptide sequences.[10]

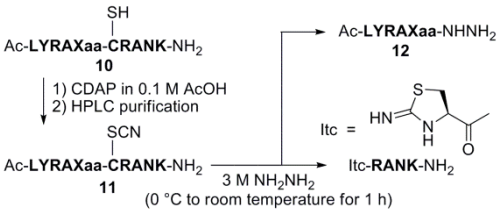
RESULTS AND DISCUSSION

Cyanylation of monocysteinyl peptide and subsequent hydrazinolysis

First, the feasibility of converting a cysteine-containing peptide to a peptide hydrazide was examined using a model cysteinyl peptide, Ac-**LYRAACRANK**-NH_2 **10a**. Treatment of **10a** with 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP)[11] in 0.1 M AcOH in H_2O afforded the corresponding *S*-cyanopeptide, Ac-**LYRAAC**(CN)**RANK**-NH_2 **11a**. After high-performance liquid chromatography (HPLC) purification, reaction of **11a** with 3 M $NH_2NH_2{\cdot}H_2O$ in H_2O

immediately went to completion to yield the desired peptide hydrazide Ac-**LYRAA**-$NHNH_2$ **12a** in 80% isolated yield as the major product. The concomitant formation of minor side products, including starting material **10a**, a disulfide peptide, and a hydrazine β-adduct peptide, was observed during hydrazinolysis. Modulation of the reaction conditions, including reaction temperature, time, and number of equivalents of reagents gave no significant improvement in the reaction outcome. We therefore concluded that the reaction in 1 to 3 M $NH_2NH_2{\cdot}H_2O$ aqueous solution is generally applicable to the conversion of *S*-cyanopeptides to the corresponding peptide hydrazides. We next examined the applicability of the S-cyanylation–hydrazinolysis sequence to naturally occurring 19-Xaa–Cys junctions using model peptides, Ac-**LYRAXaaCRANK**-NH_2 **10**; the results are shown in Table 1. The reaction of model peptides **10a–r** with CDAP in 0.1 M AcOH in H_2O, followed by HPLC purification, efficiently produced the *S*-cyanopeptides, Ac-**LYRAXaaC**(CN)**RANK**-NH_2 **11**. The purified *S*-cyanopeptides **11** were treated with 3 M $NH_2NH_2{\cdot}H_2O$ aqueous solution at 0 °C to room temperature for 1 h to convert them to the corresponding peptide hydrazides **12**; the results are summarized in Table 1.

Table 1 Examination of conversion of model monocysteine peptides to peptide hydrazides.



entry	amino acid (Xaa)	*S*-cyanopeptide	product	fraction converted[a]
1	Ala	**11a**	**12a**	0.62 (80%)[b]
2	Gly	**11b**	**12b**	0.67
3	Leu	**11c**	**12c**	0.47
4	Val	**11d**	**12d**	0.55
5	Ile	**11e**	**12e**	0.47
6	Met	**11f**	**12f**	0.67
7	Pro	**11g**	**12g**	0.57
8	Phe	**11h**	**12h**	0.59
9	Tyr	**11i**	**12i**	0.36
10	Trp	**11j**	**12j**	0.52
11	His	**11k**	**12k**	0.55
12	Lys	**11l**	**12l**	0.55
13	Arg	**11m**	**12m**	0.78

14	Ser	**11n**	**12n**	0.77
15	Thr	**11o**	**12o**	0.76
16	Asp	**11p**	**12p**	0.33
17	Glu	**11q**	**12q**	0.43
18	Asn	**11r**	**12r**	—[c]
19	Gln	**11s**	**12s**	0.64

[a]The fraction converted was determined by integration of **12** as a fraction of the sum of integration of **12** + other detected peptides. [b]Isolated yield of HPLC-purified **12a**. [c]After 1 h reaction, desired peptide **12 r** was not obtained.

It has been reported that in S-cyanylation-meditated hydrolysis and amidation, amino acids located at the N-terminal side of the cyanocysteine affect the molecular form of the cleavage products.[12] Acidic residues (Asp and Glu) were reported to cause problems arising from preferential formation of a dehydroalanine residue at the cysteine site. The junctions between bulky hydrophobic residues and cysteine are also unfavorable for the amidation reaction. The formations of aspartimide from an Asn residue seemed to be an issue in hydrazinolysis.

Except for the initially concerned residues such as Leu, Ile, Tyr, Trp, Asp, Glu, and Asn, fraction conversion of over 55% was achieved. Although the short reaction of Asn-containing *S*-cyanopeptide **11r** afforded the desired hydrazide **12r** with concomitant formation of several byproducts, including a C-terminal aspartimide peptide, continuous reaction for 1 h resulted in complete disappearance of **12r** to produce a complex mixture containing six-membered diacyl hydrazide peptide **12r'**. These results convinced us that appropriate choice of the scission junction would make S-cyanylation-mediated hydrazinolysis a naturally occurring sequence-compatible chemical method for thioester. However, one potential limitation of this method is that it is essentially limited to monocysteine-containing precursor peptides to afford non-cysteinyl thioesters, i.e., the developed protocol is only widely applicable various peptide/proteins when regioselective S-cyanylation of the scissile cysteine residue among several cysteine residues can be achieved.

Regioselective S-cyanylation using zinc-finger domain followed by hydrazinolysis

Scheme 2

We next explored a procedure applicable to the preparation of cysteinyl peptidyl thioesters, based on regioselective S-cyanylation at the Xaa–Cys junction transformable to the Xaa–thioester. As shown in Scheme 2, we used a zinc-finger peptide sequence[13,14] for the regioselective S-cyanylation, in which coordination of Cys and His residues to a zinc cation leads to temporary protection of the Cys residue that should undergo S-cyanylation (**13** to **14**). Then, protection of Cys residues in a peptide

of interest (POI) by an appropriate protecting group, followed by removal of zinc and subsequent S-cyanylation, should furnish the requisite *S*-cyanopeptide (**14** to **16**). Hydrazinolysis of the *S*-cyanopeptide **16** would result in cleavage of the desired amide bond at the Xaa–*S*-cyanocysteine junction to yield peptide hydrazide **17**. In the envisioned sequence of reactions, we initially attempted to use an *S*-sulfonate group[15] to protect Cys residues in the POI, because introduction of the sulfonate group could be performed after zinc-complex formation without isolation of the complex, and the thiol treatment involved in conversion of hydrazides to thioesters could remove the *S*-sulfonate group to regenerate the Cys residue.

Figure 1

The third C2H2-type zinc-finger sequences (-**FA*C*DI*C*GRKFARSDERKR*H*TKI*H*LRQKD**- and its **F**[1]**A**[2]-**F**[1]**Q**[2] mutant) present in the mouse transcription factor Zif268,[13] one of the best-characterized finger domains, were selected as the zinc-finger domain fused to POIs. Generally, zinc-fingers adopt a globular ββα structure, and the N-terminal residue (Phe) has been reported to be important for folding into the ββα structure to form a stable zinc-complex.[16] Taking this requirement into consideration, we attempted the preparation of conotoxin 1[17] (H-**G*CC*GA*FA***-SR) and *Stichodactyla* toxin[18] (H-**RSCIDTIPKSRCTA*FQ***-SR) thioester fragments, using the envisioned regioselective S-cyanylation-mediated hydrazinolysis protocol.

As shown in Figure 1, a chemically synthesized conotoxin/zinc-finger fusion, H-**GCCGA*FA*-*CDICGRKFARSDERKRHTKIHLRQKD***-NH_2 **19**, was initially incubated in 20 mM 3-[4-(2-hydroxyethyl)-1-piperazinyl]propanesulfonic acid (HEPPS) at pH 7.0 in the presence of 5 equiv of $ZnCl_2$ to form a zinc-finger complex, and then Na_2SO_3 (123 equiv) and $Na_2S_4O_6$ (23 equiv) were added to the reaction mixture to incorporate *S*-sulfonate groups on the cysteine residues of the conotoxin sequence. Reaction at pH 7.0 for 24 h proceeded efficiently to yield the desired di-*S*-sulfonate peptide **21**, which was then purified by HPLC using acidic eluents containing trifluoroacetic acid (TFA) for concomitant removal of zinc cations. The resulting eluents were treated with CDAP in aqueous 0.1 M AcOH, without lyophilization, to perform S-cyanylation of the cysteine residues of the zinc-finger sequence. The reaction proceeded quantitatively to produce the desired di-*S*-sulfonyl-di-*S*-cyanopeptide **22**. With the requisite regioselectively S-cyanylated peptide in hand, we subjected the resulting peptide **22** to hydrazinolysis with 3 M $NH_2NH_2{\cdot}H_2O$ at room temperature for 2.5 h. The reaction unambiguously yielded the desired hydrazide peptide, H-**GC**(S-SO_3H)**C**(S-SO_3H)**GAFA**-$NHNH_2$ **23**, along with two 2-iminothiazolidine-4-carbonyl (Itc)-capped peptides, but a complex mixture consisting of various peptides was obtained. A similar trend was observed in the case of hydrazinolysis of the di-*S*-sulfonyl-di-*S*-cyanopeptide derived from a *Stichodactyla* toxin precursor, resulting in formation of a complex mixture.

Analyses of the undesired materials in both cases indicated that one potential reason for the unsatisfactory outcome was hydrazinolysis-mediated loss of the cyano group on the cysteine residue, followed by disulfide bond formation between the *S*-sulfonylcysteine and regenerated cysteine residues.[19] Instead of the *S*-sulfonate group, we initially examined disulfide-type protections such as *S*–*S*-methyl[20] and *S*–*S*-*tert*-butyl[21] groups, because methylamine-mediated aminolysis of a disulfide-bond-containing *S*-cyanopeptide was reported to be possible.[22] However, the use of disulfide protection for hydrazinolysis failed to afford the desired peptides preferentially.

While we were performing our research, the selective S-alkylation of a zinc-finger sequence-fused cysteinyl peptide was reported for the chemoselective synthesis of a DNA-binding zinc-finger derivative,[14] in which an alkylation reaction with benzyl bromoacetate in the presence of $ZnSO_4$ in phosphate buffer was used. Using this example as a reference, we next tried to perform selective S-alkylation of the *Stichodactyla* toxin zinc-finger-fused **24** as shown in Figure 2.

Figure 2

Removal of the introduced S-protection under non-denaturing conditions, without added reagents, is desirable; therefore, we selected the 6-nitroveratryl (NV) group as a photocleavable S-protection.[23] A solution of peptide **24** in 10 mM phosphate buffer at pH 7.5 in the presence of 1.5 equiv of $ZnSO_4$ was treated with 3 equiv of 6-nitroveratryl bromide (NV-Br) at ambient temperature for 2 h. The reaction proceeded efficiently to yield the desired di-*S*-NV peptide **25**, which was then directly used in the subsequent reaction with CDAP at ambient temperature for 1.5 h to yield the desired di-*S*-NV-di-*S*-cyanopeptide **26**. HPLC purification gave the desired peptide **26** in 51% isolated yield. HPLC analysis of the reaction of the resulting peptide with 3 M NH_2NH_2 in H_2O at 0 °C for 1 h showed that it proceeded efficiently, affording the desired hydrazide **27** and Itc-capped peptides (Figure 2-B, iv).

However, in HPLC analysis of the tris(2-carboxyethyl)phosphine (TCEP)-treated reaction mixture, a di-*S*-NV peptide without two cyano groups **28** appeared as a peak separate from that of desired peptide **27** (Figure 2-B, v). This indicated that hydrazinolysis afforded the desired hydrazide **27** and di-*S*-NV disulfide peptide **28**, which was formed by intramolecular disulfide bond formation between *S*-cyanocysteine and cysteine resulting from NH_2NH_2-mediated loss of the cyano group. Although such loss of the cyano group during NH_2NH_2 treatment was also observed in previous attempts described above, regenerated thiol groups did not cause a serious problem because the *S*-NV group remained intact. Furthermore, the formed disulfide peptide was reconverted to the substrate peptide required for the hydrazinolysis reaction by reduction and subsequent S-cyanylation.

Figure 3

The desired di-*S*-NV peptide hydrazide **27** was purified by HPLC using acidic eluents, followed by lyophilization, which led to another issue of concern.

Part of the purified sample was converted to another component with the desired molecular weight minus 17 mass units. We speculated that the seven-membered cyclic diacyl hydrazide peptide **29** was formed, as observed in the case of the Asn–Cys(CN) junction. Lyophilization under acidic conditions was confirmed to be responsible for the formation of the cyclic diacyl hydrazide peptide **29**, because HPLC purification of **27** with neutral eluents (10 mM NH_4OAc buffer, pH 6.8) followed by lyophilization did not afford any cyclized diacyl hydrazide.[24] The peptide sequence producing the Gln-$NHNH_2$ was not suitable for experiments for fixing the precise reaction conditions. The *S*-NV groups were removed using the peptide mixture. Photoirradiation of the peptide mixture in a photolysis buffer[25] (50 mM semicarbazide·HCl, 20 mM ascorbic acid in MeCN-50 mM phosphate buffer (1:1), pH 6.0) at 365 nm at ambient temperature for 3 h triggered efficient removal of the *S*-NV group.

Thioester synthesis using regioselective S-cyanylation/hydrazinolysis sequence and practical application to synthesis of TsTxV toxin

As shown in Figure 4, for more precise experiments, the substrate peptide sequence was changed to a scorpion toxin, TsTxV,[26] N-terminal fragment (1–15)-zinc-finger fusion **30** (H-**KKDGYPVEGDNCA*FA***- ***CDICGRKFARSDERKRHTKIHLRQKD***-NH_2). Reaction of **30** in 10 mM phosphate buffer, pH 7.5, in the presence of 1.5 equiv of $ZnSO_4$ with 1.7 equiv of 6-NV-Br at ambient temperature for 6 h went to completion to give the desired mono-*S*-NV peptide **31** preferentially, accompanied by a small amount of tri-NV peptide. Then, CDAP in aqueous 0.1 M AcOH was added to the resulting reaction mixture and the pH was adjusted to around 5.0 to perform S-cyanylation. The sequence of reactions yielded the desired mono-*S*-NV-*S*,*S*-dicyanopeptide **32** in 54% isolated yield. Hydrazinolysis of the resulting peptide **32** with 1 M NH_2NH_2 in 1 M Gn·HCl–33 mM phosphate buffer, pH 10.4, at ambient temperature for 7 h proceeded without precipitate formation. Optimal concentration of hydrazine varies according to peptides, preliminary experiment for TsTxV indicated that the use of this reaction conditions afforded the best result so far examined. The reaction mixture was then incubated at 37 °C in the presence of 30 mM TCEP for reduction of the disulfide peptide formed during hydrazinolysis to give a mixture of the desired *S*-mono-NV hydrazide **33**, *S*-mono-NV-sulfanylpeptide **34**, and the C-terminal halves. HPLC purification of the mixture gave **33** and **34** in 38% and 16% isolated yields, respectively. Because the resulting byproduct, sulfanyl peptide **34**, can be recycled to afford **33**, loss of the cyano group is not a serious problem unless the S-protection is unsusceptible to the regenerated sulfanyl group as the NV group.

Figure 4

Photoirradiation of **33** in photolysis buffer (50 mM semicarbazide·HCl, 20 mM ascorbic acid in MeCN-50 mM phosphate buffer (1:1), pH 6.0) under a 365 nm UV lamp at ambient temperature for 3 h yielded S-deprotected peptide **35** in 59% isolated yield after HPLC purification. The resulting peptide hydrazide **35** was successfully converted to the corresponding peptide thioester, according to Liu's protocol. HPLC purification gave purified **36** in 85% isolated yield (Figure 5).

Figure 5

Thioester **36** was used to assemble a chain covering the entire sequence of TsTxV, based on *N*-sulfanylethylanilide (SEAlide) peptide-mediated N–to–C-directed one-pot/sequential NCL[27] as shown in Figure 6. The requisite peptide fragments **37** and **38**, corresponding to TsTxV 16–37 and 38–64, respectively, were prepared by 9-fluorenylmethyloxycarbonyl (Fmoc) solid-phase peptide synthesis (SPPS). The first NCL of alkyl thioester **36** with N-terminal cysteinyl SEAlide peptide **37** was performed in 6 M Gd·HCl–0.2 M HEPPS buffer, pH 7.0, in the presence of 200 mM 4-mercaptophenylacetic acid (MPAA) and 50 mM TCEP for 3 h at 37 °C to yield ligated SEAlide peptide **39** (**36** + **37**). During the first NCL, the absence of phosphate salts enabled the SEAlide moiety, as a crypto thioester, to remain intact. Then, a 1 M phosphate buffer solution of peptide **38** was added to the resulting reaction mixture to initiate the second NCL to yield the desired reduced form TsTxV **40** in 31% isolated yield after HPLC purification.

Figure 6

In the zinc-finger sequence, the two N-terminal amino acid residues adjacent to the cysteine are important for folding the ββα structure responsible for zinc-complex formation. The severe restriction on the N-terminal residues in the zinc-finger diminishes the usefulness of the developed protocol, therefore mutations were incorporated into the TsTxV fragment–zinc-finger fusion and their effects were examined (Table 2). Although not all possible mutations were investigated, substitutions of Phe or Ser for Ala, or of Ala for Phe, were acceptable for the regioselective S-cyanylation/hydrazinolysis reaction.

Table 2 Effects of the N-terminal sequence on regioselective cyanylation/hydrazinolysis sequence.

entry[a]	N-terminal sequence	regioselective cyanylation[b] (isolated yield by HPLC, %)	hydrazinolysis[c] (isolated yield by HPLC, %)

original	Phe–Ala	54	38
1	Phe–*Phe*	63	36
2	Phe–*Ser*	35[d]	65
3	*Ala*–Ala	38[d]	38

[a]Peptides, H-**KKDGYPVEGDNCA-*N-terminal sequence-CDICGRKFARSDERKRHTKIHLR-QKD***-NH_2, were subjected to regioselective cyanylation/hydrazinolysis sequence. [b]Isolated yield of H-**KKDGYPVEGDNC**(NV)**A-*N-terminal sequence-C***(CN)***DIC***(CN)***GRKFARSDERKRHTK-IHLRQKD***-NH_2. [c]Isolated yield of H-**KKDGYPVEGDNCA-*N-terminal sequence***-$NHNH_2$. [d]Although the cyanylation reaction proceeded in efficacy comparable to that of original or entry 1, loss of the desired material during HPLC purification resulted in low isolated yield.

CONCLUSION

In this study, a regioselective S-cyanylation/hydrazinolysis sequence for thioester preparation via a chemical method was developed. An important feature of this protocol is its applicability to naturally occurring peptide sequences, whereas previously developed chemical protocols require artificial chemical devices for producing thioesters. Another key point is achievement of a sequence of reactions consisting of zinc-complex formation on a zinc-finger sequence, S-6-nitroveratrylation and S-cyanylation, which guarantees regioselective S-cyanylation, enabling wide applicability to various peptide sequences. In this study, limitation of the zinc-finger N-terminal sequence required for keeping the ββα structure for zinc-complex formation was not a problem. With respect to this issue, research on application of this protocol to other peptide sequences will be continued. Further research on applications of this protocol to various peptides including recombinant ones is underway and the results will be presented in due course.

Materials and Methods

General Methods

Mass spectra were recorded on a Waters MICROMASS® LCT PREMIER™. For HPLC separations, a Cosmosil 5C_{18}-AR-II analytical column (Nacalai Tesque, 4.6 × 250 mm, flow rate 1.0 mL/min), a TSKgel Octadecyl-2PW analytical column (TOSOH CORPORATION, 4.6 × 150 mm, flow rate 1.0 mL/min), a Cosmosil 5C18-AR-II semi-preparative column (Nacalai Tesque, 10 × 250 mm, flow rate 3.0 mL/min), or a Cosmosil 5C_{18}-AR-II preparative column (Nacalai Tesque, 20 × 250 mm, flow rate 10 mL/min) was employed, and eluting products were detected by UV at 220 nm. A solvent system consisting of 0.1% TFA aqueous solution (v/v, solvent A) and 0.1% TFA in MeCN

(v/v, solvent B) was used for HPLC elution.

Preparation of model Peptides **10a–s** for Cyanylation

Protected peptide resins corresponding to the title peptides were constructed on NovaSyn® TGR resin (Rink amide type: 0.22 mmol amine/g, 0.10 g, 0.022 mmol) using standard Fmoc SPPS. TFA cleavage (TFA-*m*-cresol-thioanisole-H_2O-1,2-ethanedithiol (80:5:5:5:5 (v/v), 50 μL/1 mg resin)) of the protected resins at room temperature for 2 h followed by HPLC purification afforded the desired peptides.

10a (**X = Ala**) (45% isolated yield): Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 1% to 30% over 30 min, retention time = 24.1 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 30% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M+2H]^{2+}$) 603.8, found 603.9.

10b (**X = Gly**) (41% isolated yield): Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 1% to 30% over 30 min, retention time = 21.9 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 30% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M+2H]^{2+}$) 596.8, found 596.9.

10c (**X =Leu**) (59% isolated yield): Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 20.6 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 30% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M+2H]^{2+}$) 624.8, found 624.9.

10d (**X = Val**) (35% isolated yield): Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 17.4 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 30% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M+2H]^{2+}$) 617.8, found 617.8.

10e (**X = Ile**) (63% isolated yield): Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 19.6 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 18% to 33% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M+2H]^{2+}$) 624.8, found 624.9.

10f (**X = Met**) (54% isolated yield): Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 1% to 35% over 30 min, retention time = 25.1 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 30% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M+2H]^{2+}$) 633.8, found 633.9.

10g (**X = Pro**) (41% isolated yield): Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 1% to 30% over 30 min, retention time = 22.7 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 30%

over 30 min. MS (ESI-TOF) *m*/*z* calcd ([*M*+2H]$^{2+}$) 616.8, found 616.8.

10h (**X = Phe**) (61% isolated yield): Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 21.0 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 18% to 33% over 30 min. MS (ESI-TOF) *m*/*z* calcd ([*M*+2H]$^{2+}$) 641.8, found 641.9.

10i (**X = Tyr**) (55% isolated yield): Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 17.0 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min. MS (ESI-TOF) *m*/*z* calcd ([*M*+2H]$^{2+}$) 649.8, found 649.8.

10j (**X = Trp**) (67% isolated yield): Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 5% to 35% over 30 min, retention time = 26.1 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 12% to 32% over 30 min. MS (ESI-TOF) *m*/*z* calcd ([M + 2H]$^{2+}$) 661.3, found 661.4.

10k (**X = His**) (41% isolated yield): Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 1% to 30% over 30 min, retention time = 21.5 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min. MS (ESI-TOF) *m*/*z* calcd ([*M*+2H]$^{2+}$) 636.8, found 636.9.

10l (**X = Lys**) (70% isolated yield): Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 1% to 30% over 30 min, retention time = 21.2 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 30% over 30 min. MS (ESI-TOF) *m*/*z* calcd ([*M*+2H]$^{2+}$) 632.4, found 632.4.

10m (X = Arg) (61% isolated yield): Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 1% to 25% over 30 min, retention time = 24.9 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 30% over 30 min. MS (ESI-TOF) *m*/*z* calcd ([*M*+2H]$^{2+}$) 646.4, found 646.4.

10n (**X = Ser**) (57% isolated yield): Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 1% to 30% over 30 min, retention time = 22.3 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 30% over 30 min. MS (ESI-TOF) *m*/*z* calcd ([*M*+2H]$^{2+}$) 611.8, found 611.9.

10o (**X = Thr**) (54% isolated yield): Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 1% to 30% over 30 min, retention time = 23.0 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 30% over 30 min. MS (ESI-TOF) *m*/*z* calcd ([*M*+2H]$^{2+}$) 618.8, found 618.9.

10p (**X = Asp**) (39% isolated yield): Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 1% to 30% over 30 min, retention time = 22.3 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 30%

over 30 min. MS (ESI-TOF) *m*/*z* calcd ([*M*+2H]$^{2+}$) 625.8, found 625.9.

10q (**X = Glu**) (51% isolated yield): Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 5% to 35% over 30 min, retention time = 18.5 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 30% over 30 min. MS (ESI-TOF) *m*/*z* calcd ([*M*+2H]$^{2+}$) 632.8, found 632.8.

10r (**X = Asn**) (65% isolated yield): Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 1% to 30% over 30 min, retention time = 21.6 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 30% over 30 min. MS (ESI-TOF) *m*/*z* calcd ([*M*+2H]$^{2+}$) 625.3, found 625.4.

10s (**X = Gln**) (65% isolated yield): Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 5% to 35% over 30 min, retention time = 17.3 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 30% over 30 min. MS (ESI-TOF) *m*/*z* calcd ([*M*+2H]$^{2+}$), 632.3 found 632.3.

S-Cyanylation of Peptides **10a–s**

Representative procedure: To a solution of peptide **10a** (1.0 μmol, 1.6 mg) in 0.1 M AcOH (160 μL) was added the solution of 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) in 0.1 M AcOH (10 mg/mL, 72 μL). After being stirred at room temperature for 1 h, the solution was purified by preparative HPLC to give the purified cyanylated peptide **11a** (1.4 mg, 0.90 μmol, 88%).

11a (**X = Ala**): Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 1% to 30% over 30 min, retention time = 25.0 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 30% over 30 min. MS (ESI-TOF) *m*/*z* calcd ([*M*+2H]$^{2+}$) 616.3, found 616.3.

11b (**X = Gly**) (1.4 mg, 0.87 μmol, 79%): Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 1% to 30% over 30 min, retention time = 22.7 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 30% over 30 min. MS (ESI-TOF) *m*/*z* calcd ([*M*+2H]$^{2+}$) 609.3, found 609.3.

11c (**X =Leu**) (2.4 mg, 1.5 μmol, 75%): Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 21.5 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 17% to 32% over 30 min. MS (ESI-TOF) *m*/*z* calcd ([*M*+2H]$^{2+}$) 637.3, found 637.4.

11d (**X = Val**) (1.7 mg, 1.1 μmol, 89%): nalytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 18.4 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 17% to 32% over 30 min. MS (ESI-TOF) *m*/*z* calcd ([*M*+2H]$^{2+}$) 630.3 found 630.3.

11e (**X = Ile**) (3.3 mg, 2.0 μmol, 89%): Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 20.5 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 18% to 33% over 30 min. MS (ESI-TOF) *m*/*z* calcd ([*M*+2H]$^{2+}$) 637.3, found 637.3.

11f (**X = Met**) (3.0 mg, 1.8 μmol, 91%): Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 1% to 35% over 30 min, retention time = 25.8 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 17% to 32% over 30 min. MS (ESI-TOF) *m*/*z* calcd ([*M*+2H]$^{2+}$) 646.3, found 646.4.

11g (**X = Pro**) (2.1 mg, 1.3 μmol, 85%): Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 1% to 30% over 30 min, retention time = 23.6 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 12% to 27% over 30 min. MS (ESI-TOF) *m*/*z* calcd ([*M*+2H]$^{2+}$) 629.3, found 629.3.

11h (**X = Phe**) (1.4 mg, 0.85 μmol, 82%): Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 21.8 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 18% to 33% over 30 min. MS (ESI-TOF) *m*/*z* calcd ([*M*+2H]$^{2+}$) 654.3, found 654.2.

11i (**X = Tyr**) (2.7 mg, 1.6 μmol, 82%): Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 17.6 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 17% to 32% over 30 min. MS (ESI-TOF) *m*/*z* calcd ([*M*+2H]$^{2+}$) 662.3, found 662.3.

11j (**X = Trp**) (1.6 mg, 1.2 μmol, 87%): Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 5% to 35% over 30 min, retention time = 26.6 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 17% to 32% over 30 min. MS (ESI-TOF) *m*/*z* calcd ([*M*+2H]$^{2+}$) 673.8, found 673.9.

11k (**X = His**) (2.3 mg, 1.3 μmol, 93%). Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 1% to 25% over 30 min, retention time = 25.3 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 12% to 27% over 30 min. MS (ESI-TOF) *m*/*z* calcd ([*M*+2H]$^{2+}$) 649.3, found 649.4.

11l (**X = Lys**) (3.2 mg, 1.8 μmol, 91%): Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 1% to 30% over 30 min, retention time = 21.7 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 12% to 27% over 30 min. MS (ESI-TOF) *m*/*z* calcd ([*M*+2H]$^{2+}$) 644.9, found 644.9.

11m (**X = Arg**) (3.4 mg, 1.9 μmol, 96%): Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 1% to 25% over 30 min, retention time = 25.2 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 12% to 27% over 30 min. MS (ESI-TOF) *m*/*z* calcd ([*M*+2H]$^{2+}$) 658.9, found 658.9.

11n (**X = Ser**) (1.5 mg, 0.94 μmol, 74%): Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 1% to 30% over 30 min, retention time = 22.8. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 12% to 27% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M+2H]^{2+}$) 624.3, found 624.3.

11o (**X = Thr**) (1.7 mg, 1.1 μmol, 95%): Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 1% to 30% over 30 min, retention time = 23.6 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 30% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M+2H]^{2+}$) 631.3, found 631.3.

11p (**X = Asp**) (1.4 mg, 0.88 μmol, 83%): Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 1% to 30% over 30 min, retention time = 23.1 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 30% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M+2H]^{2+}$) 638.3, found 638.3.

11q (**X = Glu**) (2.7 mg, 1.7 μmol, 83%): Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 5% to 35% over 30 min, retention time = 19.0 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 12% to 27% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M+2H]^{2+}$) 645.3, found 645.3.

11r (**X = Asn**) (2.6 mg, 1.6 μmol, 79%). Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 1% to 30% over 30 min, retention time = 22.3 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 12% to 27% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M+2H]^{2+}$) 637.8, found 637.8.

11s (**X = Gln**) (2.8 mg, 1.7 μmol, 86%). Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 5% to 35% over 30 min, retention time = 17.9 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 12% to 27% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M+2H]^{2+}$) 644.8, found 644.8.

Hydrazinolysis of Peptides **11a–s**

Representative procedure: To a solution of cyanylated peptide **11a** (0.16 mg, 0.10 μmol) in Milli-Q water (85 μL) was added $NH_2NH_2{\cdot}H_2O$ (15 μL, final concentration 3 M) at 0 °C. The reaction was allowed to warm to room temperature. After 1 h incubation, progress of reaction was monitored by analytical HPLC. Fraction converted was determined by HPLC separation and integration of **12a** (integ. **12a**) as a fraction of the sum of integ. (**12a** + other detected peptides).

In a manner similar to that described above, reaction of cyanopeptide **11a** (0.79 mg, 0.50 μmol) in 3 M NH_2NH_2 solution at 0 °C to room temperature for 1 h, followed by semi-preparative HPLC gave the purified peptide hydrazide **12a** (0.35 mg, 0.40 μmol, 80%).

12a (**X = Ala**): Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 1% to 30% over 30 min, retention time = 22.1 min. MS (ESI-TOF) *m*/*z* calcd ([*M*+H]$^+$) 649.4, found 649.4.

12b (**X = Gly**): Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 1% to 30% over 30 min, retention time = 21.4 min. MS (ESI-TOF) *m*/*z* calcd ([*M*+H]$^+$) 635.4, found 635.4.

12c (**X = Leu**): Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 18.8 min. MS (ESI-TOF) *m*/*z* calcd ([*M*+H]$^+$) 691.4, found 691.4.

12d (**X = Val**): Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 15.2 min. MS (ESI-TOF) *m*/*z* calcd ([*M*+H]$^+$) 677.4, found 677.4.

12e (**X = Ile**): Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 17.5 min. MS (ESI-TOF) *m*/*z* calcd ([*M*+H]$^+$) 691.4, found 691.4.

12f (**X = Met**): Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 1% to 35% over 30 min, retention time = 23.5 min. MS (ESI-TOF) *m*/*z* calcd ([*M*+H]$^+$) 709.4, found 709.4.

12g (**X = Pro**): Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 12% to 32% over 30 min, retention time = 12.9 min. MS (ESI-TOF) *m*/*z* calcd ([*M*+H]$^+$) 675.4, found 675.3.

12h (**X = Phe**): Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 20.5 min. MS (ESI-TOF) *m*/*z* calcd ([*M*+H]$^+$) 725.4, found 725.4.

12i (**X = Tyr**): Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 15.5 min. MS (ESI-TOF) *m*/*z* calcd ([*M*+H]$^+$) 741.4, found 741.3.

12j (**X = Trp**): Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 5% to 35% over 30 min, retention time = 25.5 min. MS (ESI-TOF) *m*/*z* calcd ([M + H]$^+$) 764.4, found 764.4.

12k (**X = His**): Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 1% to 30% over 30 min, retention time = 20.8 min. MS (ESI-TOF) *m*/*z* calcd ([*M*+H]$^+$) 715.4, found 715.4.

12l (**X = Lys**): Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 1% to 30% over 30 min, retention time = 20.4 min. MS (ESI-TOF) *m*/*z* calcd ([*M*+H]$^+$) 706.4, found 706.4.

12m (**X = Arg**): Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 1% to 25% over 30 min, retention time = 23.8 min. MS (ESI-TOF) *m*/*z* calcd ([*M*+H]$^+$) 734.4, found 734.4.

12n (**X = Ser**): Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 1% to 30% over 30 min, retention time = 21.3 min. MS (ESI-TOF) *m*/*z* calcd ([*M*+H]$^+$) 665.4, found 665.4.

12o (**X = Thr**): Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 1% to 30% over 30 min, retention time = 21.9 min. MS (ESI-TOF) *m*/*z* calcd ([*M*+H]$^+$) 679.4, found 679.4.

12p (**X = Asp**): Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 1% to 30% over 30 min, retention time = 21.6 min. MS (ESI-TOF) *m*/*z* calcd ([*M*+H]$^+$) 693.4, found 693.3.

12q (**X = Glu**): Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 5% to 35% over 30 min, retention time = 17.5 min. MS (ESI-TOF) *m*/*z* calcd ([*M*+H]$^+$) 707.4, found 707.3.

12s (**X = Gln**): Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 5% to 35% over 30 min, retention time = 16.6 min. MS (ESI-TOF) *m*/*z* calcd ([*M*+H]$^+$), 706.4, found 706.3.

Preparation of Peptide **19**

H-**GCCGAFACDICGRKFARSDERKRHTKIHLRQKD**-NH_2

19

Protected peptide resins corresponding to the title peptides were constructed on NovaSyn® TGR resin (Rink amide type: 0.22 mmol amine/g, 0.46 g, 0.10 mmol) using standard Fmoc SPPS. TFA cleavage (TFA–*m*-cresol–thioanisole–H_2O–1,2-ethanedithiol (80:5:5:5:5 (v/v), 50 μL/1 mg resin)) of the protected resin at room temperature for 2 h followed by HPLC purification afforded the desired peptide in 41% isolated yield.

19: Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 18.0 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 20% to 30% over 30 min. MS (ESI-TOF) *m*/*z* calcd ([*M*+4H]$^{4+}$) 952.0, found 951.7.

Regioselective S-sulfonation of Peptide **19** followed by S-cyanylation

Peptide **19** (2.6 μmol, 13.5 mg) was dissolved in a $ZnCl_2$ solution (13.0 μmol, 1.26 mL of a 10.4 mM solution in 20 mM HEPPS buffer, pH = 7.0), and the mixture was stirred at room temperature for 15 min. Then, to the reaction mixture was added 20 mM HEPPS buffer containing Na_2SO_3 and $Na_2S_4O_6$ (1.35 mL, pH = 7.0, Na_2SO_3 (1.2 mmol, 150 mg), $Na_2S_4O_6$ (0.25 mmol, 67 mg)), and the solution was stirred again at room temperature for 24 h. The reaction progress was monitored by analytical HPLC, and the crude material was purified by semi-preparative HPLC to give **21**. Without lyophilization, the resulting eluents were treated with CDAP (0.12 mmol, 27 mg), and the solution was stirred at room temperature for 3 h. The reaction progress was monitored by analytical HPLC followed by purification by semi-preparative HPLC to give **22** (6.8 mg, 1.3 μmol, 2 steps 49%).

21: Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 18.1 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 20% to 30% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M+3H]^{3+}$) 1322.3, found 1322.0.

22: Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 18.1 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 20% to 30% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M+3H]^{3+}$) 1338.9, found 1338.4.

Hydrazinolysis of Peptide **22**

To a solution of peptide **22** (5.2 mg, 0.97 μmol) in Milli-Q water (0.83 mL) was added $NH_2NH_2{\cdot}H_2O$ (0.14 mL, final concentration 3 M) at 0 °C. The reaction was allowed to warm to room temperature. After 1 h incubation, progress of reaction was monitored by analytical HPLC.

23: Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 1% to 35% over 30 min, retention time = 18.7 min. MS (ESI-TOF) *m*/*z* calcd ($[M+H]^{+}$) 802.2, found 802.0.

NCL for the synthesis of **24**

Peptide thioester **S1**, H-**RSCIDTIPKSRCTAFQ**-$S(CH_2)_2CO$-**L**-NH_2, (2.2 μmol, 5.5 mg, see supporting information S2) and N-terminal cysteinyl peptide **S2**, H-**CDICGRKFARSDERKRHTKIHLRQKD**-NH_2, (2.2 μmol, 10.0 mg, see supporting information S2) were dissolved in 6 M Gn·HCl–0.2 M Na phosphate buffer containing 50 mM MPAA and 30 mM TCEP (pH 7.0, 2.2 mL, 1 mM each peptides). The reaction mixture was incubated at 37 °C for 6 h and reaction progress was monitored by analytical HPLC. The crude material was purified by preparative HPLC to give **24** (9.6 mg, 1.4 μmol, 65%).

24: Analytical HPLC conditions: TSKgel Octadecyl-2PW analytical column with a linear gradient of solvent B in solvent A, 1% to 40% over 30 min, retention time = 18.3 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 30% over 30 min. MS (ESI-TOF) *m*/*z* calcd ([*M*+5H]$^{5+}$) 1001.3, found 1000.9.

Regioselective S-cyanylation of Peptide **24**

Peptide **24** (1.3 μmol, 8.8 mg) was dissolved in 10 mM Na phosphate buffer (5.7 mL, pH = 7.5). A $ZnSO_4$ solution (2.0 μmol, 0.39 mL of a 5.0 mM solution in Milli-Q watter) was added, and the mixture was stirred at room temperature for 10 min under argon atmosphere. 6-nitroveratryl bromide (3.9 μmol, 0.47 mL of a 8.3 mM solution in CH_3CN) was added, and the solution was stirred under light-blocking conditions at room temperature for 2 h. To the reaction mixture was successively added solution of CDAP (63 μmol, 1.5 mL of a 10 mg/mL solution in 0.1 M AcOH) and 0.1% TFA aq. (2.0 mL) to carry out S-cyanylation. After being stirred under light-blocking conditions at room temperature for 1.5 h, the reaction was purified by semi-preparative HPLC to give **26** (4.7 mg, 0.66 μmol, 51%)

26: Analytical HPLC conditions: TSKgel Octadecyl-2PW analytical column with a linear gradient of solvent B in solvent A, 2% to 50% over 30 min, retention time = 19.0 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 20% to 35% over 30 min. MS (ESI-TOF) *m*/*z* calcd ([*M*+5H]$^{5+}$) 1089.3, found 1088.9.

Hydrazinolysis of Peptide **26**

To a solution of peptide **26** (0.66 μmol, 4.7 mg) in Milli-Q water (0.56 mL) was added $NH_2NH_2{\cdot}H_2O$ (98 μL, final concentration 3 M) at 0 °C. The reaction was allowed to warm to room temperature. After 1 h incubation, the reaction was treated with 6M Gn·HCl containing 30 mM TCEP (2.5 mL). Then the crude material was purified by semi-preparative HPLC to give **27** (0.43 mg, 0.15 μmol, 23%).

27: Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 23.6 min. Preparative HPLC conditions: A linear gradient of CH_3CN in NH_4OAc buffer (pH = 6.8), 10% to 40% over 30 min. MS (ESI-TOF) *m*/*z* calcd ([*M*+2H]$^{2+}$) 1115.4, found 1115.1.

28: Analytical HPLC conditions: TSKgel Octadecyl-2PW analytical column with a linear gradient of solvent B in solvent A, 2% to 50% over 30 min, retention time = 18.4 min. MS (ESI-TOF) *m*/*z* calcd ([*M*+5H]$^{5+}$) 1078.9, found 1078.5.

28': Analytical HPLC conditions: TSKgel Octadecyl-2PW analytical column with a linear gradient

of solvent B in solvent A, 2% to 50% over 30 min, retention time = 18.6 min. MS (ESI-TOF) *m*/*z* calcd ([*M*+5H]$^{5+}$) 1079.3, found 1078.9.

29: Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 25.4 min. MS (ESI-TOF) *m*/*z* calcd ([*M*+2H]$^{2+}$) 1107.0, found 1106.7.

NCL for the synthesis of **30**

Peptide thioester **S3a** (2.3 μmol, 5.0 mg, see supporting information S2) and N-terminal cysteinyl peptide **S2** (2.3 μmol, 10.5 mg) were dissolved in 6 M Gn·HCl–0.2 M Na phosphate buffer containing 30 mM MPAA and 30 mM TCEP (pH 7.0, 2.3 mL, 1 mM each peptides). The reaction mixture was incubated at 37 °C for 7 h and reaction progress was monitored by analytical HPLC. The crude material was purified by preparative HPLC to give **30** (13.0 mg, 2.0 μmol, 88%).

30: Analytical HPLC conditions: TSKgel Octadecyl-2PW analytical column with a linear gradient of solvent B in solvent A, 2% to 50% over 30 min, retention time = 15.2 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 30% over 30 min. MS (ESI-TOF) *m*/*z* calcd ([*M*+5H]$^{5+}$) 958.9, found 958.6.

Regioselective S-cyanylation of Peptide **30**

Peptide **30** (2.0 μmol, 13.0 mg) was dissolved in 10 mM Na phosphate buffer (5.7 mL, pH = 7.5). A $ZnSO_4$ solution (3.1 μmol, 0.61 mL of a 5.0 mM solution in Milli-Q watter) was added, and the mixture was stirred at room temperature for 10 min under argon atmosphere. 6-nitroveratryl bromide (3.4 μmol, 0.80 mL of a 4.2 mM solution in CH_3CN) was added, and the solution was stirred under light-blocking conditions at room temperature for 6 h. To the reaction mixture was successively added solution of CDAP (41 μmol, 0.96 mL of a 10 mg/mL solution in 0.1 M AcOH) and 0.1% TFA aq. (2.0 mL) to carry out S-cyanylation. After being stirred under light-blocking conditions at room temperature for 1.5 h, the reaction was purified by semi-preparative HPLC to give **32** (7.3 mg, 1.1 μmol, 54%)

31: Analytical HPLC conditions: TSKgel Octadecyl-2PW analytical column with a linear gradient of solvent B in solvent A, 2% to 50% over 30 min, retention time = 17.8 min. MS (ESI-TOF) *m*/*z* calcd ([*M*+5H]$^{5+}$) 997.9, found 997.6.

32: Analytical HPLC conditions: TSKgel Octadecyl-2PW analytical column with a linear gradient of solvent B in solvent A, 2% to 50% over 30 min, retention time = 17.9 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 20% to 35% over 30 min. MS (ESI-TOF) *m*/*z* calcd ([*M*+5H]$^{5+}$) 1007.9, found 1007.6.

Hydrazinolysis of Peptide **32**

Peptide **32** (0.76 μmol, 5.1 mg) was dissolved in 33 mM Na phosphate buffer containing 1 M NH_2NH_2–1 M Gn·HCl (0.76 mL, pH = 10.4) at 0 °C. Then the mixture was incubated at room temperature under light-blocking conditions for 7 h, and reaction progress was monitored by analytical HPLC. The crude material was purified by semi-preparative HPLC to give **33** (0.67 mg, 0.29 μmol, 38%) and **34** (0.82 mg, 0.12 μmol, 16%).

33: Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 5% to 35% over 30 min, retention time = 25.6 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 20% to 30% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M+2H]^{2+}$) 911.9, found 911.6.

34: Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 2% to 50% over 30 min, retention time = 20.4 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 20% to 30% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M+5H]^{5+}$) 997.9, found 997.6.

Removal of NV for the synthesis of peptide hydrazide **35**

Peptide **33** (0.93 mg, 0.41 μmol) was dissolved in a photolysis buffer (0.82 mL, 50 mM semicarbazide hydrochloride, 20 mM L-(+)-ascorbic acid in 1:1 MeCN/0.1 M Na phosphate buffer, pH = 6.0). The mixed solution was flushed with argon, sealed and placed in photoreactor. The reaction was irradiated at 365 nm for up to 3 h. After confirmation of completion of the reaction by HPLC analysis, the solution was quenched with 6 M Gn·HCl containing 30 mM TCEP (2.0 mL) and incubated at 37 °C for 2 h. The crude material was purified by semi-preparative HPLC to give the purified peptide **35** (0.50 mg, 0.24 μmol, 59%).

35: Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 5% to 35% over 30 min, retention time = 18.8 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 25% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M+2H]^{2+}$) 814.4, found 814.2.

Conversion of Peptide hydrazide **35** to Peptide Thioester **36**

Peptide **35** (0.55 mg, 0.26 μmol) was dissolved in 0.2 M Na phosphate buffer containing 6 M Gn·HCl, (pH 3.0, 87 μL). The reaction mixture was stored at –10 °C. Then, 8.7 μL of 0.2 M $NaNO_2$ aq. was added, and the reaction mixture was incubated at –10 °C for 1 h. After that, 0.2 M Na phosphate buffer containing 6 M Gn·HCl and 0.2 M sodium 2-mercaptoethanesulfonate (87 μL) was added, and pH of the mixed solution was adjusted to pH 7.0 with 4.0 M NaOH aq.. The reaction mixture was stored at room temperature for 30 min. The reaction was monitored by analytical HPLC. The crude material was purified by semi-preparative HPLC to give the purified peptide thioester **36** (0.45 mg, 0.22 μmol, 85%).

36: Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 5% to 35% over 30 min, retention time = 20.5 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 13% to 28% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M+2H]^{2+}$) 869.3, found 869.1.

Preparation of SEAlide Peptide **37**

H-**CFGYDNAYCDKLCKDKKADDGY**-SEAlide

37

The protected peptide resin was constructed on NovaSyn® TGR resin (Rink amide type: 0.25 mmol amine/g, 0.80 g, 0.20 mmol) using Fmoc SPPS (Acylation: Fmoc amino acid (5.0 equiv.), DIPCDI (5.0 equiv.) and Oxyma pure® (5.0 equiv.) in DMF or Fmoc-Tyr-incorporating SEAlinker **S9** (2.0 equiv., see supporting information S7), HATU (1.95 equiv.) and DIPEA (1.95 equiv.) in DMF for 2 h; Fmoc removal: 20% piperadine/DMF for 10 min). The resulting completed resin was treated with TFA–*m*-cresol–thioanisole–H_2O–1,2-ethanedithiol (80:5:5:5:5 (v/v), 50 μL/1 mg resin) at room temperature for 2 h. The resin in the reaction mixture was filtrated off. To the resulting filtrate was added cooled Et_2O to give precipitate. The formed precipitate was collected by centrifugation and thoroughly washed with Et_2O to afford the crude SEAlide peptide **37**. The crude SEAlide peptide was purified by preparative HPLC to give purified **37** in 36% isolated yield.

37: Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 22.3 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 20% to 35% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M+3H]^{3+}$) 942.7, found 942.5.

Preparation of N-terminal Cysteinyl TsTxV Fragment **38**

H-**CVWSPDCYCYGLPEHILKEPTKTSGRC**-OH

38

Protected peptide resins corresponding to the title peptides were constructed on Wang resin (0.70 mmol amine/g, 0.33 g, 0.23 mmol) using standard Fmoc SPPS. TFA cleavage (TFA–*m*-cresol–thioanisole–H_2O–1,2-ethanedithiol (80:5:5:5:5 (v/v), 50 μL/1 mg resin), at room temperature for 2 h followed by HPLC purification afforded the desired peptide **38** in 28% isolated yield.

38: Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 15% to 45% over 30 min, retention time = 18.3 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 20% to 30% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M+3H]^{3+}$) 1029.1, found 1028.9.

NCL for the Synthesis of Reduced Form TsTxV

Ligation of peptide thioester **36** (0.45 mg, 0.22 μmol) and SEAlide peptide **37** (0.71 mg, 0.21 μmol) was performed in 6 M Gn·HCl–0.2 M HEPPS buffer containing 200 mM MPAA and 50 mM TCEP (pH = 7.0, 220 μL, 1.0 mM each peptide). The reaction was completed within 3 h at 37 °C. After confirming the completion of the first NCL by HPLC analysis, 1.0 mM of peptide **38** (0.45 mg, 0.22 μmol) in 1.0 M Na phosphate buffer containing 50 mM TCEP (pH 7.1) was added to the reaction mixture to yield the desired reduced form TsTx-V in one-pot manner (final concentration: 3 M Gn·HCl, 0.1 M HEPPS, 0.5 M Na phosphate, 100 mM MPAA and 50 mM TCEP). After incubation at 37 °C for additional 48 h, the crude product was purified by semi-preparative HPLC to give the desired reduced form TsTxV (0.45 mg, 0.05 μmol, 31%)

39: Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 16% to 40% over 30 min, retention time = 21.6 min. MS (ESI-TOF) *m*/*z* calcd ($[M+4H]^{4+}$) 1106.0, found 1105.6.

Reduced form TsTxV: Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 16% to 40% over 30 min, retention time = 21.9 min. semi preparative HPLC conditions: A linear gradient of solvent B in solvent A, 26% to 36% over 30 min. MS (ESI-TOF) calcd (average isotopes) 7200.0, found 7199.7.

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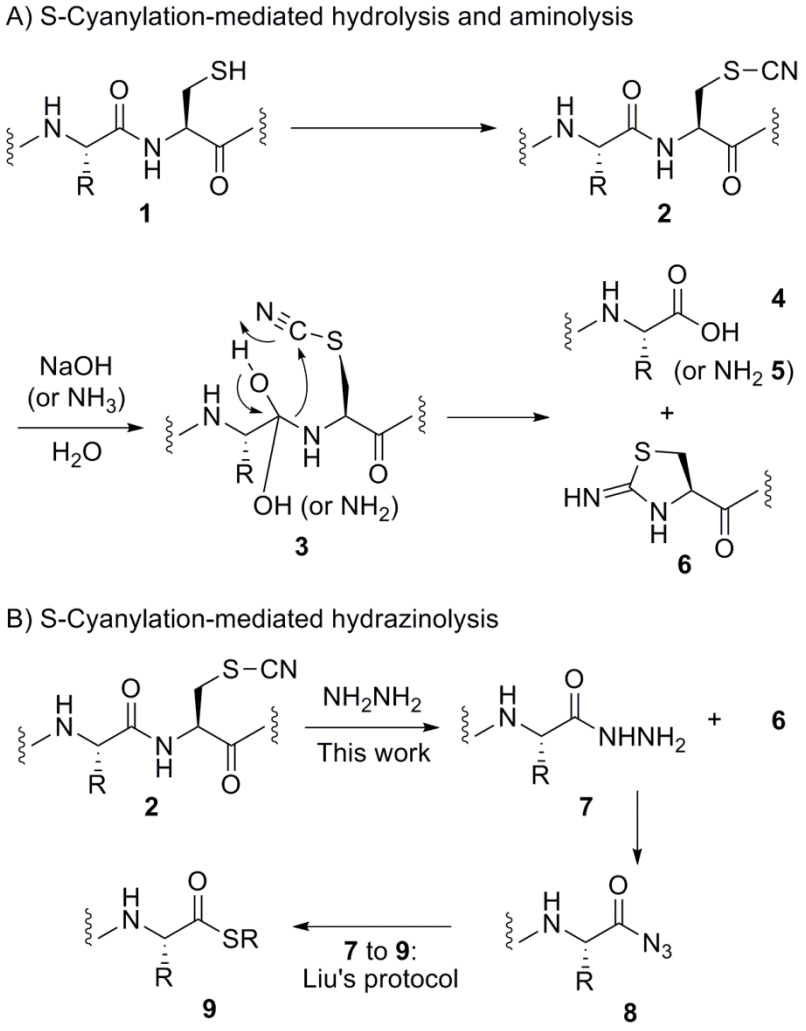
Scheme 1. S-Cyanylation-mediated reactions (A) and envisioned application to preparation of thioesters (B).



Scheme 2 Envisioned strategy for preparation of peptide thioesters using S-cyanylation/hydrazinolysis sequence

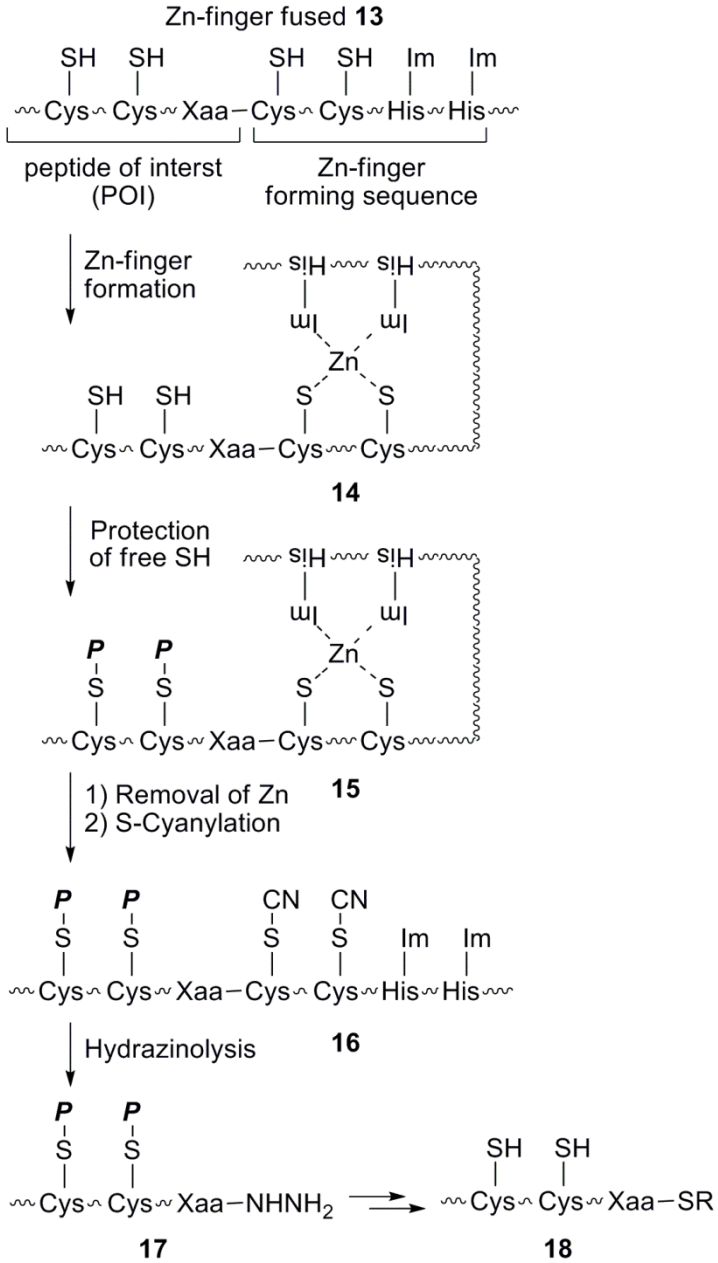


Figure 1 Production of peptide hydrazide fragment using regioselective S-cyanylation/hydrazinolysis sequence: (A) route for preparation of conotoxin thioester fragment and (B) HPLC monitoring of reactions. HPLC detected peptide **21** as a non-zinc-complexed form, because of the use of acidic eluents containing 0.1% TFA.

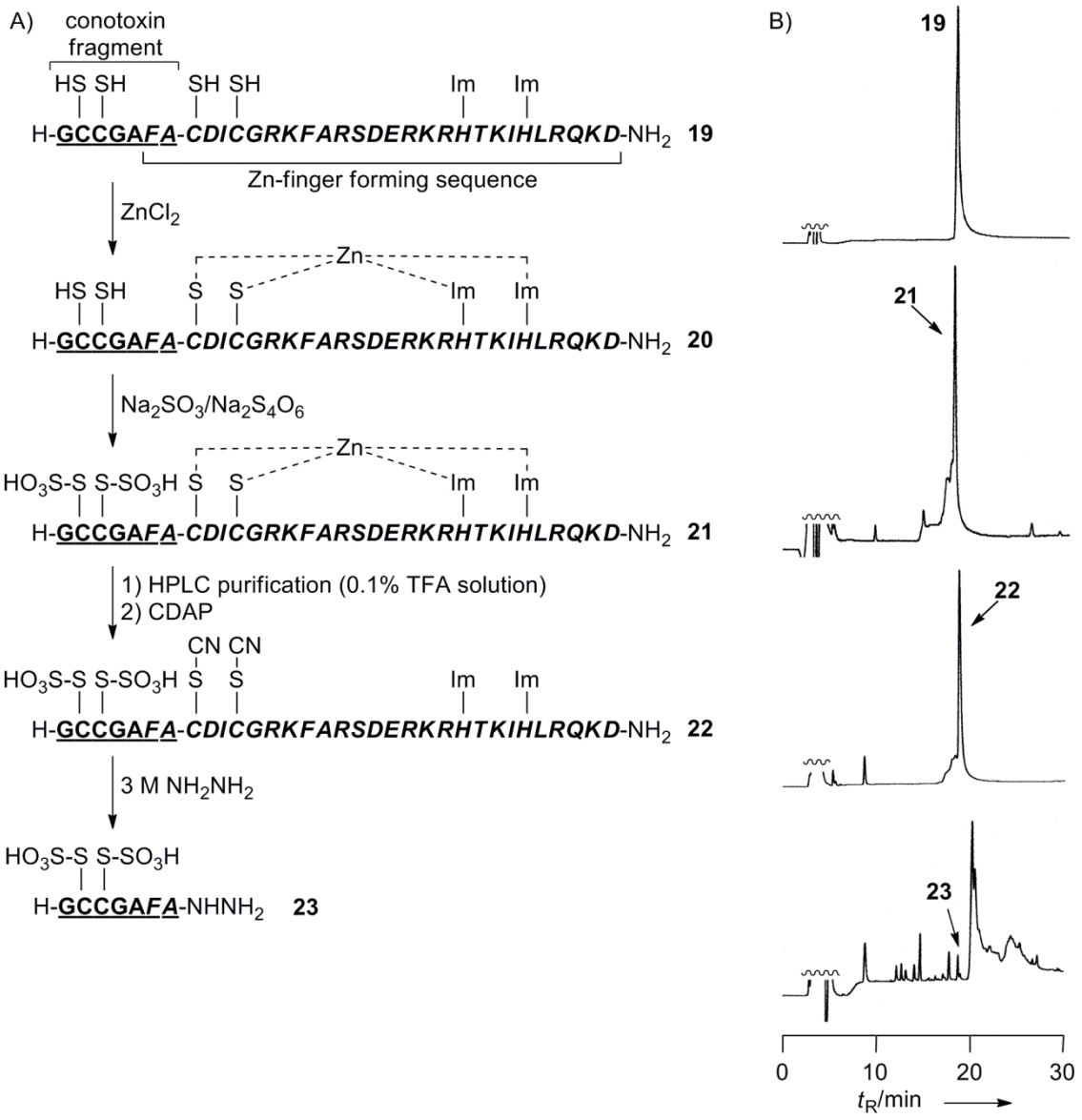


Figure 2 Reaction sequence for preparation of peptide hydrazide using 6-nitroveratryl group: (A) route for preparation of *Stichodactyla* toxin fragment and (B) HPLC monitoring of reactions. *Peptide **27** was co-eluted with peptide **28** in HPLC analysis.

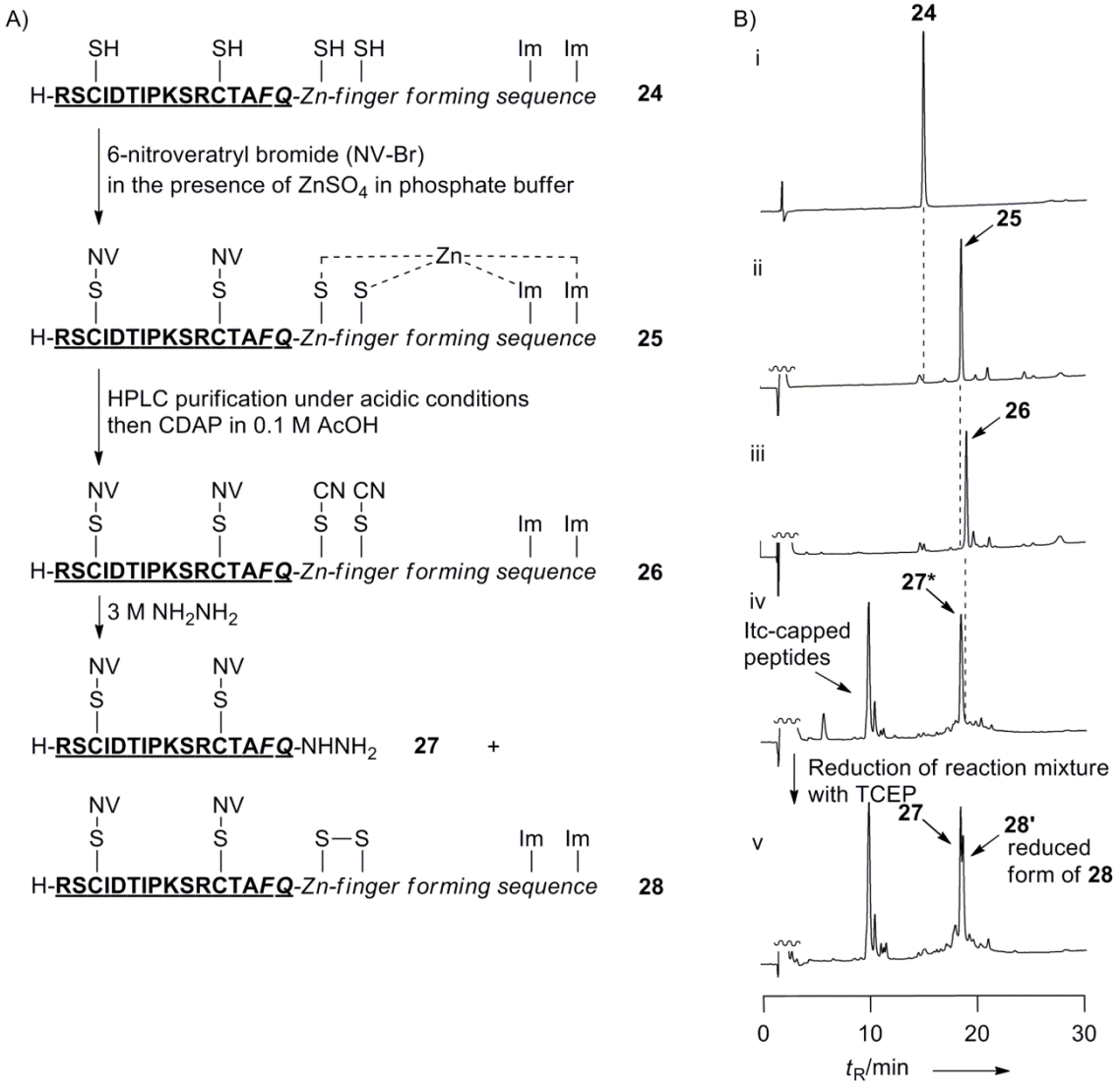


Figure 3. Formation of diacyl hydrazide peptide from Gln-$NHNH_2$ peptide under acidic conditions.

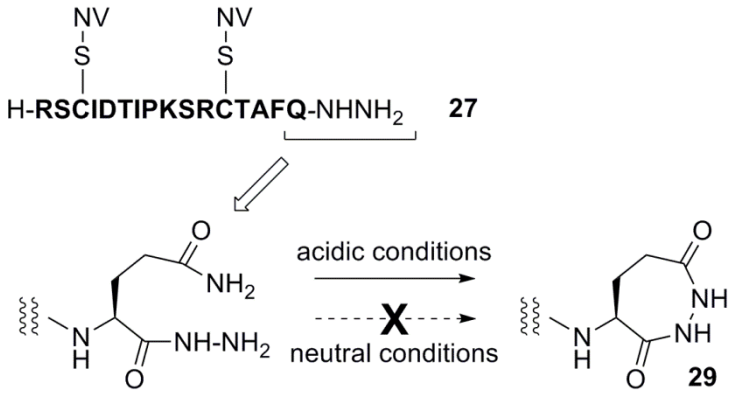


Figure 4 Conversion of TsTxV N-terminal fragment–zinc-finger fusion to peptide hydrazide: (A) route for preparation of TsTxV hydrazide fragment and (B) HPLC monitoring of reactions. *Itc-capped peptides: several peptides were generated by the presence of two cyanylation sites in peptide 32.

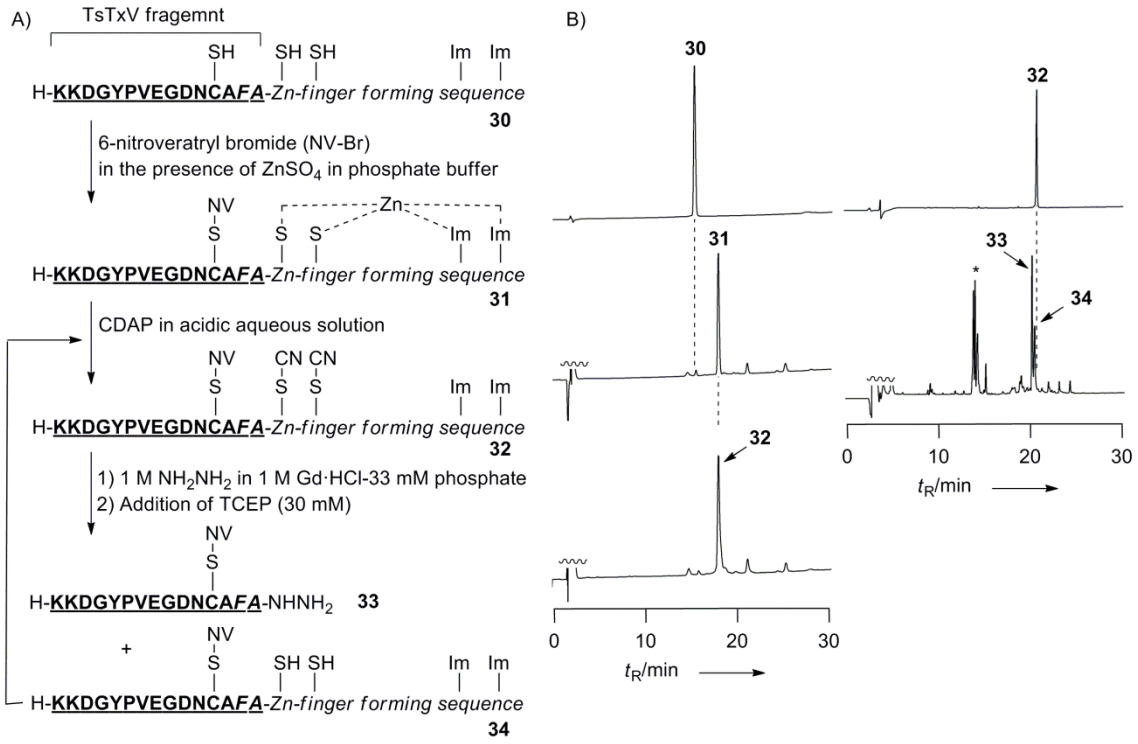


Figure 5 Conversion of TsTxV N-terminal hydrazide fragment to corresponding thioester: (A) route for preparation of TsTxV thioester fragment and (B) HPLC monitoring of reactions. *Non-peptidic material.

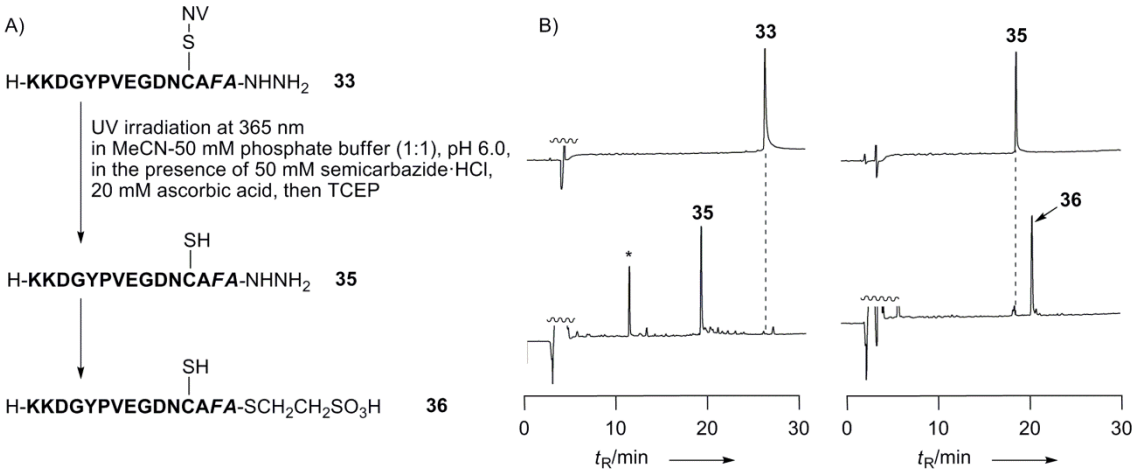


Figure 6 Synthesis of reduced form TsTxV by N–to–C-directed one-pot/sequential NCL protocol using SEAlide peptide. (A) Route for synthesis of TsTxV toxin: i) first NCL; ii) second NCL. (B) HPLC monitoring of reactions: i) first NCL, t < 1 min; ii) first NCL, t = 3 h; iii) second NCL, t < 1 min; iv) second NCL, t = 48 h